

CA2 COMPOSITIONS AND METHODS FOR TUNABLE REGULATION

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit of priority to U.S. Provisional Application No. 62/860,383, filed June 12, 2019. The entire contents of the aforementioned application are incorporated herein by reference in their entireties.

REFERENCE TO THE SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII file, created on June 11, 2020, is named 268052_469001_SL.txt and is 10,046,525 bytes in size.

FIELD

[0003] The present disclosure relates to destabilizing domains (DDs) derived from human carbonic anhydrase 2 (CA2) which can tune protein stability for at least one payload, and compositions and methods of use thereof. Provided in the present disclosure include polypeptides of CA2 biocircuit systems, CA2 effector modules, stimulus response elements (SREs), polynucleotides encoding the same, vectors and cells containing the polypeptides and/or polynucleotides for use in cancer immunotherapy.

BACKGROUND

[0004] Gene and cell therapies are revolutionizing medicine and offering new promise for the treatment of previously intractable conditions. However, most current technologies do not allow titration of the timing or levels of target protein induction. This has rendered many potential gene and cell therapy applications difficult or impossible to safely and effectively deploy.

[0005] Inadequate exogenous and/or endogenous gene control is a critical issue in many gene and cell therapy settings. This lack of tunability also makes it difficult to safely express proteins with narrow or uncertain therapeutic windows or those requiring more titrated or transient expression.

[0006] One approach to regulated protein expression or function is the use of Destabilizing Domains (DDs). Destabilizing domains are small protein domains that can be appended to a target protein of interest. DDs render the attached protein of interest unstable in the absence of a DD-binding ligand and the protein of interest is rapidly degraded by the ubiquitin-proteasome system of the cell. However, when a specific small molecule DD-binding ligand binds to the DD, the attached protein of interest is stabilized, and protein function is achieved.

[0007] DD technology forms the basis of a new class of cell and gene therapies that can deliver tunable and temporal control of gene expression and function, expanding the universe of protein therapeutics that can be safely and effectively incorporated into cell and gene therapy modalities.

SUMMARY

[0008] The present disclosure provides novel protein domains derived from human carbonic anhydrase 2 (CA2) displaying small molecule dependent stability. Such protein domains are called destabilizing domains (DDs). In the absence of its binding ligand, the DD is destabilizing and causes degradation of a payload fused to the DD (e.g., a protein of interest (POI), while in the presence of its binding ligand, the fused DD and payload can be stabilized, and its stability is dose dependent.

[0009] Provided herein are biocircuit systems comprising at least one effector module. The effector module may include (a) a stimulus response element (SRE) which may include in whole or in part, the human carbonic anhydrase 2 such as but not limited to (CA2; SEQ ID NO. 5810) and (b) at least one payload, said at least one payload which is attached, appended or associated with said SRE. The payload may be whole CD40L or a portion of CD40L.

[0010] In some embodiments, the present disclosure provides stimulus response element (SRE) which may comprise a destabilizing domain (DD) derived from human carbonic anhydrase 2 (CA2; SEQ ID NO. 5810), in whole or in part. In one embodiment, the DD may include the whole CA2 (SEQ ID NO. 5810). In another embodiment, the DD may include amino acids 2 to 260 of CA2, such as but not limited to amino acids 2 to 260 of SEQ ID NO. 5810.

[0011] In one aspect, the present disclosure provides a polypeptide comprising an effector module, said effector module comprising: i) a stimulus response element (SRE), wherein the SRE comprises a drug responsive domain (DRD), said DRD comprising human carbonic anhydrase 2 (CA2; SEQ ID NO. 5810) or a region thereof, and further comprising one or more mutations relative to the amino acid sequence of SEQ ID NO. 5810; and ii) at least one payload which is operably linked to the SRE, wherein: (a) the payload comprises CD40L (SEQ ID NO. 6) or a portion thereof; or (b) the payload comprises CD40L (SEQ ID NO. 6) or a portion thereof comprising one or more mutations relative to the amino acid sequence of SEQ ID NO. 6.

[0012] In one embodiment, the DRD comprises a H122Y mutation in the amino acid at position 122 (H122) of SEQ ID NO. 5810. In another embodiment, the DRD further comprises: (i) a R27L mutation in the amino acid at position 27 (R27) of SEQ ID NO. 5810; (ii) a T87I mutation in the amino acid at position 87 (T87) of SEQ ID NO. 5810; (iii) a N252D mutation in the amino acid at position 252 (N252) of SEQ ID NO. 5810; or (iv) a combination of (i), (ii) and/or (iii).

[0013] In one embodiment, the DRD comprises a E106D mutation in the amino acid at position 106 (E106) of SEQ ID NO. 5810. In one embodiment, the DRD comprises a W208S mutation in the amino acid at position 208 (W208) of

SEQ ID NO. 5810. In one embodiment, the DRD comprises a E106D mutation or a W208S mutation and further comprises a C205S mutation in the amino acid at position 205 (C205) of SEQ ID NO. 5810.

[0014] In one embodiment, the DRD comprises a I59N mutation in the amino acid at position 59 (I59) of SEQ ID NO. 5810. In another embodiment, the DRD further comprises a G102R mutation in the amino acid at position 102 (G102) of SEQ ID NO. 5810.

[0015] In one embodiment, the DRD comprises a L156H mutation in the amino acid at position 156 (L156) of SEQ ID NO. 5810. In another embodiment, the DRD further comprises: (i) a W4Y mutation in the amino acid at position 4 (W4) of SEQ ID NO. 5810; (ii) a F225L mutation in the amino acid at position 225 (F225) of SEQ ID NO. 5810; (iii) deletion of amino acids at positions 257-260 of SEQ ID NO. 5810; (iv) deletion of amino acids at positions 1-5 of SEQ ID NO. 5810; or (v) deletion of amino acids G234, E235 and P236 of SEQ ID NO. 5810. In another embodiment, the DRD comprises four mutations relative to SEQ ID NO. 5810, said mutations corresponding to: (i) L156H, S172C, F178Y, and E186D; or (ii) D70N, D74N, D100N, and L156H.

[0016] In one embodiment, the DRD comprises a first mutation and a second mutation relative to SEQ ID NO. 5810, wherein: (i) the first mutation is a S73N mutation in the amino acid at position 73 (S73) of SEQ ID NO. 5810; and (ii) the second mutation is a substitution of F or Y at the amino acid position 89 (R89) of SEQ ID NO. 5810.

[0017] In one embodiment, the DRD comprises a substitution of N or F at the amino acid position 56 (S56) of SEQ ID NO. 5810. In one embodiment, the DRD comprises two substitutions relative to SEQ ID NO. 5810 that correspond to S56F and D71S.

[0018] In one embodiment, the DRD comprises a S56N mutation in the amino acid at position 56 (S56) of SEQ ID NO. 5810.

[0019] In one embodiment, the DRD comprises one or more substitutions relative to SEQ ID NO. 5810, wherein at least one substitution is a substitution of D or N at the amino acid position 63 (G63) of SEQ ID NO. 5810, and wherein the one or more substitutions correspond to: G63D; G63D and M240L; G63D, E69V and N231I; or T55K, G63N and Q248N.

[0020] In one embodiment, the DRD comprises two or more substitutions relative to SEQ ID NO. 5810, wherein one of the two or more substitutions is a substitution of L or K at the amino acid position 71 (D71) of SEQ ID NO. 5810, and wherein said two or more substitutions correspond to: D71L and T87N; D71L and L250R; D71L, T87N and L250R; or D71K and T192F.

[0021] In one embodiment, the DRD comprises two or more substitutions relative to SEQ ID NO. 5810, wherein at least one of the two or more substitutions is: (i) a substitution of F at the amino acid position 241 (V241) of SEQ ID NO.

5810; or (ii) a substitution of F or L at the amino acid position 249 (P249) of SEQ ID NO. 5810; and wherein the two or more substitutions correspond to: D72F and V241F; D72F and P249L; D72F and P249F; D72F, V241F and P249L; A77I and P249F; or V241F and P249L.

[0022] In one embodiment, the DRD comprises one or more substitutions relative to SEQ ID NO. 5810, selected from Y51T, L183S, Y193I, L197P and the combination of V134F and L228F.

[0023] In some embodiments, the DRD comprises the region of human CA2 corresponding to amino acids 2 to 260 of SEQ ID NO. 5810.

[0024] In some embodiments, the DRD comprises the region of human CA2 corresponding to the full-length CA2 comprising amino acids 1 to 260 of SEQ ID NO. 5810.

[0025] In some embodiments, the SRE is responsive to one or more stimuli. In some embodiments, the stimulus is a small molecule, wherein the small molecule is selected from Acetazolamide, Celecoxib, Valdecoxib, Rofecoxib, Methazolamide, Dorzolamide, Brinzolamide, Diclofenamide, Ethoxzolamide, Zonisamide, Dansylamide, or Dichlorphenamide. In some embodiments, the small molecule is Acetazolamide.

[0026] In some embodiments, the payload is CD40L (SEQ ID NO. 6) or a portion thereof comprising at least one mutation.

[0027] In some embodiments, the payload is CD40L (SEQ ID NO. 6) or a portion thereof comprising at least two mutations, wherein the at least two mutations are selected from: (i) H224G and G226F; (ii) H224G and G226H; (iii) Y172G and G226F; (iv) H125 and G227; (v) Y120G, H224G and G226W; and (vi) S110G, F111G, E112S, M113G, Q114G, and K115S.

[0028] In some embodiments, the payload is CD40L (SEQ ID NO: 6).

[0029] In some embodiments, the DRD is N-terminally located to the payload.

[0030] In some embodiments, the DRD is separated from the payload by a linker.

[0031] In some embodiments, the effector module further comprises a signal peptide, a targeting and/or penetrating peptide, a linker, a protein tag, and/or a protein cleavage site.

[0032] In some embodiments, the present disclosure provides a composition comprising a polypeptide as described herein.

[0033] In some embodiments, the present disclosure provides a composition comprising an effector module as described herein.

[0034] In some embodiments, the present disclosure provides a polynucleotide encoding the effector module as described herein, wherein the polynucleotide is a DNA molecule or an RNA molecule. In some embodiments, the polynucleotide is monocistronic, bicistronic or multicistronic.

[0035] In one embodiment, the polynucleotide is bicistronic and encodes a second polypeptide, wherein said second polypeptide comprises an immunotherapeutic agent selected from an antibody and fragments and variants thereof, a T cell receptor (TCR) and variants thereof, a chimeric antigen receptor (CAR), a chimeric switch receptor, an inhibitor of a co-inhibitory receptor or ligand, an agonist of a co-stimulatory receptor and ligand, a cytokine, chemokine, a cytokine receptor, a chemokine receptor, cytokine-cytokine receptor fusion, a soluble growth factor, a metabolic factor, a suicide gene, or a homing receptor. In one embodiment, the polynucleotide is multicistronic and encodes at least two additional polypeptides, wherein said at least two additional polypeptides comprise an immunotherapeutic agent selected from an antibody and fragments and variants thereof, a T cell receptor (TCR) and variants thereof, a chimeric antigen receptor (CAR), a chimeric switch receptor, an inhibitor of a co-inhibitory receptor or ligand, an agonist of a co-stimulatory receptor and ligand, a cytokine, chemokine, a cytokine receptor, a chemokine receptor, cytokine-cytokine receptor fusion, a soluble growth factor, a metabolic factor, a suicide gene, or a homing receptor. In some embodiments, the second polypeptide is or the at least two additional polypeptides are operably linked to a second SRE. In some embodiments, the second polypeptide is not, or the at least two additional polypeptides are not, operably linked to any SRE.

[0036] In some embodiments, the present disclosure provides a vector comprising any of the polynucleotides described herein. In some embodiments, the vector is a viral vector or a plasmid. In some embodiments, the vector is a viral vector and the viral vector is a retroviral vector, a lentiviral vector, a gamma-retroviral vector, a recombinant AAV vector, an adenoviral vector, or an oncolytic viral vector.

[0037] In some embodiments, the present disclosure provides a cell transduced or transfected with the vector as described herein.

[0038] In some embodiments, the present disclosure provides a cell comprising at least one of: the effector module, the polynucleotide, or the vector as described herein.

[0039] In some embodiments, the cell of the present disclosure is an immune cell for adoptive cell transfer (ACT); or a CD8⁺ T cell, a CD4⁺ T cell, a helper T cell, a natural killer (NK) cell, a NKT cell, a cytotoxic T lymphocyte (CTL), a tumor infiltrating lymphocyte (TIL), a memory T cell, a regulatory T (Treg) cell, a cytokine-induced killer (CIK) cell, a dendritic cell, lymphokine activated killer (LAK) cells, a human embryonic stem cell, a mesenchymal stem cell, a hematopoietic stem cell, or a mixture thereof. In some embodiments, said cell is modified to express a chimeric antigen receptor (CAR) or an antigen-specific T cell receptor (TCR). In one embodiment, said cell is a T cell or NK cell.

[0040] In some embodiments, the present disclosure provides a cell which expresses the effector module, and/or comprises the polynucleotide, and/or is infected or transfected with the vector as described herein, wherein, said cell is a T cell modified to express an antigen-specific T cell receptor (TCR) or an antigen-specific chimeric antigen receptor (CAR).

[0041] In some embodiments, the present disclosure provides a pharmaceutical composition comprising the polypeptide, the polynucleotides, the vector, or the cell as described herein, and a pharmaceutically acceptable excipient.

[0042] In some embodiments, the present disclosure provides a method of producing a modified cell, said method comprising introducing into a cell a nucleic acid molecule comprising the polynucleotide as described herein.

[0043] In some embodiments, the present disclosure provides a method of modulating expression, function, and/or level of a payload in the cell as described herein, said method comprising administering to the cell a stimulus, wherein the SRE is responsive to the stimulus and wherein the expression, function, and/or level of the at least one payload is modulated in response to the stimulus.

[0044] In some embodiments, the present disclosure provides a method of treating a disease and/or inducing an immune response in a subject in need thereof, said method comprising: (a) administering to the subject a therapeutically effective amount of any of the compositions, polypeptides, polynucleotides, vectors, or cells described herein and administering to the subject a therapeutically effective amount of a stimulus, wherein the SRE is responsive to the stimulus and wherein expression of the at least one payload is modulated in response to the stimulus to thereby treat the disease and/or induce an immune response. In one embodiment, the disease is cancer. In some embodiments, the stimulus is selected from Acetazolamide, Celecoxib, Valdecoxib, Rofecoxib, Methazolamide, Dorzolamide, Brinzolamide, Diclofenamide, Ethoxzolamide, Zonisamide, Dansylamide, or Dichlorphenamide.

[0045] In another aspect of the present disclosure, provided is an engineered cell comprising: (i) a first polynucleotide which encodes a first polypeptide, said first polypeptide comprising: (a) a first stimulus response element (SRE), wherein the first SRE comprises a drug responsive domain (DRD), said DRD comprising human carbonic anhydrase 2 (CA2; SEQ ID NO. 5810) or a region thereof, and further comprising one or more mutations relative to the amino acid sequence of SEQ ID NO. 5810; and (b) a first payload which is operably linked to the first SRE, wherein: (I) the first payload comprises CD40L (SEQ ID NO. 6) or a portion thereof; or (II) the first payload comprises CD40L (SEQ ID NO. 6) or a portion thereof comprising one or more mutations relative to the amino acid sequence of SEQ ID NO. 6; and (ii) a second polynucleotide which encodes one or more additional polypeptides, said one or more additional polypeptides comprising an immunotherapeutic agent selected from the group consisting of: a T cell receptor (TCR) and variants thereof or a chimeric antigen receptor (CAR); wherein the DRD and the first payload are destabilized in the

absence of a first stimulus and wherein the DRD and the first payload are stabilized in the presence of the first stimulus, and the one or more additional polypeptides are expressed independently of the first payload.

[0046] In some embodiments, the first payload is (a) CD40L (SEQ ID NO. 6) or a portion thereof comprising at least one mutation; or (b) CD40L (SEQ ID NO. 6) or a portion thereof comprising at least two mutations, optionally, wherein the at least two mutations are selected from: (i) H224G and G226F; (ii) H224G and G226H; (iii) Y172G and G226F; (iv) H125 and G227; (v) Y120G, H224G and G226W; and (vi) S110G, F111G, E112S, M113G, Q114G, and K115S.

[0047] In some embodiments, the one or more additional polypeptides are linked to a second SRE comprising a second DRD, wherein the second DRD is the same or different as the DRD in the first SRE, the second DRD and the one or more additional polypeptides are destabilized in the absence of the first or a second stimulus and wherein the second DRD and the one or more additional polypeptides are stabilized in the presence of the first or the second stimulus.

[0048] In some embodiments, the present disclosure provides a DD comprising a region of or the whole human carbonic anhydrase 2 (CA2; SEQ ID NO. 5810), and further comprising a mutation relative to SEQ ID NO. 5810 selected from A115L, A116Q, A116V, A133L, A133T, A141P, A152D, A152L, A152R, A173C, A173G, A173L, A173T, A23P, A247L, A247S, A257L, A257S, A38P, A38V, A54Q, A54V, A54X, A65L, A65N, A65V, A77I, A77P, A77Q, C205M, C205R, C205V, C205W, C205Y, D101G, D101M, D110I, D129I, D138G, D138M, D138N, D161*, D161M, D161V, D164G, D164I, D174*, D174T, D179E, D179I, D179R, D189G, D189I, D19T, D19V, D242G, D242T, D32T, D34T, D41T, D52I, D52L, D71F, D71G, D71K, D71M, D71S, D71Y, D72I, D72S, D72T, D72X, D75T, D75V, D85M, E106D, E106G, E106S, E117*, E117N, E14N, E186*, E186N, E204A, E204D, E204G, E204N, E213*, E213G, E213N, E220K, E220R, E220S, E233D, E233G, E233R, E235*, E235G, E235N, E237K, E237R, E238*, E238N, E238R, E26S, E69D, E69K, E69S, F130L, F146V, F175I, F175L, F175S, F178L, F178S, F20L, F20S, F225I, F225L, F225S, F225Y, F230I, F230L, F230S, F259L, F259S, F66S, F70I, F70L, F95Y, G102D, G104R, G104V, G128R, G12D, G12E, G131E, G131R, G131W, G139D, G144D, G144V, G150A, G150S, G150W, G155A, G155C, G155D, G155S, G170A, G170D, G182A, G182W, G195A, G195R, G232R, G232W, G234L, G234V, G25E, G63D, G63V, G81E, G81V, G82D, G86A, G86D, G98V, H107I, H107Q, H119T, H119Y, H122T, H122Y, H15L, H15T, H15Y, H17D, H17I, H36I, H36Q, H64M, H94T, H96T, I145F, I145M, I166H, I166L, I209D, I209L, I215H, I215S, I22L, I255N, I255S, I33S, I59F, I59N, I59S, I91F, K111E, K111N, K112R, K113I, K113N, K126N, K132E, K132R, K148E, K148R, K153*, K153N, K158E, K158N, K167*, K169N, K169R, K171Q, K171R, K18R, K212N, K212Q, K212R, K212W, K224E, K224N, K227*, K227N, K24R, K251E, K251R, K256Q, K260F, K260L, K260Q, K39S, K45N, K45S, K80M, K80R, L118F, L120W, L140V, L140W, L143*, L147*, L147F, L156F, L156H, L156P, L156Q, L163A, L163W, L183P, L183S, L184F, L184P,

L188P, L188W, L197*, L197M, L197P, L197R, L197T, L202F, L202H, L202I, L202P, L202R, L202S, L203P, L203S, L203W, L211*, L211A, L211S, L223*, L223I, L223V, L228F, L228H, L228T, L239*, L239F, L239T, L250*, L250P, L250T, L44*, L44M, L47C, L47V, L57*, L57X, L60S, L79F, L79S, L84W, L90*, L90V, M240D, M240L, M240R, M240W, N11D, N11K, N124T, N177*, N177T, N229*, N229T, N231D, N231F, N231K, N231L, N231M, N231Q, N231T, N243Q, N243T, N252E, N252T, N61R, N61T, N61Y, N62K, N62M, N67D, N67T, P137L, P13A, P13H, P13L, P13S, P154L, P154R, P154T, P180L, P180S, P185L, P185S, P185V, P194Q, P200A, P200L, P200S, P200T, P201A, P201L, P201R, P201S, P214T, P236L, P236T, P246L, P246Q, P249A, P249F, P249H, P249I, P249X, P30L, P30S, P42L, P83A, Q103K, Q135S, Q136N, Q157R, Q157S, Q221A, Q221R, Q248F, Q248L, Q248S, Q254A, Q254K, Q28S, Q53H, Q53K, Q53N, Q74R, Q92H, Q92S, R181H, R181S, R181V, R226H, R226P, R226V, R245A, R253G, R253Q, R27A, R58G, R89D, R89F, R89I, R89X, R89Y, S105L, S105Q, S151A, S151I, S151Q, S165F, S165P, S172E, S172V, S187I, S187P, S196H, S196L, S216A, S216Q, S218A, S218Q, S219A, S219Q, S258F, S258P, S29C, S29P, S43P, S43T, S48L, S50P, S56F, S56N, S56P, S56X, S73L, S73N, S73X, S99H, T108L, T125I, T125P, T168K, T168N, T168Q, T176H, T176L, T192D, T192F, T192I, T192N, T192P, T192X, T198D, T198I, T198P, T199A, T199H, T199P, T207D, T207I, T207P, T207S, T35I, T35L, T37Q, T55L, T87L, V109M, V109W, V121F, V134C, V134F, V142F, V149G, V149L, V159L, V159S, V160C, V160L, V162A, V162C, V206*, V206C, V206M, V210C, V217L, V217R, V217S, V222A, V222C, V222G, V241G, V241W, V241X, V31L, V49F, V68L, V68W, V78C, W123G, W123R, W16G, W191*, W191G, W191L, W208G, W208L, W208S, W244*, W244G, W244L, W97C, W97G, Y114H, Y114M, Y127M, Y190*, Y190L, Y190T, Y193C, Y193F, Y193I, Y193L, Y193T, Y193V, Y193X, Y40M, Y51F, Y51M, Y51T, Y51X, Y88T, K9N, and S29A. As used herein, “*” indicates the translation of the stop codon and “X” indicates any amino acid.

[0049] In some embodiments, the present disclosure provides a DD comprising a region of or the whole human carbonic anhydrase 2 (CA2; SEQ ID NO. 5810), and further comprising a mutation relative to SEQ ID NO. 5810 selected from E106D, G63D, H122Y, I59N, L156H, L183S, L197P, S56F, S56N, W208S, Y193I, and Y51T.

[0050] In some embodiments, the present disclosure provides a DD comprising a region of or the whole human carbonic anhydrase 2 (CA2; SEQ ID NO. 5810), and further comprising two or more mutations relative to SEQ ID NO. 5810. In some embodiments, a DD may comprise CA2 (aa 2-260 of WT, R27L, H122Y), CA2 (aa 2-260 of WT, T87I, H122Y), CA2 (aa 2-260 of WT, H122Y, N252D), CA2 (aa 2-260 of WT, D72F, V241F), CA2 (aa 2-260 of WT, V241F, P249L), CA2 (aa 2-260 of WT, D72F, P249L), CA2 (aa 2-260 of WT, D71L, L250R), CA2 (aa 2-260 of WT, D72F, P249F), CA2 (aa 2-260 of WT, T55K, G63N, Q248N), CA2 (aa 2-260 of WT, L156H, A257del, S258del, F259del, K260del), CA2 (aa 2-260 of WT, L156H, S2del, H3del, H4del, W5del), CA2 (aa 2-260 of WT, W4Y, L156H), CA2 (aa 2-260 of WT, L156H, G234del, E235del, P236del), CA2 (aa 2-260 of WT, L156H, F225L), CA2 (aa 2-260 of WT,

D70N, D74N, D100N, L156H), (CA2 (aa 2-260 of WT, I59N, G102R), CA2 (aa 2-260 of WT, G63D, E69V, N231I), CA2 (aa 2-260 of WT, R27L, T87I, H122Y, N252D), CA2 (aa 2-260 of WT, D72F, V241F, P249L), CA2 (aa 2-260 of WT, D71L, T87N, L250R), CA2 (aa 2-260 of WT, L156H, S172C, F178Y, E186D), CA2 (aa 2-260 of WT, D71F, N231F), CA2 (aa 2-260 of WT, A77I, P249F), CA2 (aa 2-260 of WT, D71K, P249H), CA2 (aa 2-260 of WT, D72F, P249H), CA2 (aa 2-260 of WT, Q53N, N61Y), CA2 (aa 2-260 of WT, E106D, C205S), CA2 (aa 2-260 of WT, C205S, W208S), CA2 (aa 2-260 of WT, S73N, R89Y), CA2 (aa 2-260 of WT, D71K, T192F), CA2 (aa 2-260 of WT, Y193L, K260L), CA2 (aa 2-260 of WT, D71F, V241F, P249L), CA2 (aa 2-260 of WT, L147F, Q248F), CA2 (aa 2-260 of WT, D52I, S258P), CA2 (aa 2-260 of WT, D72S, T192N), CA2 (aa 2-260 of WT, D179E, T192I), CA2 (aa 2-260 of WT, S56N, Q103K), CA2 (aa 2-260 of WT, D71Y, Q248L), CA2 (aa 2-260 of WT, S73N, R89F), CA2 (aa 2-260 of WT, D71K, N231L, E235G, L239F), CA2 (aa 2-260 of WT, D72F, P249I), CA2 (aa 2-260 of WT, D72X, V241X, P249X), CA2 (aa 2-260 of WT, A54X, S56X, L57X, T192X), CA2 (aa 2-260 of WT, Y193V, K260F), CA2 (aa 2-260 of WT, G63D, M240L), CA2 (aa 2-260 of WT, V134F, L228F), CA2 (aa 2-260 of WT, D71G, N231K), CA2 (aa 2-260 of WT, S56F, D71S), CA2 (aa 2-260 of WT, D52L, G128R, Q248F), CA2 (aa 2-260 of WT, S73X, R89X), CA2 (aa 2-260 of WT, Y51X, D72X, V241X, P249X), CA2 (aa 2-260 of WT, D72I, W97C), CA2 (aa 2-260 of WT, D71K, T192F, N231F), CA2 (aa 2-260 of WT, H36Q, S43T, Y51F, N67D, G131W, R226H), CA2 (aa 2-260 of WT, F70I, F146V), CA2 (aa 2-260 of WT, K45N, V68L, H119Y, K169R, D179E), CA2 (aa 2-260 of WT, H15L, A54V, K111E, E220K, F225I), CA2 (aa 2-260 of WT, P13S, P83A, D101G, K111N, F230I), CA2 (aa 2-260 of WT, G63D, W123R, E220K), CA2 (aa 2-260 of WT, N11D, E69K, G86D, V109M, K113I, T125I, D138G, G155S), CA2 (aa 2-260 of WT, I59N, G102R, A173T), CA2 (aa 2-260 of WT, L79F, P180S), CA2 (aa 2-260 of WT, A77P, G102R, D138N), CA2 (aa 2-260 of WT, F20L, K45N, G63D, E69V, N231I), CA2 (aa 2-260 of WT, T199N, L202P, L228F), CA2 (aa 2-260 of WT, K9N, H122Y, T168K), CA2 (aa 2-260 of WT, Q53H, L90V, Q92H, G131E), CA2 (aa 2-260 of WT, L44M, L47V, N62K, E69D), CA2 (aa 2-260 of WT, D75V, K169N, F259L), CA2 (aa 2-260 of WT, T207S, V222A, N231D), CA2 (aa 2-260 of WT, I59F, V206M, G232R), CA2 (aa 2-260 of WT, P13A, A133T), CA2 (aa 2-260 of WT, I59N, R89I), CA2 (aa 2-260 of WT, A65N, G86D, G131R, G155D, K158N, V162A, G170D, P236L), CA2 (aa 2-260 of WT, G12R, H15Y, D19V), CA2 (aa 2-260 of WT, A65V, F95Y, E106G, H107Q, I145M, F175I), CA2 (aa 2-260 of WT, G63D, E69V, N231I), CA2 (aa 2-260 of WT, S29A, C205S) and/or CA2 (aa 2-260 of WT, S29C, C205S). As used herein, "X" indicates any amino acid.

[0051] In some embodiments, a DD may comprise CA2 (aa 2-260 of WT, R27L, H122Y), CA2 (aa 2-260 of WT, T87I, H122Y), CA2 (aa 2-260 of WT, H122Y, N252D), CA2 (aa 2-260 of WT, D72F, V241F), CA2 (aa 2-260 of WT, V241F, P249L), CA2 (aa 2-260 of WT, D72F, P249L), CA2 (aa 2-260 of WT, D71L, L250R), CA2 (aa 2-260 of WT,

D72F, P249F), CA2 (aa 2-260 of WT, T55K, G63N, Q248N), CA2 (aa 2-260 of WT, L156H, A257del, S258del, F259del, K260del), CA2 (aa 2-260 of WT, L156H, S2del, H3del, H4del, W5del), CA2 (aa 2-260 of WT, W4Y, L156H), CA2 (aa 2-260 of WT, L156H, G234del, E235del, P236del), CA2 (aa 2-260 of WT, L156H, F225L), CA2 (aa 2-260 of WT, D70N, D74N, D100N, L156H), (CA2 (aa 2-260 of WT, I59N, G102R), CA2 (aa 2-260 of WT, G63D, E69V, N231I), CA2 (aa 2-260 of WT, R27L, T87I, H122Y, N252D), CA2 (aa 2-260 of WT, D72F, V241F, P249L), CA2 (aa 2-260 of WT, D71L, T87N, L250R), CA2 (aa 2-260 of WT, L156H, S172C, F178Y, E186D), CA2 (aa 2-260 of WT, A77I, P249F), CA2 (aa 2-260 of WT, E106D, C205S), CA2 (aa 2-260 of WT, C205S, W208S), CA2 (aa 2-260 of WT, S73N, R89Y), CA2 (aa 2-260 of WT, D71K, T192F), CA2 (aa 2-260 of WT, S73N, R89F), CA2 (aa 2-260 of WT, G63D, M240L), CA2 (aa 2-260 of WT, V134F, L228F), and/or CA2 (aa 2-260 of WT, S56F, D71S).

[0052] The biocircuit systems described herein may be responsive to one or more stimuli. Such stimuli may be small molecules, such as but not limited to, Acetazolamide, Celecoxib, Valdecoxib, Rofecoxib, Methazolamide, Dorzolamide, Brinzolamide, Diclofenamide, Ethoxzolamide, Zonisamide, Dansylamide, and Dichlorphenamide. In one embodiment, the small molecule may be Acetazolamide. In one embodiment, the stimulus may be Celecoxib.

[0053] In some embodiments, the payload may be the whole or a portion of CD40L. In one embodiment, the payload may be the whole CD40L (SEQ ID NO. 6). In some embodiments, the payload may be a portion of CD40L comprising: (i) sCD40L (113-261 of WT) (SEQ ID NO. 5800); (ii) CD40L (aa 14-261 of WT) (SEQ ID NO.5802); or (iii) CD40L (aa 14-261 of WT, (S110-G116) del) (SEQ ID NO. 5804).

[0054] In some embodiments, the payload may be CD40L with one or more mutations as compared to (i) sCD40L (113-261 of WT) (SEQ ID NO. 5800); (ii) CD40L (aa 14-261 of WT) (SEQ ID NO.5802); or (iii) CD40L (aa 14-261 of WT, (S110-G116) del) (SEQ ID NO. 5804).

[0055] In some embodiments, the payload may be CD40L with one or more mutations as compared to wild-type CD40L (amino acid sequence provided as SEQ ID NO. 6). In some aspects the CD40L comprises at least one mutation such as, but not limited to, S110G, F111G, E112S, M113G, Q114G, K115S, Y120G, H125G, Y172G, H224G, G226F, G226H, G226W, and G227F. The CD40L payload may comprise two mutations, and the mutations may be, but are not limited to, H224G and G226F, H224G and G226H, Y172G and G226F, or H125 and G227. The CD40L payload may comprise three mutations, and the mutations may be, but are not limited to, Y120G, H224G and G226W. The CD40L payload may comprise four mutations. The CD40L payload may comprise five mutations. The CD40L payload may comprise six mutations and the mutations may be, but are not limited to, S110G, F111G, E112S, M113G, Q114G, and K115S.

[0056] Also provided herein are polynucleotides encoding the SREs, biocircuit systems, polypeptides and/or compositions described herein as well as vectors comprising the polynucleotides and cells comprising the polynucleotides. The present disclosure also describes pharmaceutical compositions that include components of CA2 biocircuit systems and/or the compositions described herein and a pharmaceutically acceptable excipient.

[0057] The present disclosure provides suitable examples and embodiments which describes the use of the various compositions, biocircuit systems, and components thereof, to treat a disease in a person in need thereof. For example, a method for treating a disease and/or inducing an immune response in a subject in need thereof, comprises the steps of (a) administering to the subject a therapeutically effective amount of a composition (comprising: an effector module, wherein the effector module comprises: i) a stimulus response element (SRE), wherein the SRE comprises a drug responsive domain (DRD), said DRD comprising human carbonic anhydrase 2 (CA2; SEQ ID NO. 5810) or a region thereof, and further comprising one or more mutations relative to the amino acid sequence of SEQ ID NO. 5810; and ii) at least one payload which is operably linked to the SRE, wherein (a) the payload comprises CD40L (SEQ ID NO. 6) or a portion thereof; or (b) the payload comprises CD40L (SEQ ID NO. 6) or a portion thereof comprising one or more mutations relative to the amino acid sequence of SEQ ID NO. 6), or a polynucleotide which encodes the composition components, or a vector that contains such polynucleotides which encode the components of the composition, or a cell which contains such vector, which is able to synthesize the components of the composition described herein; and (b) administering to the subject a therapeutically effective amount of a stimulus, for example, Acetazolamide, Celecoxib, Valdecoxib, Rofecoxib, Methazolamide, Dorzolamide, Brinzolamide, Diclofenamide, Ethoxzolamide, Zonisamide, Dansylamide, or Dichlorphenamide, wherein the SRE is responsive to the stimulus and wherein expression of the at least one payload is modulated in response to the stimulus to thereby treat the disease and/or induce an immune response.

[0058] Exemplary diseases that can be treated and/or prevented using the biocircuit systems and engineered cells herein can include: immune diseases, autoimmune diseases, infections diseases and hyperproliferative diseases, for example, cancer.

DETAILED DESCRIPTION

[0059] The details of one or more embodiments of the present disclosure are set forth in the accompanying description below. Although any materials and methods similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, the preferred materials and methods are now described. Other features, objects and advantages of the present disclosure will be apparent from the description. In the description, the singular forms also include the plural unless the context clearly dictates otherwise. Unless defined otherwise, all technical and

scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this present disclosure belongs. In the case of conflict, the present description will control.

I. COMPOSITIONS

Biocircuits or Biocircuit Systems

[0060] According to the present disclosure, biocircuit systems are provided which comprise, at their core, at least one effector module. Such effector module(s) are independently having associated, or integral therewith, one or more stimulus response elements (SREs). In general, a stimulus response element (SRE) may be operably linked to a payload which could be any protein of interest (POI) (e.g., an immunotherapeutic agent), to form an effector module. The SRE, when activated by a particular stimulus, e.g., a small molecule, can produce a signal or outcome, to regulate transcription and/or protein levels of the linked payload either up or down by perpetuating a stabilizing signal or destabilizing signal, or any other types of regulation. A much-detailed description of a biocircuit system are taught in co-owned U.S. Provisional Patent Application No. 62/320,864 filed April 11, 2016, 62/466,596 filed March 3, 2017 and the International Publication WO2017/180587 (the contents each of which are herein incorporated by reference in their entirety). In accordance with the present disclosure, biocircuit systems, effector modules, SREs and components that tune expression levels and activities of any agents used for immunotherapy are provided.

[0061] As used herein, a “biocircuit” or “biocircuit system” is defined as a circuit within or useful in biologic systems comprising a stimulus and at least one effector module responsive to a stimulus, where the response to the stimulus produces at least one signal or outcome within, between, as an indicator of, or on a biologic system. Biologic systems are generally understood to be any cell, tissue, organ, organ system or organism, whether animal, plant, fungi, bacterial, or viral. It is also understood that biocircuits may be artificial circuits which employ the stimuli or effector modules taught by the present disclosure and effect signals or outcomes in acellular environments such as with diagnostic, reporter systems, devices, assays or kits. The artificial circuits may be associated with one or more electronic, magnetic, or radioactive components or parts.

Effector modules

[0062] The biocircuits of the disclosure include at least one effector module. As used herein, an “effector module” is a single or multi-component construct or complex comprising at least (a) one or more stimulus response elements (SREs) and (b) one or more payloads (e.g. proteins of interest (POIs)).

[0063] Effector modules may be designed to include one or more payloads, one or more SREs, one or more cleavage sites, one or more signal sequences and one or more additional features including the presence or absence of one or more linkers. Representative effector module embodiments of the present disclosure are illustrated in Figures 2-6 in

International Publication No. WO2017/180587, the contents of which are herein incorporated by reference in their entirety. Biocircuits and components utilizing such effector molecules are given in Figures 7-12 in International Publication No. WO2017/180587, the contents of which are herein incorporated by reference in their entirety.

[0064] As shown in Figure 2 in International Publication No. WO2017/180587, representative effector module embodiments comprising one payload, i.e. one immunotherapeutic agent are illustrated. Each component of the effector module may be located or positioned in various arrangements without (A to F) or with (G to Z, and AA to DD) a cleavage site. An optional linker may be inserted between each component of the effector module.

[0065] Figures 3 to 6 in International Publication No. WO2017/180587, illustrate representative effector module embodiments comprising two payloads, i.e. two immunotherapeutic agents. In some aspects, more than two immunotherapeutic agents (payloads) may be included in the effector module under the regulation of the same SRE (e.g., the same DD). The two or more agents may be either directly linked to each other or separated. The SRE may be positioned at the N terminus of the construct, or the C terminus of the construct, or in the internal location.

[0066] The combination of the T regulatory cells, myeloid derived suppressor cells (MDSCs) and the extensive stromal networks within the tumor microenvironment (TME) can dampen the antitumor immune response by preventing T-cell infiltration and/or activation by current immunotherapies (see Ma et al. *A CD40 agonist and PD-1 antagonist antibody reprogram the microenvironment of non-immunogenic tumors to allow T cell-mediated anticancer activity*. Cancer Immunol Res Jan. 14, 2019; doi: 10.1158/2326-6066.CIR-18-0061; the contents of which are herein incorporated by reference in their entirety). Current CAR T therapies are not effective as the therapeutics have immunosuppression, tumor antigen escape, insufficient CAR T expansion and healthy tissue toxicity. The present disclosure addresses these issues with the utilization of an effector module with CD40L as an immunotherapeutic agent, fused to an SRE described herein. The CD40L may not be the only immunotherapeutic agent in the effector module. The effector module may also include a CAR construct. The combination of the CD40L and CAR as the immunotherapeutic agent and an SRE may cause any of the following alone or in combination, (1) repolarization of the CD40+ macrophages in the tumor microenvironment to a proinflammatory state, (2) activation of CD40+ dendritic cells to promote epitope spreading which can decrease tumor antigen escape (e.g., decrease the loss of CAR targeted antigens), (3) reverse signaling and cytokine production to enhance the antigen-dependent T cell expansion, and (4) regulatable protein production from the SRE which lowers toxicity of the therapeutic to healthy tissue. As a non-limiting example, an effector module including an SRE fused to a CD40L and CAR immunotherapeutic agent may be used to overcome the loss of CAR targeted antigens (e.g., antigen escape) by causing dendritic cells to recruit tumor infiltration lymphocytes (TILs) which results in the expansion of the group of anti-tumor specific T cells. As a non-limiting example, an effector module including an

SRE fused to a CD40L and CAR immunotherapeutic agent may be used to reduce the constraint of the tumor microenvironment (TME) of solid tumors by repolarizing tumor associated macrophages (TAMs) from a suppressive to an inflammatory phenotype. As a non-limiting example, an effector module including an SRE fused to a CD40L and CAR immunotherapeutic agent may be used to increase CAR T cell expansion by causing antigen-dependent T cell expansion

[0067] In some embodiments, biocircuits of the present disclosure may be modified to reduce their immunogenicity. Immunogenicity is the result of a complex series of responses to a substance that is perceived as foreign and may include the production of neutralizing and non-neutralizing antibodies, formation of immune complexes, complement activation, mast cell activation, inflammation, hypersensitivity responses, and anaphylaxis. Several factors can contribute to protein immunogenicity, including, but not limited to protein sequence, route and frequency of administration and patient population. In a preferred embodiment, protein engineering may be used to reduce the immunogenicity of the compositions of the disclosure. In some embodiments, modifications to reduce immunogenicity may include modifications that reduce binding of the processed peptides derived from the parent sequence to MHC proteins. For example, amino acid modifications may be engineered such that there are no or a minimal of number of immune epitopes that are predicted to bind with high affinity, to any prevalent MHC alleles. Several methods of identifying MHC binding epitopes of known protein sequences are known in the art and may be used to score epitopes in the compositions of the present disclosure. Such methods are disclosed in US Patent Publication No. US 20020119492, US20040230380, and US 20060148009; the contents of each of which are incorporated by reference in their entirety.

[0068] Effector modules, including their SREs and payloads, may be nucleic acid-based, protein-based or a combination thereof. They may be in the form of DNA, RNA, mRNA, proteins, fusion proteins, or any combination of the foregoing.

Stimulus response element (SRE)

[0069] As used herein a “stimulus response element” (SRE) is a component of an effector module which is joined, attached, linked to or associated with one or more payloads and in some instances, is responsible for the responsive nature of the effector module to one or more stimuli. As used herein, the “responsive” nature of an SRE to a stimulus may be characterized by a covalent or non-covalent interaction, a direct or indirect association or a structural or chemical reaction to the stimulus. Further, the response of any SRE to a stimulus may be a matter of degree or kind. The response may be a partial response. The response may be a reversible response. The response may ultimately lead to a regulated signal or output. Such output signal may be of a relative nature to the stimulus, e.g., producing a modulatory effect of

between 1% and 100% or a factored increase or decrease such as 2-fold, 3-fold, 4-fold, 5-fold, 10-fold or more. In some embodiments, the SRE is a polypeptide fused to a polypeptide payload.

[0070] In some embodiments, the present disclosure provides methods for modulating protein expression, function or level. In some aspects, the modulation of protein expression, function or level refers to modulation of expression, function or level by at least about 20%, such as by at least about 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 95% and 100%, or at least 20-30%, 20-40%, 20-50%, 20-60%, 20-70%, 20-80%, 20-90%, 20-95%, 20-100%, 30-40%, 30-50%, 30-60%, 30-70%, 30-80%, 30-90%, 30-95%, 30-100%, 40-50%, 40-60%, 40-70%, 40-80%, 40-90%, 40-95%, 40-100%, 50-60%, 50-70%, 50-80%, 50-90%, 50-95%, 50-100%, 60-70%, 60-80%, 60-90%, 60-95%, 60-100%, 70-80%, 70-90%, 70-95%, 70-100%, 80-90%, 80-95%, 80-100%, 90-95%, 90-100% or 95-100%.

[0071] Effector modules, including their SREs and payloads may individually, collectively or independently comprise peptides, polypeptides or proteins. At the protein level, such payload may be any natural or artificial peptide or polypeptide or fragment thereof. Natural peptides or polypeptide components of the payload may be derived from any known protein of any species.

[0072] Effector modules may be designed to operate in groups of one, two, three, four or more modules. When more than one effector module is utilized in a biocircuit, it is known as an effector module system of that biocircuit.

Destabilizing Domains

[0073] Destabilizing domains (DDs) are small protein domains that can be appended to a target protein of interest. The term destabilizing domain (DD) is interchangeable with the term drug responsive domain (DRD). DDs render the attached protein of interest unstable in the absence of a DD-binding ligand such that the protein is rapidly degraded by the ubiquitin-proteasome system of the cell (Stankunas, K., et al., *Mol. Cell*, 2003, 12: 1615–1624; Banaszynski, *et al.*, *Cell*, 2006, 126(5): 995–1004; reviewed in Banaszynski, L.A., and Wandless, T.J. *Chem. Biol.*, 2006, 13:11–21 and Rakhit R et al., *Chem Biol.* 2014; 21(9):1238-1252). However, when a specific small molecule ligand binds its intended DD as a ligand binding partner, the instability is reversed, and protein function is restored. The conditional nature of DD stability allows a rapid and non-perturbing switch from stable protein to unstable substrate for degradation. Moreover, its dependency on the concentration of its ligand further provides tunable control of degradation rates.

[0074] In one embodiment, the SRE is a destabilizing domain (DD). The presence, absence or an amount of a small molecule ligand that binds to or interacts with the DD, can, upon such binding or interaction modulate the stability of the payload(s) and consequently the function of the payload. Depending on the degree of binding and/or interaction the altered function of the payload may vary, hence providing a “tuning” of the payload function.

[0075] In some embodiments, the desired characteristics of the DDs may include, but are not limited to, low protein levels in the absence of a ligand of the DD (e.g., low basal stability), large dynamic range, robust and predictable dose-response behavior, and rapid kinetics of degradation. DDs that bind to a desired ligand, but not endogenous molecules may be preferred.

[0076] In some embodiments, the DDs of the present disclosure may be developed from known proteins herein referred to as the parent protein. In some embodiments, the CA2 destabilizing domains described herein or known in the art may be used as SREs in the biocircuit systems of the present disclosure in association with any of the payloads (e.g., proteins of interest or immunotherapeutic agents) taught herein.

[0077] Regions or portions or domains of wild type proteins (e.g., CA2) may be utilized as SREs/DDs in whole or in part. They may be combined or rearranged to create new peptides, proteins, regions or domains of which any may be used as SREs/DDs or the starting point for the design of further SREs and/or DDs.

[0078] In one embodiment, the SRE is derived from a region of a parent protein (e.g., CA2) or from a mutant protein. The region of the parent protein may be 5-50, 25-75, 50-100, 75-125, 100-150, 125-175, 150-200, 175-225, 200-250, 225-275, 250-300, 275-325, 300-350, 325-375, 350-400, 375-425, or 400-450 amino acids in length. As a non-limiting example, the region of the parent protein may be 250-270 amino acids in length. As a non-limiting example, the region of the parent protein may be 225-250 amino acids in length. As a non-limiting example, the region of the parent protein may be 225-260 amino acids in length.

[0079] In one embodiment, the SRE is derived from a parent protein (e.g., CA2) or from a mutant protein and includes a region of the parent protein. The SRE may include a region of the parent protein which is 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, or 100%, 5-10%, 10-15%, 15-20%, 20-25%, 25-30%, 30-35%, 35-40%, 40-45%, 45-50%, 50-55%, 55-60%, 60-65%, 65-70%, 70-75%, 75-80%, 80-85%, 85-90%, 90-95%, 95-100%, 10-20%, 20-30%, 30-40%, 40-50%, 50-60%, 60-70%, 70-80%, 80-90%, 90-100%, 10-30%, 20-40%, 30-50%, 40-60%, 50-70%, 60-80%, 70-90%, 80-100%, 10-40%, 20-50%, 30-60%, 40-70%, 50-80%, 60-90%, 70-100%, 10-50%, 20-60%, 30-70%, 40-80%, 50-90%, 60-100%, 10-60%, 20-70%, 30-80%, 40-90%, 50-100%, 10-70%, 20-80%, 30-90%, 40-100%, 10-80%, 20-90%, 30-100%, 10-90%, 20-100%, 25-50%, 50-75%, or 75-100% of the parent protein or mutant protein.

[0080] In one embodiment, the SRE is derived from a parent protein (e.g., CA2) or from a mutant protein and may have 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, or 100%, 5-10%, 10-15%, 15-20%, 20-25%, 25-30%, 30-35%, 35-40%, 40-45%, 45-50%, 50-55%, 55-60%, 60-65%, 65-70%, 70-75%, 75-80%, 80-85%, 85-90%, 90-95%, 95-100%, 10-20%, 20-30%, 30-40%, 40-50%, 50-60%, 60-

70%, 70-80%, 80-90%, 90-100%, 10-30%, 20-40%, 30-50%, 40-60%, 50-70%, 60-80%, 70-90%, 80-100%, 10-40%, 20-50%, 30-60%, 40-70%, 50-80%, 60-90%, 70-100%, 10-50%, 20-60%, 30-70%, 40-80%, 50-90%, 60-100%, 10-60%, 20-70%, 30-80%, 40-90%, 50-100%, 10-70%, 20-80%, 30-90%, 40-100%, 10-80%, 20-90%, 30-100%, 10-90%, 20-100%, 25-50%, 50-75%, or 75-100% identity to the parent protein or mutant protein.

[0081] Candidate destabilizing domain sequence identified from protein domains of parent proteins (as a template) may be mutated to generate libraries of mutants based on the template candidate domain sequence. Mutagenesis strategies used to generate DD libraries may include site-directed mutagenesis e.g. by using structure guided information; or random mutagenesis e.g. using error-prone PCR, or a combination of both. In some embodiments, destabilizing domains identified using random mutagenesis may be used to identify structural properties of the candidate DDs that may be required for destabilization, which may then be used to further generate libraries of mutations using site directed mutagenesis.

[0082] In some embodiments, DD mutant libraries may be screened for mutations with altered, preferably higher binding affinity to the ligand, as compared to the wild type protein. DD libraries may also be screened using two or more ligands and DD mutations that are stabilized by some ligands but not others may be preferentially selected. DD mutations that bind preferentially to the ligand compared to a naturally occurring protein may also be selected. Such methods may be used to optimize ligand selection and ligand binding affinity of the DD. Additionally, such approaches can be used to minimize deleterious effects caused by off-target ligand binding.

[0083] In some embodiments, suitable DDs may be identified by screening mutant libraries using barcodes. Such methods may be used to detect, identify and quantify individual mutant clones within the heterogeneous mutant library. Each DD mutant within the library may have distinct barcode sequences (with respect to each other). In other instances, the polynucleotides can also have different barcode sequences with respect to 2, 3, 4, 5, 6, 7, 8, 9, 10 or more nucleic acid bases. Each DD mutant within the library may also comprise a plurality of barcode sequences. When used in plurality may be used such that each barcode is unique to any other barcode. Alternatively, each barcode used may not be unique, but the combination of barcodes used may create a unique sequence that can be individually tracked. The barcode sequence may be placed upstream of the SRE, downstream of the SRE, or in some instances may be placed within the SRE. DD mutants may be identified by barcodes using sequencing approaches such as Sanger sequencing, and next generation sequencing, but also by polymerase chain reaction and quantitative polymerase chain reaction. In some embodiments, polymerase chain reaction primers that amplify a different size product for each barcode may be used to identify each barcode on an agarose gel. In other instances, each barcode may have a unique quantitative polymerase chain reaction probe sequence that enables targeted amplification of each barcode.

[0084] In one embodiment, the effector modules and/or SREs of the present disclosure may include at least one destabilizing domain (DD). The effector modules and/or SRE may include 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more than 10 DDs. When there are more than one DDs, each of the DDs may be derived from the same parent protein, from different parent proteins, may be a fusion of two different parent proteins, or may be artificial.

[0085] In one embodiment, the effector modules and/or SREs of the present disclosure may include 2 DDs. In one embodiment, the effector modules and/or SREs of the present disclosure may include 3 DDs. In one embodiment, the effector modules and/or SREs of the present disclosure may include 4 DDs. In one embodiment, the effector modules and/or SREs of the present disclosure may include 5 DDs. In one embodiment, the effector modules and/or SREs of the present disclosure may include 6 DDs. In one embodiment, the effector modules and/or SREs of the present disclosure may include 7 DDs. In one embodiment, the effector modules and/or SREs of the present disclosure may include 8 DDs. In one embodiment, the effector modules and/or SREs of the present disclosure may include 9 DDs. In one embodiment, the effector modules and/or SREs of the present disclosure may include 10 DDs. The DDs may be derived from any parent protein known in the art and/or described herein. In some embodiments the DDs are derived from the same parent protein. In some embodiments the DDs are derived from different regions of the same parent protein. In some embodiments, the DDs are derived from different parent proteins.

CA2 Destabilizing Domains

[0086] In some embodiments, the DDs of the present disclosure may be derived from human carbonic anhydrase 2 CA2, which is a member of the Carbonic anhydrases (CAs, EC 4.2.1.1) a superfamily of metalloenzymes present in all life kingdoms. CAs equilibrate the reaction between three chemical species: CO₂, bicarbonate, and protons. CAs have convergently evolved, with seven genetically distinct CA families that evolved independently in Bacteria, Archaea, and Eukarya, the α -, β -, γ -, δ -, ζ -, η -, and θ -CAs. In some embodiments, the DDs described herein may be derived from at least one parent protein selected from, but not limited to Carbonic Anhydrase 2 (CA2), Carbonic Anhydrase 1 (CA1), Carbonic Anhydrase 3 (CA3), Carbonic Anhydrase 4 (CA4), Carbonic Anhydrase 5A (CA5A), Carbonic Anhydrase 5B (CA5B), Carbonic Anhydrase 6 (CA6), Carbonic Anhydrase 7 (CA7), Carbonic Anhydrase 8 (CA8), Carbonic Anhydrase 9 (CA9), Carbonic Anhydrase 10 (CA10), Carbonic Anhydrase 11 (CA11), Carbonic Anhydrase 12 (CA12), Carbonic Anhydrase 13 (CA13), and Carbonic Anhydrase 14 (CA14).

[0087] In one embodiment, the DDs may be derived from cytosolic CAs such as but not limited to Carbonic Anhydrase 2 (CA2), Carbonic Anhydrase 1 (CA1), Carbonic Anhydrase 3 (CA3), Carbonic Anhydrase 7 (CA7), and Carbonic Anhydrase 13 (CA13). In one embodiment, the DDs may be derived from mitochondrial CAs such as but not limited to Carbonic Anhydrase 5A (CA5A), and Carbonic Anhydrase 5B (CA5B). In one embodiment, the DDs may be

derived from secreted CAs such as but not limited to Carbonic Anhydrase 6 (CA6). In one embodiment, the DDs may be derived from membrane associated CAs such as but not limited to Carbonic Anhydrase 4 (CA4), Carbonic Anhydrase 9 (CA9), Carbonic Anhydrase 12 (CA12), and Carbonic Anhydrase 14 (CA14). In one embodiment, the DD is derived from CA2. In another aspect, the DD may be derived from CA9.

[0088] In some embodiments, the DDs of the present disclosure may be derived from CA2 (SEQ ID NO. 5810; Uniprot ID: P00918) which may be stabilized by ligands such as small molecule inhibitors of CA2. As used herein the term “CA2 WT”, refers to the human wildtype CA2 protein sequence, which is defined as SEQ ID NO. 5810, with the GenBank Access NO. P00918, having the amino acid sequence:

MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDHTAKYDPSLKPLSVSYDQATSLRILNNGHAFNVEFDDSDKAVLKGGPLDGTYRLIQFHFHWGSLDGQGEHTVDDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAKPGKQVVDVLDSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEPEELMVDNWRPAQPLKNRQIKASFK.

[0089] In some embodiments, DDs of the present disclosure may be identified by utilizing a cocktail of CA2 inhibitors. In other instances, the suitable DDs may be identified by screening first with one CA2 inhibitor and subsequently screening with a second CA2 inhibitor.

[0090] The amino acid sequences of the destabilizing domains encompassed in the disclosure have at least about 40%, 50 or 60% identity, further at least about 70% identity, preferably at least about 75% or 80% identity, more preferably at least about 85%, 86%, 87%, 88%, 89% or 90% identity, and further preferably at least about 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identity to the amino acid sequence set forth therein. Percent identity may be determined, for example, by comparing sequence information using the advanced BLAST computer program, including version Magic-BLAST 1.2.0, available from the National Institutes of Health. The BLAST program is based on the alignment method discussed in Karl and Altschul (1990) Proc. Natl. Acad. Sci USA, 87:2264-68 (the contents of which are incorporated by reference in their entirety).

[0091] In some embodiments, DDs derived from CA2 may comprise amino acids 2-260 of the parent CA2 sequence. This is referred to herein as an M1del mutation. In one embodiment, DDs derived from CA2 may comprise amino acids 2-237 of the parent CA2 sequence.

[0092] Provided herein in Table 1, Table 2, Table 3, Table 4 and Table 6 are CA2 mutants identified by mutagenesis such as random mutagenesis screening, using a combination of nucleotide analog mutagenesis and error-prone PCR, to generate libraries of mutants; or saturation mutagenesis. CA2 destabilizing mutants may also be identified

by structure guided mutagenesis and are provided in Table 1. The position of the mutated amino acids listed in Table 1, Table 2, Table 3, Table 4, Table 5, and Table 6 are relative to the full length CA2 of SEQ ID NO. 5810.

Table 1: CA2 DDs

Library ID	Description	AA SEQUENCE	AA SEQ ID NO.	NA SEQ ID NO.
-	CA2 (aa 2-260 of WT)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSDQKAVLKGGPLDGTYRLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS PGLQKVVDVLDVSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLEC VTWIVLKEPISVSSEQLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKA SFK	5651	5652
-	CA2 (aa 2-260 of WT, W208S)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSDQKAVLKGGPLDGTYRLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS PGLQKVVDVLDVSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLEC VTSIVLKEPISVSSEQLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKA SFK	5653	5654
LibC000103	CA2 (aa 2-260 of WT, Y51N)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSND QATSLRILNNGHAFNVEFDDSDQKAVLKGGPLDGTYRLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS PGLQKVVDVLDVSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLEC VTWIVLKEPISVSSEQLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKA SFK	5655	5656
LibC000101	CA2 (aa 2-260 of WT, S56N)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATNLRILNNGHAFNVEFDDSDQKAVLKGGPLDGTYRLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS PGLQKVVDVLDVSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLEC VTWIVLKEPISVSSEQLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKA SFK	5657	5658
LibC000098	CA2 (aa 2-260 of WT, Y51T)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSTD QATSLRILNNGHAFNVEFDDSDQKAVLKGGPLDGTYRLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS PGLQKVVDVLDVSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLEC VTWIVLKEPISVSSEQLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKA SFK	5659	5660
LibC000097				5661
LibC000095	CA2 (aa 2-260 of WT, D72F, V241F, P249L)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSDQKAVLKGGPLDGTYRLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS PGLQKVVDVLDVSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLEC VTWIVLKEPISVSSEQLKFRKLNFNNGEGEPEELMFDNWRPAQPLKNRQIKA SFK	5662	5663
LibC000084	CA2 (aa 2-260 of WT, D71F, N231F)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSDQKAVLKGGPLDGTYRLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS PGLQKVVDVLDVSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLEC VTWIVLKEPISVSSEQLKFRKLNFFGEGEPEELMVDNWRPAQPLKNRQIKA SFK	5664	5665
LibC000079			5666	5667

LibC000078	CA2 (aa 2-260 of WT, S56F)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATFLRLNNGHAFNVEFDDSDQKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS PGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLEC VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKA SFK		5668
LibC000073	CA2 (aa 2-260 of WT, D71L, T87N, L250R)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRLNNGHAFNVEFLDSQDKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS PGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLEC VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPRKNRQIKA SFK	5669	5670
LibC000090	CA2 (aa 2-260 of WT, L183S)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRLNNGHAFNVEFDDSDQKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS PGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLEC VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKA SFK	5671	5672
LibC000076	CA2 (aa 2-260 of WT, A77I, P249F)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRLNNGHAFNVEFDDSDQKIVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS PGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLEC VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQFLKNRQIKA SFK	5673	5674
LibC000099	CA2 (aa 2-260 of WT, D71K, P249H)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRLNNGHAFNVEFDDSDQKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS PGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLEC VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQHLKNRQIK ASFK	5675	5676
LibC000081	CA2 (aa 2-260 of WT, D72F, P249H)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRLNNGHAFNVEFDDSDQKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS PGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLEC VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQFLKNRQIKA SFK	5677	5678
LibC000065				5679
LibC000082	CA2 (aa 2-260 of WT, Q53N, N61Y)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD NATSLRLYNNGHAFNVEFDDSDQKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS PGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLEC VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKA SFK	5680	5681
-	CA2 (aa 2-260 of WT, E106D, C205S)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRLNNGHAFNVEFDDSDQKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSDHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS KPGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLE SVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIK ASFK	5682	5683
-			5684	5685

	CA2 (aa 2-260 of WT, C205S, W208S)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSDQKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLES VTSIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKA SFK		
-	CA2 (aa 2-260 of WT, C205S)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSDQKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLES VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKA SFK	5686	5687
LibC000066	CA2 (aa 2-260 of WT, Y193I)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSDQKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLEC V TWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKAS FK	5688	5689
LibC000069				5690
LibC000056	CA2 (aa 2-260 of WT, S73N, R89Y)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDNQDKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLEC VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKA SFK	5691	5692
LibC000057	CA2 (aa 2-260 of WT, D71K, T192F)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFKDSQDKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWFYPGSLTTPPLEC VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKA SFK	5693	5694
LibC000061	CA2 (aa 2-260 of WT, E238*)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSDQKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLEC VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPE*LMVDNWRPAQPLKNRQIKA SFK	5695; 5811	5696
LibC000061	CA2 (aa 2-260 of WT, G144D)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSDQKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLDIFLKVGSAK PGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLEC VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKA SFK	5697	5698
LibC000092	CA2 (aa 2-260 of WT, Y193L, K260L)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSDQKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTLPGLSLTTPPLEC VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKA SFL	5699	5700
LibC000053	CA2 (aa 2-260 of WT, V206M)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSDQKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLEC	5701	5702

		MTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIK ASFK		
LibC000054	CA2 (aa 2-260 of WT, D71F, V241F, P249L)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSDQKAVLKGGPLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDISKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLECT VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMFDNWRPAQLLKNRQIKA SFK	5703	5704
LibC000055	CA2 (aa 2-260 of WT, Y193F)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSDQKAVLKGGPLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDISKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLECT VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKA SFK	5705	5706
LibC000058	CA2 (aa 2-260 of WT, L147F, Q248F)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSDQKAVLKGGPLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDISKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLECT VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAFPLKNRQIKA SFK	5707	5708
LibC000059	CA2 (aa 2-260 of WT, D52I, S258P)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYI QATSLRILNNGHAFNVEFDDSDQKAVLKGGPLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDISKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLECT VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKA PFK	5709	5710
LibC000060	CA2 (aa 2-260 of WT, D72S, T192N)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSDQKAVLKGGPLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDISKTKGKSADFTNFDPRGLLPESLDYWNYPGSLTTPPLECT VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKA SFK	5711	5712
LibC000062	CA2 (aa 2-260 of WT, D179E, T192I)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSDQKAVLKGGPLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDISKTKGKSADFTNFEPRGLLPESLDYWIYPGSLTTPPLECT VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKAS FK	5713	5714
LibC000063	CA2 (aa 2-260 of WT, Y193L)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSDQKAVLKGGPLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDISKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLECT VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKA SFK	5715	5716
LibC000064	CA2 (aa 2-260 of WT, S56N, Q103K)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATNLRLNNGHAFNVEFDDSDQKAVLKGGPLDGTyrLIQFHFHWGSLDGK GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDISKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLECT	5717	5718

		VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKA SFK		
LibC000067	CA2 (aa 2-260 of WT, D71Y, Q248L)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFYDSQDKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDsIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLEC VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPALPLKNRQIKA SFK	5719	5720
LibC000068	CA2 (aa 2-260 of WT, S73N, R89F)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDNQDKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDsIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLEC VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKA SFK	5721	5722
LibC000070	CA2 (aa 2-260 of WT, D71K, N231L, E235G, L239F)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFKDSQDKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDsIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLEC VTWIVLKEPISVSSEQVLKFRKLNFLGEGPEEFMVDNWRPAQPLKNRQIKA SFK	5723	5724
LibC000071	CA2 (aa 2-260 of WT, D71F)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDSQDKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDsIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLEC VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKA SFK	5725	5726
LibC000072	CA2 (aa 2-260 of WT, D72F, P249I)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDFSQDKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDsIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLEC VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQILKNRQIKA SFK	5727	5728
LibC000074	CA2 (aa 2-260 of WT, T192N)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSQDKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDsIKTKGKSADFTNFDPRGLLPESLDYWNYPGSLTTPPLEC VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKA SFK	5729	5730
LibC000075	CA2 (aa 2-260 of WT, D72X, V241X, P249X)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDXSQDKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDsIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLEC VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMXDNWRPAQXLKNRQIK ASFK	5731	5732
LibC000077	CA2 (aa 2-260 of WT, A54X, S56X, L57X, T192X)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QXTXXRILNNGHAFNVEFDDSQDKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDsIKTKGKSADFTNFDPRGLLPESLDYWXYPGSLTTPPLEC VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKA SFK	5733	5734
LibC000080	CA2 (aa 2-260 of WT,	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSQDKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK	5735	5736

	Y193V, K260F)	PGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTVPGSLTTPPLEC VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKA SFF		
LibC000085	CA2 (aa 2-260 of WT, G63D, M240L)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNDHAFNVEFDDSDQKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTVPGSLTTPPLEC VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELVDNWRPAQPLKNRQIKA SFK	5737	5738
LibC000086	CA2 (aa 2-260 of WT, V134F, L228F)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSDQKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTVPGSLTTPPLEC VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKA SFK	5739	5740
LibC000087	CA2 (aa 2-260 of WT, D71G, N231K)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFGSDQKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTVPGSLTTPPLEC VTWIVLKEPISVSSEQVLKFRKLNFKGEGEPEELMVDNWRPAQPLKNRQIKA SFK	5741	5742
LibC000088	CA2 (aa 2-260 of WT, S56F, D71S)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATFLRILNNGHAFNVEFSDSDQKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTVPGSLTTPPLEC VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKA SFK	5743	5744
LibC000089	CA2 (aa 2-260 of WT, D52L, G128R, Q248F)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYL QATSLRILNNGHAFNVEFDDSDQKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYRDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTVPGSLTTPPLEC VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAFPLKNRQIKA SFK	5745	5746
LibC000091	CA2 (aa 2-260 of WT, S73X, R89X)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDXQDKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTVPGSLTTPPLEC VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKA SFK	5747	5748
LibC000093	CA2 (aa 2-260 of WT, Y193X)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSDQKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTXPGSLTTPPLEC VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKA SFK	5749	5750
LibC000096	CA2 (aa 2-260 of WT, Y51X, D72X, V241X, P249X)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSXD QATSLRILNNGHAFNVEFDXSDQKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTVPGSLTTPPLEC	5751	5752

		VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMXDNWRPAQXLKNRQIK ASFK		
LibC000100	CA2 (aa 2-260 of WT, D72I, W97C)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSQDKAVLKGGLDGTyrLIQFHFHCGSLDGQG SEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLLEC VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKAS FK	5753	5754
LibC000102	CA2 (aa 2-260 of WT, D71K, T192F, N231F)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSQDKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLLEC VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKAS FK	5755	5756
-	CA2 (aa 2-260 of WT, 159N, G102R)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSQDKAVLKGGLDGTyrLIQFHFHWGSLDR QGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSA KPGQLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLLE C VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIK ASFK	5757	5758
LibC000210				5813
LibC000184				5814
LibC000187				5815
-	CA2 (aa 2-260 of WT, L156H)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSQDKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGHQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLLEC VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKAS FK	5759	5760
LibC000208				5816
-	CA2 (L156H)	MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSQDKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSA KPGHQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLLE C VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIK ASFK	5761	5762
LibC000061 truncated	CA2 (aa 2-237 of WT)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSQDKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAK PGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLLEC VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPE	5695	5812

[0093] Additional CA2 destabilizing domains are provided in Table 2.

Table 2: CA2 DDs

Library ID	Description	AA SEQUENCE	AA SEQ ID NO.	NA SEQ ID NO.
LibC000229	CA2 (aa 2-260 of WT, H36Q, S43T, Y51F, N67D, G131W, R226H)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPTLKPLSVSFD QATSLRILNNGHAFNVEFDDSQDKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSA KPGQLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLLE C VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIK ASFK	5817	5818
LibC000228			5819	5820

	CA2 (aa 2-260 of WT, F70I, F146V)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEIDDSQDKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS KPGKQKVVVDVLSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLLE CVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIK ASFK		
LibC000226	CA2 (aa 2-260 of WT, R27L, T87I, H122Y, N252D)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSDQDKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVYWNKYGDFGKAVQQPDGLAVLGIFLKVGS KPGKQKVVVDVLSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLLE CVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKDRQIK ASFK	5821	5822
LibC000225	CA2 (aa 2-260 of WT, K45N, V68L, H119Y, K169R, D179E)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLNPLSVSYD QATSLRILNNGHAFNLEFDDSDQDKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELYL VHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS KPGKQKVVVDVLSIKTRGKSADFTNFEPRGLLPESLDYWTYPGSLTTPPLLE CVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIK ASFK	5823	5824
LibC000224	CA2 (aa 2-260 of WT, H15L, A54V, K111E, E220K, F225I)	SHHWGYGKHNGPELWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QVTSRILNNGHAFNVEFDDSDQDKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDEKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS KPGKQKVVVDVLSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLLEC VTWIVLKEPISVSSKQVLKIRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIK SFK	5825	5826
LibC000221	CA2 (aa 2-260 of WT, P13S, P83A, D101G, K111N, F230I)	SHHWGYGKHNGSEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSDQDKAVLKGGLDGTyrLIQFHFHWGSLGGQ GSEHTVDNKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS KPGKQKVVVDVLSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLLE CVTWIVLKEPISVSSEQVLKFRKLNNGEGEPEELMVDNWRPAQPLKNRQIK ASFK	5827	5828
LibC000220	CA2 (aa 2-260 of WT, L47R)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPRSVSYD QATSLRILNNGHAFNVEFDDSDQDKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS KPGKQKVVVDVLSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLLE CVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIK ASFK	5829	5830
LibC000219	CA2 (aa 2-260 of WT, G63D, W123R, E220K)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNDHAFNVEFDDSDQDKAVLKGGLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHRNTKYGDFGKAVQQPDGLAVLGIFLKVGS KPGKQKVVVDVLSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLLEC VTWIVLKEPISVSSKQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIK ASFK	5831	5832
LibC000217	CA2 (aa 2-260 of WT, N11D, E69K, G86D, V109M, K113I, T125I, D138G, G155S)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVKFDSDQDKAVLKGGLDDTYrLIQFHFHWGSLDGQ GSEHTMDKKIYAAELHLVHWNIKYGDFGKAVQQPGGLAVLGIFLKVGS KPSLQKVVVDVLSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLLEC VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIK SFK	5833	5834
LibC000214			5835	5836

	CA2 (aa 2-260 of WT, I59N, G102R, A173T)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRNLNNGHAFNVEFDDSDQKAVLKGGPLDGTyrLIQFHFHWGSLDR QGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS AKPGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLL ECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQI KASFK		
LibC000213	CA2 (aa 2-260 of WT, L79F, P180S)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRNLNNGHAFNVEFDDSDQKAVLKGGPLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS AKPGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPP LLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQI KASFK	5837	5838
LibC000212	CA2 (aa 2-260 of WT, S73F)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRNLNNGHAFNVEFDDSDQKAVLKGGPLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS AKPGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPP LLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQI KASFK	5839	5840
LibC000211	CA2 (aa 2-260 of WT, G12R)	SHHWGYGKHNRPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRNLNNGHAFNVEFDDSDQKAVLKGGPLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS AKPGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPP LLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQI KASFK	5841	5842
LibC000209	CA2 (aa 2-260 of WT, A77P, G102R, D138N)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRNLNNGHAFNVEFDDSDQKAVLKGGPLDGTyrLIQFHFHWGSLDRQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS AKPGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPP LLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQI KASFK	5843	5844
LibC000183	CA2 (aa 2-260 of WT, F20L, K45N, G63D, E69V, N231I)	SHHWGYGKHNGPEHWHKDLPIAKGERQSPVDIDTHTAKYDPSLNPLSVSYD QATSLRILNNDHAFNVFDDSDQKAVLKGGPLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS AKPGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPP LLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQI KASFK	5845	5846
LibC000207	CA2 (aa 2-260 of WT, T199N, L202P, L228F)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSDQKAVLKGGPLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS AKPGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTNP PPLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQI KASFK	5847	5848
LibC000206	CA2 (aa 2-260 of WT, K9N, H122Y, T168K)	SHHWGYGNHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSDQKAVLKGGPLDGTyrLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVYWNNTKYGDFGKAVQQPDGLAVLGIFLKVGS AKPGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPP LLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQI KASFK	5849	5850
LibC000205	CA2 (aa 2-260 of WT, Q53H, L90V, Q92H, G131E)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD HATSLRILNNGHAFNVEFDDSDQKAVLKGGPLDGTyrVIHFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS AKPGLQKVVDVLDISIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPP LLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQI KASFK	5851	5852

		VTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKASFK		
LibC000204	CA2 (aa 2-260 of WT, L44M, L47V, N62K, E69D)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSMKPVSYSYDQATSLRILNKGHAFNVDFDSDQDKAVLKGGLDGTYRLIQFHFHWGSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS AKPGLQKVVDVLDSEIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKASFK	5853	5854
LibC000203	CA2 (aa 2-260 of WT, F20L, K45N, G104R, A116V)	SHHWGYGKHNGPEHWHKDLPIAKGERQSPVDIDTHTAKYDPSLNPLSVSYDQATSLRILNNGHAFNVEFDSDQDKAVLKGGLDGTYRLIQFHFHWGSLDGQRSEHTVDKKKYAVELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS AKPGLQKVVDVLDSEIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKASFK	5855	5856
LibC000202	CA2 (aa 2-260 of WT, D75V, K169N, F259L)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYDQATSLRILNNGHAFNVEFDSDQKAVLKGGLDGTYRLIQFHFHWGSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS AKPGLQKVVDVLDSEIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKASLK	5857	5858
LibC000182	CA2 (aa 2-260 of WT, T207S, V222A, N231D)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYDQATSLRILNNGHAFNVEFDSDQDKAVLKGGLDGTYRLIQFHFHWGSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS AKPGLQKVVDVLDSEIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLLECVSWIVLKEPISVSSEQALKFRKLNFDGEGEPEELMVDNWRPAQPLKNRQIKASFK	5859	5860
LibC000201	CA2 (aa 2-260 of WT, I59F, V206M, G232R)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYDQATSLRILNNGHAFNVEFDSDQDKAVLKGGLDGTYRLIQFHFHWGSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS AKPGLQKVVDVLDSEIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLLECMTWIVLKEPISVSSEQVLKFRKLNFNREGEPEELMVDNWRPAQPLKNRQIKASFK	5861	5862
LibC000199	CA2 (aa 2-260 of WT, P13A, A133T)	SHHWGYGKHNGAEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYDQATSLRILNNGHAFNVEFDSDQDKAVLKGGLDGTYRLIQFHFHWGSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKTQQPDGLAVLGIFLKVGS AKPGLQKVVDVLDSEIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIKASFK	5863	5864
LibC000198	CA2 (aa 2-260 of WT, N61R, K80M, K212N, N231T, L250P)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYDQATSLRILNNGHAFNVEFDSDQDKAVLMGGPLDGTYRLIQFHFHWGSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS AKPGLQKVVDVLDSEIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLLECVTWIVLNEPISVSSEQVLKFRKLNFTGEGEPEELMVDNWRPAQPPKNRQIKASFK	5865	5866
LibC000196	CA2 (aa 2-260 of WT, G63D, E69V, N231I)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYDQATSLRILNNDHAFNVDFDSDQDKAVLKGGLDGTYRLIQFHFHWGSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS AKPGLQKVVDVLDSEIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLLECVTWIVLKEPISVSSEQVLKFRKLNFIGEGEPEELMVDNWRPAQPLKNRQIKASFK	5867	5868
LibC000181	CA2 (aa 2-260 of WT, I59N, R89I)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYDQATSLRILNNGHAFNVEFDSDQDKAVLKGGLDGTYILIQFHFHWGSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS	5869	5870

		KPGLQKVVDVLSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLLE CVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIK ASFK		
LibC000194	CA2 (aa 2-260 of WT, A65N, G86D, G131R, G155D, K158N, V162A, G170D, P236L)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHNFNVEFDDSDQKAVLKGGPLDDTYRLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFRKAVQQPDGLAVLGIFLKVGS KPDQLQNVVDALDSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLLE CVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIK ASFK	5871	5872
LibC000192	CA2 (aa 2-260 of WT, G12R, H15Y, D19V)	SHHWGYGKHNRPEYWHKVFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSDQKAVLKGGPLDGTYRLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS KPGLQKVVDVLSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLLE CVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIK ASFK	5873	5874
LibC000193	CA2 (aa 2-260 of WT, L156H, S172C, F178Y, E186D)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSDQKAVLKGGPLDGTYRLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS KPGHQKVVDVLSIKTKGKCADFTNYDPRGLLPESLDYWTYPGSLTTPPLLE ECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQI KASFK	5875	5876
LibC000189	CA2 (aa 2-260 of WT, A65V, F95Y, E106G, H107Q, I145M, F175I)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHVFNVEFDDSDQKAVLKGGPLDGTYRLIQFHFHWGSLDGQ GSGQTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGMFLKVGS AKPGLQKVVDVLSIKTKGKSADITNFDPRGLLPESLDYWTYPGSLTTPPLLE ECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQI KASFK	5877	5878
LibC000186	CA2 (aa 2-260 of WT, L197P)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNGHAFNVEFDDSDQKAVLKGGPLDGTYRLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS KPGLQKVVDVLSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSPTTPPLLE CVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWRPAQPLKNRQIK ASFK	5879	5880
-	CA2 (aa 2-260 of WT, G63D, E69V, N231I)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYD QATSLRILNNDHAFNVFDDSDQKAVLKGGPLDGTYRLIQFHFHWGSLDGQ GSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS KPGLQKVVDVLSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLLE CVTWIVLKEPISVSSEQVLKFRKLNFIGEGEPEELMVDNWRPAQPLKNRQIK ASFK	5881	5882

[0094] In some embodiments, the CA2 DDs described herein may include any of the sequences provided in Table 3. In Table 3 “*” represents the translation of stop codon. When the amino acid sequence in Table 3 contains one or more stop codons, the “AA SEQ ID NO.” column provides the SEQ ID NO. of the individual components preceding and following the stop codon in the order in which they occur in the amino acid sequence.

Table 3: CA2 DDs

Library ID	Description	AA SEQUENCE	AA SEQ ID NO.	NA SEQ ID NO.
LibC000223	CA2 (aa 2-260 of WT, G12E, A38V, A65V, G98V, S99H, D101M, G102D, Q103K, G104V, S105Q, E106S, H107I, T108L, V109W, D110I, K112R, K113N, Y114M, A115L, A116Q, E117N, L118F, H119T, L120W, V121F, H122T, W123G, N124T, T125P, K126N, Y127M, D129I, F130L, K132E, A133L, V134C, Q135S, Q136N, P137L, D138M, G139D, L140W, A141P, V142F, L143*, G144V, I145F, L156Q, V162A)	SHHWGYGKHNEPEHWHKDFPIAKGERQSPVDID THTVKYDPSLKPLSVSYDQATSLRILNNGHVFNV EFDDSQDKAVLKGGLDGTyrLIQFHFHWVHL MDKVQSILWIKRNLQNFTWFTGTPNMGILGEL CSNLMDWPF*VFFLKVGSAKPGQKVVDAALDSI KTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTP PLLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEG EPEELMVDNWRPAQPLKNRQIKASFK	5883; 5884	5885
LibC000185	CA2 (aa 2-260 of WT, F20L, K45N, G104R, A116V, A173G, W191*)	SHHWGYGKHNGPEHWHKDLPIAKGERQSPVDI DTHTAKYDPSLNPLSVSYDQATSLRILNNGHAFN VEFDDSQDKAVLKGGLDGTyrLIQFHFHWGSL DGQRSEHTVDKKKYAVELHLVHWNTKYGDFG KAVQQPDGLAVLGIFLKVGSAKPGKVVVDVL DSIKTKGKSGDFTNFDPRGLLPESLDY*TYPGSLT TPPILLECVTWIVLKEPISVSSEQVLKFRKLNFNNGE GEPEELMVDNWRPAQPLKNRQIKASFK	5886; 5887	5888
LibC000215	CA2 (aa 2-260 of WT, H17D, P30S, G81V, K132R, S151I, A152D, A173G, W191*)	SHHWGYGKHNGPEHWDKDFPIAKGERQSSVDI DTHTAKYDPSLKPLSVSYDQATSLRILNNGHAFN VEFDDSQDKAVLKVGPLDGTyrLIQFHFHWGSL DGQGEHTVDKKKYAAELHLVHWNTKYGDFG RAVQQPDGLAVLGIFLKVGIDKPGKVVVDVLD SIKTKGKSGDFTNFDPRGLLPESLDY*TYPGSLTT PPLLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEG EPEELMVDNWRPAQPLKNRQIKASFK	5889; 5887	5890
LibC000191	CA2 (aa 2-260 of WT, H17D, P30S, G81V, K132R, S151I, A152D, A173G, W191*)	SHHWGYGKHNGPEHWDKDFPIAKGERQSSVDI DTHTAKYDPSLKPLSVSYDQATSLRILNNGHAFN VEFDDSQDKAVLKVGPLDGTyrLIQFHFHWGSL DGQGEHTVDKKKYAAELHLVHWNTKYGDFG RAVQQPDGLAVLGIFLKVGIDKPGKVVVDVLD SIKTKGKSGDFTNFDPRGLLPESLDY*TYPGSLTT PPLLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEG EPEELMVDNWRPAQPLKNRQIKASFK	5889; 5887	5891
LibC000200	CA2 (aa 2-260 of WT, I59N, L90*, G102R)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDI DTHTAKYDPSLKPLSVSYDQATSLRNLNNGHAF NVEFDDSQDKAVLKGGLDGTyr*IQFHFHWGS LDRQGEHTVDKKKYAAELHLVHWNTKYGDFG KAVQQPDGLAVLGIFLKVGSAKPGKVVVDVL DSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSL TTPPILLECVTWIVLKEPISVSSEQVLKFRKLNFNNG EGEPEELMVDNWRPAQPLKNRQIKASFK	5892; 5893	5894
LibC000197		SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDI DIHTAKYDPSLKPLSVSYDQATSLRILNNGHAFN	5895; 5896	5897

	CA2 (aa 2-260 of WT, T35I, Y114H, P154L, D161V, P200A, Q221R, F225L, E233D, W244*)	VEFDDSQDKAVLKGGPLDGTyrLIQFHFHWGSL DGQGEHTVDKKKHAAELHLVHWNTKYGDFG KAVQQPDGLAVLGIFLKVGS AKLGLQKV VVVL DSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSL TTAPLLEC VTWIVLKEPISVSSEVLKRLKLNFN GDGEPEELMVDN* RPAQPLKNRQIKASFK		
LibC000195	CA2 (aa 2-260 of WT, S105L, L140V, G155C, Y190*)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDI DTHTAKYDPSLKPLSVSYDQATSLRILNNGHAFN VEFDDSQDKAVLKGGPLDGTyrLIQFHFHWGSL DGQGLEHTVDKKKYAAELHLVHWNTKYGDFG KAVQQPDGVAVLGIFLKVGS AKPCLQKVVDVL DSIKTKGKSADFTNFDPRGLLPESLD*WTYPGSL TTPPLLEC VTWIVLKEPISVSSEQVLKFRKLNFN EGEPEELMVDNWRPAQPLKNRQIKASFK	5898, 5899	5900
LibC000188	CA2 (aa 2-260 of WT, T35I, Y114H, P154L, D161V, P200A, F225L, E233D, W244*)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDI DIHTAKYDPSLKPLSVSYDQATSLRILNNGHAFN VEFDDSQDKAVLKGGPLDGTyrLIQFHFHWGSL DGQGEHTVDKKKHAAELHLVHWNTKYGDFG KAVQQPDGLAVLGIFLKVGS AKLGLQKV VVVL DSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSL TTAPLLEC VTWIVLKEPISVSSEQVLKRLKLNFN GDGEPEELMVDN* RPAQPLKNRQIKASFK	5901, 5896	5902
LibC000180	CA2 (aa 2-260 of WT, K167*, R181H, E213*, V217L, L228H)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDI DTHTAKYDPSLKPLSVSYDQATSLRILNNGHAFN VEFDDSQDKAVLKGGPLDGTyrLIQFHFHWGSL DGQGEHTVDKKKYAAELHLVHWNTKYGDFG KAVQQPDGLAVLGIFLKVGS AKPGLQKVVDVL DSI*TKGKSADFTNFDPHGLLPESLDYWTYPGSL TTPPLLEC VTWIVLK*PISLSSEQVLKFRKLNFN EGEPEELMVDNWRPAQPLKNRQIKASFK	5903, 5904, 5905	5906
LibC000190	CA2 (aa 2-259 of WT, K167*, R181H, P194Q, G195A, S196H, L197*, T198P, T199P, P200L, P201L, L202F, L203W, E204N, C205V, V206*, T207P, W208G, I209L, V210C, L211S, K212R, E213N, I215S, S216A, V217S, S218A, S219A, E220S, Q221R, V222C, L223*, K224N, F225S, R226V, K227N, N229T, F230S, N231M, E233R, G234L, E235N, E237K, E238N, L239*, M240W, V241W, D242T, N243T, W244G, R245A, P246Q, A247L, Q248S, P249H, L250*, K251R, N252T, R253G, Q254K, I255S, A257L, S258P, F259S)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDI DTHTAKYDPSLKPLSVSYDQATSLRILNNGHAFN VEFDDSQDKAVLKGGPLDGTyrLIQFHFHWGSL DGQGEHTVDKKKYAAELHLVHWNTKYGDFG KAVQQPDGLAVLGIFLKVGS AKPGLQKVVDVL DSI*TKGKSADFTNFDPHGLLPESLDYWTYQAH* PPLLFWNV*PGLCSRNPASASAASRC*NSVNL TSM GRLNPKN*WWTGAQLSH*RTGSKLPS	5903, 5907, 5908, 5909, 5910, 5911, 5912	5913
LibC000227	CA2 (aa 2-259 of WT, P13H, E117*, G150S, L184F, P185L, E186N, S187P, L188W, D189I, Y190T, W191G, T192P, Y193T, P194Q, G195A, S196H, L197*, T198P, T199P, P200L, P201L, L202F, L203W, E204N, C205V, V206*, T207P, W208G, I209L, V210C, L211*, K212R, E213N, I215S, S216A, V217S, S218A, S219A, E220S, Q221R, V222C, L223*, K224N,	SHHWGYGKHNGHEHWHKDFPIAKGERQSPVDI DTHTAKYDPSLKPLSVSYDQATSLRILNNGHAFN VEFDDSQDKAVLKGGPLDGTyrLIQFHFHWGSL DGQGEHTVDKKKYAA*LHLVHWNTKYGDFG KAVQQPDGLAVLGIFLKVSS AKPGLQKVVDVLD SIKTKGKSADFTNFDPRGLFLNPWITGPTQAH*PP LLFWNV*PGLC*RNPSASAASRC*NSVNL TSMGR VNPKN*WWTGAQLSH*RTGSKLPS	5914, 5915, 5908, 5916, 5917, 5918, 5911, 5912	5919

	F225S, R226V, K227N, N229T, F230S, N231M, E233R, G234V, E235N, E237K, E238N, L239*, M240W, V241W, D242T, N243T, W244G, R245A, P246Q, A247L, Q248S, P249H, L250*, K251R, N252T, R253G, Q254K, I255S, A257L, S258P, F259S)			
LibC000218	CA2 (aa 2-260 of WT, A133T, L147F, K148E, V149G, G150W, S151Q, A152R, K153*, P154T, L156P, Q157S, K158E, V159S, V160C, D161*, V162C, L163A, D164G, S165F, I166H, K167*, T168N, K171Q, S172E, A173C, D174*, F175L, T176H, N177*, F178L, D179R, P180S, R181S, G182W, L183P, L184P, P185S, E186*, S187I, L188P, D189G, Y190L, W191L, T192D, Y193L, G195R, S196L, L197T, T198D, T199H, P201S, L202S, L203S, E204G, C205M, V206C, T207D, W208L, I209D, V210C, L211A, K212Q, E213G, P214T, I215H, S216Q, V217R, S218Q, S219Q, E220R, Q221A, V222G, L223V, K224E, F225I, R226P, K227*, L228T, N229*, F230L, N231Q, G232W, E233G, E235*, P236T, E237R, E238R, L239T, M240D, V241G, D242G, N243Q, W244L, R245A, A247S, Q248S, P249A, L250T, K251E, N252E, R253Q, Q254A, I255N, K256Q, A257S, S258F, F259L, K260Q)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDI DTHTAKYDPSLKPLSVSYDQATSLRILNNGHAFN VEFDDSQDKAVLKGGPLDGTyrLIQFHFHWGSL DGQSEHTVDKKKYAAELHLVHWNtKYGDFG KTVQQPDGLAVLGIFFEgWQR*TGpSESC*CAGF H*NKGQEC*LH*LRSSWPPS*IPGLLDLPRLTDHP SSSGMCDLDCAQGTQRQRAGVEIP*T*LQWG G*TRRTDGGQLAPSSATEEQANQSFLQ	5920,5 921; 5922, 5923; LH; 5924, 5925, T; 5926, 5927	5928
LibC000216	CA2 (aa 2-260 of WT, F20L, K45N, G104R, A116V, L147F, K148E, V149G, G150W, S151Q, A152R, K153*, P154T, L156P, Q157S, K158E, V159S, V160C, D161*, V162C, L163A, D164G, S165F, I166H, K167*, T168N, K171Q, S172E, A173C, D174*, F175L, T176H, N177*, F178L, D179R, P180S, R181S, G182W, L183P, L184P, P185S, E186*, S187I, L188P, D189G, Y190L, W191L, T192D, Y193L, G195R, S196L, L197T, T198D, T199H, P201S, L202S, L203S, E204G, C205M, V206C, T207D, W208L, I209D, V210C, L211A, K212Q, E213G, P214T, I215H, S216Q, V217R, S218Q, S219Q, E220R, Q221A, V222G, L223I, K224E, F225I, R226P, K227*, L228T, N229*, F230L, N231Q, G232W, E233G, E235*, P236T, E237R, E238R, L239T, M240D, V241G, D242G, N243Q, W244L, R245A, A247S, Q248S, P249A, L250T, K251E, N252E, R253Q, Q254A, I255N, K256Q, A257S, S258F, F259L, K260Q)	SHHWGYGKHNGPEHWHKDLPIAKGERQSPVDI DTHTAKYDPSLNPLSVSYDQATSLRILNNGHAFN VEFDDSQDKAVLKGGPLDGTyrLIQFHFHWGSL DGQRSEHTVDKKKYAVELHLVHWNtKYGDFG KAVQQPDGLAVLGIFFEgWQR*TGpSESC*CAGF H*NKGQEC*LH*LRSSWPPS*IPGLLDLPRLTDHP SSSGMCDLDCAQGTQRQRAGIEIP*T*LQWG G*TRRTDGGQLAPSSATEEQANQSFLQ	5929; 5921, 5922, 5923; LH; 5924, 5930, T; 5926, 5927	5931

LibC000222	CA2 (aa 2-259 of WT, N11K, G12D, P13L, E14N, H15T, W16G, H17I, K18R, D19T, F20S, I22L, A23P, K24R, G25E, E26S, R27A, Q28S, S29P, P30L, V31L, D32T, I33S, D34T, T35L, H36I, T37Q, A38P, K39S, Y40M, D41T, P42L, S43P, L44*, K45S, L47C, S48L, V49F, S50P, Y51M, D52I, Q53K, A54Q, T55L, S56P, L57*, R58G, I59S, L60S, N61T, N62M, G63V, H64M, A65L, F66S, N67T, V68W, E69S, F70L, D71M, D72T, S73L, Q74R, D75T, A77Q, V78C, L79S, K80R, G81E, G82D, L84W, D85M, G86A, T87L, Y88T, R89D, L90*, I91F, Q92S, H94T, H96T, W97G, G98V, S99H, D101M, G102D, Q103K, G104V, S105Q, E106S, H107I, T108L, V109W, D110I, K112R, K113N, Y114M, A115L, A116Q, E117N, L118F, H119T, L120W, V121F, H122T, W123G, N124T, T125P, K126N, Y127M, D129I, F130L, A133L, V134C, Q135S, Q136N, P137L, D138M, G139D, L140W, A141P, V142F, L143*, G144V, I145F, L147*, K148R, V149L, G150A, S151A, A152L, K153N, P154R, G155A, L156F, Q157R, V159L, V160L, D161M, V162C, L163W, D164I, S165P, I166L, T168Q, K169R, G170A, K171R, S172V, A173L, D174T, F175S, T176L, N177T, F178S, D179I, P180L, R181V, G182A, L183S, L184F, P185V, E186N, S187P, L188W, D189I, Y190T, W191G, T192P, Y193T, P194Q, G195A, S196H, L197*, T198P, T199P, P200L, P201L, L202F, L203W, E204N, C205V, V206*, T207P, W208G, I209L, V210C, L211S, K212W, E213N, I215S, S216A, V217S, S218A, S219A, E220S, Q221R, V222C, L223*, K224N, F225Y, R226V, K227N, N229T, F230S, N231M, E233R, G234V, E235N, E237K, E238N, L239*, M240R, V241W, D242T, N243T, W244G, R245A, P246L, A247L, Q248S, P249H, L250*, K251R, N252T, R253G, Q254K, I255S, A257L, S258P, F259S)	SHHWGYGKHKDLNTGIRTSPLPRESASPLLTSTLI QPSMTLP*SPCLFPMIKQLP*GSSTMVMLSTWSL MTLR TKQCSREDP WMALTD*FSFTFTGVHLM DK VQSILWIKRNMLQNFTWFTGTPNMGILGKLCSN LMDWPF*VFF*RLAALNRAFRKLLMCWIPLKQR ARVLTSLTSILVASFVNPWITGPTQAH*PPLLFWN V*PGLCSWNPSASAASRC*NYVNL TSMGRVNP K N*RWTTGALLSH*RTGKSKLPS	5932; 5933; 5934; 5935; VFF; 5936; 5908; 5937; 5938; 5939; 5912	5940
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[0095] Additional CA2 destabilizing domains are provided in Table 4. CA2 destabilizing mutants provided in Table 4 are identified as described above, such as by structure guided mutagenesis or by combining single mutants.

Table 4: CA2 DDs

Description	AA Sequence	AA SEQ ID NO.	NA SEQ ID NO.
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CA2 (M1del, E106D) - variant 1	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV SYDQATSLRILNNGHAFNVEFDDSDKAVLKGGLDGTyrLIQFHFW GSLDGQGSHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVL GIFLKVGSAKPGQLQKVVDVLDSIKTKGKSADFTNFDPRGLLPESLDYWT YPGSLTTPPILLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVD NWRPAQPLKNRQIKASFK	6073	6109
CA2 (M1del, I59N, G102R) - variant 2	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV SYDQATSLRNLNNGHAFNVEFDDSDKAVLKGGLDGTyrLIQFHFW GSLDRQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVL GIFLKVGSAKPGQLQKVVDVLDSIKTKGKSADFTNFDPRGLLPESLDYWT YPGSLTTPPILLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVD NWRPAQPLKNRQIKASFK	6074	6110
CA2 (M1del, L197P) - variant 1	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV SYDQATSLRILNNGHAFNVEFDDSDKAVLKGGLDGTyrLIQFHFW GSLDGQGSHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVL GIFLKVGSAKPGQLQKVVDVLDSIKTKGKSADFTNFDPRGLLPESLDYWT YPGSPTTPPILLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVD NWRPAQPLKNRQIKASFK	6075	6111
CA2 (M1del, L156H, S172C, F178Y, E186D) - variant 1	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV SYDQATSLRILNNGHAFNVEFDDSDKAVLKGGLDGTyrLIQFHFW GSLDGQGSHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVL GIFLKVGSAKPGHQKVVDVLDSIKTKGKCADFTNYDPRGLLPDSLDYW TYPGSLTTPPILLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMV DNWRPAQPLKNRQIKASFK	6076	6112
CA2 (M1del, L156H) - variant 2	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV SYDQATSLRILNNGHAFNVEFDDSDKAVLKGGLDGTyrLIQFHFW GSLDGQGSHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVL GIFLKVGSAKPGHQKVVDVLDSIKTKGKSADFTNFDPRGLLPESLDYWT YPGSLTTPPILLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVD NWRPAQPLKNRQIKASFK	6077	6113
CA2 (M1del, R27L, T87I, H122Y, N252D) - variant 1	SHHWGYGKHNGPEHWHKDFPIAKGELQSPVDIDTHTAKYDPSLKPLSV SYDQATSLRILNNGHAFNVEFDDSDKAVLKGGLDGIYrLIQFHFW GSLDGQGSHTVDKKKYAAELHLVYWNNTKYGDFGKAVQQPDGLAVL GIFLKVGSAKPGQLQKVVDVLDSIKTKGKSADFTNFDPRGLLPESLDYWT YPGSLTTPPILLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVD NWRPAQPLKDRQIKASFK	6078	6114
CA2 (I59N) - variant 1	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV SYDQATSLRNLNNGHAFNVEFDDSDKAVLKGGLDGTyrLIQFHFW GSLDGQGSHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVL GIFLKVGSAKPGQLQKVVDVLDSIKTKGKSADFTNFDPRGLLPESLDYWT YPGSLTTPPILLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVD NWRPAQPLKNRQIKASFK	6079	6115
CA2 (G63D) - variant 1	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV SYDQATSLRILNNDHAFNVEFDDSDKAVLKGGLDGTyrLIQFHFW	6080	6116

	GSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVL GIFLKVGSAKPGQLQKVVDVLDSIKTKGKSADFTNFDPRGLLPESLDYWT YPGSLTTPPILLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVD NWRPAQPLKNRQIKASFK		
CA2 (M1del, H122Y) - variant 1	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV SYDQATSLRILNNGHAFNVEFDDSDQKAVLKGGPLDGTyrLIQFHFHW GSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVL GIFLKVGSAKPGQLQKVVDVLDSIKTKGKSADFTNFDPRGLLPESLDYWT YPGSLTTPPILLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVD NWRPAQPLKNRQIKASFK	6081	6117
CA2 (M1del, G63D, M240L) - variant 1	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV SYDQATSLRILNNDHAFNVEFDDSDQKAVLKGGPLDGTyrLIQFHFHW GSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVL GIFLKVGSAKPGQLQKVVDVLDSIKTKGKSADFTNFDPRGLLPESLDYWT YPGSLTTPPILLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELLVD NWRPAQPLKNRQIKASFK	6082	6118
CA2 (M1del, A77I, P249F) - variant 1	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV SYDQATSLRILNNGHAFNVEFDDSDQKIVLKGGPLDGTyrLIQFHFHWG SLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGI FLKVGSAPGLQKVVDVLDSIKTKGKSADFTNFDPRGLLPESLDYWTY PGSLTTPPILLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDN WRPAQFLKNRQIKASFK	6083	6119
CA2 (M1del, D71K, T192F) - variant 1	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV SYDQATSLRILNNGHAFNVEFKDSQKAVLKGGPLDGTyrLIQFHFHW GSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVL GIFLKVGSAKPGQLQKVVDVLDSIKTKGKSADFTNFDPRGLLPESLDYWF YPGSLTTPPILLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVD NWRPAQPLKNRQIKASFK	6084	6120
CA2 (M1del, L156H)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV SYDQATSLRILNNGHAFNVEFDDSDQKAVLKGGPLDGTyrLIQFHFHW GSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVL GIFLKVGSAKPGHQKVVDVLDSIKTKGKSADFTNFDPRGLLPESLDYWT YPGSLTTPPILLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVD NWRPAQPLKNRQIKASFK	6085	6121
CA2 (M1del, R27L, H122Y) - variant 1	SHHWGYGKHNGPEHWHKDFPIAKGELQSPVDIDTHTAKYDPSLKPLSV SYDQATSLRILNNGHAFNVEFDDSDQKAVLKGGPLDGTyrLIQFHFHW GSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVL GIFLKVGSAKPGQLQKVVDVLDSIKTKGKSADFTNFDPRGLLPESLDYWT YPGSLTTPPILLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVD NWRPAQPLKNRQIKASFK	6086	6122
CA2 (M1del, T87I, H122Y) - variant 1	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV SYDQATSLRILNNGHAFNVEFDDSDQKAVLKGGPLDGIYrLIQFHFHW GSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVL GIFLKVGSAKPGQLQKVVDVLDSIKTKGKSADFTNFDPRGLLPESLDYWT	6087	6123

	YPGSLTTPPLLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVD NWRPAQPLKNRQIKASFK		
CA2 (M1del, H122Y, N252D) - variant 1	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV SYDQATSLRILNNGHAFNVEFDDSDQKAVLKGGPLDGTyrLIQFHFHW GSLDGQGSEHTVDKKKYAAELHLVYWNTKYGDFGKAVQQPDGLAVL GIFLKVGSAKPGQLQKVVDVLDSIKTKGKSADFTNFDPRGLLPESLDYWT YPGSLTTPPLLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVD NWRPAQPLKDRQIKASFK	6088	6124
CA2 (M1del, D72F, V241F) - variant 1	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV SYDQATSLRILNNGHAFNVEFDDSDQKAVLKGGPLDGTyrLIQFHFHW GSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVL GIFLKVGSAKPGQLQKVVDVLDSIKTKGKSADFTNFDPRGLLPESLDYWT YPGSLTTPPLLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMFD NWRPAQPLKNRQIKASFK	6089	6125
CA2 (M1del, V241F, P249L) - variant 1	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV SYDQATSLRILNNGHAFNVEFDDSDQKAVLKGGPLDGTyrLIQFHFHW GSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVL GIFLKVGSAKPGQLQKVVDVLDSIKTKGKSADFTNFDPRGLLPESLDYWT YPGSLTTPPLLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMFD NWRPAQLLKNRQIKASFK	6090	6126
CA2 (M1del, D72F, P249L) - variant 1	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV SYDQATSLRILNNGHAFNVEFDDSDQKAVLKGGPLDGTyrLIQFHFHW GSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVL GIFLKVGSAKPGQLQKVVDVLDSIKTKGKSADFTNFDPRGLLPESLDYWT YPGSLTTPPLLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVD NWRPAQLLKNRQIKASFK	6091	6127
CA2 (M1del, D71L, T87N) - variant 1	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV SYDQATSLRILNNGHAFNVEFLDSQKAVLKGGPLDGNrLIQFHFHW GSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVL GIFLKVGSAKPGQLQKVVDVLDSIKTKGKSADFTNFDPRGLLPESLDYWT YPGSLTTPPLLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVD NWRPAQPLKNRQIKASFK	6092	6128
CA2 (M1del, D71L, L250R) - variant 1	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV SYDQATSLRILNNGHAFNVEFLDSQKAVLKGGPLDGTyrLIQFHFHW GSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVL GIFLKVGSAKPGQLQKVVDVLDSIKTKGKSADFTNFDPRGLLPESLDYWT YPGSLTTPPLLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVD NWRPAQPRKNRQIKASFK	6093	6129
CA2 (Y51T) - variant 1	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV STDQATSLRILNNGHAFNVEFDDSDQKAVLKGGPLDGTyrLIQFHFHW GSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVL GIFLKVGSAKPGQLQKVVDVLDSIKTKGKSADFTNFDPRGLLPESLDYWT YPGSLTTPPLLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVD NWRPAQPLKNRQIKASFK	6094	6130

CA2 (S73N, R89F) - variant 1	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV SYDQATSLRILNNGHAFNVEFDDNQDKAVLKGGPLDGTYRLIQHFHFW GSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVL GIFLKVGSAKPGQLQKVVDVLDSIKTKGKSADFTNFDPRGLLPESLDYWT YPGSLTTPPILLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVD NWRPAQPLKNRQIKASFK	6095	6131
CA2 (D72F, P249F) - variant 1	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV SYDQATSLRILNNGHAFNVEFDDNQDKAVLKGGPLDGTYRLIQHFHFW GSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVL GIFLKVGSAKPGQLQKVVDVLDSIKTKGKSADFTNFDPRGLLPESLDYWT YPGSLTTPPILLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVD NWRPAQPLKNRQIKASFK	6096	6132
CA2 (T55K, G63N, Q248N) - variant 1	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV SYDQAKSLRILNNHAFNVEFDDNQDKAVLKGGPLDGTYRLIQHFHFW GSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVL GIFLKVGSAKPGQLQKVVDVLDSIKTKGKSADFTNFDPRGLLPESLDYWT YPGSLTTPPILLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVD NWRPANPLKNRQIKASFK	6097	6133
CA2 (Y193I) - variant 1	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV SYDQATSLRILNNGHAFNVEFDDNQDKAVLKGGPLDGTYRLIQHFHFW GSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVL GIFLKVGSAKPGQLQKVVDVLDSIKTKGKSADFTNFDPRGLLPESLDYWT IPGSLTTPPILLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVD NWRPAQPLKNRQIKASFK	6098	6134
CA2 (S56F) - variant 1	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV SYDQATFLRILNNGHAFNVEFDDNQDKAVLKGGPLDGTYRLIQHFHFW GSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVL GIFLKVGSAKPGQLQKVVDVLDSIKTKGKSADFTNFDPRGLLPESLDYWT YPGSLTTPPILLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVD NWRPAQPLKNRQIKASFK	6099	6135
CA2 (S56F, D71S) - variant 1	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV SYDQATFLRILNNGHAFNVEFDDNQDKAVLKGGPLDGTYRLIQHFHFW GSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVL GIFLKVGSAKPGQLQKVVDVLDSIKTKGKSADFTNFDPRGLLPESLDYWT YPGSLTTPPILLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVD NWRPAQPLKNRQIKASFK	6100	6136
CA2 (S73N, R89Y) - variant 1	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV SYDQATSLRILNNGHAFNVEFDDNQDKAVLKGGPLDGTYYLIQHFHFW GSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVL GIFLKVGSAKPGQLQKVVDVLDSIKTKGKSADFTNFDPRGLLPESLDYWT YPGSLTTPPILLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVD NWRPAQPLKNRQIKASFK	6101	6137

CA2 (V134F, L228F) - variant 1	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV SYDQATSLRILNNGHAFNVEFDDSQDKAVLKGGLDGTyrLIQFHFHW GSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAFQPPDGLAVL GIFLKVGSAKPGHQKVVVDVLSIKTKGKSADFTNFDPRGLLPESLDYWT YPGSLTTPPILLECVTWIVLKEPISVSSEQVLKFRKFNNGEGEPEELMVD NWRPAQPLKNRQIKASFK	6102	6138
CA2 (M1del, L156H, A256del, S257del, F258del, K259del) - variant 1	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV SYDQATSLRILNNGHAFNVEFDDSQDKAVLKGGLDGTyrLIQFHFHW GSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQPPDGLAVL GIFLKVGSAKPGHQKVVVDVLSIKTKGKSADFTNFDPRGLLPESLDYWT YPGSLTTPPILLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVD NWRPAQPLKNRQIK	6103	6139
CA2 (M1del, S2del, H3del, H4del, W5del, L156H) - variant 1	GYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYDQ ATSLRILNNGHAFNVEFDDSQDKAVLKGGLDGTyrLIQFHFHWGSLD GQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQPPDGLAVLGIFLK VGSAPGHQKVVVDVLSIKTKGKSADFTNFDPRGLLPESLDYWTYPGS LTPPILLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVDNWR PAQPLKNRQIKASFK	6104	6140
CA2 (M1del, W5Y, L156H) - variant 1	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV SYDQATSLRILNNGHAFNVEFDDSQDKAVLKGGLDGTyrLIQFHFHW GSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQPPDGLAVL GIFLKVGSAKPGHQKVVVDVLSIKTKGKSADFTNFDPRGLLPESLDYWT YPGSLTTPPILLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVD NWRPAQPLKNRQIKASFK	6105	6141
CA2 (M1del, L156H, G234del, E235del, P236del) - variant 1	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV SYDQATSLRILNNGHAFNVEFDDSQDKAVLKGGLDGTyrLIQFHFHW GSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQPPDGLAVL GIFLKVGSAKPGHQKVVVDVLSIKTKGKSADFTNFDPRGLLPESLDYWT YPGSLTTPPILLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEEELMVDNW RPAQPLKNRQIKASFK	6106	6142
CA2 (M1del, L156H, F225L) - variant 1	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV SYDQATSLRILNNGHAFNVEFDDSQDKAVLKGGLDGTyrLIQFHFHW GSLDGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQPPDGLAVL GIFLKVGSAKPGHQKVVVDVLSIKTKGKSADFTNFDPRGLLPESLDYWT YPGSLTTPPILLECVTWIVLKEPISVSSEQVLKLRKLNFNNGEGEPEELMVD NWRPAQPLKNRQIKASFK	6107	6143
CA2 (M1del, D71N, D75N, D101N, L156H) - variant 1	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSV SYDQATSLRILNNGHAFNVEFNDSQNKAVLKGGLDGTyrLIQFHFHW GSLNGQGSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQPPDGLAVL GIFLKVGSAKPGHQKVVVDVLSIKTKGKSADFTNFDPRGLLPESLDYWT YPGSLTTPPILLECVTWIVLKEPISVSSEQVLKFRKLNFNNGEGEPEELMVD NWRPAQPLKNRQIKASFK	6108	6144

[0096] In some embodiments a region or a portion of the CA2 WT may be used as template for generating CA2 DDs. In some embodiments, the CA2 DDs may exclude the lysine at position 260 of SEQ ID NO. 5810. In some aspects, the CA2 regions may include but are not limited to those described in Table 5.

Table 5: CA2 regions

Description	AA SEQUENCE	AA SEQ ID NO.	NA SEQ ID NO.
CA2 (aa 1-142 of WT)	MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYDQATSLRI LNNGHAFNVEFDDSDQKAVLKGGLDGTyrLIQFHFHWGSLDGQGSEHTVDKKKYAA ELHLVHWNTKYGDFGKAVQQPDGLAV	5941	-
CA2 (aa 2-142 of WT)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYDQATSLRIL NNGHAFNVEFDDSDQKAVLKGGLDGTyrLIQFHFHWGSLDGQGSEHTVDKKKYAAE LHLVHWNTKYGDFGKAVQQPDGLAV	5942	-
CA2 (aa 1-190 of WT)	MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYDQATSLRI LNNGHAFNVEFDDSDQKAVLKGGLDGTyrLIQFHFHWGSLDGQGSEHTVDKKKYAA ELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAPGLQKVVDVLDSIKTKGKSAD FTNFDPRGLLPESLDY	5943	-
CA2 (aa 2-190 of WT)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYDQATSLRIL NNGHAFNVEFDDSDQKAVLKGGLDGTyrLIQFHFHWGSLDGQGSEHTVDKKKYAAE LHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAPGLQKVVDVLDSIKTKGKSADF TNFDPRGLLPESLDY	5944	-
CA2 (aa 1-89 of WT)	MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYDQATSLRI LNNGHAFNVEFDDSDQKAVLKGGLDGTyr	5945	-
CA2 (aa 2-89 of WT)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYDQATSLRIL NNGHAFNVEFDDSDQKAVLKGGLDGTyr	5946	-
CA2 (aa 1-243 of WT)	MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYDQATSLRI LNNGHAFNVEFDDSDQKAVLKGGLDGTyrLIQFHFHWGSLDGQGSEHTVDKKKYAA ELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAPGLQKVVDVLDSIKTKGKSAD FTNFDPRGLLPESLDYWTYPGSLTTPPLECWTWIVLKEPISVSSEQVLKFRKLNFNNGEGE PEELMVDN	5947	-
CA2 (aa 2-243 of WT)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYDQATSLRIL NNGHAFNVEFDDSDQKAVLKGGLDGTyrLIQFHFHWGSLDGQGSEHTVDKKKYAAE LHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAPGLQKVVDVLDSIKTKGKSADF TNFDPRGLLPESLDYWTYPGSLTTPPLECWTWIVLKEPISVSSEQVLKFRKLNFNNGEGE EELMVDN	5948	-
CA2 (aa 1-166 of WT)	MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYDQATSLRI LNNGHAFNVEFDDSDQKAVLKGGLDGTyrLIQFHFHWGSLDGQGSEHTVDKKKYAA ELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAPGLQKVVDVLDSI	5949	-
CA2 (aa 2-166 of WT)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYDQATSLRIL NNGHAFNVEFDDSDQKAVLKGGLDGTyrLIQFHFHWGSLDGQGSEHTVDKKKYAAE LHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAPGLQKVVDVLDSI	5903	5950
CA2 (aa 1-116 of WT)	MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYDQATSLRI LNNGHAFNVEFDDSDQKAVLKGGLDGTyrLIQFHFHWGSLDGQGSEHTVDKKKYAA	5951	-

CA2 (aa 2-116 of WT)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYDQATSLRILNNGHAFNVEFDDSQDKAVLKGGLDGTYRLIQFHFHWGSLDGGQSEHTVDKKKYAA	5952	-
CA2 (aa 1-152 of WT)	MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYDQATSLRLNNGHAFNVEFDDSQDKAVLKGGLDGTYRLIQFHFHWGSLDGGQSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS	5953	-
CA2 (aa 2-152 of WT)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYDQATSLRILNNGHAFNVEFDDSQDKAVLKGGLDGTYRLIQFHFHWGSLDGGQSEHTVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGS	5954	-
CA2 (aa 1-43 of WT)	MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPS	5955	-
CA2 (aa 2-43 of WT)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPS	5956	-

[0097] Any of the CA2 regions described herein may be utilized to generate CA2 DD. Table 6 provides CA2 DDs derived from CA2 regions.

Table 6: CA2 DDs derived from CA2 regions

Description	AA SEQUENCE	AA SEQ ID NO.	NA SEQ ID NO.
CA2 (aa 2-142 of WT, G12E, A38V, A65V, G98V, S99H, D101M, G102D, Q103K, G104V, S105Q, E106S, H107I, T108L, V109W, D110I, K112R, K113N, Y114M, A115L, A116Q, E117N, L118F, H119T, L120W, V121F, H122T, W123G, N124T, T125P, K126N, Y127M, D129I, F130L, K132E, A133L, V134C, Q135S, Q136N, P137L, D138M, G139D, L140W, A141P, V142F)	SHHWGYGKHNEPEHWHKDFPIAKGERQSPVDIDTHTVKYDPSLKPLSVSYDQATSLRILNNGHAFNVEFDDSQDKAVLKGGLDGTYRLIQFHFHWVHLMKQVSLWKRNMLQNFTWFTGTPNMGILGELCSNLMWPF	5883	5957
CA2 (aa 2-190 of WT, F20L, K45N, G104R, A116V, A173G)	SHHWGYGKHNGPEHWHKDLPIAKGERQSPVDIDTHTAKYDPSLNPLSVSYDQATSLRILNNGHAFNVEFDDSQDKAVLKGGLDGTYRLIQFHFHWGSLDGGQSEHTVDKKKYAVELHLVHWNTKYGDFGKAVQQPDGLAVLGI FLKVGSAKPLGKVVVDVLDISKTKGKSGDFTNFDPRG LLPESLDY	5886	5958
CA2 (aa 2-190 of WT, H17D, P30S, G81V, K132R, S151I, A152D, A173G)	SHHWGYGKHNGPEHWDKDFPIAKGERQSSVDIDTHTAKYDPSLKPLSVSYDQATSLRILNNGHAFNVEFDDSQDKAVLKGGLDGTYRLIQFHFHWGSLDGGQSEHTVDKKKYAAELHLVHWNTKYGDFGRAVQQPDGLAVLGI FLKVGIDKPLGKVVVDVLDISKTKGKSGDFTNFDPRG LLPESLDY	5889	5959
CA2 (aa 2-89 of WT, I59N)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYDQATSLRNLNNGHAFNVEFDDSQDKAVLKGGLDGTYR	5892	5960
CA2 (aa 2-243 of WT, T35I, Y114H, P154L, D161V, P200A, Q221R, F225L, E233D)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDHTAKYDPSLKPLSVSYDQATSLRILNNGHAFNVEFDDSQDKAVLKGGLDGTYRLIQFHFHWGSLDGGQSEHTVDKKKHAAELHLVHWNTKYGDFGKAVQQPDGLAVLGI FLKVGSAKGLQKVVVVDVLDISKTKGSADFTNFDPRG	5895	5961

	LLPESLDYWTYPGSLTTAPLLEC VTWIVLKEPISVSSE RVLKLRKLNFGDGEPEELMVDN		
CA2 (aa 2-189 of WT, S105L, L140V, G155C)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHT AKYDPSLKPLSVSYDQATSLRILNNGHAFNVEFDDSQ DKAVLKGGLDGT YRLIQFHFHWGSLDGGGLEHTVD KKKYAAELHLVHWNTKYGDFGKAVQQPDGLAVLGI FLKVGSAKPCLKQVVDVLSIKTKGKSADFTNFDPRG LLPESLD	5898	5962
CA2 (aa 2-243 of WT, T35I, Y114H, P154L, D161V, P200A, F225L, E233D)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDHT AKYDPSLKPLSVSYDQATSLRILNNGHAFNVEFDDSQ DKAVLKGGLDGT YRLIQFHFHWGSLDGGGSEHTVD KKKHAAELHLVHWNTKYGDFGKAVQQPDGLAVLGI FLKVGSAKGLQKVVVVVLSIKTKGKSADFTNFDPRG LLPESLDYWTYPGSLTTAPLLEC VTWIVLKEPISVSSE QVLKLRKLNFGDGEPEELMVDN	5901	5963
CA2 (aa 2-116 of WT, P13H)	SHHWGYGKHNGHEHWHKDFPIAKGERQSPVDIDTHT AKYDPSLKPLSVSYDQATSLRILNNGHAFNVEFDDSQ DKAVLKGGLDGT YRLIQFHFHWGSLDGGGSEHTVD KKKYAA	5914	5964
CA2 (aa 2-152 of WT, A133T, L147F, K148E, V149G, G150W, S151Q, A152R)	SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHT AKYDPSLKPLSVSYDQATSLRILNNGHAFNVEFDDSQ DKAVLKGGLDGT YRLIQFHFHWGSLDGGGSEHTVD KKKYAAELHLVHWNTKYGDFGKT VQPPDGLAVLGI FFEGWQR	5920	5965
CA2 (aa 2-152 of WT, F20L, K45N, G104R, A116V, L147F, K148E, V149G, G150W, S151Q, A152R)	SHHWGYGKHNGPEHWHKDLPIAKGERQSPVDIDTHT AKYDPSLNPLSVSYDQATSLRILNNGHAFNVEFDDSQ DKAVLKGGLDGT YRLIQFHFHWGSLDGGQRSEHTVD KKKYAVELHLVHWNTKYGDFGKAVQQPDGLAVLGI FFEGWQR	5929	5966
CA2 (aa 2-43 of WT, N11K, G12D, P13L, E14N, H15T, W16G, H17I, K18R, D19T, F20S, I22L, A23P, K24R, G25E, E26S, R27A, Q28S, S29P, P30L, V31L, D32T, I33S, D34T, T35L, H36I, T37Q, A38P, K39S, Y40M, D41T, P42L, S43P)	SHHWGYGKHKDLNTGIRTSPLPRESASPLLTSTLIQPS MTLP	5932	5967

[0098] In some embodiments, DDs derived from CA2 may include one, two, three, four, five, or more of the mutations described in the previous Tables.

[0099] In some embodiments, a DD derived from CA2 comprises at least one mutation relative to the amino acid sequence of wildtype CA2, which mutation, in the absence of a stimulus, destabilizes the DD and at least one payload that is operably linked to the DD or an SRE comprising the DD. In the presence of the stimulus, the DD and the at least one payload are stabilized. In some embodiments, the DD derived from CA2 includes one, two, three, four or more mutations that, in the absence of the stimulus, destabilizes the DD and the at least one operably linked payload. In some embodiments, the destabilization ratio of a DD derived from CA2 comprising the at least one mutation is lower than the

destabilization ratio of wildtype CA2. In some embodiments, the stabilization ratio of a DD derived from CA2 comprising the at least one mutation is higher than the stabilization ratio of wildtype CA2. In some embodiments, a DD may comprise one or more additional mutations that do not significantly affect the destabilization and stabilization ratios.

[0100] In some embodiments, the mutation may be a conserved (with similar physicochemical properties as the amino acid at the mutation site), a semi conserved (e.g., negatively to positively charge amino acid) or a non-conserved (amino acid with different physicochemical properties than the amino acid at the mutation site). In some embodiments, the amino acid lysine may be mutated to glutamic acid or arginine; the amino acid phenylalanine may be mutated to leucine; the amino acid leucine may be mutated to phenylalanine; or the amino acid asparagine may be mutated to serine. Regions or portions or domains of wild type proteins may be utilized as SREs/DDs in whole or in part. They may be combined or rearranged to create new peptides, proteins, regions or domains of which any may be used as SREs/DDs or the starting point for the design of further SREs and/or DDs.

[0101] The destabilization domains described herein may also include amino acid and nucleotide substitutions that do not affect stability, including conservative, non-conservative substitutions and or polymorphisms. In some embodiments, CA2 DDs described herein may also be fragments of the above destabilizing domains, including fragments containing variant amino acid sequences. Preferred fragments are unstable in the absence of the stimulus and stabilized upon addition of the stimulus. Preferred fragments retain the ability to interact with the stimulus with similar efficiency as the DDs described herein.

[0102] In one embodiment, the SRE comprises a region of the CA2 protein. The region of the CA2 protein may be 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260 or more than 260 amino acids in length. The region of the parent protein may be 5-50, 25-75, 50-100, 75-125, 100-150, 125-175, 150-200, 175-225, 200-250, 225-260 amino acids in length.

[0103] In some embodiments, the present disclosure provides a DD comprising a region of or the whole human carbonic anhydrase 2 (CA2; SEQ ID NO. 5810), and further comprising a mutation relative to SEQ ID NO. 5810 selected from A115L, A116Q, A116V, A133L, A133T, A141P, A152D, A152L, A152R, A173C, A173G, A173L, A173T, A23P, A247L, A247S, A257L, A257S, A38P, A38V, A54Q, A54V, A54X, A65L, A65N, A65V, A77I, A77P, A77Q, C205M, C205R, C205V, C205W, C205Y, D101G, D101M, D110I, D129I, D138G, D138M, D138N, D161*, D161M, D161V, D164G, D164I, D174*, D174T, D179E, D179I, D179R, D189G, D189I, D19T, D19V, D242G, D242T, D32T, D34T, D41T, D52I, D52L, D71F, D71G, D71K, D71M, D71S, D71Y, D72I, D72S, D72T, D72X, D75T, D75V, D85M, E106D, E106G, E106S, E117*, E117N, E14N, E186*, E186N, E204A, E204D, E204G, E204N, E213*, E213G, E213N, E220K, E220R, E220S, E233D, E233G, E233R, E235*, E235G, E235N, E237K, E237R, E238*, E238N, E238R, E26S, E69D, E69K, E69S, F130L, F146V, F175I, F175L, F175S, F178L, F178S, F20L, F20S, F225I, F225L, F225S, F225Y, F230I, F230L, F230S, F259L, F259S, F66S, F70I, F70L, F95Y, G102D, G104R, G104V, G128R, G12D, G12E, G131E, G131R, G131W, G139D, G144D, G144V, G150A, G150S, G150W, G155A, G155C, G155D, G155S, G170A, G170D, G182A, G182W, G195A, G195R, G232R, G232W, G234L, G234V, G25E, G63D, G63V, G81E, G81V, G82D, G86A, G86D, G98V, H107I, H107Q, H119T, H119Y, H122T, H122Y, H15L, H15T, H15Y, H17D, H17I, H36I, H36Q, H64M, H94T, H96T, I145F, I145M, I166H, I166L, I209D, I209L, I215H, I215S, I22L, I255N, I255S, I33S, I59F, I59N, I59S, I91F, K111E, K111N, K112R, K113I, K113N, K126N, K132E, K132R, K148E, K148R, K153*, K153N, K158E, K158N, K167*, K169N, K169R, K171Q, K171R, K18R, K212N, K212Q, K212R, K212W, K224E, K224N, K227*, K227N, K24R, K251E, K251R, K256Q, K260F, K260L, K260Q, K39S, K45N, K45S, K80M, K80R, L118F, L120W, L140V, L140W, L143*, L147*, L147F, L156F, L156H, L156P, L156Q, L163A, L163W, L183P, L183S, L184F, L184P, L188P, L188W, L197*, L197M, L197P, L197R, L197T, L202F, L202H, L202I, L202P, L202R, L202S, L203P, L203S, L203W, L211*, L211A, L211S, L223*, L223I, L223V, L228F, L228H, L228T, L239*, L239F, L239T, L250*, L250P, L250T, L44*, L44M, L47C, L47V, L57*, L57X, L60S, L79F, L79S, L84W, L90*, L90V, M240D, M240L, M240R, M240W, N11D, N11K, N124T, N177*, N177T, N229*, N229T, N231D, N231F, N231K, N231L, N231M, N231Q, N231T, N243Q, N243T, N252E, N252T, N61R, N61T, N61Y, N62K, N62M, N67D, N67T, P137L, P13A, P13H, P13L, P13S, P154L, P154R, P154T, P180L, P180S, P185L, P185S, P185V, P194Q, P200A, P200L, P200S, P200T, P201A, P201L, P201R, P201S, P214T, P236L, P236T, P246L, P246Q, P249A, P249F, P249H, P249I, P249X, P30L, P30S, P42L, P83A, Q103K, Q135S, Q136N, Q157R, Q157S, Q221A, Q221R, Q248F, Q248L, Q248S, Q254A, Q254K, Q28S, Q53H, Q53K, Q53N, Q74R, Q92H, Q92S, R181H, R181S, R181V, R226H, R226P, R226V, R245A, R253G, R253Q, R27A, R58G, R89D, R89F, R89I, R89X, R89Y, S105L, S105Q, S151A, S151I, S151Q, S165F, S165P, S172E, S172V, S187I, S187P, S196H, S196L, S216A, S216Q, S218A, S218Q, S219A, S219Q, S258F, S258P, S29C, S29P, S43P, S43T,

S48L, S50P, S56F, S56N, S56P, S56X, S73L, S73N, S73X, S99H, T108L, T125I, T125P, T168K, T168N, T168Q, T176H, T176L, T192D, T192F, T192I, T192N, T192P, T192X, T198D, T198I, T198P, T199A, T199H, T199P, T207D, T207I, T207P, T207S, T35I, T35L, T37Q, T55L, T87L, V109M, V109W, V121F, V134C, V134F, V142F, V149G, V149L, V159L, V159S, V160C, V160L, V162A, V162C, V206*, V206C, V206M, V210C, V217L, V217R, V217S, V222A, V222C, V222G, V241G, V241W, V241X, V31L, V49F, V68L, V68W, V78C, W123G, W123R, W16G, W191*, W191G, W191L, W208G, W208L, W208S, W244*, W244G, W244L, W97C, W97G, Y114H, Y114M, Y127M, Y190*, Y190L, Y190T, Y193C, Y193F, Y193I, Y193L, Y193T, Y193V, Y193X, Y40M, Y51F, Y51M, Y51T, Y51X, Y88T, K9N, and S29A. As used herein, “*” indicates the translation of the stop codon and “X” indicates any amino acid.

[0104] In some embodiments, the present disclosure provides a DD comprising a region of or the whole human carbonic anhydrase 2 (CA2; SEQ ID NO. 5810), and further comprising a mutation relative to SEQ ID NO. 5810 selected from E106D, G63D, H122Y, I59N, L156H, L183S, L197P, S56F, S56N, W208S, Y193I, and Y51T.

[0105] In some embodiments, the present disclosure provides a DD comprising a region of or the whole human carbonic anhydrase 2 (CA2; SEQ ID NO. 5810), and further comprising two or more mutations relative to SEQ ID NO. 5810. In some embodiments, a DD may comprise CA2 (aa 2-260 of WT, R27L, H122Y), CA2 (aa 2-260 of WT, T87I, H122Y), CA2 (aa 2-260 of WT, H122Y, N252D), CA2 (aa 2-260 of WT, D72F, V241F), CA2 (aa 2-260 of WT, V241F, P249L), CA2 (aa 2-260 of WT, D72F, P249L), CA2 (aa 2-260 of WT, D71L, L250R), CA2 (aa 2-260 of WT, D72F, P249F), CA2 (aa 2-260 of WT, T55K, G63N, Q248N), CA2 (aa 2-260 of WT, L156H, A257del, S258del, F259del, K260del), CA2 (aa 2-260 of WT, L156H, S2del, H3del, H4del, W5del), CA2 (aa 2-260 of WT, W4Y, L156H), CA2 (aa 2-260 of WT, L156H, G234del, E235del, P236del), CA2 (aa 2-260 of WT, L156H, F225L), CA2 (aa 2-260 of WT, D70N, D74N, D100N, L156H), (CA2 (aa 2-260 of WT, I59N, G102R), CA2 (aa 2-260 of WT, G63D, E69V, N231I), CA2 (aa 2-260 of WT, R27L, T87I, H122Y, N252D), CA2 (aa 2-260 of WT, D72F, V241F, P249L), CA2 (aa 2-260 of WT, D71L, T87N, L250R), CA2 (aa 2-260 of WT, L156H, S172C, F178Y, E186D), CA2 (aa 2-260 of WT, D71F, N231F), CA2 (aa 2-260 of WT, A77I, P249F), CA2 (aa 2-260 of WT, D71K, P249H), CA2 (aa 2-260 of WT, D72F, P249H), CA2 (aa 2-260 of WT, Q53N, N61Y), CA2 (aa 2-260 of WT, E106D, C205S), CA2 (aa 2-260 of WT, C205S, W208S), CA2 (aa 2-260 of WT, S73N, R89Y), CA2 (aa 2-260 of WT, D71K, T192F), CA2 (aa 2-260 of WT, Y193L, K260L), CA2 (aa 2-260 of WT, D71F, V241F, P249L), CA2 (aa 2-260 of WT, L147F, Q248F), CA2 (aa 2-260 of WT, D52I, S258P), CA2 (aa 2-260 of WT, D72S, T192N), CA2 (aa 2-260 of WT, D179E, T192I), CA2 (aa 2-260 of WT, S56N, Q103K), CA2 (aa 2-260 of WT, D71Y, Q248L), CA2 (aa 2-260 of WT, S73N, R89F), CA2 (aa 2-260 of WT, D71K, N231L, E235G, L239F), CA2 (aa 2-260 of WT, D72F, P249I), CA2 (aa 2-260 of WT, D72X, V241X, P249X), CA2 (aa 2-260 of WT, A54X, S56X, L57X, T192X), CA2 (aa 2-260 of WT, Y193V, K260F), CA2 (aa 2-260 of WT,

G63D, M240L), CA2 (aa 2-260 of WT, V134F, L228F), CA2 (aa 2-260 of WT, D71G, N231K), CA2 (aa 2-260 of WT, S56F, D71S), CA2 (aa 2-260 of WT, D52L, G128R, Q248F), CA2 (aa 2-260 of WT, S73X, R89X), CA2 (aa 2-260 of WT, Y51X, D72X, V241X, P249X), CA2 (aa 2-260 of WT, D72I, W97C), CA2 (aa 2-260 of WT, D71K, T192F, N231F), CA2 (aa 2-260 of WT, H36Q, S43T, Y51F, N67D, G131W, R226H), CA2 (aa 2-260 of WT, F70I, F146V), CA2 (aa 2-260 of WT, K45N, V68L, H119Y, K169R, D179E), CA2 (aa 2-260 of WT, H15L, A54V, K111E, E220K, F225I), CA2 (aa 2-260 of WT, P13S, P83A, D101G, K111N, F230I), CA2 (aa 2-260 of WT, G63D, W123R, E220K), CA2 (aa 2-260 of WT, N11D, E69K, G86D, V109M, K113I, T125I, D138G, G155S), CA2 (aa 2-260 of WT, I59N, G102R, A173T), CA2 (aa 2-260 of WT, L79F, P180S), CA2 (aa 2-260 of WT, A77P, G102R, D138N), CA2 (aa 2-260 of WT, F20L, K45N, G63D, E69V, N231I), CA2 (aa 2-260 of WT, T199N, L202P, L228F), CA2 (aa 2-260 of WT, K9N, H122Y, T168K), CA2 (aa 2-260 of WT, Q53H, L90V, Q92H, G131E), CA2 (aa 2-260 of WT, L44M, L47V, N62K, E69D), CA2 (aa 2-260 of WT, D75V, K169N, F259L), CA2 (aa 2-260 of WT, T207S, V222A, N231D), CA2 (aa 2-260 of WT, I59F, V206M, G232R), CA2 (aa 2-260 of WT, P13A, A133T), CA2 (aa 2-260 of WT, I59N, R89I), CA2 (aa 2-260 of WT, A65N, G86D, G131R, G155D, K158N, V162A, G170D, P236L), CA2 (aa 2-260 of WT, G12R, H15Y, D19V), CA2 (aa 2-260 of WT, A65V, F95Y, E106G, H107Q, I145M, F175I), CA2 (aa 2-260 of WT, G63D, E69V, N231I), CA2 (aa 2-260 of WT, S29A, C205S) and/or CA2 (aa 2-260 of WT, S29C, C205S). As used herein, “X” indicates any amino acid.

[0106] In some embodiments, a DD may comprise CA2 (aa 2-260 of WT, R27L, H122Y), CA2 (aa 2-260 of WT, T87I, H122Y), CA2 (aa 2-260 of WT, H122Y, N252D), CA2 (aa 2-260 of WT, D72F, V241F), CA2 (aa 2-260 of WT, V241F, P249L), CA2 (aa 2-260 of WT, D72F, P249L), CA2 (aa 2-260 of WT, D71L, L250R), CA2 (aa 2-260 of WT, D72F, P249F), CA2 (aa 2-260 of WT, T55K, G63N, Q248N), CA2 (aa 2-260 of WT, L156H, A257del, S258del, F259del, K260del), CA2 (aa 2-260 of WT, L156H, S2del, H3del, H4del, W5del), CA2 (aa 2-260 of WT, W4Y, L156H), CA2 (aa 2-260 of WT, L156H, G234del, E235del, P236del), CA2 (aa 2-260 of WT, L156H, F225L), CA2 (aa 2-260 of WT, D70N, D74N, D100N, L156H), (CA2 (aa 2-260 of WT, I59N, G102R), CA2 (aa 2-260 of WT, G63D, E69V, N231I), CA2 (aa 2-260 of WT, R27L, T87I, H122Y, N252D), CA2 (aa 2-260 of WT, D72F, V241F, P249L), CA2 (aa 2-260 of WT, D71L, T87N, L250R), CA2 (aa 2-260 of WT, L156H, S172C, F178Y, E186D), CA2 (aa 2-260 of WT, A77I, P249F), CA2 (aa 2-260 of WT, E106D, C205S), CA2 (aa 2-260 of WT, C205S, W208S), CA2 (aa 2-260 of WT, S73N, R89Y), CA2 (aa 2-260 of WT, D71K, T192F), CA2 (aa 2-260 of WT, S73N, R89F), CA2 (aa 2-260 of WT, G63D, M240L), CA2 (aa 2-260 of WT, V134F, L228F), and/or CA2 (aa 2-260 of WT, S56F, D71S).

[0107] In some embodiments, the CA2 may be derived from carbonic anhydrases of Homo sapiens. In some embodiments, the CA2 DDs described herein may have at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%,

90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% but less than 100% sequence identity to a particular reference polynucleotide or polypeptide as determined by sequence alignment programs and parameters described herein and known to those skilled in the art. In some embodiments the reference polypeptide may be SEQ ID NO. 5810. Tools for alignment may include those of the BLAST suite (Stephen F. Altschul, et al. (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402).

[0108] In some embodiments, the CA2 DDs may be derived from carbonic anhydrases of species other than Homo sapiens. In some embodiments, the CA2 DDs may be derived from carbonic anhydrases of species such as but not limited to Acinonyx jubatus, Ailuropoda melanoleuca, Balaenoptera acutorostrata scammoni, Callithrix jacchus, Callorhinus ursinus, Camelus bactrianus, Camelus dromedarius, Camelus ferus, Canis lupus dingo, Canis lupus familiaris, Carlito syrichta, Castor canadensis, Cebus capucinus imitator, Ceratotherium simum simum, Cercocebus atys, Chinchilla lanigera, Chlorocebus sabaeus, Colobus angolensis palliatus, Delphinapterus leucas, Dipodomys ordii, Enhydra lutris kenyoni, Equus asinus, Equus caballus, Equus przewalskii, Erinaceus europaeus, Eumetopias jubatus, Felis catus, Galeopterus variegatus, Gorilla gorilla gorilla, Homo sapiens, Ictidomys tridecemlineatus, Jaculus jaculus, Lagenorhynchus obliquidens, Lemur catta, Leptonychotes weddellii, Lipotes vexillifer, Loxodonta africana, Macaca fascicularis, Macaca mulatta, Macaca nemestrina, Mandrillus leucophaeus, Manis javanica, Marmota flaviventris, Marmota marmota marmota, Microcebus murinus, Mus caroli, Mus musculus, Mus pahari, Mustela putorius furo, Nannospalax galili, Neomonachus schauinslandi, Neophocaena asiaeorientalis asiaeorientalis, Nomascus leucogenys, Odobenus rosmarus divergens, Orcinus orca, Oryctolagus cuniculus, Oryzomys latipes, Pan paniscus, Pan troglodytes, Panthera pardus, Panthera tigris altaica, Papio anubis, Phrynosoma macleodi, Piliocolobus tephrosceles, Pongo abelii, Propithecus coquereli, Puma concolor, Rhinopithecus bieti, Rhinopithecus roxellana, Saimiri boliviensis boliviensis, Sus scrofa, Theropithecus gelada, Trichechus manatus latirostris, Tupaia chinensis, Tursiops truncatus, Urocyon v. parryi, Ursus arctos horribilis, Ursus maritimus, Vulpes vulpes, and/or Zalophus californianus.

[0109]

Stabilization and Destabilization Ratio of SRE

[0110] In some embodiments, the present disclosure provides methods for modulating protein expression, function or level by measuring the stabilization ratio and destabilization ratio. As used herein, the stabilization ratio may be defined as the ratio of expression, function or level of a protein of interest in response to the stimulus to the expression, function or level of the protein of interest in the absence of the stimulus specific to the SRE. In some aspects, the stabilization ratio is at least 1, such as by at least 1-10, 1-20, 1-30, 1-40, 1-50, 1-60, 1-70, 1-80, 1-90, 1-100, 20-30, 20-40, 20-50, 20-60, 20-70, 20-80, 20-90, 20-95, 20-100, 30-40, 30-50, 30-60, 30-70, 30-80, 30-90, 30-95, 30-100, 40-50,

40-60, 40-70, 40-80, 40-90, 40-95, 40-100, 50-60, 50-70, 50-80, 50-90, 50-95, 50-100, 60-70, 60-80, 60-90, 60-95, 60-100, 70-80, 70-90, 70-95, 70-100, 80-90, 80-95, 80-100, 90-95, 90-100 or 95-100. As used herein, the destabilization ratio may be defined as the ratio of expression, function or level of a protein of interest in the absence of the stimulus specific to the SRE to the expression, function or level of the protein of interest, that is expressed constitutively and in the absence of the stimulus specific to the SRE. As used herein “constitutively” refers to the expression, function or level of a protein of interest that is not linked to an SRE and is therefore expressed both in the presence and absence of the stimulus. In some aspects, the destabilization ratio is at least 0, such as by at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, or at least, 0-0.1, 0-0.2, 0-0.3, 0-0.4, 0-0.5, 0-0.6, 0-0.7, 0-0.8, 0-0.9, 0.1-0.2, 0.1-0.3, 0.1-0.4, 0.1-0.5, 0.1-0.6, 0.1-0.7, 0.1-0.8, 0.1-0.9, 0.2-0.3, 0.2-0.4, 0.2-0.5, 0.2-0.6, 0.2-0.7, 0.2-0.8, 0.2-0.9, 0.3-0.4, 0.3-0.5, 0.3-0.6, 0.3-0.7, 0.3-0.8, 0.3-0.9, 0.4-0.5, 0.4-0.6, 0.4-0.7, 0.4-0.8, 0.4-0.9, 0.5-0.6, 0.5-0.7, 0.5-0.8, 0.5-0.9, 0.6-0.7, 0.6-0.8, 0.6-0.9, 0.7-0.8, 0.7-0.9 or 0.8-0.9.

[0111] In some embodiments, the SRE of the effector module may stabilize the payload of interest by a stabilization ratio of 1 or more, wherein the stabilization ratio may comprise the ratio of expression, function or level of the payload of interest in the presence of the stimulus to the expression, function or level of the payload of interest in the absence of the stimulus.

[0112] In some embodiments, the SRE may destabilize the payload by a destabilization ratio between 0 and 0.09, wherein the destabilization ratio may comprise the ratio of expression, function or level of the payload of interest in the absence of the stimulus specific to the SRE to the expression, function or level of the payload of interest that is expressed constitutively, and in the absence of the stimulus specific to the SRE.

Payloads

[0113] As used herein a “payload” or “target payload” or “payload of interest (POI)” is defined as any protein or nucleic acid whose function is to be altered.

[0114] Payloads may include any coding or non-coding gene or any protein or fragment thereof.

[0115] Payloads are often associated with one or more SREs and may be encoded alone or in combination with one or more SRE in a polynucleotide of the disclosure. Payloads themselves may be altered (at the protein or nucleic acid level) thereby providing for an added layer of tenability of the effector module. For example, payloads may be engineered or designed to contain mutations, single or multiple, which affect the stability of the payload or its susceptibility to degradation, cleavage or trafficking. The combination of an SRE which can have a spectrum of responses to a stimulus with a payload which is altered to exhibit a variety of responses or gradations of output signals, e.g., expression levels,

produce biocircuits which are superior to those in the art. The ability to independently tune both the SRE and the payload greatly increases the scope of uses of the effector modules of the present disclosure.

[0116] As used herein, the phrase “derived from” as it relates to effector modules, SRE’s or payloads means that the effector module, SRE or payload originates at least in part from the stated parent molecule or sequence. For example, in designing an SRE, such SRE may be derived from an epitope or region of a naturally occurring protein but then have been modified in any of the ways taught herein to optimize the SRE function.

[0117] In one embodiment, the payload is derived from a region of parent protein or from a mutant protein. The region of the parent protein may be 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, or more than 450 amino acids in length. The region of the parent protein may be 5-50, 25-75, 50-100, 75-125, 100-150, 125-175, 150-200, 175-225, 200-250, 225-275, 250-300, 275-325, 300-350, 325-375, 350-400, 375-425, or 400-450 amino acids in length.

[0118] In one embodiment, the payload is derived from a region of parent protein or from a mutant protein and includes a region of the parent protein. The payload may include a region of the parent protein which is 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, or 100%, 5-

10%, 10-15%, 15-20%, 20-25%, 25-30%, 30-35%, 35-40%, 40-45%, 45-50%, 50-55%, 55-60%, 60-65%, 65-70%, 70-75%, 75-80%, 80-85%, 85-90%, 90-95%, 95-100%, 10-20%, 20-30%, 30-40%, 40-50%, 50-60%, 60-70%, 70-80%, 80-90%, 90-100%, 10-30%, 20-40%, 30-50%, 40-60%, 50-70%, 60-80%, 70-90%, 80-100%, 10-40%, 20-50%, 30-60%, 40-70%, 50-80%, 60-90%, 70-100%, 10-50%, 20-60%, 30-70%, 40-80%, 50-90%, 60-100%, 10-60%, 20-70%, 30-80%, 40-90%, 50-100%, 10-70%, 20-80%, 30-90%, 40-100%, 10-80%, 20-90%, 30-100%, 10-90%, 20-100%, 25-50%, 50-75%, or 75-100% of the parent protein or mutant protein.

[0119] In one embodiment, the payload is derived from a parent protein or from a mutant protein and may have 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, or 100%, 5-10%, 10-15%, 15-20%, 20-25%, 25-30%, 30-35%, 35-40%, 40-45%, 45-50%, 50-55%, 55-60%, 60-65%, 65-70%, 70-75%, 75-80%, 80-85%, 85-90%, 90-95%, 95-100%, 10-20%, 20-30%, 30-40%, 40-50%, 50-60%, 60-70%, 70-80%, 80-90%, 90-100%, 10-30%, 20-40%, 30-50%, 40-60%, 50-70%, 60-80%, 70-90%, 80-100%, 10-40%, 20-50%, 30-60%, 40-70%, 50-80%, 60-90%, 70-100%, 10-50%, 20-60%, 30-70%, 40-80%, 50-90%, 60-100%, 10-60%, 20-70%, 30-80%, 40-90%, 50-100%, 10-70%, 20-80%, 30-90%, 40-100%, 10-80%, 20-90%, 30-100%, 10-90%, 20-100%, 25-50%, 50-75%, or 75-100% identity to the parent protein or mutant protein.

[0120] In one embodiment, the transmembrane domain region of a first payload may be replaced with a transmembrane domain, variant or fragment thereof, from a second parent protein.

Polypeptides and Polypeptides as Payloads

[0121] The stimuli, biocircuit components, effector modules, including their SREs and payloads of the present disclosure may exist as a whole polypeptide, a plurality of polypeptides or fragments of polypeptides, which independently may be encoded by one or more nucleic acids, a plurality of nucleic acids, fragments of nucleic acids or variants of any of the aforementioned.

[0122] As used herein, the term “polypeptide” refers to a polymer of amino acid residues (natural or unnatural) linked together most often by peptide bonds. The term, as used herein, refers to proteins, polypeptides, and peptides of any size, structure, or function. In some instances, the polypeptide encoded is smaller than about 50 amino acids and the polypeptide is then termed a peptide. If the polypeptide is a peptide, it will be at least about 2, 3, 4, or at least 5 amino acid residues long. Thus, polypeptides include gene products, naturally occurring polypeptides, synthetic polypeptides, homologs, orthologs, paralogs, fragments and other equivalents, variants, and analogs of the foregoing. A polypeptide may be a single molecule or may be a multi-molecular complex such as a dimer, trimer or tetramer. They may also comprise single chain or multichain polypeptides and may be associated or linked. The term polypeptide may also apply

to amino acid polymers in which one or more amino acid residues are an artificial chemical analogue of a corresponding naturally occurring amino acid.

[0123] As used herein, the term “polypeptide variant” refers to molecules which differ in their amino acid sequence from a native or reference sequence. The amino acid sequence variants may possess substitutions, deletions, and/or insertions at certain positions within the amino acid sequence, as compared to a native or reference sequence. Ordinarily, variants will possess at least about 50% identity (homology) to a native or reference sequence, and preferably, they will be at least about 80%, more preferably at least about 90% identical (homologous) to a native or reference sequence.

[0124] In some embodiments “variant mimics” are provided. As used herein, the term “variant mimic” refers to a variant which contains one or more amino acids which would mimic an activated sequence. For example, glutamate may serve as a mimic for phospho-threonine and/or phospho-serine. Alternatively, variant mimics may result in deactivation or in an inactivated product containing the mimic, e.g., phenylalanine may act as an inactivating substitution for tyrosine; or alanine may act as an inactivating substitution for serine. The amino acid sequences of the pharmaceutical compositions, biocircuits, biocircuit components, effector modules including their SREs or payloads of the disclosure may comprise naturally occurring amino acids and as such may be considered to be proteins, peptides, polypeptides, or fragments thereof. Alternatively, the pharmaceutical compositions, biocircuits, biocircuit components, effector modules including their SREs or payloads may comprise both naturally and non-naturally occurring amino acids.

[0125] As used herein, the term “amino acid sequence variant” refers to molecules with some differences in their amino acid sequences as compared to a native or starting sequence. The amino acid sequence variants may possess substitutions, deletions, and/or insertions at certain positions within the amino acid sequence. As used herein, the terms “native” or “starting” when referring to sequences are relative terms referring to an original molecule against which a comparison may be made. Native or starting sequences should not be confused with wild type sequences. Native sequences or molecules may represent the wild-type (that sequence found in nature) but do not have to be identical to the wild-type sequence.

[0126] Ordinarily, variants will possess at least about 70% homology to a native sequence, and preferably, they will be at least about 80%, more preferably at least about 90% homologous to a native sequence.

[0127] As used herein, the term “homology” as it applies to amino acid sequences is defined as the percentage of residues in the candidate amino acid sequence that are identical with the residues in the amino acid sequence of a second sequence after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology. Methods and computer programs for the alignment are well known in the art. It is understood that homology depends on a calculation of percent identity but may differ in value due to gaps and penalties introduced in the calculation.

[0128] As used herein, the term "homolog" as it applies to amino acid sequences is meant the corresponding sequence of other species having substantial identity to a second sequence of a second species.

[0129] As used herein, the term "analog" is meant to include polypeptide variants which differ by one or more amino acid alterations, e.g., substitutions, additions or deletions of amino acid residues that still maintain the properties of the parent polypeptide.

[0130] As used herein, the term "derivative" is used synonymously with the term "variant" and refers to a molecule that has been modified or changed in any way relative to a reference molecule or starting molecule.

[0131] The present disclosure contemplates several types of pharmaceutical compositions, biocircuits, biocircuit components, effector modules including their SREs or payloads which are amino acid based including variants and derivatives. These include substitutional, insertional, deletional and covalent variants and derivatives. As such, included within the scope of this disclosure are pharmaceutical compositions, biocircuits, biocircuit components, effector modules including their SREs or payloads comprising substitutions, insertions, additions, deletions and/or covalent modifications. For example, sequence tags or amino acids, such as one or more lysines, can be added to peptide sequences of the disclosure (e.g., at the N-terminal or C-terminal ends). Sequence tags can be used for peptide purification or localization. Lysines can be used to increase peptide solubility or to allow for biotinylation. Alternatively, amino acid residues located at the carboxy and amino terminal regions of the amino acid sequence of a peptide or protein may optionally be deleted providing for truncated sequences. Certain amino acids (e.g., C-terminal or N-terminal residues) may alternatively be deleted depending on the use of the sequence, as for example, expression of the sequence as part of a larger sequence which is soluble or linked to a solid support.

[0132] "Substitutional variants" when referring to proteins are those that have at least one amino acid residue in a native or starting sequence removed and a different amino acid inserted in its place at the same position. The substitutions may be single, where only one amino acid in the molecule has been substituted, or they may be multiple, where two or more amino acids have been substituted in the same molecule.

[0133] As used herein, the term "conservative amino acid substitution" refers to the substitution of an amino acid that is normally present in the sequence with a different amino acid of similar size, charge, or polarity. Examples of conservative substitutions include the substitution of a non-polar (hydrophobic) residue such as isoleucine, valine and leucine for another non-polar residue. Likewise, examples of conservative substitutions include the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, and between glycine and serine. Additionally, the substitution of a basic residue such as lysine, arginine or histidine for another, or the substitution of one acidic residue such as aspartic acid or glutamic acid for another acidic residue are

additional examples of conservative substitutions. Examples of non-conservative substitutions include the substitution of a non-polar (hydrophobic) amino acid residue such as isoleucine, valine, leucine, alanine, methionine for a polar (hydrophilic) residue such as cysteine, glutamine, glutamic acid or lysine and/or a polar residue for a non-polar residue.

[0134] As used herein, the term "insertional variants" when referring to proteins are those with one or more amino acids inserted immediately adjacent to an amino acid at a particular position in a native or starting sequence. As used herein, the term "immediately adjacent" refers to an adjacent amino acid that is connected to either the alpha-carboxy or alpha-amino functional group of a starting or reference amino acid.

[0135] As used herein, the term "deletional variants" when referring to proteins, are those with one or more amino acids in the native or starting amino acid sequence removed. Ordinarily, deletional variants will have one or more amino acids deleted in a particular region of the molecule.

[0136] As used herein, the term "derivatives," as referred to herein includes variants of a native or starting protein comprising one or more modifications with organic proteinaceous or non-proteinaceous derivatizing agents, and post-translational modifications. Covalent modifications are traditionally introduced by reacting targeted amino acid residues of the protein with an organic derivatizing agent that is capable of reacting with selected side-chains or terminal residues, or by harnessing mechanisms of post-translational modifications that function in selected recombinant host cells. The resultant covalent derivatives are useful in programs directed at identifying residues important for biological activity, for immunoassays, or for the preparation of anti-protein antibodies for immunoaffinity purification of the recombinant glycoprotein. Such modifications are within the ordinary skill in the art and are performed without undue experimentation.

[0137] As used herein, the terms "site," as it pertains to amino acid-based embodiments is used synonymously with "amino acid residue" and "amino acid side chain". A site represents a position within a peptide or polypeptide that may be modified, manipulated, altered, derivatized or varied within the polypeptide-based molecules of the present disclosure.

[0138] As used herein, the terms "termini" or "terminus," when referring to proteins refers to an extremity of a peptide or polypeptide. Such extremity is not limited only to the first or final site of the peptide or polypeptide but may include additional amino acids in the terminal regions. The polypeptide-based molecules of the present disclosure may be characterized as having both an N-terminus (terminated by an amino acid with a free amino group (NH₂)) and a C-terminus (terminated by an amino acid with a free carboxyl group (COOH)).

[0139] Polypeptides or proteins of the disclosure are in some cases made up of multiple polypeptide chains brought together by disulfide bonds or by non-covalent forces (multimers, oligomers). These sorts of proteins will have multiple

N-and C-termini. Alternatively, the termini of the polypeptides may be modified such that they begin or end, as the case may be, with a non-polypeptide-based moiety such as an organic conjugate.

[0140] Once any of the features have been identified or defined as a component of a biocircuit system component, stimulus, effector module including the SREs or payloads of the disclosure, any of several manipulations and/or modifications of these features may be performed by moving, swapping, inverting, deleting, randomizing or duplicating. Furthermore, it is understood that manipulation of features may result in the same outcome as a modification to the compositions of the disclosure. For example, a manipulation which involved deleting a domain would result in the alteration of the length of a molecule just as modification of a nucleic acid to encode less than a full-length molecule would.

[0141] Modifications and manipulations can be accomplished by methods known in the art such as site directed mutagenesis. The resulting modified molecules may then be tested for activity using in vitro or in vivo assays such as those described herein, or any other suitable screening assay known in the art.

[0142] In some embodiments, compositions of the present disclosure may comprise one or more atoms that are isotopes. As used herein, the term “isotope” refers to a chemical element that has one or more additional neutrons. In some embodiments, compounds of the present disclosure may be deuterated. As used herein, the term “deuterate” refers to the process of replacing one or more hydrogen atoms in a substance with deuterium isotopes. Deuterium isotopes are isotopes of hydrogen. The nucleus of hydrogen contains one proton while deuterium nuclei contain both a proton and a neutron. The pharmaceutical compositions, biocircuits, biocircuit components, effector modules including their SREs or payloads of the present disclosure may be deuterated in order to change one or more physical property, such as stability, or to allow pharmaceutical compositions, biocircuits, biocircuit components, effector modules including their SREs or payloads to be used in diagnostic and/or experimental applications.

[0143] At the protein level, any of the biocircuit components may comprise one or more post-translational modifications (PTM). Such PTMs may occur intracellularly after administration of a protein-based biocircuit component or upon or after translation of a biocircuit component administered as a nucleic acid encoding said biocircuit component.

[0144] Post translational modifications (PTMs) of the present disclosure include, but are not limited to acetylation, phosphorylation, ubiquitination, carboxylation, deamidation, deamination, deacetylation, dihydroxylation, dephosphorylation, formylation, gamma-carboxyglutamation, glutathionylation, glycation, hydroxylation, methylation, nitration, sumoylation, N-or O-transglutamination, glycosylation and farnesylation.

[0145] Effector modules, including their SREs and payloads, may independently have 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more PTMs which are the same or different.

[0146] Effector modules may be designed to include one or more structural or functional domain, repeat, or motif of a protein family. Such domains, repeats and motifs are categorized by protein family; and representative families are given in the EMBL-EBI database, located at <http://www.ebi.ac.uk/>.

[0147] In some embodiments, protein modifications engineered into the structure of the compositions of the disclosure to interfere with antigen processing and peptide loading such as glycosylation and PEGylation, may also be useful in the present disclosure. Compositions of the disclosure may also be engineered to include non-classical amino acid sidechains to design less immunogenic compositions. Any of the methods discussed in International Patent Publication No. WO2005051975 for reducing immunogenicity may be useful in the present disclosure (the contents of which are incorporated by reference in their entirety).

[0148] The SRE may be, but is not limited to, a peptide, peptide complex, peptide-protein complex, protein, fusion protein, protein complex, protein-protein complex. The SRE may include one or more regions derived from any natural or mutated protein, or antibody. In this aspect, the SRE is an element, when responding to a stimulus, can tune intracellular localization, intramolecular activation, and/or degradation of payloads.

[0149] In some embodiments, effector modules of the present disclosure may comprise additional features that facilitate the expression and regulation of the effector module, such as one or more signal sequences (SSs), one or more cleavage and/or processing sites, one or more targeting and/or penetrating peptides, one or more tags, and/or one or more linkers. Additionally, effector modules of the present disclosure may further comprise other regulatory moieties such as inducible promoters, enhancer sequences, microRNA sites, and/or microRNA targeting sites. Each aspect or tuned modality may bring to the effector module or biocircuit a differentially tuned feature. For example, an SRE may represent a destabilizing domain, while mutations in the protein payload may alter its cleavage sites or dimerization properties or half-life and the inclusion of one or more microRNA or microRNA binding site may impart cellular detargeting or trafficking features. Consequently, the present disclosure embraces biocircuits which are multifactorial in their tenability. Such biocircuits may be engineered to contain one, two, three, four or more tuned features.

[0150] In some embodiments, effector modules of the present disclosure may include one or more degrons to tune expression. As used herein, a "degron" refers to a minimal sequence within a protein that is sufficient for the recognition and the degradation by the proteolytic system. An important property of degrons is that they are transferrable, that is, appending a degron to a sequence confers degradation upon the sequence. In some embodiments, the degron may be appended to the destabilizing domains, the payload or both. Incorporation of the degron within the effector module of the disclosure, confers additional protein instability to the effector module and may be used to minimize basal expression. In some embodiments, the degron may be an N degron, a phospho degron, a heat inducible degron, a photosensitive degron,

an oxygen dependent degron. As a non-limiting example, the degron may be an Ornithine decarboxylase degron as described by Takeuchi et al. (Takeuchi J et al. (2008). Biochem J. 2008 Mar 1;410(2):401-7; the contents of which are incorporated by reference in their entirety). Other examples of degrons useful in the present disclosure include degrons described in International patent publication Nos. WO2017004022, WO2016210343, and WO2011062962; the contents of each of which are incorporated by reference in their entirety.

Immunotherapeutic Agents

[0151] In some embodiments, payloads of the present disclosure may be immunotherapeutic agents that induce immune responses in an organism. The immunotherapeutic agent may be a co-stimulatory molecule such as CD40L and fragments and variants thereof. In one embodiment, the immunotherapeutic agent induces an anti-cancer immune response in a cell, or in a subject.

[0152] In some embodiments, payloads of the present disclosure may comprise whole or a portion of human CD40L WT (SEQ ID NO. 6; Uniprot ID: P29965). As used herein the term “CD40L WT”, refers to the human wild-type CD40L protein sequence, which is defined as SEQ ID NO. 6, with accession number P29965, having the amino acid sequence: MIETYNQTSRPSAATGLPISMKIFMYLLTVFLITQMIGSALFAVYLHRRDLKIEDERNLH EDFVFMKTIQRCNTGERSLSLLNCEEIKSQFEGFVKDIMLNKEETKKENSFEMQKGDQNPQIAAHVISEASSKTTS VLQWAEKGYTMSNNLVLENGKQLTVKRQGLYYIYAQVTFCNREASSQAPFIASLCLKSPGRFERILLRAAN THSSAKPCGQQSIHLGGVFELQPGASVFN VTDPSQVSHGTGFTSFGLLKL.

Cytokines and co-stimulatory molecules

[0153] In some embodiments, payloads of the present disclosure may be cytokines, and fragments, variants, analogs and derivatives thereof, including but not limited to interleukins, tumor necrosis factors (TNFs), interferons (IFNs), TGF beta and chemokines. It is understood in the art that certain gene and/or protein nomenclature for the same gene or protein may be inclusive or exclusive of punctuation such as a dash “-” or symbolic such as Greek letters. Whether these are included or excluded herein, the meaning is not meant to be changed as would be understood by one of skill in the art. For example, CD40L, CD40 L and CD40LG refer to the same protein.

[0154] In some embodiments, cytokines of the present disclosure may be utilized to improve expansion, survival, persistence, and potency of immune cells such as CD8+TEM, natural killer cells and tumor infiltrating lymphocytes (TIL) cells used for immunotherapy. In other embodiments, T cells engineered with two or more DD regulated cytokines are utilized to provide kinetic control of T cell activation and tumor microenvironment remodeling. In one aspect, the present disclosure provides biocircuits and compositions to minimize toxicity related to cytokine therapy. Despite its success in mitigating tumor burden, systemic cytokine therapy often results in the development of severe dose limiting

side effects. Two factors contribute to the observed toxicity (a) Pleiotropism, wherein cytokines affect different cells types and sometimes produce opposing effects on the same cells depending on the context (b) Cytokines have short serum half-life and thus need to be administered at high doses to achieve therapeutic effects, which exacerbates the pleiotropic effects. In one aspect, cytokines of the present disclosure may be utilized to modulate cytokine expression in the event of adverse effects. In some embodiments, cytokines of the present disclosure may be designed to have prolonged life span or enhanced specificity to minimize toxicity.

[0155] In some embodiments, the immunotherapeutic agent may be CD40L (also referred to as CD154 and TNFSF5). CD40L belongs to the TNF super family and is primarily expressed on T cells. CD40L binds to CD40 expressed on a multitude of immune cells and initiates a cascade of cellular responses depending on the cell type. CD40L may also bind to $\alpha 5\beta 1$ integrin and $\alpha IIb\beta 3$ integrins. In some embodiments, the CD40L of the present disclosure may be engineered to bind to only one of its binding partners e.g. CD40. In some aspects, the CD40L described herein may be capable of binding to all of its cognate binding partners.

[0156] CD40L may bind to CD40 expressed in but not limited to Antigen Presenting Cells (APCs), B cells, monocytes, macrophages, platelets, neutrophils, dendritic cells, endothelial cells, and αSMC (smooth muscle cells). Binding of CD40L to CD40 expressed on dendritic cells may promote dendritic cell (DC) licensing. DCs may be converted to a functional state by an antigen-specific T helper cell in order to activate cytotoxic CD8⁺ T cells, a process referred to as DC licensing. CD40 engagement on DCs results in DC stimulation as evidenced by the surface expression of costimulatory and MHC molecules; proinflammatory cytokine production (e.g. IL12 and TNF) as well as epitope spreading.

[0157] In some embodiments, CD40L regulated by the biocircuit systems described herein may be utilized for the therapy of solid, immunogenic tumors. CD40L may improve the efficacy of solid tumor targeted T cells in immunogenic tumors by activating adaptive and innate immune responses in situ. Regulatable CD40L based biocircuit systems described herein may be desirable since the expression of the endogenous CD40L in T cells is transient. Further, the tumor microenvironment is rich in sheddases that may cleave the endogenous CD40L expressed by T cells. Exogenously expressed constitutive CD40L expression may result in liver toxicity and excessive B cell proliferation resulting in lymphomas (Schmitz et al (2006) Hepatology 44(2):430-9, Vonderheide et al. (2007) J Clin Oncol. 1;25(7):876-83, Sacco et al (2000) Cancer Gene Ther.;7(10):1299-306; the contents of each of which are incorporated by reference in their entirety). Constitutive (unregulated) expression may lead to CRS, thromboembolic syndromes, autoimmune

reactions, AICD due to hyper-immune stimulation and tumor angiogenesis, thereby creating a need for the biocircuits of the disclosure.

[0158] In some embodiments, the immunotherapeutic agent may be a multimer of CD40L molecules such as but not limited to a dimer, a trimer, a tetramer, a pentamer, a hexamer, a septamer, or a heptamer. In one embodiment, the CD40L may form a trimer. Multimerization of CD40L may enhance the signaling via the CD40L/CD40 axis. Binding of trimeric CD40L to CD40 may also initiate CD40 clustering and TRAF activation ultimately leading to NF- κ B activation.

[0159] CD40L described herein may be resistant to proteinases and sheddases such as those found in the tumor microenvironment e.g. ADAM10, or ADAM17. The heightened activity of ADAM17 in the tumor microenvironment has been associated with diminished signaling via the CD40/CD40L axis (see Lowe and Corvaia (2016), *Int J Cancer Clin Res*, 3:058; the contents of which are incorporated by reference in their entirety).

[0160] CD40L is involved in dendritic cell antigen presentation; production of IL12, and the generation of CD8+ T-cell immunity. Any of the methods described by Curren et al. to enhance antitumor efficacy of CARs using CD40L may be useful in the present disclosure (Curren et al. *Mol Ther.* 2015 Apr; 23(4): 769–778; the contents of which are incorporated by reference in their entirety). In one embodiment, agonistic CD40 antibodies may be useful in the present disclosure. CD40 monoclonal antibodies have shown clinical activity in the absence of disabling toxicity.

[0161] In one embodiment, the CD40L immunotherapeutic agent may be derived from UniProt ID: P29965 (also referred to herein as the “WT”). The payloads of the present disclosure may be a region or portion of CD40L. Non-limiting examples of regions of CD40L include but are not limited to amino acids 113-261 of UniProt ID: P29965, wherein the cytoplasmic domain, the transmembrane domain and a portion of the extracellular domain have been removed from UniProt ID: P29965 leaving a portion of the extracellular domain and the receptor binding domain intact. In one embodiment, the payload may be amino acids 14-261 of UniProt ID: P29965, which excludes the cytoplasmic tail of CD40L, thereby may potentially reduce internalization. In one aspect, the payload may be amino acids 14-261 of UniProt ID: P29965 with a deletion in amino acids S110-G116, which renders the CD40L resistant to cleavage by proteolytic enzymes.

[0162] In some embodiments, the mutations may be engineered within CD40L payload such that it does not bind to or bind with reduced affinity to CD40L endogenously expressed by cells described herein. CD40L is a type II transmembrane protein that forms a trimer on the cell surface. In some embodiments, trimerization occurs through the interaction of amino acid residues 47 – 261 of SEQ ID NO. 6. In some embodiments, residues within 47 – 261 of SEQ ID NO. 6 may be mutated in the CD40L payload to prevent trimerization (herein referred to as “trimerization mutants.” In some embodiments the residues within 116-261 of SEQ ID NO. 6 may be mutated. In some aspects, mutations may

allow selective trimerization such that a CD40L trimerization mutant may be able to bind to another CD40L trimerization mutant protein but not to a CD40L protein lacking the mutations. Trimerization mutations sites may be sites within the CD40L protein that are involved in the trimerization as determined by the crystal structure of the CD40L trimer. Positions within CD40L that may be mutated include but are not limited to amino acids at position 125, 170, 172, 224, 226 and/or 227 of SEQ ID NO. 6. In some embodiments, the mutations to CD40L payload to prevent its trimerization with the endogenous CD40L may include but are not limited to Y170G, Y172G, H224G, G226F, G226H, G226W, and/or G227F.

[0163] Sheddases e.g. ADAM10/17 present in the tumor microenvironment can cleave CD40L thereby preventing the successful activation of CD40 by CD40L. Analysis of the sequence of CD40L reveals an ADAM10/17 proteolytic cleavage site. In some embodiments, a deletion of amino acids 1-13 of CD40L may be engineered to reduce internalization. A deletion of amino acids 110-116 of CD40L may also be designed to remove the ADAM10/17 sites. Deletion or mutation of the methionine residue at amino acid position 113 of CD40L may also be utilized to reduce cleavage by ADAM10/17 enzymes. In one embodiment, a region or portion of the human CD40L protein may be replaced by the murine CD40L protein sequence to generate a CD40L protein that is resistant to cleavage by ADAM10/17. Any of the CD40L sequences aimed at reducing its shedding as described in US Patent Publication US20180085451A1 and/or US Patent US 7,495,090B2 may be used in the effector modules and biocircuits described herein (the contents of each of which are incorporated by reference in their entirety). CD40L may be tethered to the membrane using a transmembrane domain. In one embodiment, CD40L may be tethered to the membrane using CD8 derived domains such as but not limited to CD8 transmembrane domain, CD8 hinge domain and/or CD8 cytoplasmic tail.

[0164] In some embodiments the payload may be but are not limited to CD40L (SEQ ID NO. 5-6) and their coding sequences i.e. SEQ ID NO. 11-12 respectively.

Chimeric antigen receptors (CARs)

[0165] In some embodiments, biocircuits of the present disclosure may include chimeric antigen receptors (CARs) which when transduced into immune cells (e.g., T cells and NK cells), can re-direct the immune cells against the target (e.g., a tumor cell) which expresses a molecule recognized by the extracellular target moiety of the CAR.

[0166] As used herein, the term “chimeric antigen receptor (CAR)” refers to a synthetic receptor that mimics the TCR on the surface of T cells. In general, a CAR is composed of an extracellular targeting domain, a transmembrane domain/region and an intracellular signaling/activation domain. In a standard CAR receptor, the components: the extracellular targeting domain, transmembrane domain and intracellular signaling/activation domain, are linearly constructed as a single fusion protein. The extracellular region comprises a targeting domain/moiety (e.g., a scFv) that

recognizes a specific tumor antigen or other tumor cell-surface molecules. The intracellular region may contain a signaling domain of TCR complex (e.g., the signal region of CD3 ζ), and/or one or more costimulatory signaling domains, such as those from CD28, 4-1BB (CD137) and OX-40 (CD134). For example, a “first-generation CAR” only has the CD3 ζ signaling domain. In an effort to augment T-cell persistence and proliferation, costimulatory intracellular domains are added, giving rise to second generation CARs having a CD3 ζ signal domain plus one costimulatory signaling domain, and third generation CARs having CD3 ζ signal domain plus two or more costimulatory signaling domains. A CAR, when expressed by a T cell, endows the T cell with antigen specificity determined by the extracellular targeting moiety of the CAR. Recently, it is also desirable to add one or more elements such as homing and suicide genes to develop a more competent and safer architecture of CAR, so called the fourth-generation CAR.

[0167] In some embodiments, the immunotherapeutic agent of the effector module is a chimeric antigen receptor (CAR). The chimeric antigen may comprise an extracellular target moiety; a transmembrane domain; an intracellular signaling domain; and optionally, one or more co-stimulatory domains.

[0168] In some embodiments, the extracellular targeting domain is joined through the hinge (also called space domain or spacer) and transmembrane regions to an intracellular signaling domain. The hinge connects the extracellular targeting domain to the transmembrane domain which transverses the cell membrane and connects to the intracellular signaling domain. The hinge may need to be varied to optimize the potency of CAR expressing cells toward cancer cells due to the size of the target protein where the targeting moiety binds, and the size and affinity of the targeting domain itself. Upon recognition and binding of the targeting moiety to the target cell, the intracellular signaling domain leads to an activation signal for the CAR T cell, which is further amplified by the “second signal” from one or more intracellular costimulatory domains. The CAR T cell, once activated, can destroy the target cell.

[0169] In some embodiments, the CAR of the present disclosure may be split into two parts, each part is linked to a dimerizing domain, such that an input that triggers the dimerization promotes assembly of the intact functional receptor. Wu and Lim recently reported a split CAR in which the extracellular CD19 binding domain and the intracellular signaling element are separated and linked to the FKBP domain and the FRB* (T2089L mutant of FKBP-rapamycin binding) domain that heterodimerize in the presence of the rapamycin analog AP21967. The split receptor is assembled in the presence of AP21967 and together with the specific antigen binding, activates T cells (Wu et al., Science, 2015, 625(6258): aab4077).

[0170] In some embodiments, the CAR of the present disclosure may be designed as an inducible CAR. Sakemura et al recently reported the incorporation of a Tet-On inducible system to the CD19 CAR construct. The CD19 CAR is activated only in the presence of doxycycline (Dox). Sakemura reported that Tet-CD19CAR T cells in the presence of

Dox were equivalently cytotoxic against CD19+ cell lines and had equivalent cytokine production and proliferation upon CD19 stimulation, compared with conventional CD19CAR T cells (Sakemura et al., Cancer Immuno. Res., 2016, Jun 21, Epub ahead of print). In one example, the biocircuit may include a Tet-CAR. In another example, a Tet-CAR may be the payload of the CA2 effector module under the control of SREs (e.g., CA2 DDs) described herein. The dual systems provide more flexibility to turn-on and off the CAR expression in transduced T cells.

[0171] According to the present disclosure, the CAR may be a first-generation CAR, or a second-generation CAR, or a third-generation CAR, or a fourth-generation CAR. In some embodiments, the payload of the present disclosure may be a full CAR construct composed of the extracellular domain, the hinge and transmembrane domain and the intracellular signaling region. In other embodiments, a component of the full CAR construct including an extracellular targeting moiety, a hinge region, a transmembrane domain, an intracellular signaling domain, one or more co-stimulatory domain, and other additional elements that improve CAR architecture and functionality including but not limited to a leader sequence, a homing element and a safety switch, or the combination of such components may be included in the biocircuits.

Extracellular targeting domain/moiety

[0172] In accordance with the disclosure, the extracellular target moiety of a CAR may be any agent that recognizes and binds to a given target molecule, for example, a neoantigen on tumor cells, with high specificity and affinity. The target moiety may be an antibody and variants thereof that specifically binds to a target molecule on tumor cells, or a peptide aptamer selected from a random sequence pool based on its ability to bind to the target molecule on tumor cells, or a variant or fragment thereof that can bind to the target molecule on tumor cells, or an antigen recognition domain from native T-cell receptor (TCR) (e.g. CD4 extracellular domain to recognize HIV infected cells), or exotic recognition components such as a linked cytokine that leads to recognition of target cells bearing the cytokine receptor, or a natural ligand of a receptor.

[0173] In some embodiments, the targeting domain of a CAR may be a Ig NAR, a Fab fragment, a Fab' fragment, a F(ab)'2 fragment, a F(ab)'3 fragment, Fv, a single chain variable fragment (scFv), a bis-scFv, a (scFv)2, a minibody, a diabody, a tribody, a tetrabody, a disulfide stabilized Fv protein (dsFv), a unibody, a nanobody, or an antigen binding region derived from an antibody that specifically recognizes a target molecule, for example a tumor specific antigen (TSA). In one embodiment, the targeting moiety is a scFv. The scFv domain, when it is expressed on the surface of a CAR T cell and subsequently binds to a target protein on a cancer cell, is able to maintain the CAR T cell in proximity to the cancer cell and to trigger the activation of the T cell. A scFv can be generated using routine recombinant DNA technology techniques and is discussed in the present disclosure.

[0174] In one embodiment, the targeting moiety of the CAR may recognize CD19. CD19 is a well-known B cell surface molecule, which upon B cell receptor activation enhances B-cell antigen receptor induced signaling and expansion of B cell populations. CD19 is broadly expressed in both normal and neoplastic B cells. Malignancies derived from B cells such as chronic lymphocytic leukemia, acute lymphocytic leukemia and many non-Hodgkin lymphomas frequently retain CD19 expression. This near universal expression and specificity for a single cell lineage has made CD19 an attractive target for immunotherapies. Human CD19 has 14 exons wherein exon 1-4 encode the extracellular portion of the CD19, exon 5 encodes the transmembrane portion of CD19 and exons 6-14 encode the cytoplasmic tail. In one embodiment, the targeting moiety may comprise scFvs derived from the variable regions of the FMC63 antibody. FMC63 is an IgG2a mouse monoclonal antibody clone specific to the CD19 antigen that reacts with CD19 antigen on cells of the B lineage. The epitope of CD19 recognized by the FMC63 antibody is in exon 2 (Sotillo et al (2015) Cancer Discov ;5(12):1282-95; the contents of which are incorporated by reference in their entirety). In some embodiments, the targeting moiety of the CAR may be derived from the variable regions of other CD19 monoclonal antibody clones including but not limited to 4G7, SJ25C1, CVID3/429, CVID3/155, HIB19, and J3-119.

[0175] In one aspect, the extracellular target moiety may be an scFv derived from an antibody. In one aspect, the scFv may specifically bind to a CD19 antigen.

Intracellular signaling domains

[0176] The intracellular domain of a CAR fusion polypeptide, after binding to its target molecule, transmits a signal to the effector immune cell, activating at least one of the normal effector functions of effector immune cells, including cytolytic activity (e.g., cytokine secretion) or helper activity. Therefore, the intracellular domain comprises an "intracellular signaling domain" of a T cell receptor (TCR).

[0177] In some aspects, the entire intracellular signaling domain can be employed. In other aspects, a truncated portion of the intracellular signaling domain may be used in place of the intact chain as long as it transduces the effector function signal.

[0178] In some embodiments, the intracellular signaling domain of the present disclosure may contain signaling motifs which are known as immunoreceptor tyrosine-based activation motifs (ITAMs). Examples of ITAM containing cytoplasmic signaling sequences include those derived from TCR CD3zeta, FcR gamma, FcR beta, CD3 gamma, CD3 delta, CD3 epsilon, CD5, CD22, CD79a, CD79b, and CD66d. In one example, the intracellular signaling domain is a CD3 zeta (CD3ζ) signaling domain.

[0179] In some embodiments, the intracellular region of the present disclosure further comprises one or more costimulatory signaling domains which provide additional signals to the effector immune cells. These costimulatory

signaling domains, in combination with the signaling domain can further improve expansion, activation, memory, persistence, and tumor-eradicating efficiency of CAR engineered immune cells (e.g., CAR T cells). In some cases, the costimulatory signaling region contains 1, 2, 3, or 4 cytoplasmic domains of one or more intracellular signaling and /or costimulatory molecules. The costimulatory signaling domain may be the intracellular/cytoplasmic domain of a costimulatory molecule, including but not limited to CD2, CD7, CD27, CD28, 4-1BB (CD137), OX40 (CD134), CD30, CD40, ICOS (CD278), GITR (glucocorticoid-induced tumor necrosis factor receptor), LFA-1 (lymphocyte function-associated antigen-1), LIGHT, NKG2C, B7-H3. In one example, the costimulatory signaling domain is derived from the cytoplasmic domain of CD28. In another example, the costimulatory signaling domain is derived from the cytoplasmic domain of 4-1BB (CD137). In another example, the co-stimulatory signaling domain may be an intracellular domain of GITR as taught in U.S. Pat. NO. 9, 175, 308; the contents of which are incorporated herein by reference in its entirety.

Transmembrane domains and hinge regions

[0180] In some embodiments, the CAR of the present disclosure may comprise a transmembrane domain. As used herein, the term “Transmembrane domain (TM)” refers broadly to an amino acid sequence of about 15 residues in length which spans the plasma membrane. More preferably, a transmembrane domain includes at least 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, or 45 amino acid residues and spans the plasma membrane. In some embodiments, the transmembrane domain of the present disclosure may be derived either from a natural or from a synthetic source. The transmembrane domain of a CAR may be derived from any naturally membrane-bound or transmembrane protein. For example, the transmembrane region may be derived from (i.e. comprise at least the transmembrane region(s) of) the alpha, beta or zeta chain of the T-cell receptor, CD3 epsilon, CD4, CD5, CD8, CD8 α , CD9, CD16, CD22, CD33, CD28, CD37, CD45, CD64, CD80, CD86, CD134, CD137, CD152, or CD154. In some embodiments, the transmembrane domain of the present disclosure may be selected from the group consisting of a CD8 α transmembrane domain, a CD4 transmembrane domain, a CD 28 transmembrane domain, a CTLA-4 transmembrane domain, a PD-1 transmembrane domain, and a human IgG4 Fc region.

[0181] Alternatively, the transmembrane domain of the present disclosure may be synthetic. In some aspects, the synthetic sequence may comprise predominantly hydrophobic residues such as leucine and valine.

[0182] In some embodiments, the transmembrane domain of the present disclosure may be selected from the group consisting of a CD8 α transmembrane domain, a CD4 transmembrane domain, a CD 28 transmembrane domain, a CTLA-4 transmembrane domain, a PD-1 transmembrane domain, and a human IgG4 Fc region. As non-limiting examples, the transmembrane domain may be a CTLA-4 transmembrane domain comprising the amino acid sequences of SEQ ID NOs. 1-5 of International Patent Publication NO. WO2014/100385; and a PD-1 transmembrane domain comprising the amino

acid sequences of SEQ ID NOs. 6-8 of International Patent Publication NO. WO2014100385; the contents of each of which are incorporated herein by reference in their entirety.

[0183] In some embodiments, the CAR of the present disclosure may comprise an optional hinge region (also called spacer). A hinge sequence is a short sequence of amino acids that facilitates flexibility of the extracellular targeting domain that moves the target binding domain away from the effector cell surface to enable proper cell/cell contact, target binding and effector cell activation (Patel et al., Gene Therapy, 1999; 6: 412-419). The hinge sequence may be positioned between the targeting moiety and the transmembrane domain.

[0184] In some embodiments, the CAR of the present disclosure may comprise one or more linkers between any of the domains of the CAR. The linker may be between 1-30 amino acids long.

[0185] In some embodiments, the components including the targeting moiety, transmembrane domain and intracellular signaling domains of the present invention may be constructed in a single fusion polypeptide.

[0186] In one embodiment, the CAR construct comprises a CD19 scFv (e.g., CAT13.1E10 or FMC63), a CD8 α spacer or transmembrane domain, and a 4-1BB and CD3 ζ endodomain. These constructs with CAT13.1E10 may have increased proliferation after stimulation in vitro, increased cytotoxicity against the CD19+ targets, and increased effector and target interactions as compared to constructs with FMC63.

[0187] In some embodiments, the payload of the disclosure may be any of the co-stimulatory molecules and/or intracellular domains described herein. In some embodiments, one or more co-stimulatory molecules, each under the control of different SRE may be used in the present disclosure. SRE regulated co-stimulatory molecules may also be expressed in conjunction with a first-generation CAR, a second-generation CAR, a third generation CAR, a fourth generation, or any other CAR design described herein.

Tandem CAR (TanCAR)

[0188] In some embodiments, the CAR of the present disclosure may be a tandem chimeric antigen receptor (TanCAR) which is able to target two, three, four, or more tumor specific antigens. In some aspects, the CAR is a bispecific TanCAR including two targeting domains which recognize two different TSAs on tumor cells. The bispecific CAR may be further defined as comprising an extracellular region comprising a targeting domain (e.g., an antigen recognition domain) specific for a first tumor antigen and a targeting domain (e.g., an antigen recognition domain) specific for a second tumor antigen. In other aspects, the CAR is a multispecific TanCAR that includes three or more targeting domains configured in a tandem arrangement. The space between the targeting domains in the TanCAR may be between about 5 and about 30 amino acids in length, for example, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 and 30 amino acids.

Split CAR

[0189] In some embodiments, the components including the targeting moiety, transmembrane domain and intracellular signaling domains of the present disclosure may be split into two or more parts such that it is dependent on multiple inputs that promote assembly of the intact functional receptor. In one embodiment, the split synthetic CAR system can be constructed in which the assembly of an activated CAR receptor is dependent on the binding of a ligand to the SRE (e.g. a small molecule) and a specific antigen to the targeting moiety. As a non-limiting example, the split CAR consists of two parts that assemble in a small molecule-dependent manner; one part of the receptor features an extracellular antigen binding domain (e.g. scFv) and the other part has the intracellular signaling domains, such as the CD3 ζ intracellular domain.

[0190] In other aspects, the split parts of the CAR system can be further modified to increase signal. In one example, the second part of cytoplasmic fragment may be anchored to the plasma membrane by incorporating a transmembrane domain (e.g., CD8 α transmembrane domain) to the construct. An additional extracellular domain may also be added to the second part of the CAR system, for instance an extracellular domain that mediates homodimerization. These modifications may increase receptor output activity, i.e., T cell activation.

[0191] In some aspects, the two parts of the split CAR system contain heterodimerization domains that conditionally interact upon binding of a heterodimerizing small molecule. As such, the receptor components are assembled in the presence of the small molecule, to form an intact system which can then be activated by antigen engagement. Any known heterodimerizing components can be incorporated into a split CAR system. Other small molecule dependent heterodimerization domains may also be used, including, but not limited to, gibberellin-induced dimerization system (GID1-GAI), trimethoprim-SLF induced ecDHFR and FKBP dimerization (Czlapinski et al., J Am Chem Soc., 2008, 130(40): 13186-13187) and ABA (abscisic acid) induced dimerization of PP2C and PYL domains (Cutler et al., Annu Rev Plant Biol. 2010, 61: 651-679). The dual regulation using inducible assembly (e.g., ligand dependent dimerization) and degradation (e.g., destabilizing domain induced CAR degradation) of the split CAR system may provide more flexibility to control the activity of the CAR modified T cells.

Switchable CAR

[0192] In some embodiments, the CAR of the disclosure may be a switchable CAR. Juillerat et al (Juillerat et al., Sci. Rep., 2016, 6: 18950; the contents of which are incorporated herein by reference in their entirety) recently reported controllable CARs that can be transiently switched on in response to a stimulus (e.g. a small molecule). In this CAR design, a system is directly integrated in the hinge domain that separate the scFv domain from the cell membrane domain in the CAR. Such system is possible to split or combine different key functions of a CAR such as activation and

costimulation within different chains of a receptor complex, mimicking the complexity of the TCR native architecture. This integrated system can switch the scFv and antigen interaction between on/off states controlled by the absence/presence of the stimulus.

Reversible CAR

[0193] In other embodiments, the CAR of the disclosure may be a reversible CAR system. In this CAR architecture, a LID domain (ligand-induced degradation) is incorporated into the CAR system. The CAR can be temporarily down-regulated by adding a ligand of the LID domain. The combination of LID and DD mediated regulation provides tunable control of continually activated CAR T cells, thereby reducing CAR mediated tissue toxicity.

Activation-conditional CAR

[0194] In some embodiments, the biocircuits described herein may include an activation-conditional chimeric antigen receptor, which is only expressed in an activated immune cell. The expression of the CAR may be coupled to activation conditional control region which refers to one or more nucleic acid sequences that induce the transcription and/or expression of a sequence e.g. a CAR under its control. Such activation conditional control regions may be promoters of genes that are upregulated during the activation of the effector immune cell e.g. IL2 promoter or NFAT binding sites. In some embodiments, activation of the immune cell may be achieved by a constitutively expressed CAR (International Publication NO. WO2016126608; the contents of which are incorporated herein by reference in their entirety).

Polynucleotides

[0195] Biocircuit components including effector modules, their SREs and payloads, may be nucleic acid-based. The term “nucleic acid,” in its broadest sense, includes any compound and/or substance that comprise a polymer of nucleotides, e.g., linked nucleosides. These polymers are often referred to as polynucleotides. Exemplary nucleic acids or polynucleotides of the disclosure include, but are not limited to, ribonucleic acids (RNAs), deoxyribonucleic acids (DNAs), threose nucleic acids (TNAs), glycol nucleic acids (GNAs), peptide nucleic acids (PNAs), locked nucleic acids (LNAs, including LNA having a β -D-ribo configuration, α -LNA having an α -L-ribo configuration (a diastereomer of LNA), 2'-amino-LNA having a 2'-amino functionalization, and 2'-amino- α -LNA having a 2'-amino functionalization) or hybrids thereof.

[0196] In some embodiments, the nucleic acid molecule is a messenger RNA (mRNA). As used herein, the term “messenger RNA” (mRNA) refers to any polynucleotide which encodes a polypeptide of interest and which is capable of being translated to produce the encoded polypeptide of interest in vitro, in vivo, in situ or ex vivo. Polynucleotides described herein may be mRNA or any nucleic acid molecule and may or may not be chemically modified.

[0197] Traditionally, the basic components of an mRNA molecule include at least a coding region, a 5'UTR, a 3'UTR, a 5' cap and a poly-A tail. Building on this wild type modular structure, the present disclosure expands the scope of functionality of traditional mRNA molecules by providing payload constructs which maintain a modular organization, but which comprise one or more structural and/or chemical modifications or alterations which impart useful properties to the polynucleotide, for example tenability of function. As used herein, a “structural” feature or modification is one in which two or more linked nucleosides are inserted, deleted, duplicated, inverted or randomized in a polynucleotide without significant chemical modification to the nucleosides themselves. Because chemical bonds will necessarily be broken and reformed to effect a structural modification, structural modifications are of a chemical nature and hence are chemical modifications. However, structural modifications will result in a different sequence of nucleotides. For example, the polynucleotide “ATCG” may be chemically modified to “AT-5meC-G”. The same polynucleotide may be structurally modified from “ATCG” to “ATCCCG”. Here, the dinucleotide “CC” has been inserted, resulting in a structural modification to the polynucleotide.

[0198] In some embodiments, polynucleotides of the present disclosure may harbor 5'UTR sequences which play a role in translation initiation. 5'UTR sequences may include features such as Kozak sequences which are commonly known to be involved in the process by which the ribosome initiates translation of genes, Kozak sequences have the consensus XCCR(A/G) CCAUG, where R is a purine (adenine or guanine) three bases upstream of the start codon (AUG) and X is any nucleotide. In one embodiment, the Kozak sequence is ACCGCC. By engineering the features that are typically found in abundantly expressed genes of target cells or tissues, the stability and protein production of the polynucleotides of the disclosure can be enhanced.

[0199] Further provided are polynucleotides, which may contain an internal ribosome entry site (IRES) which play an important role in initiating protein synthesis in the absence of 5' cap structure in the polynucleotide. An IRES may act as the sole ribosome binding site or may serve as one of the multiple binding sites. Polynucleotides of the disclosure containing more than one functional ribosome binding site may encode several peptides or polypeptides that are translated independently by the ribosomes giving rise to bicistronic and/or multicistronic nucleic acid molecules.

[0200] In one embodiment, polynucleotides of the present disclosure may encode variant polypeptides which have a certain identity with a reference polypeptide sequence. As used herein, a “reference polypeptide sequence” refers to a starting polypeptide sequence. Reference sequences may be wild type sequences or any sequence to which reference is made in the design of another sequence.

[0201] The term “identity” as known in the art, refers to a relationship between two or more sequences, as determined by comparing the sequences. In the art, identity also means the degree of sequence relatedness between

sequences, as determined by the number of matches between strings of two or more residues (amino acid or nucleic acid). Identity measures the percent of identical matches between two or more sequences with gap alignments (if any) addressed by a particular mathematical model or computer program (i.e., “algorithms”). Identity of related sequences can be readily calculated by known methods. Such methods include, but are not limited to, those described in Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part 1, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M. Stockton Press, New York, 1991; and Carillo et al., SIAM J. Applied Math. 48, 1073 (1988).

[0202] In some embodiments, the variant sequence may have the same or a similar activity as the reference sequence. Alternatively, the variant may have an altered activity (e.g., increased or decreased) relative to a reference sequence. Generally, variants of a particular polynucleotide or polypeptide of the disclosure will have at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% but less than 100% sequence identity to that particular reference polynucleotide or polypeptide as determined by sequence alignment programs and parameters described herein and known to those skilled in the art. Such tools for alignment include those of the BLAST suite (Stephen F. Altschul, Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.)

Chemical modifications to polynucleotides

[0203] According to the present disclosure, the terms “modification” or, as appropriate, “modified” polynucleotides refer to modification with respect to A, G, U (T in DNA) or C nucleotides.

[0204] Modifications of the polynucleotides of the disclosure may be on the nucleoside base and/or sugar portion of the nucleosides which comprise the polynucleotide. In some embodiments, multiple modifications are included in the modified nucleic acid or in one or more individual nucleoside or nucleotide. For example, modifications to a nucleoside may include one or more modifications to the nucleobase and the sugar. Modifications to the polynucleotides of the present disclosure may include any of those taught in, for example, International Publication WO2013052523, the contents of which are incorporated herein by reference in its entirety.

[0205] As described herein “nucleoside” is defined as a compound containing a sugar molecule (e.g., a pentose or ribose) or a derivative thereof in combination with an organic base (e.g., a purine or pyrimidine) or a derivative thereof

(also referred to herein as “nucleobase”). As described herein, “nucleotide” is defined as a nucleoside including a phosphate group.

[0206] The modified nucleotides, which may be incorporated into a polynucleotide can be modified on the internucleoside linkage (e.g., phosphate backbone). Herein, in the context of the polynucleotide backbone, the phrases “phosphate” and “phosphodiester” are used interchangeably. Backbone phosphate groups can be modified by replacing one or more of the oxygen atoms with a different substituent. Further, the modified nucleosides and nucleotides can include the wholesale replacement of an unmodified phosphate moiety with another internucleoside linkage. Examples of modified phosphate groups include, but are not limited to, phosphorothioate, phosphoroselenates, boranophosphates, boranophosphate esters, hydrogen phosphonates, phosphoramidates, phosphorodiamidates, alkyl or aryl phosphonates, and phosphotriesters. Phosphorodithioates have both non-linking oxygens replaced by sulfur. The phosphate linker can also be modified by the replacement of a linking oxygen with nitrogen (bridged phosphoramidates), sulfur (bridged phosphorothioates), and carbon (bridged methylene-phosphonates). Other modifications which may be used are taught in, for example, International Application WO2013052523, the contents of which are incorporated herein by reference in their entirety.

[0207] Different sugar modifications, nucleotide modifications, and/or internucleoside linkages (e.g., backbone structures) may exist at various positions in the polynucleotide. One of ordinary skill in the art will appreciate that the nucleotide analogs or other modification(s) may be located at any position(s) of a polynucleotide such that the function of the polynucleotide is not substantially decreased. A modification may also be a 5' or 3' terminal modification. The polynucleotide may contain from about 1% to about 100% modified nucleotides (either in relation to overall nucleotide content, or in relation to one or more types of nucleotide, i.e. any one or more of A, G, U or C) or any intervening percentage (e.g., from 1% to 20%, from 1% to 25%, from 1% to 50%, from 1% to 60%, from 1% to 70%, from 1% to 80%, from 1% to 90%, from 1% to 95%, from 10% to 20%, from 10% to 25%, from 10% to 50%, from 10% to 60%, from 10% to 70%, from 10% to 80%, from 10% to 90%, from 10% to 95%, from 10% to 100%, from 20% to 25%, from 20% to 50%, from 20% to 60%, from 20% to 70%, from 20% to 80%, from 20% to 90%, from 20% to 95%, from 20% to 100%, from 50% to 60%, from 50% to 70%, from 50% to 80%, from 50% to 90%, from 50% to 95%, from 50% to 100%, from 70% to 80%, from 70% to 90%, from 70% to 95%, from 70% to 100%, from 80% to 90%, from 80% to 95%, from 80% to 100%, from 90% to 95%, from 90% to 100%, and from 95% to 100%).

[0208] In some embodiments, the polynucleotide includes a modified pyrimidine or purine. In some embodiments, the pyrimidine or purine in the polynucleotide molecule may be replaced with from about 1% to about 100% of a modified uracil or modified uridine (e.g., from 1% to 20%, from 1% to 25%, from 1% to 50%, from 1% to 60%, from 1%

to 70%, from 1% to 80%, from 1% to 90%, from 1% to 95%, from 10% to 20%, from 10% to 25%, from 10% to 50%, from 10% to 60%, from 10% to 70%, from 10% to 80%, from 10% to 90%, from 10% to 95%, from 10% to 100%, from 20% to 25%, from 20% to 50%, from 20% to 60%, from 20% to 70%, from 20% to 80%, from 20% to 90%, from 20% to 95%, from 20% to 100%, from 50% to 60%, from 50% to 70%, from 50% to 80%, from 50% to 90%, from 50% to 95%, from 50% to 100%, from 70% to 80%, from 70% to 90%, from 70% to 95%, from 70% to 100%, from 80% to 90%, from 80% to 95%, from 80% to 100%, from 90% to 95%, from 90% to 100%, and from 95% to 100% of a modified pyrimidine or purine.

[0209] In some embodiments, the polynucleotides may comprise two or more effector module component sequences which are in a pattern such as ABABAB or AABBAABBAABB or ABCABCABC or variants thereof repeated once, twice, or more than three times. In these patterns, each letter, A, B, or C represent a different effector module component.

[0210] In yet another embodiment, the polynucleotides may comprise two or more effector module component sequences with each component having one or more sequences. As a non-limiting example, the sequences may be in a pattern such as ABABAB or AABBAABBAABB or ABCABCABC or variants thereof repeated once, twice, or more than three times in each of the regions. As another non-limiting example, the sequences may be in a pattern such as ABABAB or AABBAABBAABB or ABCABCABC or variants thereof repeated once, twice, or more than three times across the entire polynucleotide. In these patterns, each letter, A, B, or C represent a different sequence or component.

Codon Selection

[0211] In some embodiments, one or more codons of the polynucleotides of the present disclosure may be replaced with other codons encoding the native amino acid sequence to tune the expression of the SREs, through a process referred to as codon selection. Since mRNA codon, and tRNA anticodon pools tend to vary among organisms, cell types, sub cellular locations and over time, the codon selection described herein is a spatiotemporal (ST) codon selection.

[0212] In some embodiments of the disclosure, certain polynucleotide features may be codon optimized. Codon optimization refers to a process of modifying a nucleic acid sequence for enhanced expression in the host cell by replacing at least 1, 2, 3, 4, 5, 10, 15, 20, 25, 50 or more codons of the native sequence with codons that are most frequently used in the genes of that host cell while maintaining the native amino acid sequence. Codon usage may be measured using the Codon Adaptation Index (CAI) which measures the deviation of a coding polynucleotide sequence from a reference gene set. Codon usage tables are available at the Codon Usage Database (<http://www.kazusa.or.jp/codon/>) and the CAI can be calculated by EMBOSS CAI program (<http://emboss.sourceforge.net/>). Codon optimization methods are known in the art and may be useful in efforts to

achieve one or more of several goals. These goals include to match codon frequencies in target and host organisms to ensure proper folding, bias nucleotide content to alter stability or reduce secondary structures, minimize tandem repeat codons or base runs that may impair gene construction or expression, customize transcriptional and translational control regions, insert or remove protein signaling sequences, remove/add post translation modification sites in encoded protein (e.g. glycosylation sites), add, remove or shuffle protein domains, insert or delete restriction sites, modify ribosome binding sites and degradation sites, to adjust translational rates to allow the various domains of the protein to fold properly, or to reduce or eliminate problem secondary structures within the polynucleotide. In one embodiment, a polynucleotide sequence or portion thereof is codon optimized using optimization algorithms. Codon options for each amino acid are well-known in the art as are various species table for optimizing for expression in that particular species.

[0213] In some embodiments of the disclosure, certain polynucleotide features may be codon optimized. For example, a preferred region for codon optimization may be upstream (5') or downstream (3') to a region which encodes a polypeptide. These regions may be incorporated into the polynucleotide before and/or after codon optimization of the payload encoding region or open reading frame (ORF).

[0214] After optimization (if desired), the polynucleotides components are reconstituted and transformed into a vector such as, but not limited to, plasmids, viruses, cosmids, and artificial chromosomes.

[0215] The stop codon of the polynucleotides of the present disclosure may be modified to include sequences and motifs to alter the expression levels of the SREs, payloads and effector modules of the present disclosure. Such sequences may be incorporated to induce stop codon readthrough, wherein the stop codon may specify amino acids e.g. selenocysteine or pyrrolysine. In other instances, stop codons may be skipped altogether to resume translation through an alternate open reading frame. Stop codon read through may be utilized to tune the expression of components of the effector modules at a specific ratio (e.g. as dictated by the stop codon context). Examples of preferred stop codon motifs include UGAN, UAAN, and UAGN, where N is either C or U.

[0216] Suppression of termination occurs during translation of many viral mRNAs as a means of generating a second protein with extended carboxy terminus. In retroviruses, gag and pol genes are encoded by a single mRNA and separated by an amber termination codon UAG. Translational suppression of the amber codon allows synthesis of the gag pol precursor. Translation suppression is mediated by suppressor tRNAs that can recognize termination codons and insert a specific amino acid. In some embodiments, effector modules described herein may incorporate amber termination codons. Such codons may be used in lieu of or in addition to IRES and p2A sequences in bicistronic constructs. Stop codon read through may be combined with P2A to obtain low level expression of a downstream gene. In some

embodiments, the amber stop codons may be combined with tRNA expression or amino-acyl tRNA synthetase for further control.

Conjugates

[0217] It is contemplated by the present disclosure that the compositions of the present invention may be complexed, conjugated or combined with one or more homologous or heterologous molecules. As used herein, the term “homologous molecule” refers to a molecule which is similar in at least one of structure or function relative to a starting molecule while a “heterologous molecule” is one that differs in at least one of structure or function relative to a starting molecule. Structural homologs are therefore molecules which may be substantially structurally similar. In some embodiments, such homologs may be identical. Functional homologs are molecules which may be substantially functionally similar. In some embodiments, such homologs may be identical.

[0218] Pharmaceutical compositions, biocircuits, biocircuit components, effector modules including their SREs or payloads of the present disclosure may comprise conjugates. Such conjugates of the disclosure may include naturally occurring substances or ligands, such as proteins (e.g., human serum albumin (HSA), low-density lipoprotein (LDL), high-density lipoprotein (HDL), or globulin); carbohydrates (e.g., a dextran, pullulan, chitin, chitosan, inulin, cyclodextrin or hyaluronic acid); or lipids. Conjugates may also be recombinant or synthetic molecules, such as synthetic polymers, e.g., synthetic polyamino acids, an oligonucleotide (e.g. an aptamer). Examples of polyamino acids may include polylysine (PLL), poly L-aspartic acid, poly L-glutamic acid, styrene-maleic acid anhydride copolymer, poly(L-lactide-co-glycolide) copolymer, divinyl ether-maleic anhydride copolymer, N-(2-hydroxypropyl) methacrylamide copolymer (HMPA), polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyurethane, poly (2-ethylacrylic acid), N-isopropylacrylamide polymers, or polyphosphazene. Example of polyamines include: polyethylenimine, polylysine (PLL), spermine, spermidine, polyamine, pseudopeptide-polyamine, peptidomimetic polyamine, dendrimer polyamine, arginine, amidine, protamine, cationic lipid, cationic porphyrin, quaternary salt of a polyamine, or an alpha helical peptide.

[0219] In some embodiments, conjugates may also include targeting groups. As used herein, the term “targeting group” refers to a functional group or moiety attached to an agent that facilitates localization of the agent to a desired region, tissue, cell and/or protein. Such targeting groups may include but are not limited to cell or tissue targeting agents or groups (e.g. lectins, glycoproteins, lipids, proteins, an antibody that binds to a specified cell type such as a kidney cell or other cell type). In some embodiments, targeting groups may comprise thyrotropins, melanotropins, lectins, glycoproteins, surfactant protein A, mucin carbohydrates, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-gulcosamine, multivalent mannose, multivalent fucose, glycosylated polyaminoacids,

multivalent galactose, transferrin, bisphosphonate, polyglutamate, polyaspartate, lipids, cholesterol, steroids, bile acids, folates, vitamin B12, biotin, an RGD peptide, an RGD peptide mimetic or an aptamer.

[0220] In some embodiments, targeting groups may be proteins, e.g., glycoproteins, or peptides, e.g., molecules having a specific affinity for a co-ligand, or antibodies e.g., an antibody, that binds to a specified cell type such as a cancer cell, endothelial cell, or bone cell. Targeting groups may also comprise hormones and/or hormone receptors.

[0221] In some embodiments, targeting groups may be any ligand capable of targeting specific receptors. Examples include, without limitation, folate, GalNAc, galactose, mannose, mannose-6-phosphate, aptamers, integrin receptor ligands, chemokine receptor ligands, transferrin, biotin, serotonin receptor ligands, PSMA, endothelin, GCPII, somatostatin, LDL, and HDL ligands. In some embodiments, targeting groups are aptamers. Such aptamers may be unmodified or comprise any combination of modifications disclosed herein.

[0222] In still other embodiments, pharmaceutical compositions, biocircuits, biocircuit components, effector modules including their SREs or payloads of the present disclosure may be covalently conjugated to cell penetrating polypeptides. In some embodiments, cell-penetrating peptides may also include signal sequences. In some embodiments, conjugates of the disclosure may be designed to have increased stability, increased cell transfection and/or altered biodistribution (e.g., targeted to specific tissues or cell types.)

[0223] In some embodiments, conjugating moieties may be added to pharmaceutical compositions, biocircuits, biocircuit components, effector modules including their SREs or payloads of the present disclosure such that they allow the attachment of detectable labels to targets for clearance. Such detectable labels include, but are not limited to biotin labels, ubiquitins, fluorescent molecules, human influenza hemagglutinin (HA), c-myc, histidine (His), flag, glutathione S-transferase (GST), V5 (a paramyxovirus of simian virus 5 epitope), biotin, avidin, streptavidin, horse radish peroxidase (HRP) and digoxigenin.

[0224] In some embodiments, pharmaceutical compositions, biocircuits, biocircuit components, effector modules including their SREs or payloads of the present disclosure may be combined with one another or other molecules in the treatment of diseases and/or conditions.

Additional Effector Module Features

[0225] The effector module of the present disclosure may further comprise a signal sequence which regulates the distribution of the payload of interest, a cleavage and/or processing feature which facilitate cleavage of the payload from the effector module construct, a targeting and/or penetrating signal which can regulate the cellular localization of the effector module, a tag, and/or one or more linker sequences which link different components of the effector module.

Signal sequences

[0226] In addition to the SRE and payload region, effector modules of the disclosure may further comprise one or more additional features such as one or more signal sequences.

[0227] Signal sequences (sometimes referred to as signal peptides, targeting signals, target peptides, localization sequences, transit peptides, leader sequences or leader peptides) direct proteins (e.g., the effector module of the present disclosure) to their designated cellular and/or extracellular locations. Protein signal sequences play a central role in the targeting and translocation of nearly all secreted proteins and many integral membrane proteins.

[0228] A signal sequence is a short (5-30 amino acids long) peptide present at the N-terminus of the majority of newly synthesized proteins that are destined towards a particular location. Signal sequences can be recognized by signal recognition particles (SRPs) and cleaved using type I and type II signal peptide peptidases. Signal sequences derived from human proteins can be incorporated as a regulatory module of the effector module to direct the effector module to a particular cellular and/or extracellular location. These signal sequences are experimentally verified and can be cleaved (Zhang Z. and Henzel W.J.; "Signal peptide prediction based on analysis of experimentally verified cleavage sites."; *Protein Sci.* 2004, 13:2819-2824).

[0229] In some embodiments, a signal sequence may be, although not necessarily, located at the N-terminus or C-terminus of the effector module, and may be, although not necessarily, cleaved off the desired effector module to yield a "mature" payload.

[0230] In some embodiments, the signal sequence used herein may exclude the methionine at the position 1 of amino acid sequence of the signal sequence. This may be referred to as an M1del mutation.

[0231] In addition to signal sequences naturally occurring such as from a secreted protein, a signal sequence may be a variant modified from a known signal sequence of a protein. For example, U.S. Pat. NOs.: 8,258,102 and 9,133,265 to Sleep disclose a modified albumin signal sequence having a secretion signal and an additional X1-X2-X3-X4-X5-motif which can increase protein secretion; U.S. Pat. NO.: 9,279,007 to Do discloses signal sequences of modified fragments of human immunoglobulin heavy chain binding protein (Bip) that can enhance protein expression and secretion; U.S. Pat. NO.: 8,148,494 to Leonhartsberger et al., discloses a signal peptide with a cleavage site that can be fused with a recombinant protein; the contents of each of which are incorporated by reference in their entirety.

[0232] In some instances, the secreted signal sequences may be cytokine signal sequences such as, but not limited to, IL2 signal sequence or a p40 signal sequence.

[0233] In some instances, signal sequences directing the payload of interest to the surface membrane of the target cell may be used. Expression of the payload on the surface of the target cell may be useful to limit the diffusion of the payload to non-target *in vivo* environments, thereby potentially improving the safety profile of the payloads.

Additionally, the membrane presentation of the payload may allow for physiologically and qualitative signaling as well as stabilization and recycling of the payload for a longer half-life. Membrane sequences may be the endogenous signal sequence of the N terminal component of the payload of interest. Optionally, it may be desirable to exchange this sequence for a different signal sequence. Signal sequences may be selected based on their compatibility with the secretory pathway of the cell type of interest so that the payload is presented on the surface of the T cell. In some embodiments, the signal sequence may be IgE signal sequence, CD8a signal sequence (also referred to as CD8a leader), or IL15Ra signal sequence (also referred to as IL15Ra leader) or M1del CD8a signal sequence (also referred to as M1del CD8 leader sequence).

[0234] Other signal sequence variants may be used in the present effector module may include those discussed in U.S. patent application publication NOs.: 2007/0141666; PCT patent application publication NOs.: 1993/018181; the contents of each of which are incorporated herein by reference in their entirety.

[0235] Other examples of signal sequences variant may be a modified signal sequence discussed in U.S. Pat. NOs. 8,148,494; 8,258,102; 9,133,265; 9,279,007; and U.S. patent application publication NO. 20070141666; and International patent application publication NO. WO1993018181; the contents of each of which are incorporated herein by reference in their entirety.

[0236] In other examples, a signal sequence may be a heterogeneous signal sequence from other organisms such as virus, yeast and bacteria, which can direct an effector module to a particular cellular site, such as a nucleus (e.g., EP 1209450). Other examples may include Aspartic Protease (NSP24) signal sequences from *Trichoderma* that can increase secretion of fused protein such as enzymes (e.g., U. S. Pat. NO. 8,093,016 to Cervin and Kim), bacterial lipoprotein signal sequences (e.g., PCT application publication NO. WO199109952 to Lau and Rioux), *E.coli* enterotoxin II signal peptides (e.g., U.S. Pat. NO. 6,605,697 to Kwon et al.), *E.coli* secretion signal sequence (e.g., U.S. patent publication NO. US2016090404 to Malley et al.), a lipase signal sequence from a methylotrophic yeast (e.g., U.S. Pat. NO. 8,975,041), and signal peptides for DNases derived from *Coryneform bacteria* (e.g., U.S. Pat. NO. 4,965,197); the contents of each of which are incorporated herein by reference in their entirety.

[0237] Signal sequences may also include nuclear localization signals (NLSs), nuclear export signals (NESs), polarized cell tubulo-vesicular structure localization signals (See, e.g., U.S. Pat. NO. 8,993,742; Cour et al., *Nucleic Acids Res.* 2003, 31(1): 393-396; the contents of each of which are incorporated herein by reference in their entirety), extracellular localization signals, signals to subcellular locations (e.g. lysosome, endoplasmic reticulum, golgi, mitochondria, plasma membrane and peroxisomes, etc.) (See, e.g., U.S. Pat. NO. 7,396,811; and Negi et al., *Database*, 2015, 1-7; the contents of each of which are incorporated herein by reference in their entirety).

Cleavage sites

[0238] In some embodiments, the effector module comprises a cleavage and/or processing feature.

[0239] The effector module of the present disclosure may include at least one protein cleavage signal/site. The protein cleavage signal/site may be located at the N-terminus, the C-terminus, at any space between the N- and the C-termini such as, but not limited to, half-way between the N- and C-termini, between the N-terminus and the half-way point, between the half-way point and the C-terminus, and combinations thereof.

[0240] The effector module may include one or more cleavage signal(s)/site(s) of any proteinases. The proteinases may be a serine proteinase, a cysteine proteinase, an endopeptidase, a dipeptidase, a metalloproteinase, a glutamic proteinase, a threonine proteinase and an aspartic proteinase. In some aspects, the cleavage site may be a signal sequence of furin, actinidain, calpain-1, carboxypeptidase A, carboxypeptidase P, carboxypeptidase Y, caspase-1, caspase-2, caspase-3, caspase-4, caspase-5, caspase-6, caspase-7, caspase-8, caspase-9, caspase-10, cathepsin B, cathepsin C, cathepsin G, cathepsin H, cathepsin K, cathepsin L, cathepsin S, cathepsin V, clostripain, chymase, chymotrypsin, elastase, endoproteinase, enterokinase, factor Xa, formic acid, granzyme B, Matrix metalloproteinase-2, Matrix metalloproteinase-3, pepsin, proteinase K, SUMO protease, subtilisin, TEV protease, thermolysin, thrombin, trypsin and TAGZyme.

Tags

[0241] In some embodiments, the effector module comprises a protein tag.

[0242] The protein tag may be used for detecting and monitoring the process of the effector module. The effector module may include one or more tags such as an epitope tag (e.g., a FLAG or hemagglutinin (HA) tag). A large number of protein tags may be used for the present effector modules. They include, but are not limited to, self-labeling polypeptide tags (e.g., haloalkane dehalogenase (halotag2 or halotag7), ACP tag, clip tag, MCP tag, snap tag), epitope tags (e.g., FLAG, HA, His, and Myc), fluorescent tags (e.g., green fluorescent protein (GFP), red fluorescent protein (RFP), yellow fluorescent protein (YFP), and its variants), bioluminescent tags (e.g. luciferase and its variants), affinity tags (e.g., maltose-binding protein (MBP) tag, glutathione-S-transferase (GST) tag), immunogenic affinity tags (e.g., protein A/G, IRS, AU1, AU5, glu-glu, KT3, S-tag, HSV, VSV-G, Xpress and V5), and other tags (e.g., biotin (small molecule), StrepTag (StreptII), SBP, biotin carboxyl carrier protein (BCCP), eXact, CBP, CYD, HPC, CBD intein-chitin binding domain, Trx, NorpA, and NusA).

[0243] In other embodiments, a tag may also be selected from those disclosed in U.S. Pat. NOs. 8,999,897; 8,357,511; 7,094, 568; 5,011,912; 4,851,341; and 4,703,004; U.S patent application publication NOs. US2013115635 and

US2013012687; and International application publication NO. WO2013091661; the contents of each of which are incorporated herein by reference in their entirety.

[0244] In some aspects, a multiplicity of protein tags, either the same or different tags, may be used; each of the tags may be located at the same N or C terminus, whereas in other cases these tags may be located at each terminus.

Linkers

[0245] In some embodiments, the effector module comprises a linker.

[0246] In some embodiments, the effector module of the disclosure may further comprise a linker sequence. The linker region serves primarily as a spacer between two or more polypeptides within the effector module. The "linker" or "spacer", as used herein, refers to a molecule or group of molecules that connects two molecules, or two parts of a molecule such as two domains of a recombinant protein.

[0247] In some embodiments, "Linker" (L) or "linker domain" or "linker region" or "linker module" or "peptide linker" as used herein refers to an oligo- or polypeptide region of from about 1 to 100 amino acids in length, which links together any of the domains/regions of the effector module (also called peptide linker). The peptide linker may be 1-40 amino acids in length, or 2-30 amino acids in length, or 20-80 amino acids in length, or 50-100 amino acids in length. Linker length may also be optimized depending on the type of payload utilized and based on the crystal structure of the payload. In some instances, a shorter linker length may be preferably selected. In some aspects, the peptide linker is made up of amino acids linked together by peptide bonds, preferably from 1 to 20 amino acids linked by peptide bonds, wherein the amino acids are selected from the 20 naturally occurring amino acids: Glycine (G), Alanine (A), Valine (V), Leucine (L), Isoleucine (I), Serine (S), Cysteine (C), Threonine (T), Methionine (M), Proline (P), Phenylalanine (F), Tyrosine (Y), Tryptophan (W), Histidine (H), Lysine (K), Arginine (R), Aspartate (D), Glutamic acid (E), Asparagine (N), and Glutamine (Q). One or more of these amino acids may be glycosylated, as is understood by those in the art.

[0248] A linker sequence may be a natural linker derived from a multi-domain protein. A natural linker is a short peptide sequence that separates two different domains or motifs within a protein.

[0249] In some aspects, linkers may be flexible or rigid. In other aspects, linkers may be cleavable or non-cleavable. As used herein, the terms "cleavable linker domain or region" or "cleavable peptide linker" are used interchangeably. In some embodiments, the linker sequence may be cleaved enzymatically and/or chemically.

[0250] The linkers of the present disclosure may also be non-peptide linkers. For example, alkyl linkers such as —NH—(CH₂)_a—C(O)—, wherein a=2-20 can be used. These alkyl linkers may further be substituted by any non-sterically hindering group such as lower alkyl (e.g., C₁-C₆) lower acyl, halogen (e.g., Cl, Br), CN, NH₂, phenyl, etc.

Targeting or penetrating peptides

[0251] In some embodiments, the effector module comprises a targeting and/or penetrating peptide.

[0252] Small targeting and/or penetrating peptides that selectively recognize cell surface markers (e.g. receptors, trans-membrane proteins, and extra-cellular matrix molecules) can be employed to target the effector module to the desired organs, tissues or cells. Short peptides (5-50 amino acid residues) synthesized *in vitro* and naturally occurring peptides, or analogs, variants, derivatives thereof, may be incorporated into the effector module for homing the effector module to the desired organs, tissues and cells, and/or subcellular locations inside the cells.

[0253] In some embodiments, a targeting sequence and/or penetrating peptide may be included in the effector module to drive the effector module to a target organ, or a tissue, or a cell (e.g., a cancer cell). In other embodiments, a targeting and/or penetrating peptide may direct the effector module to a specific subcellular location inside a cell. As non-limiting examples, such targeting sequences and/or penetrating peptides may include those for targeting the effector module to desired region of the central nervous system (e.g., U.S. Pat. NO.: 9,259,432; U.S. application publication NO.: 2015/259392); or adipose tissue (e.g., U.S. Pat. NOs.: 8,067,377 and 8,710,017); or prostate (e.g., U.S. patent publication NO.: 2016/0046668); the contents of each of which are incorporated herein by reference in their entirety.

[0254] In other embodiments, a targeting and/or penetrating peptide may direct the effector module to a specific subcellular location inside a cell. As a non-limiting example, a mitochondrion targeting peptide and/or a mitochondria membrane penetrating peptide may be included in the effector module to drive the effector module to the mitochondria of a cell. See e.g., U.S. Pat. NOs.: 9,260,495; 9,173,952 and 9,132,198; and U.S. application publication NO.: 2015/361140; the contents of each of which are incorporated herein by reference in their entirety.

[0255] A targeting peptide has any number of amino acids from about 6 to about 30 inclusive. The peptide may have 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 amino acids. Generally, a targeting peptide may have 25 or fewer amino acids, for example, 20 or fewer, for example 15 or fewer.

[0256] Naturally occurring small targeting and/or penetrating peptides that recognize specific tissues or cells bind cell surface molecules (e.g. receptors, trans-membrane proteins) with high affinity, which make them attractive trafficking moieties. Such peptides may include peptide toxins from microbes, insects (e.g. scorpion, honey bee, spider), animals (e.g. snake) and plants, and analogs, variants and derivatives thereof; and secreted peptide hormones, ligands and signal peptides.

[0257] In some aspects, analogs, variants and derivatives from natural toxins that abolish their cytotoxic activities may be used as targeting peptides. Exotoxin is a toxin secreted by bacteria. Many exotoxins have been shown to bind

specific cell molecules. For example, enterotoxins, a group of protein toxins produced and secreted from bacterial organisms bind the mucosal (epithelial) cells of the intestinal wall. Enterotoxins may include, but are not limited to, *E. coli* heat stable enterotoxin (ST), Cholera toxin (CT), *E. coli* heat-labile enterotoxin (LT), *Bordetella pertussis*-derived pertussis toxin (PT), *Pseudomonas aeruginosa* exotoxin A (ETA), *Staphylococcus* enterotoxins, *Corynebacterium diphtheria*-derived diphtheria toxin, enterotoxin NSP4 from rotavirus. Other exotoxins include neurotoxins which affect the nervous system, cardiotoxins which affect the heart, pseudomonas exotoxins, Botulinum neurotoxins, shiga toxin, shiga-like toxin 1 and 2, Clostridium difficile toxins, Clostridium perfringens epsilon toxin and anthrax toxin.

[0258] In addition to exotoxins, other toxins may include those isolated from plants such as maize RIP, gelonin, pokeweed antiviral protein, saporin, trichsanthin, ricin, abrin; scorpions such as Charybdotoxin; spider such as PcTx1; cone snail such as PcTx1; sea anemone such as gigantoxin 1; honey bees such as mellitins, a group of water-soluble, cationic, amphipathic 26 amino acid alpha-helical peptides isolated from the venoms of honey bee *Apis mellifera* (western or European or big honey bee), *Apis florea* (little or dwarf honey bee), *Apis dorsata* (giant honey bee) and *Apis cerana* (oriental honey bee); snake venom toxins, bombesin which is originally isolated from the skin of toad, which binds g-protein couple gastrin releasing peptide receptors (e.g. BBR-1/2/3) in the gastric tract and brain. See e.g. Suchanek, G., et al., *PNAS* (1978) 75:701-704; the contents of which are incorporated by reference in its entirety.

[0259] Peptides hormones and other signal peptides transfer important messages for cell to cell communications, which selectively bind cells that express their receptors with high affinity. In some aspects, peptide hormones may be included in the effector module. Such small peptide hormones and signal peptides may include, but are not limited to, adiponectin, adipose-derived hormone, agouti signaling peptide, allatostatin, amylin, angiotensin, atrial natriuretic peptide, bomben-like peptide, big gastrin, betatrophin, bradykinin, calcitonin, corticotrophin releasing hormone, cosyntrophin, endothelin, enteroglucagon, FGF, FNDC5, follicle-stimulating hormone, gastrin, ghrelin, glucagon and glucagon-like peptide, gonadotrophin, granulocyte colony stimulating factor, growth hormone, growth hormone releasing hormone, hepcidin, human chorionic gonadotrophin, human placental lactogen, incretin, insulin and insulin analogs, insulin-like growth factor, leptin, little gastrin, liraglutide, luteinizing hormone, melanocortin, minigastrin, alpha-melanocyte-stimulating hormone, neuropeptide Y, nerve growth factor (NGF), neurotrophin-3/4, NPH insulin, orexin, obestatin, osteocalcin, pancreatic hormone, parathyroid hormone, peptide hormone, peptide YY, prolactin, preprohormone, relaxin, renin, salcatonin, somatostatin (SST), secretin, substance P, sincalide, teleost leptins, temporin, tesamorelin, thyroid stimulating hormone, urocortin, vasoactive intestinal peptide (VIP), VGF and Vitellogenin.

[0260] Targeting and penetrating peptides may also be engineered biomimetic peptides and/or chemically modified small peptides. Numerous peptides with specific motifs and sequences that target specific cells and tissues with high

affinity and selectivity in normal or diseased conditions are identified. A synthetic targeting peptide may be up to 30 amino acids in length or may be longer. A targeting peptide generally has at least about 5 amino acids but may have fewer, for example, 4 amino acids, or 3 amino acids. Generally, a targeting peptide has any number of amino acids from about 6 to about 30 inclusive. Generally, a targeting peptide may have 25 or fewer amino acids, for example, 20 or fewer, for example 15 or fewer.

[0261] A chimeric peptide may also be synthesized with fused amino acids from naturally occurring proteins and artificial amino acid sequences.

Stimuli

[0262] Biocircuits of the present disclosure are triggered by one or more stimuli. Stimuli include a ligand, an externally added or endogenous metabolite, the presence or absence of a defined ligand, the presence or action of one or more effector modules, or a concentration gradient of ions or biomolecules or the like.

Ligands

[0263] In some embodiments, the stimulus is a ligand. Ligands may be nucleic acid-based, protein-based, lipid-based, organic, inorganic or any combination of the foregoing.

[0264] In some embodiments, the ligand may be, but is not limited to, a protein, peptide, nucleic acid, lipid, lipid derivative, sterol, steroid, metabolite, metabolite derivative, and small molecule.

[0265] In some embodiments, the stimulus is a small molecule. In some embodiments, the small molecules are cell permeable. In some embodiments, the small molecules are FDA-approved, safe and orally administered.

[0266] In some embodiments, the ligands bind to carbonic anhydrases. In some embodiments, the ligand binds to and inhibits carbonic anhydrase function and is herein referred to as carbonic anhydrase inhibitor.

[0267] In some embodiments, the ligand is a small molecule that binds to carbonic anhydrase 2. In one embodiment, the small molecule is CA2 inhibitor. Examples of CA2 inhibitors include, but are not limited to Celecoxib, Valdecoxib, Rofecoxib, Acetazolamide, Methazolamide, Dorzolamide, Brinzolamide, Diclofenamide, Ethoxzolamide, Zonisamide, Dansylamide, and Dichlorphenamide.

[0268] In some embodiments, the ligands may comprise portions of small molecules known to mediate binding to CA2. Ligands may also be modified to reduce off-target binding to carbonic anhydrases other than CA2 and increase specific binding to CA2.

[0269] Ligands may also be selected from the analysis of the dependence of a known CA2 ligand's activity on its molecular/ chemical structure, through Structure Activity Relationships (SAR) study. Any of the methods related to SAR, known in art may be utilized to identify stabilizing ligands of the disclosure. SAR may be utilized to improve properties

of the ligand such as specificity, potency, pharmacokinetics, bioavailability, and safety. SAR analysis of known CA2 inhibitors may also be combined with high resolution X ray structures of CA2 complexed with ligands.

[0270] In one embodiment, the stimuli of the present disclosure may be FDA approved ligands capable of binding to the specific DDs or target regions within the DDs.

[0271] In some embodiments, ligands that do not affect the activity of the immune cell, and/or the chimeric antigen receptor, in the absence of the SREs may be preferably selected.

[0272] In some embodiments, two or more ligands may be utilized to stabilize the same stimulus response element.

Ligand conjugates

[0273] In some embodiments, the ligand may be complexed or bound to another molecule such as, but not limited to, another ligand, a protein, peptide, nucleic acid, lipid, lipid derivative, sterol, steroid, metabolite, metabolite derivative or small molecule. In some embodiments, the ligand stimulus is complexed to or bound to one or more other molecules. In some embodiments, the ligand stimulus is complexed or bound to one or more different kinds and/or numbers of other molecules. In some embodiments, the ligand stimulus is a multimer of the same kind of ligand. In some embodiments, the ligand stimulus multimer comprises 2, 3, 4, 5, 6, or more monomers.

[0274] Ligands such as small molecules that are well known to bind candidate proteins can be tested for their regulation in protein responses. The small molecules may be clinically approved to be safe and have appropriate pharmaceutical kinetics and distribution. In some embodiments, the stimulus is a ligand of a destabilizing domain (DD), for example, a small molecule that binds a destabilizing domain and stabilizes the POI fused to the destabilizing domain.

Promoters

[0275] In some embodiments, compositions of the disclosure comprise a promoter.

[0276] As used herein a promoter is defined as a DNA sequence recognized by transcription machinery of the cell, required to initiate specific transcription of the polynucleotide sequence of the present disclosure. Vectors can comprise native or non-native promoters operably linked to the polynucleotides of the disclosure. The promoters selected may be strong, weak, constitutive, inducible, tissue specific, development stage-specific, and/or organism specific. One example of a suitable promoter is the immediate early cytomegalovirus (CMV) promoter such as, but not limited to SEQ ID NO: 5635-5637. This promoter sequence is a strong constitutive promoter sequence capable of driving high levels of expression of polynucleotide sequence that is operatively linked to it. Another example of a promoter is Elongation Growth Factor-1 Alpha (EF-1 alpha) such as, but not limited to, SEQ ID NO: 5638-5642. Other constitutive promoters may also be used, including, but not limited to simian virus 40 (SV40), mouse mammary tumor virus (MMTV), human immunodeficiency virus (HIV), long terminal repeat (LTR), promoter, an avian leukemia virus promoter, an Epstein-Barr

virus immediate early promoter, a Rous sarcoma virus promoter as well as human gene promoters including, but not limited to the phosphoglycerate kinase (PGK) promoter (non-limiting examples include SEQ ID NO: 5643-5650), actin promoter, the myosin promoter, the hemoglobin promoter, the Ubiquitin C (Ubc) promoter, the human U6 small nuclear protein promoter and the creatine kinase promoter. In some instances, inducible promoters such as but not limited to metallothionein promoter, glucocorticoid promoter, a progesterone promoter, and a tetracycline promoter may be used.

[0277] In some embodiments, the optimal promoter may be selected based on its ability to achieve minimal expression of the SREs and payloads of the disclosure in the absence of the ligand and detectable expression in the presence of the ligand.

[0278] Additional promoter elements e.g. enhancers may be used to regulate the frequency of transcriptional initiation. Such regions may be located 10-100 base pairs upstream or downstream of the start site. In some instances, two or more promoter elements may be used to cooperatively or independently activate transcription.

[0279] In some embodiments, the promoter of the disclosure may be a Tet-ON promoter. Combination of the transcription regulation Tet system with the DDs permits simultaneous control of gene expression and protein stability. Any of the dual-Tet ON-DD systems described by Pedone et al. (2018) doi: <https://doi.org/10.1101/404699> may be useful in the present disclosure (the contents of which are herein incorporated by reference in their entirety).

Other Regulatory Features

[0280] In some embodiments, compositions of the disclosure may include optional proteasome adaptors. As used herein, the term "proteasome adaptor" refers to any nucleotide/ amino acid sequence that targets the appended payload for degradation. In some aspects, the adaptors target the payload for degradation directly thereby circumventing the need for ubiquitination reactions. Proteasome adaptors may be used in conjunction with destabilizing domains to reduce the basal expression of the payload. Exemplary proteasome adaptors include the UbL domain of Rad23 or hHR23b, HPV E7 which binds to both the target protein Rb and the S4 subunit of the proteasome with high affinity, which allows direct proteasome targeting, bypassing the ubiquitination machinery; the protein gankyrin which binds to Rb and the proteasome subunit S6.

Exemplary Effector Module Constructs

[0281] Biocircuits of the present disclosure may comprise at least one effector module which may comprise at least one SRE derived from CA2 (referred to as "CA2 SREs") which may be operably linked to at least one payload of interest. These types of biocircuits and effector modules are referred to as "CA2 biocircuits" and "CA2 effector modules". Additionally, the CA2 effector module may comprise additional features including, but not limited to, signal sequences, linker, spacers, tags, flags, cleavage sites, and IRES. Any of the exemplary SREs (e.g., DDs), payloads of

interest, signal sequences, linker, spacers, tags, flags, cleavage sites, and IRES taught herein or known in the art may be combined to create the CA2 effector modules of the present disclosure.

Payloads of Interest

[0282] In one embodiment, the CA2 effector module comprises a payload of interest. The payload of interest may be a wild-type polypeptide, a fragment of a wild-type polypeptide and/or comprise one or more mutations relative to a wild-type polypeptide. Non-limiting examples of the payload of interest are shown in Table 7.

Table 7. Payloads of Interest

Payload	Amino Acid SEQ ID NO.	Nucleic Acid SEQ ID NO.
CD40L	6	5799
sCD40L (113-261 of WT)	5800	5801
CD40L (aa 14-261 of WT)	5802	5803
CD40L (aa 14-261 of WT, (S110-G116) del)	5804	5805
CD40L (H224G, G226F)	6011	6012
CD40L (H224G, G226H)	6013	6014
CD40L (Y172G, G226F)	6015	6016

[0283] In some embodiments, the payloads described herein may be co-expressed with a chimeric antigen receptor.

[0284] In some embodiments, the payloads described herein may be co-expressed with an antigen-specific T cell receptor (TCR).

[0285] The CA2 biocircuits and/or effector modules of the present disclosure may be monocistronic or multicistronic meaning one (monocistronic) or more than one (multicistronic) message (e.g., payload of interest) is produced. If two messages are produced, the CA2 biocircuit or effector module is considered bicistronic.

[0286] In one embodiment, at least one CA2 effector module of the present disclosure is monocistronic.

[0287] In one embodiment, at least one CA2 effector module of the present disclosure is multicistronic.

[0288] In one embodiment, at least one CA2 effector module of the present disclosure is bicistronic.

[0289] In one embodiment, the CA2 biocircuit of the present disclosure is monocistronic.

[0290] In one embodiment, the CA2 biocircuit of the present disclosure is multicistronic.

[0291] In one embodiment, the CA2 biocircuit of the present disclosure is bicistronic.

[0292] In some embodiments, the payload may be a fusion protein comprising any of the immunotherapeutic agents described and ubiquitin. Within the fusion protein, the ubiquitin may be positioned at the N terminus and the immunotherapeutic agent may be positioned at the C terminus. In one aspect, the immunotherapeutic agent may itself be a fusion protein and the ubiquitin may be located in between the proteins that are fused. The payloads may include a

single ubiquitin protein or a chain of ubiquitin proteins. The ubiquitin protein may be linked to the immunotherapeutic agent through a single amino acid. The selection of the single amino acid may depend on the desired half-life of the fusion protein. In one embodiment, the immunotherapeutic agent may be IL12.

[0293] In some embodiments, the payload may be the whole or a portion of CD40L. In one embodiment, the payload may be the whole CD40L (SEQ ID NO. 6). In some aspects, the payload may be a portion of CD40L comprising: (i) sCD40L (113-261 of WT) (SEQ ID NO. 5800); (ii) CD40L (aa 14-261 of WT) (SEQ ID NO. 5802); or (iii) CD40L (aa 14-261 of WT, (S110-G116) del) (SEQ ID NO. 5804).

[0294] In some embodiments, the payload may be CD40L with one or more mutations as compared to (i) sCD40L (113-261 of WT) (SEQ ID NO. 5800); (ii) CD40L (aa 14-261 of WT) (SEQ ID NO. 5802); or (iii) CD40L (aa 14-261 of WT, (S110-G116) del) (SEQ ID NO. 5804).

[0295] In some embodiments, the payload may be CD40L with one or more mutations as compared to wild-type CD40L (amino acid sequence provided as SEQ ID NO. 6). In some aspects the CD40L comprises at least one mutation such as, but not limited to, S110G, F111G, E112S, M113G, Q114G, K115S, Y120G, H125G, Y172G, H224G, G226F, G226H, G226W, and G227F. The CD40L payload may comprise two mutations, and the mutations may be, but are not limited to, H224G and G226F, H224G and G226H, Y172G and G226F, or H125 and G227. The CD40L payload may comprise three mutations, and the mutations may be, but are not limited to, Y120G, H224G and G226W. The CD40L payload may comprise four mutations. The CD40L payload may comprise five mutations. The CD40L payload may comprise six mutations and the mutations may be, but are not limited to, S110G, F111G, E112S, M113G, Q114G, and K115S.

CA2 CD40L effector modules

[0296] In some embodiments, CA2 DDs described herein may be appended to a payload using a linker. In some embodiments, an effector module of the present disclosure comprises a linker between the payload and a CA2 DD. The payload may comprise CD40L (SEQ ID NO. 6) or a portion thereof or CD40L (SEQ ID NO. 6) or a portion thereof comprising one or more mutations relative to the amino acid sequence of SEQ ID NO. 6. In some embodiments, an effector module comprises CA2 (aa 2-260 of WT, E106D)-linker-CD40L (SEQ ID NO. 6). In some embodiments, an effector module comprises CA2 (aa 2-260 of WT, G63D)-linker-CD40L (SEQ ID NO. 6). In some embodiments, an effector module comprises CA2 (aa 2-260 of WT, H122Y)-linker-CD40L (SEQ ID NO. 6). In some embodiments, an effector module comprises CA2 (aa 2-260 of WT, I59N)-linker-CD40L (SEQ ID NO. 6). In some embodiments, an effector module comprises CA2 (aa 2-260 of WT, L156H)-linker-CD40L (SEQ ID NO. 6). In some embodiments, an effector module comprises CA2 (aa 2-260 of WT, L183S)-linker-CD40L (SEQ ID NO. 6). In some embodiments, an

effector module comprises CA2 (aa 2-260 of WT, L197P)-linker-CD40L (SEQ ID NO. 6). In some embodiments, an effector module comprises CA2 (aa 2-260 of WT, S56F)-linker-CD40L (SEQ ID NO. 6). In some embodiments, an effector module comprises CA2 (aa 2-260 of WT, S56N)-linker-CD40L (SEQ ID NO. 6). In some embodiments, an effector module comprises CA2 (aa 2-260 of WT, W208S)-linker-CD40L (SEQ ID NO. 6). In some embodiments, an effector module comprises CA2 (aa 2-260 of WT, Y193I)-linker-CD40L (SEQ ID NO. 6). In some embodiments, an effector module comprises CA2 (aa 2-260 of WT, Y51T)-linker-CD40L (SEQ ID NO. 6). As used herein, “aa 2-260 of WT” refers to amino acid positions 2-260 of wildtype CA2 (SEQ ID NO. 5810) and the position of the mutated amino acid in the CA2 DD is relative to SEQ ID NO. 5810. In some embodiments, an effector module comprises CA2 (aa 1-260 of WT, E106D)-linker-CD40L (SEQ ID NO. 6). In some embodiments, an effector module comprises CA2 (aa 1-260 of WT, G63D)-linker-CD40L (SEQ ID NO. 6). In some embodiments, an effector module comprises CA2 (aa 1-260 of WT, H122Y)-linker-CD40L (SEQ ID NO. 6). In some embodiments, an effector module comprises CA2 (aa 1-260 of WT, I59N)-linker-CD40L (SEQ ID NO. 6). In some embodiments, an effector module comprises CA2 (aa 1-260 of WT, L156H)-linker-CD40L (SEQ ID NO. 6). In some embodiments, an effector module comprises CA2 (aa 1-260 of WT, L183S)-linker-CD40L (SEQ ID NO. 6). In some embodiments, an effector module comprises CA2 (aa 1-260 of WT, L197P)-linker-CD40L (SEQ ID NO. 6). In some embodiments, an effector module comprises CA2 (aa 1-260 of WT, S56F)-linker-CD40L (SEQ ID NO. 6). In some embodiments, an effector module comprises CA2 (aa 1-260 of WT, S56N)-linker-CD40L (SEQ ID NO. 6). In some embodiments, an effector module comprises CA2 (aa 1-260 of WT, W208S)-linker-CD40L (SEQ ID NO. 6). In some embodiments, an effector module comprises CA2 (aa 1-260 of WT, Y193I)-linker-CD40L (SEQ ID NO. 6). In some embodiments, an effector module comprises CA2 (aa 1-260 of WT, Y51T)-linker-CD40L (SEQ ID NO. 6). As used herein, “aa 1-260 of WT” refers to amino acid positions 1-260 of wildtype CA2 (SEQ ID NO. 5810) and the position of the mutated amino acid in the CA2 DD is relative to SEQ ID NO. 5810.

[0297] In some embodiments, CA2 DDs described herein may be appended to CD40L using any of the components described in Table 8. Table 9 provides CA2 DDs appended to CD40L payloads. CA2 CD40L effector modules may further be operably linked to any of the CARs described herein. CA2 CD40L constructs in tandem with CD19 CAR are provided in Table 9. Any of the DD described herein may be combined with the construct components in Table 8 to prepare regulated CD40L constructs listed in Table 9. In Table 9, “*” represents the translation of the stop codon.

Table 8. CA2 CD40L and CA2 CD40L with CAR construct components

Component	AA SEQ ID NO.	NA SEQ ID NO.
CD8 α leader (No Met)	6004	6005

Linker (GS)	GS	GGATCA; GGATCC
Linker (G4S)3	3528	3532
Linker (GS)15	5968	5969
Linker (GSG) (BamHI-Gly)	GSG	GGATCAGGA
Linker (MH)	MH	ATGCAT
Linker (GSGGGSGGGSG)	5806	5807;6009 6009
Linker (GGS)	GGS	GGAGGATCC
eGFP (A206K)	6021	6022
CD40L	6	5799
CD40L (H224G, G226F)	6011	6012
CD40L (H224G, G226H)	6013	6014
CD40L (Y172G, G226F)	6015	6016
CD40L (Y170G, H224G, G226W)	6017	6018
CD40L (H125G, G227F)	6019	6020
CD40L (S110G, F111G, E112S, M113G, Q114G, K115S)	6069	6070
CA2 (L156H)	5761	5762
CA2 (aa 2-260 of WT, I59N, G102R)	5757	5758
CA2 (aa 2-260 of WT, L156H)	5759	5760
CA2 (aa 2-260 of WT, G63D, E69V, N231I)	5867	5868
CA2 (aa 2-260 of WT, S56N)	5657	5658
CA2 (aa 2-260 of WT, D72F, V241F, P249L)	5662	5663
CA2 (aa 2-260 of WT, D71L, T87N, L250R)	5669	5670
CA2 (aa 2-260 of WT, L183S)	5671	5672
CA2 (aa 2-260 of WT, L156H, S172C, F178Y, E186D)	5875	5876
CA2 (aa 2-260 of WT, R27L, T87I, H122Y, N252D)	5821	5822
CA2 (aa 2-260 of WT, L197P)	5879	5880
FLAG-tag	3481	3482
HA Tag	3493	3494
CD19 scFV	204	210
CD8a Hinge and Transmembrane Domain	1021	1022; 1023
CD8a Transmembrane domain	1224	1225
CD8 Hinge	1011	1012
4-1BB intracellular domain	1259	1266
CD3 zeta intracellular domain	1145	1151
P2A Cleavage Site	3449	3450

Table 9. CA2 CD40L and CA2 CD40L with CAR constructs

ID	AA Sequence	AA SEQ	NA SEQ
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		ID NO.	ID NO.
OT-001990 (CA2 (L156H); Linker (GGSGGGSGG GSG); CD40L; stop)	MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAK YDPSLKPLSVSYDQATSLRILNNGHAFNVEFDDSDKAVL KGGPLDGTYRLIQFHFHWGSLDGGQSEHTVDKKKYAAEL HLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAPGHQ KVVDVLSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSL TTPPLLECWTWIVLKEPISVSSEQVLKFRKLNFNGEPEPEEL MVDNWRPAQPLKNRQIKASFKGSGGGSGGGSGMIETYN QTSRPSAATGLPISMKIFMYLLTVFLITQMIGSALFAVYLHR RLDKIEDERNLHEDFVFMKTIQRCNTGERSLSLLNCEEIKS QFEGFVKDIMLNKEETKKENSFEMQKGDQNPQIAAHVISE ASSKTTSVLQWAEKGYTMSNNLVLENGKQLTVKRQGL YYIYAQVTFCNREASSQAPFIASLCLKSPGRFERILLRAAN THSSAKPCGQCSIHLGGVFELQPGASVFVNVDPSQVSHGT GFTSFGLLKL*	5808	5809
OT-002072 (Met, CA2 (aa 2- 260 of WT, I59N, G102R); Linker (GSG); CD40L; stop)	MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAK YDPSLKPLSVSYDQATSLRILNNGHAFNVEFDDSDKAVL KGGPLDGTYRLIQFHFHWGSLDRQGSEHTVDKKKYAAEL HLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAPGLQ KVVDVLSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSL TTPPLLECWTWIVLKEPISVSSEQVLKFRKLNFNGEPEPEEL MVDNWRPAQPLKNRQIKASFKGSGMIETYNQTSRPSAATG LPISMKIFMYLLTVFLITQMIGSALFAVYLHRRDLKIEDERN LHEDFVFMKTIQRCNTGERSLSLLNCEEIKSQFEGFVKDIM LNKEETKKENSFEMQKGDQNPQIAAHVISEASSKTTSVLQ WAEKGYTMSNNLVLENGKQLTVKRQGLYYIYAQVTFC SNREASSQAPFIASLCLKSPGRFERILLRAANTHSSAKPCGQ QSIHLGGVFELQPGASVFVNVDPSQVSHGTGFTSFGLLKL *	6023	6024
OT-002073(Met, CA2 (aa 2- 260 of WT, L156H); Linker (GSG); CD40L; stop)	MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAK YDPSLKPLSVSYDQATSLRILNNGHAFNVEFDDSDKAVL KGGPLDGTYRLIQFHFHWGSLDGGQSEHTVDKKKYAAEL HLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAPGHQ KVVDVLSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSL TTPPLLECWTWIVLKEPISVSSEQVLKFRKLNFNGEPEPEEL MVDNWRPAQPLKNRQIKASFKGSGMIETYNQTSRPSAATG LPISMKIFMYLLTVFLITQMIGSALFAVYLHRRDLKIEDERN LHEDFVFMKTIQRCNTGERSLSLLNCEEIKSQFEGFVKDIM LNKEETKKENSFEMQKGDQNPQIAAHVISEASSKTTSVLQ WAEKGYTMSNNLVLENGKQLTVKRQGLYYIYAQVTFC SNREASSQAPFIASLCLKSPGRFERILLRAANTHSSAKPCGQ QSIHLGGVFELQPGASVFVNVDPSQVSHGTGFTSFGLLKL *	6025	6026
OT-001968 (CA2 (aa 2-260 of WT, G63D, E69V, N231I); linker (GGSGGGSGG GSG); CD40L; stop)	MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAK YDPSLKPLSVSYDQATSLRILNNDHAFNVFDDSDKAVL KGGPLDGTYRLIQFHFHWGSLDGGQSEHTVDKKKYAAEL HLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAPGLQ KVVDVLSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSL TTPPLLECWTWIVLKEPISVSSEQVLKFRKLNFIGEPEPEEL MVDNWRPAQPLKNRQIKASFKGSGGGSGGGSGMIETYN QTSRPSAATGLPISMKIFMYLLTVFLITQMIGSALFAVYLHR RLDKIEDERNLHEDFVFMKTIQRCNTGERSLSLLNCEEIKS QFEGFVKDIMLNKEETKKENSFEMQKGDQNPQIAAHVISE	6027	6028

	ASSKTTSVLQWAEKGYTMSNNLVTLENGKQLTVKRQGL YYIYAQVTFCSNREASSQAPFIASLCLKSPGRFERILLRAAN THSSAKPCGQQSIHLGGVFELQPGASVFVNVTDPSPQVSHGT GFTSFGLLKL*		
OT-002074 (Met, CA2 (aa 2- 260 of WT, S56N); Linker ((GGSG)3); CD40L; stop)	MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDHTAK YDPSLKPLSVSYDQATNLRILNNGHAFNVEFDDSDQKAVL KGGPLDGTYRLIQFHHWGS LDGQSGEHTVDKKKYAAEL HLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAPGLQ KVVDVLDSEIKTKGSADFTNFDPRGLLPESLDYWTYPGSL TTPPLLEC VTWIVLKEPISVSSEQVLKFRKLNFNGEGEPEEL MVDNWRPAQPLKNRQIKASFKGSGGGSGGGSGMIETYN QTSRPSAATGLPISMKIFMYLLTVFLITQMIGSALFAVYLHR RLDKIEDERNLHEDFVFMKTIQRCNTGERSLLNCEEIKS QFEGFVKDIMLNKEETKKENSFEMQKGDQNPQIAAHVISE ASSKTTSVLQWAEKGYTMSNNLVTLENGKQLTVKRQGL YYIYAQVTFCSNREASSQAPFIASLCLKSPGRFERILLRAAN THSSAKPCGQQSIHLGGVFELQPGASVFVNVTDPSPQVSHGT GFTSFGLLKL*	6029	6030
OT-002075 (Met, CA2 (aa 2- 260 of WT, D72F, V241F, P249L) Linker ((GGSG)3); CD40L; stop)	MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDHTAK YDPSLKPLSVSYDQATSLRILNNGHAFNVEFDFSQDKAVL KGGPLDGTYRLIQFHHWGS LDGQSGEHTVDKKKYAAEL HLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAPGLQ KVVDVLDSEIKTKGSADFTNFDPRGLLPESLDYWTYPGSL TTPPLLEC VTWIVLKEPISVSSEQVLKFRKLNFNGEGEPEEL MFDNWRPAQLLKNRQIKASFKGSGGGSGGGSGMIETYN QTSRPSAATGLPISMKIFMYLLTVFLITQMIGSALFAVYLHR RLDKIEDERNLHEDFVFMKTIQRCNTGERSLLNCEEIKS QFEGFVKDIMLNKEETKKENSFEMQKGDQNPQIAAHVISE ASSKTTSVLQWAEKGYTMSNNLVTLENGKQLTVKRQGL YYIYAQVTFCSNREASSQAPFIASLCLKSPGRFERILLRAAN THSSAKPCGQQSIHLGGVFELQPGASVFVNVTDPSPQVSHGT GFTSFGLLKL*	6031	6032
OT-002076 (Met, CA2 (aa 2- 260 of WT, D71L, T87N, L250R); Linker ((GGSG)3); CD40L; stop)	MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDHTAK YDPSLKPLSVSYDQATSLRILNNGHAFNVEFLDSQDKAVL KGGPLDGNRYRLIQFHHWGS LDGQSGEHTVDKKKYAAEL HLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAPGLQ KVVDVLDSEIKTKGSADFTNFDPRGLLPESLDYWTYPGSL TTPPLLEC VTWIVLKEPISVSSEQVLKFRKLNFNGEGEPEEL MVDNWRPAQPRKNRQIKASFKGSGGGSGGGSGMIETYN QTSRPSAATGLPISMKIFMYLLTVFLITQMIGSALFAVYLHR RLDKIEDERNLHEDFVFMKTIQRCNTGERSLLNCEEIKS QFEGFVKDIMLNKEETKKENSFEMQKGDQNPQIAAHVISE ASSKTTSVLQWAEKGYTMSNNLVTLENGKQLTVKRQGL YYIYAQVTFCSNREASSQAPFIASLCLKSPGRFERILLRAAN THSSAKPCGQQSIHLGGVFELQPGASVFVNVTDPSPQVSHGT GFTSFGLLKL*	6033	6034
OT-002077 (Met, CA2 (aa 2- 260 of WT, L183S); Linker ((GGSG)3); CD40L; stop)	MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDHTAK YDPSLKPLSVSYDQATSLRILNNGHAFNVEFDDSDQKAVL KGGPLDGTYRLIQFHHWGS LDGQSGEHTVDKKKYAAEL HLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAPGLQ KVVDVLDSEIKTKGSADFTNFDPRGSLPESLDYWTYPGSL TTPPLLEC VTWIVLKEPISVSSEQVLKFRKLNFNGEGEPEEL MVDNWRPAQPLKNRQIKASFKGSGGGSGGGSGMIETYN QTSRPSAATGLPISMKIFMYLLTVFLITQMIGSALFAVYLHR	6035	6036

	RLDKIEDERNLHEDFVFMKTIQRCNTGERSLSLLNCEEIKS QFEGFVKDIMLNKEETKKENSFEMQKGDQNPQIAAHVISE ASSKTTSVLQWAEKGYTMSNNLVTLENGKQLTVKRQGL YYIYAQVTFCSNREASSQAPFIASLCLKSPGRFERILLRAAN THSSAKPCGQQSIHLGGVFELQPGASVFVNVTDPQVSHGT GFTSFGLLKL*		
OT-001970 (Met; CA2 (aa 2- 260 of WT, L156H, S172C, F178Y, E186D); linker (GGSGGGSGG GSG); CD40L; stop)	MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDHTAK YDPSLKPLSVSYDQATSLRILNNGHAFNVEFDDSDQKAVL KGGPLDGTYRLIQFHFHWGSLDGQGEHTVDKCKYAAEL HLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAPGHQ KVVDVLSIKTKGKCADFTNYDPRGLLPDSLDYWTYPGSL TTPPLLEC VTWIVLKEPISVSSEQVLKFRKLNFNGEPEEL MVDNWRPAQPLKNRQIKASFSGSGGGSGGSGMIETYN QTSRPSAATGLPISMKIFMYLLTVFLITQMIGSALFAVYLHR RLDKIEDERNLHEDFVFMKTIQRCNTGERSLSLLNCEEIKS QFEGFVKDIMLNKEETKKENSFEMQKGDQNPQIAAHVISE ASSKTTSVLQWAEKGYTMSNNLVTLENGKQLTVKRQGL YYIYAQVTFCSNREASSQAPFIASLCLKSPGRFERILLRAAN THSSAKPCGQQSIHLGGVFELQPGASVFVNVTDPQVSHGT GFTSFGLLKL*	6037	6038
OT-001971 (Met; CA2 (aa 2- 260 of WT, R27L, T87I, H122Y, N252D); linker (GGSGGGSGG GSG); CD40L; stop)	MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDHTAK YDPSLKPLSVSYDQATSLRILNNGHAFNVEFDDSDQKAVL KGGPLDGTYRLIQFHFHWGSLDGQGEHTVDKCKYAAELH LVYWNKYGDFGKAVQQPDGLAVLGIFLKVGSAPGLQK VVDVLSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTT PPLLEC VTWIVLKEPISVSSEQVLKFRKLNFNGEPEELM VDNWRPAQPLKDRQIKASFSGSGGGSGGSGMIETYNQ TSRPSAATGLPISMKIFMYLLTVFLITQMIGSALFAVYLHRR LDKIEDERNLHEDFVFMKTIQRCNTGERSLSLLNCEEIKSQF EGFVKDIMLNKEETKKENSFEMQKGDQNPQIAAHVISEAS SKTTSVLQWAEKGYTMSNNLVTLENGKQLTVKRQGLYY IYAQVTFCSNREASSQAPFIASLCLKSPGRFERILLRAANTH SSAKPCGQQSIHLGGVFELQPGASVFVNVTDPQVSHGTGF TSFGLLKL*	6039	6040
OT-001969 (Met; CA2 (aa 2- 260 of WT, L197P); linker (GGSGGGSGG GSG); CD40L; stop)	MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDHTAK YDPSLKPLSVSYDQATSLRILNNGHAFNVEFDDSDQKAVL KGGPLDGTYRLIQFHFHWGSLDGQGEHTVDKCKYAAEL HLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAPGLQ KVVDVLSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSP TTPPLLEC VTWIVLKEPISVSSEQVLKFRKLNFNGEPEEL MVDNWRPAQPLKNRQIKASFSGSGGGSGGSGMIETYN QTSRPSAATGLPISMKIFMYLLTVFLITQMIGSALFAVYLHR RLDKIEDERNLHEDFVFMKTIQRCNTGERSLSLLNCEEIKS QFEGFVKDIMLNKEETKKENSFEMQKGDQNPQIAAHVISE ASSKTTSVLQWAEKGYTMSNNLVTLENGKQLTVKRQGL YYIYAQVTFCSNREASSQAPFIASLCLKSPGRFERILLRAAN THSSAKPCGQQSIHLGGVFELQPGASVFVNVTDPQVSHGT GFTSFGLLKL*	6041	6042
OT-002155 (Met; CA2 (aa 2- 260 of WT); Linker (GGSGG3); CD40L; stop)	MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDHTAK YDPSLKPLSVSYDQATSLRILNNGHAFNVEFDDSDQKAVL KGGPLDGTYRLIQFHFHWGSLDGQGEHTVDKCKYAAEL HLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAPGLQ KVVDVLSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSL TTPPLLEC VTWIVLKEPISVSSEQVLKFRKLNFNGEPEEL	6043	6044

	MVDNWRPAQPLKNRQIKASFKGSGGGSGGGSGMIETYN QTSRPSAATGLPISMKIFMYLLTVFLITQMIGSALFAVYLHR RLDKIEDERNLHEDFVFMKTIQRCNTGERSLLNCEEIKS QFEGFVKDIMLNKEETKKENSFEMQKGDQNPQIAAHVISE ASSKTTSVLQWAEKGYTMSNNLVTLENGKQLTVKRQGL YYIYAQVTFCNREASSQAPFIASLCLKSPGRFERILLRAAN THSSAKPCGQQSIHLGGVFELQPGASVFVNVTDPQSQVSHGT GFTSFGLLKL*		
OT-002083 (Met, CA2 (aa 2- 260 of WT, L156H); Linker ((GGSG)3); CD40L (H224G, G226F); stop	MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAK YDPSLKPLSVSYDQATSLRILNNGHAFNVEFDDSDQKAVL KGGPLDGTYRLIQFHHWGS LDGQGEHTVDKKKYAAEL HLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAPGHQ KVVDVLSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSL TTPPILLECVTWIVLKEPISVSSEQVLKFRKLNFNGEPEPEEL MVDNWRPAQPLKNRQIKASFKGSGGGSGGGSGMIETYN QTSRPSAATGLPISMKIFMYLLTVFLITQMIGSALFAVYLHR RLDKIEDERNLHEDFVFMKTIQRCNTGERSLLNCEEIKS QFEGFVKDIMLNKEETKKENSFEMQKGDQNPQIAAHVISE ASSKTTSVLQWAEKGYTMSNNLVTLENGKQLTVKRQGL YYIYAQVTFCNREASSQAPFIASLCLKSPGRFERILLRAAN THSSAKPCGQQSIGLFGVFELQPGASVFVNVTDPQSQVSHGT GFTSFGLLKL*	6045	6046
OT-002084 (Met, CA2 (aa 2- 260 of WT, L156H); Linker ((GGSG)3); CD40L (H224G, G226H); stop	MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAK YDPSLKPLSVSYDQATSLRILNNGHAFNVEFDDSDQKAVL KGGPLDGTYRLIQFHHWGS LDGQGEHTVDKKKYAAEL HLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAPGHQ KVVDVLSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSL TTPPILLECVTWIVLKEPISVSSEQVLKFRKLNFNGEPEPEEL MVDNWRPAQPLKNRQIKASFKGSGGGSGGGSGMIETYN QTSRPSAATGLPISMKIFMYLLTVFLITQMIGSALFAVYLHR RLDKIEDERNLHEDFVFMKTIQRCNTGERSLLNCEEIKS QFEGFVKDIMLNKEETKKENSFEMQKGDQNPQIAAHVISE ASSKTTSVLQWAEKGYTMSNNLVTLENGKQLTVKRQGL YYIYAQVTFCNREASSQAPFIASLCLKSPGRFERILLRAAN THSSAKPCGQQSIGLHGVFELQPGASVFVNVTDPQSQVSHGT GFTSFGLLKL*	6047	6048
OT-002085 (Met, CA2 (aa 2- 260 of WT, L156H); Linker (GGS); EGFP (A206K); Linker ((GGSG)3); CD40L; stop)	MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAK YDPSLKPLSVSYDQATSLRILNNGHAFNVEFDDSDQKAVL KGGPLDGTYRLIQFHHWGS LDGQGEHTVDKKKYAAEL HLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAPGHQ KVVDVLSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSL TTPPILLECVTWIVLKEPISVSSEQVLKFRKLNFNGEPEPEEL MVDNWRPAQPLKNRQIKASFKGSGMVSKEELFTGVVPIL VELDGDVNGHKFSVSGEGEGDATYGKLTCLKFICTTGKLPV PWPTLVTTLYGVQCFSRYPDHMKQHDFFKSAMPEGYVQ ERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGN ILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGS VQLADHYQQNTPIGDGPVLLPDNHYLSTQSKLSKDPNEKR DHMVLEFVTAAGITLGMDELKGGSGGGSGGGSGMIET YNQTSRPSAATGLPISMKIFMYLLTVFLITQMIGSALFAVYL HRRDKIEDERNLHEDFVFMKTIQRCNTGERSLLNCEEI KSQFEGFVKDIMLNKEETKKENSFEMQKGDQNPQIAAHVI SEASSKTTSVLQWAEKGYTMSNNLVTLENGKQLTVKRQ GLYYIYAQVTFCNREASSQAPFIASLCLKSPGRFERILLRA	6049	6050

	ANTHSSAKPCGQQSIHLGGVFELQPGASVFVNVTDPQSQS HGTGFTSFGLLKL*		
OT-002086 (Met; CA2 (aa 2-260 of WT, L156H); Linker (GGS); Flag-tag; Linker ((GSG)3); CD40L; HA-tag; stop)	MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAK YDPSLKPLSVSYDQATSLRILNNGHAFNVEFDDSDQKAVL KGGPLDGTYRLIQFHHWGSGLDGGSEHTVDKKKYAAEL HLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAPGHQ KVVDVLSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSL TTPPLECVTWIVLKEPISVSSEQVLKFRKLNFNGEGEPEEL MVDNWRPAQPLKNRQIKASFKGGSYKDDDDKGGSGGG SGGSGSMIETYNQTSRPSAATGLPISMKIFMYLLTVFLITQ MIGSALFAVYLHRRLDKIEDERNLHEDFVFMKTIQRCNTG ERSLLNCEEIKSQFEGFVKDIMLNKEETKKENSFEMQKG DQNPQIAAHVISEASSKTTSVLQWAEKGYTMSNNLVTL NGKQLTVKRQGLYYIYAQVTFCSNREASSQAPFIASLCLKS PGRFERILLRAANTHSSAKPCGQQSIHLGGVFELQPGASVF VNVTDPQSQVSHGTGFTSFGLLKL*PYDVPDYA*	6051	6052
OT-002156 (Met; CD8α leader (no met); CD19 scFv; CD8a Hinge and Transmembrane Domain; CD28 co-stimulatory domain/4-1BB intracellular domain; CD3 zeta intracellular domain; Linker (GSG); P2A cleavage site; Linker (GS); Met; CA2 (aa 2-260 of WT, L156H); CD40L; stop)	MALPVTALLPLALLHAARPDQMTQTSSLSASLGDRVT ISCRASQDISKYLNWYQQKPDGTVKLLIYHTSRLHSGVPSR FSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGT KLEITGGGGSGGGSGGGSEVKLQESGPGLVAPSQSLSV TCTVSGVSLPDYGVSWIRQPPRKGLEWLGVIWGSETTYYN SALKSRLTIKDNSKSQVFLKMNSLQTDITAIIYCAKHYY YGGSYAMDYWGQTSVTVSSTTTPAPRPPTAPTASQPLS LRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLL SLVITLYCKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFP EEEEGGCEL RVKFSRSADAPAYKQQNQLYNELNLGRREE YDVLDKRRGRDPGEMGKPRRKNPQEGLYNELQKDKMAE AYSEIGMKGERRRGKGHDLGYQGLSTATKDTYDALHMQ ALPPRSGSATNFSLLKQAGDVEENPGPGSMHHWGYGKH NGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSY DQATSLRILNNGHAFNVEFDDSDQKAVLKGGPLDGTYRLI QFHHWGSGLDGGSEHTVDKKKYAAELHLVHWNTKYGD FGKAVQQPDGLAVLGIFLKVGSAPGHQKVVDVLSIKTK GKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLECVTWIV LKEPISVSSEQVLKFRKLNFNGEGEPEELMVDNWRPAQPL KNRQIKASFKGSGGGSGGGSGSMIETYNQTSRPSAATGLPI SMKIFMYLLTVFLITQMIGSALFAVYLHRRLDKIEDERNLH EDFVFMKTIQRCNTGERSLLNCEEIKSQFEGFVKDIMLN KEETKKENSFEMQKGQNPQIAAHVISEASSKTTSVLQWA EKGYTMSNNLVTLENGKQLTVKRQGLYYIYAQVTFCSN REASSQAPFIASLCLKSPGRFERILLRAANTHSSAKPCGQQS IHLGGVFELQPGASVFVNVTDPQSQVSHGTGFTSFGLLKL*	6053	6054
OT-001605 (Met CD8α leader (no met); CD19 scFv; CD8a Hinge and Transmembrane Domain; CD28 co-stimulatory domain/4-1BB intracellular domain; CD3 zeta intracellular	MALPVTALLPLALLHAARPDQMTQTSSLSASLGDRVT ISCRASQDISKYLNWYQQKPDGTVKLLIYHTSRLHSGVPSR FSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGT KLEITGGGGSGGGSGGGSEVKLQESGPGLVAPSQSLSV TCTVSGVSLPDYGVSWIRQPPRKGLEWLGVIWGSETTYYN SALKSRLTIKDNSKSQVFLKMNSLQTDITAIIYCAKHYY YGGSYAMDYWGQTSVTVSSTTTPAPRPPTAPTASQPLS LRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLL SLVITLYCKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFP EEEEGGCEL RVKFSRSADAPAYKQQNQLYNELNLGRREE YDVLDKRRGRDPGEMGKPRRKNPQEGLYNELQKDKMAE AYSEIGMKGERRRGKGHDLGYQGLSTATKDTYDALHMQ	6055	6056

domain; Linker (GS); P2A cleavage site; Linker (GS); Linker (MH); CD40L; stop)	ALPPRGSATNFSLLKQAGDVEENPGPGSMHMIETYNQTSP RSAATGLPISMKIFMYLLTVFLITQMIGSALFAVYLHRRLD KIEDERNLHEDFVFMKTIQRCNTGERSLSLLNCEEIKSQFEG FVKDIMLNKEETKKENSFEMQKGDQNPQIAAHVISEASSK TTSVLQWAEKGYTMSNNLVTLENGKQLTVKRQGLYYIY AQVTFCSNREASSQAPFIASLCLKSPGRFERILLRAANTHSS AKPCGQQSIHLGGVFELQPGASVFNVTDPQSQVSHGTGFTS FGLLKL*		
OT-002172 (Met, CA2 (aa 2-260 of WT, L156H); Linker (GGSGGGSGG GSG); CD40L (S110G, F11G, E112S, M113G, Q114G, K115S); stop)	MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAK YDPSLKPLSVSYDQATSLRILNNGHAFNVEFDDSDKAVL KGGPLDGTYRLIQFHHWGS LDGQGEHTVDKKKYAAEL HLVHWNTKYGDFGKAVQQPDGLAVLGIFLKVGSAPGHQ KVVDVLDISKTKGKSADFTNFDPRGLLPESLDYWTYPGSL TTPPLECVTWIVLKEPISVSSEQVLKFRKLNFNGEPEPEEL MVDNWRPAQPLKNRQIKASFKGSGGGSGGGSGGMIETYN QTSRPSAATGLPISMKIFMYLLTVFLITQMIGSALFAVYLHR RLDKIEDERNLHEDFVFMKTIQRCNTGERSLSLLNCEEIKS QFEGFVKDIMLNKEETKKENGSGSGGDQNPQIAAHVISE ASSKTTSVLQWAEKGYTMSNNLVTLENGKQLTVKRQGL YYIYAQVTFCSNREASSQAPFIASLCLKSPGRFERILLRAAN THSSAKPCGQQSIHLGGVFELQPGASVFNVTDPQSQVSHGT GFTSFGLLKL*	6071	6072

[0298] In some embodiments, the effector modules described herein may include one or more cleavage sites between the DD and CD40L. Inclusion of cleavage sites may uncouple the proteolytic turnover of the DD from the payload, thereby altering the levels of expression of the payload independent of the DD. In some embodiments, the addition of the cleavage site may increase expression of the payload. In other aspects, addition of cleavage site may reduce the expression of the payload.

[0299] In some embodiments, the effector modules described herein may include payloads where regions of the payloads have been substituted for a sequence comprising G and S. As a non-limiting example, in regards to the amino acid sequence, the region may be 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more than 15 amino acids in length. The amino acids of the payload may be replaced with a repeating pattern of GG, GS, SG, SS, GGG, GGS, GSS, GSG, SGG, SGS, SSG, SSS or a combination thereof.

[0300] In some embodiments, the effector modules described herein may include payloads where regions of the payloads have been substituted for a sequence comprising G and S. As a non-limiting example, in regards to the amino acid sequence, the region may be 6 amino acids in length. The amino acids of the payload may be replaced with GGS repeated once.

II. PHARMACEUTICAL COMPOSITIONS AND FORMULATIONS

[0301] The present teachings further comprise pharmaceutical compositions comprising one or more of the stimuli, CA2 biocircuits, CA2 effector modules or systems of the present disclosure, and optionally at least one pharmaceutically acceptable excipient or inert ingredient.

[0302] As used herein the term “pharmaceutical composition” refers to a preparation of one or more of the CA2 biocircuits or components described herein, or pharmaceutically acceptable salts thereof, optionally with other chemical components such as physiologically suitable carriers and excipients.

[0303] The term “excipient” or “inactive ingredient” refers to an inert or inactive substance added to a pharmaceutical composition to further facilitate administration of a compound. Non-limiting examples of such inert ingredients are disclosed herein under Formulations.

[0304] In some embodiments, compositions are administered to humans, human patients or subjects. For the purposes of the present disclosure, the phrase “active ingredient” generally refers to any one or more CA2 biocircuit system component to be delivered as described herein.

[0305] Although the descriptions of pharmaceutical compositions provided herein are principally directed to pharmaceutical compositions which are suitable for administration to humans, it will be understood by the skilled artisan that such compositions are generally suitable for administration to any other animal, e.g., to non-human animals, e.g. non-human mammals. Subjects to which administration of the pharmaceutical compositions is contemplated include, but are not limited to, non-human mammals, including agricultural animals such as cattle, horses, chickens and pigs, domestic animals such as cats, dogs, or research animals such as mice, rats, rabbits, dogs and non-human primates.

[0306] A pharmaceutical composition in accordance with the present disclosure may be prepared, packaged, and/or sold in bulk, as a single unit dose, and/or as a plurality of single unit doses. As used herein, a “unit dose” is discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient. The amount of the active ingredient is generally equal to the dosage of the active ingredient which would be administered to a subject and/or a convenient fraction of such a dosage such as, for example, one-half or one-third of such a dosage.

[0307] Relative amounts of the active ingredient, the pharmaceutically acceptable excipient or inert ingredient, and/or any additional ingredients in a pharmaceutical composition in accordance with the disclosure will vary, depending upon the identity, size, and/or condition of the subject treated and further depending upon the route by which the composition is to be administered. By way of example, the composition may comprise between 0.1% and 100%, e.g., between 0.5 and 50%, between 1-30%, between 5-80%, at least 80% (w/w) active ingredient.

[0308] Efficacy of treatment or amelioration of disease can be assessed, for example by measuring disease progression, disease remission, symptom severity, reduction in pain, quality of life, dose of a medication required to sustain a treatment effect, level of a disease marker or any other measurable parameter appropriate for a given disease being treated or targeted for prevention. It is well within the ability of one skilled in the art to monitor efficacy of treatment or prevention by measuring any one of such parameters, or any combination of parameters. In connection with the administration of compositions of the present disclosure, "effective against" for example a cancer, indicates that administration in a clinically appropriate manner results in a beneficial effect for at least a statistically significant fraction of patients, such as an improvement of symptoms, a cure, a reduction in disease load, reduction in tumor mass or cell numbers, extension of life, improvement in quality of life, or other effect generally recognized as positive by medical doctors familiar with treating the particular type of cancer.

[0309] A treatment or preventive effect is evident when there is a statistically significant improvement in one or more parameters of disease status, or by a failure to worsen or to develop symptoms where they would otherwise be anticipated. As an example, a favorable change of at least 10% in a measurable parameter of disease, and preferably at least 20%, 30%, 40%, 50% or more can be indicative of effective treatment. Efficacy for a given composition or formulation of the present disclosure can also be judged using an experimental animal model for the given disease as known in the art. When using an experimental animal model, efficacy of treatment is evidenced when a statistically significant change is observed.

Formulations

[0310] The compositions of the present disclosure may be formulated in any manner suitable for delivery. The formulation may be, but is not limited to, nanoparticles, poly (lactic-co-glycolic acid) (PLGA) microspheres, lipidoids, lipoplex, liposome, polymers, carbohydrates (including simple sugars), cationic lipids and combinations thereof.

[0311] In one embodiment, the formulation is a nanoparticle which may comprise at least one lipid. The lipid may be selected from, but is not limited to, DLin-DMA, DLin-K-DMA, 98N12-5, C12-200, DLin-MC3-DMA, DLin-KC2-DMA, DODMA, PLGA, PEG, PEG-DMG and PEGylated lipids. In another aspect, the lipid may be a cationic lipid such as, but not limited to, DLin-DMA, DLin-D-DMA, DLin-MC3-DMA, DLin-KC2-DMA and DODMA.

[0312] For polynucleotides described herein, the formulation may be selected from any of those taught, for example, in International Application PCT/US2012/069610, the contents of which are incorporated herein by reference in its entirety.

Inactive ingredients

[0313] In some embodiments, pharmaceutical or other formulations may comprise at least one excipient which is an inactive ingredient. As used herein, the term “inactive ingredient” refers to one or more inactive agents included in formulations. In some embodiments, all, none or some of the inactive ingredients which may be used in the formulations of the present disclosure may be approved by the US Food and Drug Administration (FDA). III. DOSING, DELIVERY AND ADMINISTRATIONS

[0314] The compositions described herein may be delivered to a cell or a subject through one or more routes and modalities. The viral vectors containing one or more CA2 effector modules, SREs, payloads and other components described herein may be used to deliver them to a cell and/or a subject. Other modalities may also be used such as mRNAs, plasmids, and as recombinant proteins.

Delivery

Naked delivery

[0315] Pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads of the present disclosure may be delivered to cells, tissues, organs and/or organisms in naked form. As used herein, the term “naked” refers to pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads delivered free from agents or modifications which promote transfection or permeability. The naked pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads may be delivered to the cells, tissues, organs and/or organisms using routes of administration known in the art and described herein. In some embodiments, naked delivery may include formulation in a simple buffer such as saline or PBS.

Formulated delivery

[0316] In some embodiments, pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads of the present disclosure may be formulated, using methods described herein. Formulations may comprise pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads which may be modified and/or unmodified. Formulations may further include, but are not limited to, cell penetration agents, pharmaceutically acceptable carriers, delivery agents, bioerodible or biocompatible polymers, solvents, and/or sustained-release delivery depots. Formulations of the present disclosure may be delivered to cells using routes of administration known in the art and described herein.

[0317] Pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads may also be formulated for direct delivery to organs or tissues in any of several ways in the art including, but not limited to, direct soaking or bathing, via a catheter, by gels, powder, ointments, creams, gels,

lotions, and/or drops, by using substrates such as fabric or biodegradable materials coated or impregnated with compositions, and the like.

Delivery to Cells

[0318] In another aspect of the present disclosure, polynucleotides encoding CA2 biocircuits, CA2 effector modules, SREs (e.g., CA2 DDs), payloads of interest (e.g., immunotherapeutic agents) and compositions described herein and vectors comprising said polynucleotides may be introduced into cells. As a non-limiting example, the cells may be effector immune cells.

[0319] In some aspects of the present disclosure, polynucleotides encoding CA2 biocircuits, CA2 effector modules, SREs (e.g., CA2 DDs), payloads of interest (e.g., immunotherapeutic agents) and compositions of the disclosure, may be packaged into viral vectors or integrated into viral genomes allowing transient or stable expression of the polynucleotides. Preferable viral vectors are retroviral vectors including lentiviral vectors. In order to construct a retroviral vector, a polynucleotide molecule encoding a CA2 biocircuit, an CA2 effector module, a CA2 SRE (e.g., CA2 DD) or a payload of interest (e.g., an immunotherapeutic agent) is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. The recombinant viral vector is then introduced into a packaging cell line containing the gag, pol, and env genes, but without the LTR and packaging components. The recombinant retroviral particles are secreted into the culture media, then collected, optionally concentrated, and used for gene transfer. Lentiviral vectors are especially preferred as they are capable of infecting both dividing and non-dividing cells.

[0320] Vectors may also be transferred to cells by non-viral methods by physical methods such as needles, electroporation, sonoporation, hydroporation; chemical carriers such as inorganic particles (e.g. calcium phosphate, silica, gold) and/or chemical methods. In some embodiments, synthetic or natural biodegradable agents may be used for delivery such as cationic lipids, lipid nano emulsions, nanoparticles, peptide-based vectors, or polymer-based vectors.

[0321] In some embodiments, the polypeptides described herein may be delivered to the cell directly. In one embodiment, the polypeptides of the disclosure may be delivered using synthetic peptides comprising an endosomal leakage domain (ELD) fused to a cell penetration domain (CLD). The polypeptides of the present disclosure are co introduced into the cell with the ELD-CLD-synthetic peptide. ELDs facilitate the escape of proteins that are trapped in the endosome, into the cytosol. Such domains are derived proteins of microbial and viral origin and have been described in the art. CPDs allow the transport of proteins across the plasma membrane and have also been described in the art. The ELD-CLD fusion proteins synergistically increase the transduction efficiency when compared to the co-transduction with either domain alone. In some embodiments, a histidine rich domain may optionally be added to the shuttle construct as an

additional method of allowing the escape of the cargo from the endosome into the cytosol. The shuttle may also include a cysteine residue at the N or C terminus to generate multimers of the fusion peptide. Multimers of the ELD-CLD fusion peptides generated by the addition of cysteine residue to the terminus of the peptide show even greater transduction efficiency when compared to the single fusion peptide constructs. The polypeptides of the invention may also be appended to appropriate localization signals to direct the cargo to the appropriate sub-cellular location e.g. nucleus. In some embodiments any of the ELDs, CLDs or the fusion ELD-CLD synthetic peptides taught in the International Patent Publication, WO2016161516 and WO2017175072 may be useful in the present invention (the contents of each of which are herein incorporated by reference in their entirety).

Delivery Modalities and/or Vectors

[0322] The CA2 biocircuit systems, CA2 effector modules, SREs and/or payloads of the present disclosure may be delivered using one or more modalities. The present disclosure also provides vectors that package polynucleotides described herein encoding CA2 biocircuits, CA2 effector modules, SREs (e.g., CA2 DDs) and payloads, and combinations thereof. Vectors of the present disclosure may also be used to deliver the packaged polynucleotides to a cell, a local tissue site or a subject. These vectors may be of any kind, including DNA vectors, RNA vectors, plasmids, viral vectors and particles. Viral vector technology is well known and described in Sambrook et al. (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York). Viruses, which are useful as vectors include, but are not limited to lentiviral vectors, adenoviral vectors, adeno-associated viral (AAV) vectors, herpes simplex viral vectors, retroviral vectors, oncolytic viruses, and the like.

[0323] In general, vectors contain an origin of replication functional in at least one organism, a promoter sequence and convenient restriction endonuclease site, and one or more selectable markers e.g. a drug resistance gene.

[0324] In some embodiments, the recombinant expression vector may comprise regulatory sequences, such as transcription and translation initiation and termination codons, which are specific to the type of host cell into which the vector is to be introduced.

[0325] In some embodiments, the vector described herein may comprise one or more payloads taught herein, wherein the two or more payloads may be included in one CA2 effector module. In this case, the two or more payloads are tuned by the same stimulus simultaneously. In other embodiments, the vector of the invention may comprise two or more CA2 effector modules, wherein each CA2 effector module comprises a different payload. In this case, the two or more CA2 effector modules and payloads are tuned by different stimuli, providing separately independent regulation of the two or more components.

Lentiviral vehicles/particles

[0326] In some embodiments, lentiviral vehicles/particles may be used as delivery modalities. Lentiviruses are a subgroup of the *Retroviridae* family of viruses, named because reverse transcription of viral RNA genomes to DNA is required before integration into the host genome. As such, the most important features of lentiviral vehicles/particles are the integration of their genetic material into the genome of a target/host cell. Some examples of lentivirus include the Human Immunodeficiency Viruses: HIV-1 and HIV-2, the Simian Immunodeficiency Virus (SIV), feline immunodeficiency virus (FIV), bovine immunodeficiency virus (BIV), Jembrana Disease Virus (JDV), equine infectious anemia virus (EIAV), equine infectious anemia virus, visna-maedi and caprine arthritis encephalitis virus (CAEV).

[0327] Typically, lentiviral particles making up the gene delivery vehicle are replication defective on their own (also referred to as “self-inactivating”). Lentiviruses are able to infect both dividing and non-dividing cells by virtue of the entry mechanism through the intact host nuclear envelope (Naldini L et al., *Curr. Opin. Biotechnol.*, 1998, 9: 457-463). Recombinant lentiviral vehicles/particles have been generated by multiply attenuating the HIV virulence genes, for example, the genes Env, Vif, Vpr, Vpu, Nef and Tat are deleted making the vector biologically safe. Correspondingly, lentiviral vehicles, for example, derived from HIV-1/HIV-2 can mediate the efficient delivery, integration and long-term expression of transgenes into non-dividing cells. As used herein, the term “recombinant” refers to a vector or other nucleic acid containing both lentiviral sequences and non-lentiviral retroviral sequences.

[0328] Lentiviral particles may be generated by co-expressing the virus packaging elements and the vector genome itself in a producer cell such as human HEK293T cells. These elements are usually provided in three or four separate plasmids. The producer cells are co-transfected with plasmids that encode lentiviral components including the core (i.e. structural proteins) and enzymatic components of the virus, and the envelope protein(s) (referred to as the packaging systems), and a plasmid that encodes the genome including a foreign transgene, to be transferred to the target cell, the vehicle itself (also referred to as the transfer vector). In general, the plasmids or vectors are included in a producer cell line. The plasmids/vectors are introduced via transfection, transduction or infection into the producer cell line. Methods for transfection, transduction or infection are well known by those of skill in the art. As non-limiting example, the packaging and transfer constructs can be introduced into producer cell lines by calcium phosphate transfection, lipofection or electroporation, generally together with a dominant selectable marker, such as neo, DHFR, Gln synthetase or ADA, followed by selection in the presence of the appropriate drug and isolation of clones.

[0329] The producer cell produces recombinant viral particles that contain the foreign gene, for example, the CA2 effector module of the present disclosure. The recombinant viral particles are recovered from the culture media and titrated by standard methods used by those of skill in the art. The recombinant lentiviral vehicles can be used to infect target cells.

[0330] Cells that can be used to produce high-titer lentiviral particles may include, but are not limited to, HEK293T cells, 293G cells, STAR cells (Relander et al., *Mol. Ther.*, 2005, 11: 452-459), FreeStyle™ 293 Expression System (ThermoFisher, Waltham, MA), and other HEK293T-based producer cell lines (e.g., Stewart et al., *Hum Gene Ther.* 2011, 22(3):357-369; Lee et al., *Biotechnol Bioeng*, 2012, 10996): 1551-1560; Throm et al., *Blood*, 2009, 113(21): 5104-5110; the contents of each of which are incorporated herein by reference in their entirety).

[0331] In some aspects, the envelope proteins may be heterologous envelope proteins from other viruses, such as the G protein of vesicular stomatitis virus (VSV G) or baculoviral gp64 envelop proteins. The VSV-G glycoprotein may especially be chosen among species classified in the vesiculovirus genus: *Carajas virus* (CJSV), *Chandipura virus* (CHPV), *Cocal virus* (COCV), *Isfahan virus* (ISFV), *Maraba virus* (MARAV), *Piry virus* (PIRYV), *Vesicular stomatitis Alagoas virus* (VSAV), *Vesicular stomatitis Indiana virus* (VSIV) and *Vesicular stomatitis New Jersey virus* (VSNJV) and/or stains provisionally classified in the vesiculovirus genus as *Grass carp rhabdovirus*, *BeAn 157575 virus* (BeAn 157575), *Boteke virus* (BTKV), *Calchaqui virus* (CQIV), *Eel virus American* (EVA), *Gray Lodge virus* (GLOV), *Jurona virus* (JURY), *Klamath virus* (KLAV), *Kwatta virus* (KWAV), *La Joya virus* (LJV), *Malpais Spring virus* (MSPV), *Mount Elgon bat virus* (MEBV), *Perinet virus* (PERV), *Pike fry rhabdovirus* (PFRV), *Porton virus* (PORV), *Radi virus* (RADIV), *Spring viremia of carp virus* (SVCV), *Tupaia virus* (TUPV), *Ulcerative disease rhabdovirus* (UDRV) and *Yug Bogdanovac virus* (YBV). The gp64 or other baculoviral env protein can be derived from *Autographa californica* nucleopolyhedrovirus (AcMNPV), *Anagrapha falcifera* nuclear polyhedrosis virus, *Bombyx mori* nuclear polyhedrosis virus, *Choristoneura fumiferana* nucleopolyhedrovirus, *Orgyia pseudotsugata* single capsid nuclear polyhedrosis virus, *Epiphyas postvittana* nucleopolyhedrovirus, *Hyphantria cunea* nucleopolyhedrovirus, *Galleria mellonella* nuclear polyhedrosis virus, Dhori virus, Thogoto virus, *Antheraea pernyi* nucleopolyhedrovirus or Batken virus.

[0332] Other elements provided in lentiviral particles may comprise retroviral LTR (long-terminal repeat) at either 5' or 3' terminus, a retroviral export element, optionally a lentiviral reverse response element (RRE), a promoter or active portion thereof, and a locus control region (LCR) or active portion thereof. The CA2 effector module is linked to the vector.

[0333] Methods for generating recombinant lentiviral particles are discussed in the art, for example, U.S. Pat. NOs.: 8, 846, 385; 7,745, 179; 7,629,153; 7,575,924; 7,179, 903; and 6, 808, 905; the contents of each of which are incorporated herein by reference in their entirety.

[0334] Lentivirus vectors used may be selected from, but are not limited to pLVX, pLenti, pLenti6, pLJM1, FUGW, pWPXL, pWPI, pLenti CMV puro DEST, pLJM1-EGFP, pULTRA, pInducer20, pHIV-EGFP, pCW57.1, pTRPE, pELPS, pRRL, and pLionII.

Lentiviral vectors and Cell Engineering

[0335] Lentiviral vectors are used for introducing transgenes into T cells (e.g., primary human T cells or Jurkat cells) for preclinical research and clinical applications, including recently approved products such as Tisagenlecleucel (KYMRIA[®]) for relapsed/refractory B-cell lymphoma. VSV-G pseudotyped 3rd generation lentiviral vectors offer high titers, high transduction efficiency and safety, and have become the vectors of choice for T cell engineering. While not wishing to be bound by theory, T cell engineering usually involves T cell activation by CD3/CD28 antibodies, followed by lentivirus transduction, and then cell expansion which can last from 5 to 30 days (e.g., 9 to 14 days or 9 to 15 days). In general, lentivirus transgene integration may take over 7 days to fully stabilize in T cells (e.g., primary human T cells or Jurkat cells). While longer cultures can increase the cell numbers, the longer cultures can also change the T cell phenotype to a more differentiated state. Therefore, the duration of *ex vivo* culture can impact the persistence and efficacy of CAR T cells. For example, cells cultured for shorter duration may display a less differentiated phenotype and can be highly efficacious in preclinical models.

[0336] While not wishing to be bound by theory, the state of T cell differentiation may influence the engraftment and persistence of T cells following adoptive transfer. Ghassemi et al (Reducing *Ex Vivo* Culture Improves the Antileukemic Activity of Chimeric Antigen Receptor (CAR) T Cells. Cancer Immunol Res; 6(9) Sept. 2018; the contents of which are herein incorporated by reference in their entirety) describe primary human T cell differentiation over time and saw that early harvested CAR T cells exhibited enhanced effector function and proliferation, as well as enhanced potency and persistence *in vivo*.

[0337] Lentivirus dynamics such as transduction, integration and/or expression kinetics of lentivirally introduced transgenes in T cells (e.g., primary human T cells or Jurkat cells) *ex vivo* may impact the efficacy and durability of *in vivo* anti-tumor responses. Some types of T cells may produce different results. For example, the Jurkat cell line may not provide the dynamic range of expression as primary human T cells. Methods to evaluate these lentivirus dynamics are known in the art and are described herein.

[0338] In some embodiments, to determine the transgene expression kinetics CD3/CD28 activated primary human T cells can be transduced with lentivirus carrying a transgene (e.g., a regulated transgene or constitutive transgene such as CD19 CAR, IL12, fluorescent protein or any transgene (e.g., payload) described herein). The cells may be analyzed by methods described herein and/or known in the art for viability, viral genomic integration (e.g., by using quantitative PCR), transcript levels (e.g., by using quantitative RT-PCR), and cell surface expression of the transgene if applicable (e.g., if the transgene is or includes CD19 CAR then the surface expression of the CD19 CAR can be evaluated). The cells may be analyzed prior to transduction and/or after transduction such as 1 day, 2 days, 3 days, 4 days, 5 days, 6 days,

7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, 21 days, 22 days, 23 days, 24 days, 25 days, 26 days, 27 days, 28 days, 29 days, 30 or more than 30 days after transduction.

[0339] In some embodiments, the CD3/CD28 activated primary human T cells can be reactivated with CD3/CD28 beads after transduction. The cells may be reactivated 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, 21 days, 22 days, 23 days, 24 days, 25 days, 26 days, 27 days, 28 days, 29 days, 30 or more than 30 days after transduction. The cells may be analyzed by methods described herein and/or known in the art for viability, viral genomic integration (e.g., by using quantitative PCR), transcript levels (e.g., by using quantitative RT-PCR), cell surface expression of the transgene if applicable (e.g., if the transgene is or includes CD19 CAR then the surface expression of the CD19 CAR can be evaluated), copy number, and/or mRNA levels.

[0340] In some embodiments, the cell viability of activated primary human T cells transduced with lentivirus carrying a transgene is greater than 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, or 99%. As a non-limiting example, the cell viability is greater than 90%. As a non-limiting example, the cell viability is greater than 85%.

[0341] In some embodiments, the cell viability of Jurkat cells transduced with lentivirus carrying a transgene is greater than 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, or 99%. As a non-limiting example, the cell viability is greater than 90%. As a non-limiting example, the cell viability is greater than 85%.

[0342] In some embodiments, the integration of the transgene into the genome of the cell may be at or above the saturation point. As a non-limiting example, the saturation point may be 3 copies per cell.

[0343] In some embodiments, the integration of the transgene into the genome may be high in the initial timepoints evaluated and then decline to a lower integration value before becoming stable for the remainder of the culture. As a non-limiting example, the integration may be up to 20 copies per cell of the transgene into the genome during the early timepoints before declining to 2 copies per cell and being stable throughout the remainder of the culture.

[0344] In some embodiments, the transduction ability of T cells may be evaluated. T cells from at least one donor may be transduced with a lentivirus containing a transgene at a dose that is predicted to reach the saturating levels (e.g., enough virus that each cell should contain a copy if a Poisson distribution is expected) and a higher lentivirus dose that exceeds saturation 5 times. Copies per cell, percentage and MFI of cells (or concentration in media of transgene) may be detected in order to determine if all cells are expressing transgene. As a non-limiting example, T cells from two distinct donors may be transduced with lentivirus which includes a transgene. The transduction may be at two doses, saturation and 5x saturation, and show that 5-10 days after transduction that all groups may reach or exceed a predicted saturating

level of integrated transgene and similar expression intensity across groups but not all cells are expressing the transgene. Not all T cells may have equal transduction susceptibility, even when sourced from the same donor. The fraction of total cells that express GFP (above the detection threshold) may vary between donors, lots and/or viral dose. The percent of total cells that express GFP from a single donor may be between 70% and 95%.

[0345] In some embodiments, a percentage of the cultured T cells (e.g., primary human T cells and/or Jurkat cells) may express the transgene. The percentage of cultured T cells expressing the transgene may be, but is not limited to, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 99% or greater than 99%. As a non-limiting example, the percentage may be greater than 70%. As a non-limiting example, the percentage may be greater than 75%. As a non-limiting example, the percentage may be greater than 80%. As a non-limiting example, the percentage may be greater than 85%. As a non-limiting example, the percentage may be greater than 90%. As a non-limiting example, the percentage may be greater than 95%.

[0346] In some embodiments, the mRNA levels from the culture may decline over the duration of the study. The decline may not be limited to a specific transgene and the trend may be seen across multiple classes of expressed proteins. In order to increase the mRNA levels, the cells may be reactivated after the mRNA levels decrease from the initial levels. The cells may be reactivated 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, 21 days, 22 days, 23 days, 24 days, 25 days, 26 days, 27 days, 28 days, 29 days, 30 or more than 30 days after transduction. As a non-limiting example, in order to increase mRNA levels in the culture, the cells may be reactivated with CD3/CD28 beads 13 days after transduction.

[0347] In some embodiments, the surface expression from the culture may decline over the duration of the study. For example, the surface expression may decline between days 3 to 13 days, 3 to 14 days, or 3 to 15 days after transduction. In order to increase the surface expression, the cells may be reactivated after the surface expression decrease from the initial levels. The cells may be reactivated 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, 21 days, 22 days, 23 days, 24 days, 25 days, 26 days, 27 days, 28 days, 29 days, 30 or more than 30 days after transduction.

[0348] In some embodiments, the transgene is a CAR such as, but not limited to, CD19 CAR. As a non-limiting example, the CAR is CD19 CAR. The cell viability may be greater than 90% in cells transduced with a CD19 CAR. The cell viability may be greater than 85% in cells transduced with a CD19 CAR. If the cells are primary T cells transduced with a CD19 CAR, then number of viable cells may increase over the initial timepoints before decreasing. If the cells are Jurkat cells transduced with a CD19 CAR, then the number of viable cells may increase for at least 10 days. The number of copies per cell for CD19 CAR transduced cells may be higher for the initial timepoints before decreasing by 50% or

more for the later timepoints. The cell surface expression of CD19 CAR may decrease during the course of the study from about 20000 CAR MFI to less than 5000 CAR MFI over a period of 10 days (e.g., day 3 to day 13). After restimulation on day 15 the MFI may increase to above 5000 CAR MFI. The percentage of primary human T cells expressing CAR may be between 40% and 60% for 3-13 days after transduction. The percentage of Jurkat cells expressing CAR may be between 30% and 70% for 3-13 days after transduction. An initial decline of about 20% may be seen between days 3 and 6 after transduction. Restimulation of the T cells may increase the percent of CAR positive cells back to initial percentage levels (e.g., around 60%).

[0349] In some embodiments, the transgene encodes a fluorescent protein such as, but not limited to cytosolic green fluorescence protein (GFP), luciferase, and mCherry. As a non-limiting example, the fluorescent protein is GFP. The cell viability may be greater than 90% in cells transduced with GFP. The cell viability may be greater than 85% in cells transduced with GFP. If the cells are primary T cells transduced with GFP, then the number of viable cells may increase over the initial timepoints before decreasing. If the cells are Jurkat cells transduced with GFP, then the number of viable cells may increase for at least 10 days. The number of copies per cell for GFP transduced cells may be higher for the initial timepoints before decreasing by 50% or more for the later timepoints. The surface expression of the cells may have a steady and rapid decline bottoming out at day 10 with a slight increase if restimulated. The highest level of cell surface expression of GFP in Jurkat cells may be at day 10 (about 35000 GFP MFI) before decreasing for the rest of the study. The percentage of primary human T cells expressing GFP may be around 80% for 3-13 days after transduction. The percentage of Jurkat cells expressing GFP may be around 90% for 3-13 days after transduction.

[0350] In some embodiments, lentivirally engineered cells described herein have genomic DNA integration that stabilizes after an initial decline of copy number, decreasing RNA and surface expression levels over time, and an increase in RNA and surface expression after re-stimulation.

[0351] In some embodiments, lentivirally engineering cells may be evaluated using the following 14-day method where samples are collected 5 times throughout the culture. On day -1 the T cells (e.g., primary human T cells or Jurkat cells) may be thawed and the CD3/CD28 beads are added. On day 0, the lentivirus for each of the conditions is added (e.g., 4 mL of cells at 0.5e6/mL) and there is a control of non-transduced cells. Double media to 8 mL on day 1 and then double the media to 16 mL on day 2. On day 3, harvest 4 mL and then double media to 24 mL on day 4. Harvest 4 mL on day 6 before doubling media to 40 mL. The cells can be split (e.g., 14 mL 0.5e6 cells/mL) on day 8 and then on day 6 harvest 4 mL before doubling media to 40 mL. 4mL may be harvested on day 10 before the media is doubled to 20 mL. On day 13, 4 mL are harvested before doubling the media to 32 mL. The culture is split in half and half of the culture is activated (CD3/CD28 activation beads 1:1) and stimulated overnight. On day 14, 4 mL of each stimulated and non-

stimulated cells are harvested and the culture is ended. Transgene copy number per cell are assayed by harvesting cells and extracting genomic DNA then quantifying with standard curve qPCR against the endogenous genome and against the transgene sequence, then converting the detected quantities to a ratio. Mean Fluorescence Intensity (MFI) is assayed by flow cytometry with appropriate staining for each group. Percent expressing may also be assayed by flow cytometry quantifying the percent of cells fluorescing above threshold. Soluble payloads can be quantified by harvesting culture supernatant at each marked timepoint and running MesoScale Discovery plate assay (MSD) then normalizing for cell density.

Adeno-associated viral particles

[0352] Delivery of any of the CA2 biocircuits, CA2 biocircuit components, CA2 effector modules, SREs or payloads of the present disclosure may be achieved using recombinant adeno-associated viral (rAAV) vectors. Such vectors or viral particles may be designed to utilize any of the known serotype capsids or combinations of serotype capsids.

[0353] AAV vectors include not only single stranded vectors but self-complementary AAV vectors (scAAVs). scAAV vectors contain DNA which anneals together to form double stranded vector genome. By skipping second strand synthesis, scAAVs allow for rapid expression in the cell.

[0354] The rAAV vectors may be manufactured by standard methods in the art such as by triple transfection, in sf9 insect cells or in suspension cell cultures of human cells such as HEK293 cells.

[0355] The CA2 biocircuits, CA2 biocircuit components, CA2 effector modules, SREs or payloads may be encoded in one or more viral genomes to be packaged in the AAV capsids taught herein.

[0356] Such vector or viral genomes may also include, in addition to at least one or two ITRs (inverted terminal repeats), certain regulatory elements necessary for expression from the vector or viral genome. Such regulatory elements are well known in the art and include for example promoters, introns, spacers, stuffer sequences, and the like.

[0357] The CA2 biocircuits, CA2 biocircuit components, CA2 effector modules, SREs or payloads described herein may be administered in one or more AAV particles.

[0358] In some embodiments, the CA2 effector modules may be administered in one or more AAV particles. In some embodiments, more than one CA2 effector module or SRE may be encoded in a viral genome.

Retroviral vehicles/particles (γ -retroviral vectors)

[0359] In some embodiments, retroviral vehicles/particles may be used to deliver the CA2 biocircuits, CA2 biocircuit components, CA2 effector modules, SREs or payloads of the present disclosure. Retroviral vectors (RVs) allow the permanent integration of a transgene in target cells. In addition to lentiviral vectors based on complex HIV-1/2,

retroviral vectors based on simple gamma-retroviruses have been widely used to deliver therapeutic genes and demonstrated clinically as one of the most efficient and powerful gene delivery systems capable of transducing a broad range of cell types. Example species of Gamma retroviruses include the murine leukemia viruses (MLVs) and the feline leukemia viruses (FeLV).

[0360] In some embodiments, gamma-retroviral vectors derived from a mammalian gamma-retrovirus such as murine leukemia viruses (MLVs), are recombinant. The MLV families of gamma retroviruses include the ecotropic, amphotropic, xenotropic and polytropic subfamilies. Ecotropic viruses are able to infect only murine cells using mCAT-1 receptor. Examples of ecotropic viruses are Moloney MLV and AKV. Amphotropic viruses infect murine, human and other species through the Pit-2 receptor. One example of an amphotropic virus is the 4070A virus. Xenotropic and polytropic viruses utilize the same (Xpr1) receptor but differ in their species tropism. Xenotropic viruses such as NZB-9-1 infect human and other species but not murine species, whereas polytropic viruses such as focus-forming viruses (MCF) infect murine, human and other species.

[0361] Gamma-retroviral vectors may be produced in packaging cells by co-transfecting the cells with several plasmids including one encoding the retroviral structural and enzymatic (gag-pol) polyprotein, one encoding the envelope (env) protein, and one encoding the vector mRNA comprising polynucleotide encoding the compositions of the present disclosure that is to be packaged in newly formed viral particles.

[0362] In some aspects, the recombinant gamma-retroviral vectors are pseudotyped with envelope proteins from other viruses. Envelope glycoproteins are incorporated in the outer lipid layer of the viral particles which can increase/alter the cell tropism. Exemplary envelop proteins include the gibbon ape leukemia virus envelope protein (GALV) or vesicular stomatitis virus G protein (VSV-G), or Simian endogenous retrovirus envelop protein, or Measles Virus H and F proteins, or Human immunodeficiency virus gp120 envelop protein, or coxal vesiculovirus envelop protein (See, e.g., U.S. application publication NO.: 2012/164118; the contents of which are incorporated herein by reference in its entirety). In other aspects, envelope glycoproteins may be genetically modified to incorporate targeting/binding ligands into gamma-retroviral vectors, binding ligands including, but not limited to, peptide ligands, single chain antibodies and growth factors (Waehler et al., *Nat. Rev. Genet.* 2007, 8(8):573–587; the contents of which are incorporated herein by reference in its entirety). These engineered glycoproteins can retarget vectors to cells expressing their corresponding target moieties. In other aspects, a “molecular bridge” may be introduced to direct vectors to specific cells. The molecular bridge has dual specificities: one end can recognize viral glycoproteins, and the other end can bind to the molecular determinant on the target cell. Such molecular bridges, for example ligand-receptor, avidin-biotin, and chemical conjugations, monoclonal antibodies and engineered fusogenic proteins, can direct the attachment of viral

vectors to target cells for transduction (Yang et al., *Biotechnol. Bioeng.*, 2008, 101(2): 357-368; and Maetzig et al., *Viruses*, 2011, 3, 677-713; the contents of each of which are incorporated herein by reference in their entirety).

[0363] In some embodiments, the recombinant gamma-retroviral vectors are self-inactivating (SIN) gammaretroviral vectors. The vectors are replication incompetent. SIN vectors may harbor a deletion within the 3' U3 region initially comprising enhancer/promoter activity. Furthermore, the 5' U3 region may be replaced with strong promoters (needed in the packaging cell line) derived from Cytomegalovirus or RSV, or an internal promoter of choice, and/or an enhancer element. The choice of the internal promoters may be made according to specific requirements of gene expression needed for a particular purpose of the present disclosure.

[0364] In some embodiments, polynucleotides encoding the CA2 biocircuit, CA2 biocircuit components, CA2 effector module, SRE are inserted within the recombinant viral genome. The other components of the viral mRNA of a recombinant gamma-retroviral vector may be modified by insertion or removal of naturally occurring sequences (e.g., insertion of an IRES, insertion of a heterologous polynucleotide encoding a polypeptide or inhibitory nucleic acid of interest, shuffling of a more effective promoter from a different retrovirus or virus in place of the wild-type promoter and the like). In some examples, the recombinant gamma-retroviral vectors may comprise modified packaging signal, and/or primer binding site (PBS), and/or 5'-enhancer/promoter elements in the U3-region of the 5'-long terminal repeat (LTR), and/or 3'-SIN elements modified in the U3-region of the 3'-LTR. These modifications may increase the titers and the ability of infection.

[0365] Gammaretroviral vectors suitable for delivering CA2 biocircuit components, CA2 effector modules, SREs or payloads of the present disclosure may be selected from those disclosed in U.S. Pat. NOs.: 8,828,718; 7,585,676; 7,351,585; U.S. application publication NO.: 2007/048285; PCT application publication NOs.: WO2010/113037; WO2014/121005; WO2015/056014; and EP Pat. NOs.: EP1757702; EP1757703 (the contents of each of which are incorporated herein by reference in their entirety).

Oncolytic Viral vector

[0366] In some embodiments, polynucleotides of present disclosure may be packaged into oncolytic viruses. As used herein, the term "oncolytic virus" refers to a virus that preferentially infects and kills cancer cells such as vaccine viruses. An oncolytic virus can occur naturally or can be a genetically modified virus such as oncolytic adenovirus, and oncolytic herpes virus.

[0367] In some embodiments, oncolytic vaccine viruses may include viral particles of a thymidine kinase (TK)-deficient, granulocyte macrophage (GM)-colony stimulating factor (CSF)-expressing, replication-competent vaccinia

virus vector sufficient to induce oncolysis of cells in the tumor; See e.g., US Pat. NO.: 9,226,977; the contents of which are incorporated herein by reference in their entirety.

Messenger RNA (mRNA)

[0368] In some embodiments, the CA2 effector modules described herein may be designed as a messenger RNA (mRNA). As used herein, the term “messenger RNA” (mRNA) refers to any polynucleotide which encodes a polypeptide of interest and which is capable of being translated to produce the encoded polypeptide of interest *in vitro*, *in vivo*, *in situ* or *ex vivo*. Such mRNA molecules may have the structural components or features of any of those taught in International Application number PCT/US2013/030062, the contents of which are incorporated herein by reference in its entirety.

[0369] In some embodiments, the CA2 effector modules may be designed as self-amplifying RNA. “Self-amplifying RNA” as used herein refers to RNA molecules that can replicate in the host resulting in the increase in the amount of the RNA and the protein encoded by the RNA. Such self-amplifying RNA may have structural features or components of any of those taught in International Patent Application Publication No. WO2011005799 (the contents of which are incorporated herein by reference in their entirety).

Dosing

[0370] The present disclosure provides methods comprising administering any one or more or component of a CA2 biocircuit system to a subject in need thereof. These may be administered to a subject using any amount and any route of administration effective for preventing or treating or imaging a disease, disorder, and/or condition (e.g., a disease, disorder, and/or condition relating to cancer or an autoimmune disease). The exact amount required will vary from subject to subject, depending on the species, age, and general condition of the subject, the severity of the disease, the particular composition, its mode of administration, its mode of activity, and the like.

[0371] Compositions in accordance with the present disclosure are typically formulated in dosage unit form for ease of administration and uniformity of dosage. It will be understood, however, that the total daily usage of the compositions of the present disclosure may be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective, prophylactically effective, or appropriate imaging dose level for any particular patient will depend upon a variety of factors including the disorder being treated and the severity of the disorder; the activity of the specific compound employed; the specific composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific compound employed; and like factors well known in the medical arts.

[0372] In some embodiments, compositions described herein may be used in varying doses to avoid T cell exhaustion, prevent cytokine release syndrome and minimize toxicity associated with immunotherapy. For example, low doses of the compositions of the present disclosure may be used to initially treat patients with high tumor burden, while patients with low tumor burden may be treated with high and repeated doses of the compositions described herein to ensure recognition of a minimal tumor antigen load. In another instance, the compositions of the present invention may be delivered in a pulsatile fashion to reduce tonic T cell signaling and enhance persistence *in vivo*. In some aspects, toxicity may be minimized by initially using low doses of the compositions of the invention, prior to administering high doses. Dosing may be modified if serum markers such as ferritin, serum C-reactive protein, IL6, IFN- γ , and TNF- α are elevated.

[0373] In some embodiments, the neurotoxicity may be associated with CAR or TIL therapy. Such neurotoxicity may be associated CD19-CARs. Toxicity may be due to excessive T cell infiltration into the brain. In some embodiments, neurotoxicity may be alleviated by preventing the passage of T cells through the blood brain barrier. This can be achieved by the targeted gene deletion of the endogenous α -4 integrin inhibitors such as tysabri/natalizumab may also be useful in the present invention.

[0374] Also provided herein are methods of administering ligands in accordance with the invention to a subject in need thereof. The ligand may be administered to a subject or to cells, using any amount and any route of administration effective for tuning the CA2 biocircuits of the invention. The exact amount required will vary from subject to subject, depending on the species, age, and general condition of the subject, the severity of the disease, the particular composition, its mode of administration, its mode of activity, and the like. The subject may be a human, a mammal, or an animal. Compositions in accordance with the invention are typically formulated in unit dosage form for ease of administration and uniformity of dosage. It will be understood, however, that the total daily usage of the compositions of the present invention may be decided by the attending physician within the scope of sound medical judgment. In certain embodiments, the ligands in accordance with the present invention may be administered at dosage levels sufficient to deliver from about 0.0001 mg/kg to about 100 mg/kg, from about 0.001 mg/kg to about 0.05 mg/kg, from about 0.005 mg/kg to about 0.05 mg/kg, from about 0.001 mg/kg to about 0.005 mg/kg, from about 0.05 mg/kg to about 0.5 mg/kg, from about 0.01 mg/kg to about 50 mg/kg, from about 0.1 mg/kg to about 40 mg/kg, from about 0.5 mg/kg to about 30 mg/kg, from about 0.01 mg/kg to about 10 mg/kg, from about 0.1 mg/kg to about 10 mg/kg, or from about 1 mg/kg to about 25 mg/kg, from about 10 mg/kg to about 100 mg/kg, from about 50 mg/kg to about 500 mg/kg, from about 100 mg/kg to about 1000 mg/kg, of subject body weight per day, one or more times a day, to obtain the desired effect. In some embodiments, the dosage levels may be 1mg/kg, 5 mg/kg, 10mg/kg, 20mg/kg, 30 mg/kg, 40 mg/kg, 50 mg/kg, 60

mg/kg, 70 mg/kg, 80 mg/kg, 90 mg/kg, 100 mg/kg, 100 mg/kg, 110 mg/kg, 120 mg/kg, 130 mg/kg, 140 mg/kg, 150 mg/kg, 160 mg/kg, 170 mg/kg, 180 mg/kg, 190 mg/kg or mg/kg of subject body weight per day, or more times a day, to obtain the desired effect.

[0375] The present disclosure provides methods for delivering to a cell or tissue any of the ligands described herein, comprising contacting the cell or tissue with said ligand and can be accomplished *in vitro*, *ex vivo*, or *in vivo*. In certain embodiments, the ligands in accordance with the present invention may be administered to cells at dosage levels sufficient to deliver from about 1 nM to about 10 nM, from about 5 nM to about 50 nM, from about 10 nM to about 100 nM, from about 50 nM to about 500 nM, from about 100 nM to about 1000 nM, from about 1 μ M to about 10 μ M, from about 5 μ M to about 50 μ M from about 10 μ M to about 100 μ M from about 25 μ M to about 250 μ M from about 50 μ M to about 500 μ M. In some embodiments, the ligand may be administered to cells at doses selected from but not limited to 0.00064 μ M, 0.0032 μ M, 0.016 μ M, 0.08 μ M, 0.4 μ M, 1 μ M 2 μ M, 10 μ M, 50 μ M, 75 μ M, 100 μ M, 150 μ M, 175 μ M, 200 μ M, 250 μ M.

[0376] The desired dosage of the ligands of the present invention may be delivered only once, three times a day, two times a day, once a day, every other day, every third day, every week, every two weeks, every three weeks, or every four weeks. In certain embodiments, the desired dosage may be delivered using multiple administrations (e.g., two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, or more administrations). When multiple administrations are employed, split dosing regimens such as those described herein may be used. As used herein, a “split dose” is the division of “single unit dose” or total daily dose into two or more doses, e.g., two or more administrations of the “single unit dose”. As used herein, a “single unit dose” is a dose of any therapeutic administered in one dose/at one time/single route/single point of contact, i.e., single administration event. The desired dosage of the ligand of the present disclosure may be administered as a “pulse dose” or as a “continuous flow”. As used herein, a “pulse dose” is a series of single unit doses of any therapeutic administered with a set frequency over a period of time. As used herein, a “continuous flow” is a dose of therapeutic administered continuously for a period of time in a single route/single point of contact, i.e., continuous administration event. A total daily dose, an amount given or prescribed in 24-hour period, may be administered by any of these methods, or as a combination of these methods, or by any other methods suitable for a pharmaceutical administration.

Administration

[0377] In some embodiments, the compositions for immunotherapy may be administered to cells *ex vivo* and subsequently administered to the subject. Immune cells can be isolated and expanded *ex vivo* using a variety of methods known in the art. For example, methods of isolating cytotoxic T cells are described in U.S. Pat. NOs. 6,805,861 and

6,531, 451; the contents of each of which are incorporated herein by reference in their entirety. Isolation of NK cells is described in U.S. Pat. NOs. 7,435, 596; the contents of which are incorporated by reference herein in its entirety.

[0378] In some embodiments, depending upon the nature of the cells, the cells may be introduced into a host organism e.g. a mammal, in a wide variety of ways including by injection, transfusion, infusion, local instillation or implantation. In some aspects, the cells described herein may be introduced at the site of the tumor. The number of cells that are employed will depend upon a number of circumstances, the purpose for the introduction, the lifetime of the cells, the protocol to be used, for example, the number of administrations, the ability of the cells to multiply, or the like. The cells may be in a physiologically-acceptable medium.

[0379] In some embodiments, the cells described herein may be administered in multiple doses to subjects having a disease or condition. The administrations generally effect an improvement in one or more symptoms of cancer or a clinical condition and/or treat or prevent cancer or clinical condition or symptom thereof.

[0380] In some embodiments, the compositions for immunotherapy may be administered *in vivo*. In some embodiments, polypeptides of the present disclosure comprising CA2 biocircuits, CA2 effector molecules, SREs, payloads of interest (e.g., immunotherapeutic agents) and compositions described herein may be delivered *in vivo* to the subject. *In vivo* delivery of immunotherapeutic agents is well described in the art. For example, methods of delivery of cytokines are described in the E.P. Pat. NO. EP0930892 A1, the contents of which are incorporated herein by reference.

Routes of delivery

[0381] The pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs (e.g., CA2 DDs), payloads (e.g., immunotherapeutic agents), vectors and cells of the present disclosure may be administered by any route to achieve a therapeutically effective outcome.

These include, but are not limited to enteral (into the intestine), gastroenteral, epidural (into the dura matter), oral (by way of the mouth), transdermal, peridural, intracerebral (into the cerebrum), intracerebroventricular (into the cerebral ventricles), epicutaneous (application onto the skin), intradermal, (into the skin itself), subcutaneous (under the skin), nasal administration (through the nose), intravenous (into a vein), intravenous bolus, intravenous drip, intraarterial (into an artery), intramuscular (into a muscle), intracardiac (into the heart), intraosseous infusion (into the bone marrow), intrathecal (into the spinal canal), intraperitoneal, (infusion or injection into the peritoneum), intravesical infusion, intravitreal, (through the eye), intracavernous injection (into a pathologic cavity) intracavitary (into the base of the penis), intravaginal administration, intrauterine, extra-amniotic administration, transdermal (diffusion through the intact skin for systemic distribution), transmucosal (diffusion through a mucous membrane), transvaginal, insufflation (snorting), sublingual, sublabial, enema, eye drops (onto the conjunctiva), in ear drops, auricular (in or by way of the ear), buccal

(directed toward the cheek), conjunctival, cutaneous, dental (to a tooth or teeth), electro-osmosis, endocervical, endosinusal, endotracheal, extracorporeal, hemodialysis, infiltration, interstitial, intra-abdominal, intra-amniotic, intra-articular, intrabiliary, intrabronchial, intrabursal, intracartilaginous (within a cartilage), intracaudal (within the cauda equine), intracisternal (within the cisterna magna cerebellomedullaris), intracorneal (within the cornea), dental intracornal, intracoronary (within the coronary arteries), intracorporus cavernosum (within the dilatable spaces of the corpus cavernosa of the penis), intradiscal (within a disc), intraductal (within a duct of a gland), intraduodenal (within the duodenum), intradural (within or beneath the dura), intraepidermal (to the epidermis), intraesophageal (to the esophagus), intragastric (within the stomach), intragingival (within the gingivae), intraileal (within the distal portion of the small intestine), intralesional (within or introduced directly to a localized lesion), intraluminal (within a lumen of a tube), intralymphatic (within the lymph), intramedullary (within the marrow cavity of a bone), intrameningeal (within the meninges), intramyocardial (within the myocardium), intraocular (within the eye), intraovarian (within the ovary), intrapericardial (within the pericardium), intrapleural (within the pleura), intraprostatic (within the prostate gland), intrapulmonary (within the lungs or its bronchi), intrasinal (within the nasal or periorbital sinuses), intraspinal (within the vertebral column), intrasynovial (within the synovial cavity of a joint), intratendinous (within a tendon), intratesticular (within the testicle), intrathecal (within the cerebrospinal fluid at any level of the cerebrospinal axis), intrathoracic (within the thorax), intratubular (within the tubules of an organ), intratumor (within a tumor), intratympanic (within the auris media), intravascular (within a vessel or vessels), intraventricular (within a ventricle), iontophoresis (by means of electric current where ions of soluble salts migrate into the tissues of the body), irrigation (to bathe or flush open wounds or body cavities), laryngeal (directly upon the larynx), nasogastric (through the nose and into the stomach), occlusive dressing technique (topical route administration which is then covered by a dressing which occludes the area), ophthalmic (to the external eye), oropharyngeal (directly to the mouth and pharynx), parenteral, percutaneous, periarticular, peridural, perineural, periodontal, rectal, respiratory (within the respiratory tract by inhaling orally or nasally for local or systemic effect), retrobulbar (behind the pons or behind the eyeball), intramyocardial (entering the myocardium), soft tissue, subarachnoid, subconjunctival, submucosal, topical, transplacental (through or across the placenta), transtracheal (through the wall of the trachea), transtympanic (across or through the tympanic cavity), ureteral (to the ureter), urethral (to the urethra), vaginal, caudal block, diagnostic, nerve block, biliary perfusion, cardiac perfusion, photopheresis or spinal.

Parenteral and injectable administration

[0382] In some embodiments, pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads of the present disclosure may be administered parenterally. Liquid

dosage forms for oral and parenteral administration include, but are not limited to, pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups, and/or elixirs. In addition to active ingredients, liquid dosage forms may comprise inert diluents commonly used in the art such as, for example, water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. Besides inert diluents, oral compositions can include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, and/or perfuming agents. In certain embodiments for parenteral administration, compositions are mixed with solubilizing agents such as CREMOPHOR[®], alcohols, oils, modified oils, glycols, polysorbates, cyclodextrins, polymers, and/or combinations thereof. In other embodiments, surfactants are included such as hydroxypropylcellulose.

[0383] Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions may be formulated according to the known art using suitable dispersing agents, wetting agents, and/or suspending agents. Sterile injectable preparations may be sterile injectable solutions, suspensions, and/or emulsions in nontoxic parenterally acceptable diluents and/or solvents, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, U.S.P., and isotonic sodium chloride solution. Sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil can be employed including synthetic mono- or diglycerides. Fatty acids such as oleic acid can be used in the preparation of injectables.

[0384] Injectable formulations may be sterilized, for example, by filtration through a bacterial-retaining filter, and/or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium prior to use.

[0385] In order to prolong the effect of active ingredients, it is often desirable to slow the absorption of active ingredients from subcutaneous or intramuscular injections. This may be accomplished by the use of liquid suspensions of crystalline or amorphous material with poor water solubility. The rate of absorption of active ingredients depends upon the rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle. Injectable depot forms are made by forming microencapsulated matrices of the drug in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of drug to polymer and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters)

and poly(anhydrides). Depot injectable formulations are prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissues.

Ophthalmic or otic administration

[0386] In some embodiments, pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads of the present disclosure may be prepared, packaged, and/or sold in formulations suitable for ophthalmic and/or otic administration. Such formulations may, for example, be in the form of eye and/or ear drops including, for example, a 0.1/1.0% (w/w) solution and/or suspension of the active ingredient in aqueous and/or oily liquid excipients. Such drops may further comprise buffering agents, salts, and/or one or more other of any additional ingredients described herein. Other ophthalmically-administrable formulations which are useful include those which comprise active ingredients in microcrystalline form and/or in liposomal preparations. Subretinal inserts may also be used as forms of administration.

Detectable agents and Labels

[0387] The stimuli, CA2 biocircuit systems and components, CA2 effector modules including the SREs and payloads may be associated with or bound to one or more radioactive agents or detectable agents.

[0388] These agents include various organic small molecules, inorganic compounds, nanoparticles, enzymes or enzyme substrates, fluorescent materials, luminescent materials (e.g., luminol), bioluminescent materials (e.g., luciferase, luciferin, and aequorin), chemiluminescent materials, radioactive materials (e.g., ^{18}F , ^{67}Ga , $^{81\text{m}}\text{Kr}$, ^{82}Rb , ^{111}In , ^{123}I , ^{133}Xe , ^{201}Tl , ^{125}I , ^{35}S , ^{14}C , ^3H , or $^{99\text{m}}\text{Tc}$ (e.g., as pertechnetate (technetate(VII), TcO_4^-)), and contrast agents (e.g., gold (e.g., gold nanoparticles), gadolinium (e.g., chelated Gd), iron oxides (e.g., superparamagnetic iron oxide (SPIO), monocrySTALLINE iron oxide nanoparticles (MIONS), and ultrasmall superparamagnetic iron oxide (USPIO)), manganese chelates (e.g., Mn-DPDP), barium sulfate, iodinated contrast media (iohexol), microbubbles, or perfluorocarbons).

[0389] In some embodiments, the detectable agent may be a non-detectable precursor that becomes detectable upon activation (e.g., fluorogenic tetrazine-fluorophore constructs (e.g., tetrazine-BODIPY FL, tetrazine-Oregon Green 488, or tetrazine-BODIPY TMR-X) or enzyme activatable fluorogenic agents (e.g., PROSENSE® (VisEn Medical))). In vitro assays in which the enzyme labeled compositions can be used include, but are not limited to, enzyme linked immunosorbent assays (ELISAs), immunoprecipitation assays, immunofluorescence, enzyme immunoassays (EIA), radioimmunoassays (RIA), and Western blot analysis.

Kits

[0390] The present disclosure includes a variety of kits for conveniently and/or effectively carrying out methods of the present disclosure. Typically, kits will comprise sufficient amounts and/or numbers of components to allow a user to perform one or multiple treatments of a subject(s) and/or to perform one or multiple experiments.

[0391] In one embodiment, the present disclosure provides kits for inhibiting genes in vitro or in vivo, comprising a CA2 biocircuit of the present disclosure or a combination of CA2 biocircuits of the present disclosure, optionally in combination with any other suitable active agents.

[0392] The kit may further comprise packaging and instructions and/or a delivery agent to form a formulation composition. The delivery agent may comprise, for example, saline, a buffered solution.

[0393] In additional embodiments, assay screening kits are provided. The kit includes a container for the screening assay. An instruction for the use of the assay and the information about the screening method are to be included in the kit.

IV. APPLICATIONS

[0394] The CA2 biocircuits, CA2 effector modules, SREs, stimuli, compositions or systems comprising one or more of the stimuli, CA2 biocircuits, CA2 effector modules of the present disclosure may be utilized in a large variety of applications including, but not limited to, therapeutics, diagnosis and prognosis, bioengineers, bioprocessing, biofactory, research agents, metabolomics, gene expression, enzyme replacement, etc.

Therapeutic Uses

Cancer Immunotherapy

[0395] Cancer immunotherapy aims at the induction or restoration of the reactivity of the immune system towards cancer. Significant advances in immunotherapy research have led to the development of various strategies which may broadly be classified into active immunotherapy and passive immunotherapy. In general, these strategies may be utilized to directly kill cancer cells or to counter the immunosuppressive tumor microenvironment. Active immunotherapy aims at induction of an endogenous, long-lasting tumor-antigen specific immune response. The response can further be enhanced by non-specific stimulation of immune response modifiers such as cytokines. In contrast, passive immunotherapy includes approaches where effector immune molecules such as tumor-antigen specific cytotoxic T cells or antibodies are administered to the host. This approach is short lived and requires multiple applications.

[0396] Despite significant advances, the efficacy of current immunotherapy strategies is limited by associated toxicities. These are often related to the narrow therapeutic window associated with immunotherapy, which in part, emerges from the need to push therapy dose to the edge of potentially fatal toxicity to get a clinically meaningful

treatment effect. Further, dose expands *in vivo* since adoptively transferred immune cells continue to proliferate within the patient, often unpredictably.

[0397] A major risk involved in immunotherapy is the on-target but off tumor side effects resulting from T-cell activation in response to normal tissue expression of the tumor associated antigen (TAA). Clinical trials utilizing T cells expressing T-cell receptor against specific TAA reported skin rash, colitis and hearing loss in response to immunotherapy.

[0398] Immunotherapy may also produce on target, on-tumor toxicities that emerge when tumor cells are killed in response to the immunotherapy. The adverse effects include tumor lysis syndrome, cytokine release syndrome and the related macrophage activation syndrome. Importantly, these adverse effects may occur during the destruction of tumors, and thus even a successful on-tumor immunotherapy might result in toxicity. Approaches to regulatably control immunotherapy are thus highly desirable since they have the potential to reduce toxicity and maximize efficacy.

[0399] The present disclosure provides systems, compositions, immunotherapeutic agents and methods for cancer immunotherapy. These compositions provide tunable regulation of gene expression and function in immunotherapy. The present disclosure also provides CA2 biocircuit systems, CA2 effector modules, stimulus response elements (SREs) and payloads, as well as polynucleotides encoding any of the foregoing. In one aspect, the systems, compositions, immunotherapeutic agents and other components described herein can be controlled by a separately added stimulus, which provides a significant flexibility to regulate cancer immunotherapy. Further, the systems, compositions and the methods of the present disclosure may also be combined with therapeutic agents such as chemotherapeutic agents, small molecules, gene therapy, and antibodies.

[0400] The tunable nature of the systems and compositions described herein has the potential to improve the potency and duration of the efficacy of immunotherapies. Reversibly silencing the biological activity of adoptively transferred cells using compositions of the present disclosure allows maximizing the potential of cell therapy without irretrievably killing and terminating the therapy.

[0401] The present disclosure provides methods for fine tuning of immunotherapy after administration to patients. This in turn improves the safety and efficacy of immunotherapy and increases the subject population that may benefit from immunotherapy.

[0402] In one embodiment, the CA2 biocircuit systems, CA2 effector modules, SREs, and components that tune expression levels and activities of any agents may be used for immunotherapy. As non-limiting examples, an immunotherapeutic agent may be an antibody and fragments and variants thereof, a cancer specific T cell receptor (TCR) and variants thereof, an anti-tumor specific chimeric antigen receptor (CAR), a chimeric switch receptor, an inhibitor of a

co-inhibitory receptor or ligand, an agonist of a co-stimulatory receptor and ligand, a cytokine, chemokine, a cytokine receptor, a chemokine receptor, a soluble growth factor, a metabolic factor, a suicide gene, a homing receptor, or any agent that induces an immune response in a cell and a subject.

[0403] In some embodiments, the composition for inducing an immune response may comprise an CA2 effector module. In some embodiments, the CA2 effector module may comprise a stimulus response element (SRE) operably linked to at least one payload. In one aspect, the payload may be an immunotherapeutic agent.

[0404] In some embodiments, CA2 biocircuit systems, CA2 effector modules, and compositions of the present disclosure relate to post-translational regulation of protein (payload) function anti-tumor immune responses of immunotherapeutic agents.

1. Adoptive cell transfer (adoptive immunotherapy)

[0405] In some embodiments, cells which are genetically modified to express at least one CA2 biocircuit system, CA2 effector module, SRE, and/or payload of interest (e.g., immunotherapeutic agent) may be used for adoptive cell therapy (ACT). As used herein, Adoptive cell transfer refers to the administration of immune cells (from autologous, allogenic or genetically modified hosts) with direct anticancer activity. ACT has shown promise in clinical application against malignant and infectious disease. For example, T cells genetically engineered to recognize CD19 have been used to treat follicular B cell lymphoma (Kochenderfer et al., *Blood*, 2010, 116:4099-4102; and Kochenderfer and Rosenberg, *Nat Rev Clin Oncol.*, 2013, 10(5): 267-276) and ACT using autologous lymphocytes genetically-modified to express anti-tumor T cell receptors has been used to treat metastatic melanoma (Rosenberg and Dudley, *Curr. Opin. Immunol.* 2009, 21: 233-240).

[0406] According to the present disclosure, the CA2 biocircuits and systems may be used in the development and implementation of cell therapies such as adoptive cell therapy. Certain effector modules useful in cell therapy are given in Figures 7-12 in International Publication No. WO2017/180587, the contents of which are herein incorporated by reference in their entirety. The CA2 biocircuits, CA2 effector modules and their SREs and payloads may be used in cell therapies to effect CAR therapies, in the manipulation or regulation of TILs, in allogeneic cell therapy, in combination T cell therapy with other treatment lines (e.g. radiation, cytokines), to encode engineered TCRs, or modified TCRs, or to enhance T cells other than TCRs (e.g. by introducing cytokine genes, genes for the checkpoint inhibitors PD1, CTLA4).

[0407] Provided herein are methods for use in adoptive cell therapy. The methods involve preconditioning a subject in need thereof, modulating immune cells with SRE, CA2 biocircuits and compositions of the present disclosure, administering to a subject, engineered immune cells expressing compositions described herein and the successful engraftment of engineered cells within the subject.

[0408] In some embodiments, SREs, CA2 biocircuits and compositions of the present disclosure may be used to minimize preconditioning regimens associated with adoptive cell therapy. As used herein “preconditioning” refers to any therapeutic regimen administered to a subject to improve the outcome of adoptive cell therapy. Preconditioning strategies include but are not limited to total body irradiation and/or lymphodepleting chemotherapy. Adoptive therapy clinical trials without preconditioning have failed to demonstrate any clinical benefit, indicating its importance in ACT. Yet, preconditioning is associated with significant toxicity and limits the subject cohort that is suitable for ACT. In some instances, immune cells for ACT may be engineered to express cytokines such as IL12 and IL15 as payload using SREs of the present disclosure to reduce the need for preconditioning (Pengram et al. (2012) Blood 119 (18): 4133-41; the contents of which are incorporated by reference in their entirety).

[0409] In some embodiments, immune cells for ACT may be dendritic cells, T cells such as CD8⁺ T cells and CD4⁺ T cells, natural killer (NK) cells, NK T cells, Cytotoxic T lymphocytes (CTLs), tumor infiltrating lymphocytes (TILs), lymphokine activated killer (LAK) cells, memory T cells, regulatory T cells (Tregs), helper T cells, cytokine-induced killer (CIK) cells, and any combination thereof. In other embodiments, immune stimulatory cells for ACT may be generated from embryonic stem cell (ESC) and induced pluripotent stem cell (iPSC). In some embodiments, autologous or allogeneic immune cells are used for ACT.

[0410] In some embodiments, cells used for ACT may be T cells engineered to express CARs comprising an antigen-binding domain specific to an antigen on tumor cells of interest. In other embodiments, cells used for ACT may be NK cells engineered to express CARs comprising an antigen-binding domain specific to an antigen on tumor cells of interest. In addition to adoptive transfer of genetically modified T cells (e.g., CAR T cells) for immunotherapy, alternate types of CAR-expressing leukocytes, either alone, or in combination with CAR T cells may be used for adoptive immunotherapy. In one example, a mixture of T cells and NK cells may be used for ACT. The expression level of CARs in T cells and NK cells, according to the present disclosure, is tuned and controlled by a small molecule that binds to the DD(s) operably linked to the CAR in the CA2 effector module.

[0411] In some embodiments, the CARs of the present disclosure may be placed under the transcriptional control of the T cell receptor alpha constant (TRAC) locus in the T cells to achieve uniform CAR expression while enhancing T cell potency. The TRAC locus may be disrupted using the CRISPR/Cas 9, zinc finger nucleases (ZFNs), TALENs followed by the insertion of the CAR construct. Methods of engineering CAR constructs directed to the TRAC locus are described in Eyquem J. et al (2017) Nature.543(7643):113-117 (the contents of which are incorporated herein by reference in their entirety).

[0412] In some embodiments, NK cells engineered to express the present compositions may be used for ACT. NK cell activation induces perforin/granzyme-dependent apoptosis in target cells. NK cell activation also induces cytokine secretion such as IFN γ , TNF- α and GM-CSF. These cytokines enhance the phagocytic function of macrophages and their antimicrobial activity and augment the adaptive immune response via up-regulation of antigen presentation by antigen presenting cells such as dendritic cells (DCs) (Reviewed by Vivier et al., *Nat. Immunol.*, 2008, 9(5): 503-510).

[0413] Other examples of genetic modification may include the introduction of chimeric antigen receptors (CARs) and the down-regulation of inhibitory NK cell receptors such as NKG2A.

[0414] NK cells may also be genetically reprogrammed to circumvent NK cell inhibitory signals upon interaction with tumor cells. For example, using CRISPR, ZFN, or TALEN to genetically modify NK cells to silence their inhibitory receptors may enhance the anti-tumor capacity of NK cells.

[0415] Immune cells can be isolated and expanded *ex vivo* using a variety of methods known in the art. For example, methods of isolating and expanding cytotoxic T cells are described in U.S. Pat. NOs. 6,805,861 and 6,531,451; US Patent Publication NO. US20160348072A1 and International Patent Publication NO. WO2016168595A1; the contents of each of which are incorporated herein by reference in their entirety. Isolation and expansion of NK cells is described in US Patent Publication NO. US20150152387A1, U.S. Patent NO. 7,435,596; and Oyer, J.L. (2016). *Cytotherapy*. 18(5):653-63; the contents of each of which are incorporated by reference herein in its entirety. Specifically, human primary NK cells may be expanded in the presence of feeder cells e.g. a myeloid cell line that has been genetically modified to express membrane bound IL15, IL21, IL12 and 4-1BBL.

[0416] In some instances, sub populations of immune cells may be enriched for ACT. Methods for immune cell enrichment are taught in International Patent Publication NO. WO2015039100A1. In another example, T cells positive for B and T lymphocyte attenuator marker BTLA) may be used to enrich for T cells that are anti-cancer reactive as described in U.S. Pat. NO. 9,512,401 (the content of each of which are incorporated herein by reference in their entirety).

[0417] In some embodiments, immune cells for ACT may be depleted of select sub populations to enhance T cell expansion. For example, immune cells may be depleted of Foxp3+ T lymphocytes to minimize the anti-tumor immune response using methods taught in US Patent Publication NO. US 20160298081A1; the contents of which are incorporated by reference herein in their entirety.

[0418] In some embodiments, activation and expansion of T cells for ACT is achieved antigenic stimulation of a transiently expressed Chimeric Antigen Receptor (CAR) on the cell surface. Such activation methods are taught in International Patent NO. WO2017015427, the content of which are incorporated herein by reference in their entirety.

[0419] In some embodiments, immune cells may be activated by antigens associated with antigen presenting cells (APCs). In some embodiments, the APCs may be dendritic cells, macrophages or B cells that are antigen specific or nonspecific. The APCs may be autologous or homologous in their organ. In some embodiments, the APCs may be artificial antigen presenting cells (aAPCs) such as cell based aAPCs or acellular aAPCs. Cell based aAPCs may be selected from either genetically modified allogeneic cells such as human erythroleukemia cells or xenogeneic cells such as murine fibroblasts and *Drosophila* cells. Alternatively, the APCs may be acellular wherein the antigens or costimulatory domains are presented on synthetic surfaces such as latex beads, polystyrene beads, lipid vesicles or exosomes.

[0420] In some embodiments, cells described herein, specifically T cells may be expanded using artificial cell platforms. In one embodiment, the mature T cells may be generated using artificial thymic organoids (ATOs) described by Seet CS et al. 2017. *Nat Methods*. 14, 521–530 (the contents of which are incorporated herein by reference in their entirety). ATOs are based on a stromal cell line expressing delta like canonical notch ligand (DLL1). In this method, stromal cells are aggregated with hematopoietic stem and progenitor cells by centrifugation and deployed on a cell culture insert at the air–fluid interface to generate organoid cultures. ATO-derived T cells exhibit naive phenotypes, a diverse T cell receptor (TCR) repertoire and TCR-dependent function.

[0421] In some embodiments, adoptive cell therapy is carried out by autologous transfer, wherein the cells are derived from a subject in need of a treatment and the cells, following isolation and processing are administered to the same subject. In other instances, ACT may involve allogenic transfer wherein the cells are isolated and/or prepared from a donor subject other than the recipient subject who ultimately receives cell therapy. The donor and recipient subject may be genetically identical, or similar or may express the same HLA class or subtype.

[0422] In some embodiments, the multiple immunotherapeutic agents introduced into the immune cells for ACT (e.g., T cells and NK cells) may be controlled by the same CA2 biocircuit system. In other embodiments, the multiple immunotherapeutic agents introduced into the immune cells for ACT (e.g., T cells and NK cells) may be controlled by different biocircuit systems. In another example, a suicide gene and a CAR construct may be linked to two separate effector modules.

[0423] Following genetic modulation using SREs, CA2 biocircuits and compositions described herein, cells are administered to the subject in need thereof. Methods for administration of cells for adoptive cell therapy are known and may be used in connection with the provided methods and compositions. For example, adoptive T cell therapy methods are described, e.g., in US Patent Application Publication No. 2003/0170238 to Gruenberg et al; US Patent No. 4,690,915 to Rosenberg; Rosenberg (2011) *Nat Rev Clin Oncol*. 8(10):577-85). See, e.g., Themeli et al. (2013) *Nat Biotechnol*.

31(10): 928-933; Tsukahara et al. (2013) Biochem Biophys Res Commun 438(1): 84-9; Davila et al. (2013) PLoS ONE 8(4): e61338; the contents of each of which are incorporated herein by reference in their entirety.

[0424] In some embodiments, immune cells for ACT may be modified to express one or more immunotherapeutic agents which facilitate immune cells activation, infiltration, expansion, survival and anti-tumor functions. The immunotherapeutic agents may be a second CAR or TCR specific to a different target molecule; a cytokine or a cytokine receptor; a chimeric switch receptor that converts an inhibitory signal to a stimulatory signal; a homing receptor that guides adoptively transferred cells to a target site such as the tumor tissue; an agent that optimizes the metabolism of the immune cell; or a safety switch gene (e.g., a suicide gene) that kills activated T cells when a severe event is observed after adoptive cell transfer or when the transferred immune cells are no-longer needed.

[0425] In some embodiments, immune cells used for adoptive cell transfer can be genetically manipulated to improve their persistence, cytotoxicity, tumor targeting capacity, and ability to home to disease sites *in vivo*, with the overall aim of further improving upon their capacity to kill tumors in cancer patients. One example is to introduce CA2 effector modules described herein comprising cytokines such as gamma-cytokines (IL2 and IL15) into immune cells to promote immune cell proliferation and survival. Transduction of cytokine genes (e.g., gamma-cytokines IL2 and IL15) into cells will be able to propagate immune cells without addition of exogenous cytokines and cytokine expressing NK cells have enhanced tumor cytotoxicity.

[0426] In some embodiments, CA2 biocircuits, SREs or CA2 effector modules may be utilized to prevent T cell exhaustion. As used herein, "T cell exhaustion" refers to the stepwise and progressive loss of T cell function caused by chronic T cell activation. T cell exhaustion is a major factor limiting the efficacy of antiviral and antitumor immunotherapies. Exhausted T cells have low proliferative and cytokine producing capabilities concurrent with high rates of apoptosis and high surface expression of multiple inhibitory receptors. T cell activation leading to exhaustion may occur either in the presence or absence of the antigen.

[0427] In some embodiments, the CA2 biocircuits, and their components may be utilized to prevent T cell exhaustion in the context of Chimeric Antigen Receptor-T cell therapy (CAR-T). In this context, exhaustion in some instances, may be caused by the oligomerization of the scFvs of the CAR on the cell surface which leads to continuous activation of the intracellular domains of the CAR. As a non-limiting example, CARs of the present disclosure may include scFvs that are unable to oligomerize. As another non-limiting example, CARs that are rapidly internalized and re-expressed following antigen exposure may also be selected to prevent chronic scFv oligomerization on cell surface. In one embodiment, the framework region of the scFvs may be modified to prevent constitutive CAR signaling (Long et al. 2014. Cancer Research. 74(19) S1; the contents of which are incorporated by reference in their entirety). Tunable CA2

biocircuit systems of the present disclosure may also be used to regulate the surface expression of the CAR on the T cell surface to prevent chronic T cell activation. The CARs described herein may also be engineered to minimize exhaustion. As a non-limiting example, the 41-BB signaling domain may be incorporated into CAR design to ameliorate T cell exhaustion. In some embodiments, any of the strategies disclosed by Long H A et al. may be utilized to prevent exhaustion (Long A H et al. (2015) Nature Medicine 21, 581–590; the contents of which are incorporated herein by reference in their entirety).

[0428] In some embodiments, the tunable nature of the CA2 biocircuits of the present disclosure may be utilized to reverse human T cell exhaustion observed with tonic CAR signaling. Reversibly silencing the biological activity of adoptively transferred cells using compositions of the present disclosure may be used to reverse tonic signaling which, in turn, may reinvigorate the T cells. Reversal of exhaustion may be measured by the downregulation of multiple inhibitory receptors associated with exhaustion.

[0429] In some embodiments, T cell metabolic pathways may be modified to diminish the susceptibility of T cells to exhaustion. Metabolic pathways may include, but are not limited to glycolysis, urea cycle, citric acid cycle, beta oxidation, fatty acid biosynthesis, pentose phosphate pathway, nucleotide biosynthesis, and glycogen metabolic pathways. As a non-limiting example, payloads that reduce the rate of glycolysis may be utilized to restrict or prevent T cell exhaustion (Long et al. Journal for Immunotherapy of Cancer 2013, 1(Suppl 1): P21; the contents of which are incorporated by reference in their entirety). In one embodiment, T cells of the present disclosure may be used in combination with inhibitors of glycolysis such as 2-deoxyglucose, and rapamycin.

[0430] In some embodiments, CA2 effector modules of the present disclosure, useful for immunotherapy may be placed under the transcriptional control of the T cell receptor alpha locus constant (TRAC) locus in the T cells. Eyquem et al. have shown that expression of the CAR from the TRAC locus prevents T cell exhaustion and the accelerated differentiation of T cells caused by excessive T cell activation (Eyquem J. et al (2017) Nature.543(7643):113-117; the contents of which are incorporated herein by reference in their entirety).

[0431] In some embodiments, payloads described herein may be used in conjunction with antibodies or fragments that target T cell surface markers associated with T cell exhaustion. T-cell surface markers associated with T cell exhaustion that may be used include, but are not limited to, CTLA-1, PD-1, TGIT, LAG-3, 2B4, BTLA, TIM3, VISTA, and CD96.

[0432] In one embodiment, the payload described herein may be a CD276 CAR (with CD28, 4-1BB, and CD3 zeta intracellular domains), that does not show an upregulation of the markers associated with early T cell exhaustion (see

International patent publication No. WO2017044699; the contents of which are incorporated by reference in their entirety).

[0433] In some embodiments, the compositions of the present disclosure may be utilized to alter TIL (tumor infiltrating lymphocyte) populations in a subject. In one embodiment, any of the payloads described herein may be utilized to change the ratio of CD4 positive cells to CD8 positive populations. In some embodiments, TILs may be sorted ex vivo and engineered to express any of the cytokines described herein. Payloads described herein may be used to expand CD4 and/or CD8 populations of TILs to enhance TIL mediated immune response.

2. Cancer vaccines

[0434] In some embodiments, CA2 biocircuits, CA2 effector modules, payloads of interest (e.g., immunotherapeutic agents), vectors, cells and compositions of the present disclosure may be used in conjunction with cancer vaccines.

[0435] In some embodiments, cancer vaccine may comprise peptides and/or proteins derived from tumor associated antigen (TAA). Such strategies may be utilized to evoke an immune response in a subject, which in some instances may be a cytotoxic T lymphocyte (CTL) response. Peptides used for cancer vaccines may also modified to match the mutation profile of a subject. For example, EGFR derived peptides with mutations matched to the mutations found in the subject in need of therapy have been successfully used in patients with lung cancer (Li F et al. (2016) Oncoimmunology. Oct 7;5(12): e1238539; the contents of which are incorporated herein by reference in their entirety).

[0436] In one embodiment, cancer vaccines of the present disclosure may be superagonist altered peptide ligands (APL) derived from TAAs. These are mutant peptide ligands that deviate from the native peptide sequence by one or more amino acids, which activate specific CTL clones more effectively than native epitopes. These alterations may allow the peptide to bind better to the restricting Class I MHC molecule or interact more favorably with the TCR of a given tumor-specific CTL subset. APLs may be selected using methods taught in US Patent Publication NO. US20160317633A1, the contents of which are incorporated herein by reference in their entirety.

3. Combination treatments

[0437] In some embodiments, it is desirable to combine compositions, vectors and cells described herein for administration to a subject. Compositions described herein comprising different immunotherapeutic agents may be used in combination for enhancement of immunotherapy.

[0438] In some embodiments, it is desirable to combine compositions described herein with adjuvants, that can enhance the potency and longevity of antigen-specific immune responses. Adjuvants used as immunostimulants in combination therapy include biological molecules or delivery carriers that deliver antigens. As non-limiting examples,

the compositions of the present disclosure may be combined with biological adjuvants such as cytokines, Toll Like Receptors, bacterial toxins, and/or saponins. In other embodiments, the compositions of the present disclosure may be combined with delivery carriers. Exemplary delivery carriers include, polymer microspheres, immune stimulating complexes, emulsions (oil-in-water or water-in-oil), aluminum salts, liposomes or virosomes.

[0439] In some embodiments, effector immune cells modified to express CA2 biocircuits, CA2 effector modules, SREs and payloads described herein may be combined with the biological adjuvants described herein. Dual regulation of CAR and cytokines and ligands to segregate the kinetic control of target-mediated activation from intrinsic cell T cell expansion. Such dual regulation also minimizes the need for pre-conditioning regimens in patients.

[0440] In some embodiments, effector immune cells modified to express one or more antigen-specific TCRs or CARs may be combined with compositions described herein comprising immunotherapeutic agents that convert the immunosuppressive tumor microenvironment.

[0441] In one aspect, effector immune cells modified to express CARs specific to different target molecules on the same cell may be combined. In another aspect, different immune cells modified to express the same CAR construct such as NK cells and T cells may be used in combination for a tumor treatment, for instance, a T cell modified to express a CD19 CAR may be combined with a NK cell modified to express the same CD19 CAR to treat B cell malignancy.

[0442] In other embodiments, immune cells modified to express CARs may be combined with checkpoint blockade agents.

[0443] In some embodiments, effector immune cells modified to expressed CA2 biocircuits, CA2 effector modules, SREs and payloads described herein may be combined with cancer vaccines described herein.

[0444] In some embodiments, methods of the present disclosure may include combination of the compositions of the present disclosure with other agents effective in the treatment of cancers, infectious diseases and other immunodeficient disorders, such as anti-cancer agents. As used herein, the term “anti-cancer agent” refers to any agent which is capable of negatively affecting cancer in a subject, for example, by killing cancer cells, inducing apoptosis in cancer cells, reducing the growth rate of cancer cells, reducing the incidence or number of metastases, reducing tumor size, inhibiting tumor growth, reducing the blood supply to a tumor or cancer cells, promoting an immune response against cancer cells or a tumor, preventing or inhibiting the progression of cancer, or increasing the lifespan of a subject with cancer.

[0445] In some embodiments, anti-cancer agent or therapy may be a chemotherapeutic agent, or radiotherapy, immunotherapeutic agent, surgery, or any other therapeutic agent which, in combination with the present disclosure, improves the therapeutic efficacy of treatment.

[0446] In one embodiment, a CA2 effector module comprising a CD19 CAR may be used in combination with amino pyrimidine derivatives such as the Burkitt's tyrosine receptor kinase (BTK) inhibitor using methods taught in International Patent Application NO. WO2016164580, the contents of which are incorporated herein by reference in their entirety.

[0447] In some embodiments, compositions of the present disclosure may be used in combination with immunotherapeutics other than the inventive therapy described herein, such as antibodies specific to some target molecules on the surface of a tumor cell.

[0448] Exemplary chemotherapies include, without limitation, Acivicin; Aclarubicin; Acodazole hydrochloride; Acronine; Adozelesin; Aldesleukin; Altretamine; Ambomycin; Ametrantrone acetate; Amsacrine; Anastrozole; Anthramycin; Asparaginase; Asperin, Sulindac, Curcumin, alkylating agents including: Nitrogen mustards such as mechlor-ethamine, cyclophosphamide, ifosfamide, melphalan and chlorambucil; nitrosoureas such as carmustine (BC U), lomustine (CCNU), and semustine (methyl-CC U); thyleneimines/methylmelamine such as triethylenemelamine (TEM), triethylene, thiophosphoramidate (thiotepa), hexamethylmelamine (HMM, altretamine); alkyl sulfonates such as busulfan; triazines such as dacarbazine (DTIC); antimetabolites including folic acid analogs such as methotrexate and trimetrexate, pyrrolidine analogs such as 5-fluorouracil, fluorodeoxyuridine, gemcitabine, cytosine arabinoside (AraC, cytarabine), 5-azacytidine, 2,2'-difluorodeoxycytidine, purine analogs such as 6-mercaptopurine, 6-thioguanine, azathioprine, 2'-deoxycoformycin (pentostatin), erythrohydroxynonyladenine (EHNA), fludarabine phosphate, and 2-chlorodeoxyadenosine (cladribine, 2-CdA); natural products including antimetabolic drugs such as paclitaxel, vinca alkaloids including vinblastine (VLB), vincristine, and vinorelbine, taxotere, estramustine, and estramustine phosphate; epipodophyllotoxins such as etoposide and teniposide; antibiotics, such as actinomycin D, daunomycin (rubidomycin), doxorubicin, mitoxantrone, idarubicin, bleomycins, plicamycin (mithramycin), mitomycinC, and actinomycin; enzymes such as L-asparaginase, cytokines such as interferon (IFN)-gamma, tumor necrosis factor (TNF)-alpha, TNF-beta and GM-CSF, anti-angiogenic factors, such as angiostatin and endostatin, inhibitors of FGF or VEGF such as soluble forms of receptors for angiogenic factors, including soluble VGF/VEGF receptors, platinum coordination complexes such as cisplatin and carboplatin, anthracenediones such as mitoxantrone, substituted urea such as hydroxyurea, methylhydrazine derivatives including N-methylhydrazine (MIFf) and procarbazine, adrenocortical suppressants such as mitotane (o,p'-DDD) and aminoglutethimide; hormones and antagonists including adrenocorticosteroid antagonists such as prednisone and equivalents, dexamethasone and aminoglutethimide; progestin such as hydroxyprogesterone caproate, medroxyprogesterone acetate and megestrol acetate; estrogen such as diethylstilbestrol and ethinyl estradiol equivalents; antiestrogen such as tamoxifen; androgens including testosterone propionate and fluoxymesterone/equivalents;

antiandrogens such as flutamide, gonadotropin-releasing hormone analogs and leuprolide; non-steroidal antiandrogens such as flutamide; kinase inhibitors, histone deacetylase inhibitors, methylation inhibitors, proteasome inhibitors, monoclonal antibodies, oxidants, anti-oxidants, telomerase inhibitors, BH3 mimetics, ubiquitin ligase inhibitors, stat inhibitors and receptor tyrosin kinase inhibitors such as imatinib mesylate (marketed as Gleevec or Glivec) and erlotinib (an EGF receptor inhibitor) now marketed as Tarveca; anti-virals such as oseltamivir phosphate, Amphotericin B, and palivizumab; Sdi 1 mimetics; Semustine; Senescence derived inhibitor 1; Sparfosic acid; Spicamycin D; Spiromustine; Splenopentin; Spongistatin 1; Squalamine; Stipiamide; Stromelysin inhibitors; Sulfinosine; Superactive vasoactive intestinal peptide antagonist; Velaresol; Veramine; Verdins; Verteporfin; Vinorelbine; Vinxaltine; Vitaxin; Vorozole; Zanolterone; Zeniplatin; Zilascorb; and Zinostatin stimalamer; PI3K β small-molecule inhibitor, GSK2636771; pan-PI3K inhibitor (BKM120); BRAF inhibitors. Vemurafenib (Zelboraf) and dabrafenib (Tafinlar); or any analog or derivative and variant of the foregoing.

[0449] Radiotherapeutic agents and factors include radiation and waves that induce DNA damage for example, γ -irradiation, X-rays, UV-irradiation, microwaves, electronic emissions, radioisotopes, and the like. Therapy may be achieved by irradiating the localized tumor site with the above described forms of radiations. It is most likely that all of these factors effect a broad range of damage DNA, on the precursors of DNA, the replication and repair of DNA, and the assembly and maintenance of chromosomes. Dosage ranges for X-rays range from daily doses of 50 to 200 roentgens for prolonged periods of time (3 to 4 weeks), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely, and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells.

[0450] In some embodiments, the chemotherapeutic agent may be an immunomodulatory agent such as lenalidomide (LEN). Recent studies have demonstrated that lenalidomide can enhance antitumor functions of CAR modified T cells (Otahal et al., *Oncoimmunology*, 2015, 5(4): e1115940). Some examples of anti-tumor antibodies include tocilizumab, siltuximab.

[0451] Other agents may be used in combination with compositions of the present disclosure may also include, but not limited to, agents that affect the upregulation of cell surface receptors and their ligands such as Fas/Fas ligand, DR4 or DR5/TRAIL and GAP junctions, cytostatic and differentiation agents, inhibitors of cell adhesion such as focal adhesion kinase (FAKs) inhibitors and Lovastatin, or agents that increase the sensitivity of the hyper proliferative cells to apoptotic inducers such as the antibody C225.

[0452] The combinations may include administering the compositions of the present disclosure and other agents at the same time or separately. Alternatively, the present immunotherapy may precede or follow the other agent/therapy by intervals ranging from minutes, days, weeks to months.

4. Diseases

[0453] Provided in the present disclosure is a method of reducing a tumor volume or burden in a subject in need, the method comprising introducing into the subject a composition described herein.

[0454] The present disclosure also provides methods for treating a cancer in a subject, comprising administering to the subject an effective amount of an effector immune cell genetically modified to express at least one CA2 effector module described herein.

Cancer

[0455] Various cancers may be treated with pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads of the present disclosure. As used herein, the term “cancer” refers to any of various malignant neoplasms characterized by the proliferation of anaplastic cells that tend to invade surrounding tissue and metastasize to new body sites and also refers to the pathological condition characterized by such malignant neoplastic growths. Cancers may be tumors or hematological malignancies, and include but are not limited to, all types of lymphomas/leukemias, carcinomas and sarcomas, such as those cancers or tumors found in the anus, bladder, bile duct, bone, brain, breast, cervix, colon/rectum, endometrium, esophagus, eye, gallbladder, head and neck, liver, kidney, larynx, lung, mediastinum (chest), mouth, ovaries, pancreas, penis, prostate, skin, small intestine, stomach, spinal marrow, tailbone, testicles, thyroid and uterus.

[0456] Types of carcinomas which may be treated with the compositions of the present disclosure include, but are not limited to, papilloma/carcinoma, choriocarcinoma, endodermal sinus tumor, teratoma, adenoma/adenocarcinoma, melanoma, fibroma, lipoma, leiomyoma, rhabdomyoma, mesothelioma, angioma, osteoma, chondroma, glioma, lymphoma/leukemia, squamous cell carcinoma, small cell carcinoma, large cell undifferentiated carcinomas, basal cell carcinoma and sinonasal undifferentiated carcinoma.

[0457] Types of sarcomas which may be treated with the compositions of the present disclosure include, but are not limited to, soft tissue sarcoma such as alveolar soft part sarcoma, angiosarcoma, dermatofibrosarcoma, desmoid tumor, desmoplastic small round cell tumor, extraskeletal chondrosarcoma, extraskeletal osteosarcoma, fibrosarcoma, hemangiopericytoma, hemangiosarcoma, Kaposi's sarcoma, leiomyosarcoma, liposarcoma, lymphangiosarcoma, lymphosarcoma, malignant fibrous histiocytoma, neurofibrosarcoma, rhabdomyosarcoma, synovial sarcoma, and Askin's

tumor, Ewing's sarcoma (primitive neuroectodermal tumor), malignant hemangioendothelioma, malignant schwannoma, osteosarcoma, and chondrosarcoma.

[0458] As a non-limiting example, the carcinoma which may be treated may be Acute granulocytic leukemia, Acute lymphocytic leukemia, Acute myelogenous leukemia, Adenocarcinoma, Adenosarcoma, Adrenal cancer, Adrenocortical carcinoma, Anal cancer, Anaplastic astrocytoma, Angiosarcoma, Appendix cancer, Astrocytoma, Basal cell carcinoma, B-Cell lymphoma), Bile duct cancer, Bladder cancer, Bone cancer, Bowel cancer, Brain cancer, Brain stem glioma, Brain tumor, Breast cancer, Carcinoid tumors, Cervical cancer, Cholangiocarcinoma, Chondrosarcoma, Chronic lymphocytic leukemia, Chronic myelogenous leukemia, Colon cancer, Colorectal cancer, Craniopharyngioma, Cutaneous lymphoma, Cutaneous melanoma, Diffuse astrocytoma, Ductal carcinoma in situ, Endometrial cancer, Ependymoma, Epithelioid sarcoma, Esophageal cancer, Ewing sarcoma, Extrahepatic bile duct cancer, Eye cancer, Fallopian tube cancer, Fibrosarcoma, Gallbladder cancer, Gastric cancer, Gastrointestinal cancer, Gastrointestinal carcinoid cancer, Gastrointestinal stromal tumors, General, Germ cell tumor, Glioblastoma multiforme, Glioma, Hairy cell leukemia, Head and neck cancer, Hemangioendothelioma, Hodgkin lymphoma, Hodgkin's disease, Hodgkin's lymphoma, Hypopharyngeal cancer, Infiltrating ductal carcinoma, Infiltrating lobular carcinoma, Inflammatory breast cancer, Intestinal Cancer, Intrahepatic bile duct cancer, Invasive / infiltrating breast cancer, Islet cell cancer, Jaw cancer, Kaposi sarcoma, Kidney cancer, Laryngeal cancer, Leiomyosarcoma, Leptomeningeal metastases, Leukemia, Lip cancer, Liposarcoma, Liver cancer, Lobular carcinoma in situ, Low-grade astrocytoma, Lung cancer, Lymph node cancer, Lymphoma, Male breast cancer, Medullary carcinoma, Medulloblastoma, Melanoma, Meningioma, Merkel cell carcinoma, Mesenchymal chondrosarcoma, Mesenchymous, Mesothelioma, Metastatic breast cancer, Metastatic melanoma, Metastatic squamous neck cancer, Mixed gliomas, Mouth cancer, Mucinous carcinoma, Mucosal melanoma, Multiple myeloma, Nasal cavity cancer, Nasopharyngeal cancer, Neck cancer, Neuroblastoma, Neuroendocrine tumors, Non-Hodgkin lymphoma, Non-Hodgkin's lymphoma, Non-small cell lung cancer, Oat cell cancer, Ocular cancer, Ocular melanoma, Oligodendroglioma, Oral cancer, Oral cavity cancer, Oropharyngeal cancer, Osteogenic sarcoma, Osteosarcoma, Ovarian cancer, Ovarian epithelial cancer, Ovarian germ cell tumor, Ovarian primary peritoneal carcinoma, Ovarian sex cord stromal tumor, Paget's disease, Pancreatic cancer, Papillary carcinoma, Paranasal sinus cancer, Parathyroid cancer, Pelvic cancer, Penile cancer, Peripheral nerve cancer, Peritoneal cancer, Pharyngeal cancer, Pheochromocytoma, Pilocytic astrocytoma, Pineal region tumor, Pineoblastoma, Pituitary gland cancer, Primary central nervous system lymphoma, Prostate cancer, Rectal cancer, Renal cell cancer, Renal pelvis cancer, Rhabdomyosarcoma, Salivary gland cancer, Sarcoma, Sarcoma, bone, Sarcoma, soft tissue, Sarcoma, uterine, Sinus cancer, Skin cancer, Small cell lung cancer, Small intestine cancer, Soft tissue sarcoma, Spinal cancer, Spinal column cancer, Spinal cord cancer,

Spinal tumor, Squamous cell carcinoma, Stomach cancer, Synovial sarcoma, T-cell lymphoma), Testicular cancer, Throat cancer, Thymoma / thymic carcinoma, Thyroid cancer, Tongue cancer, Tonsil cancer, Transitional cell cancer, Transitional cell cancer, Transitional cell cancer, Triple-negative breast cancer, Tubal cancer, Tubular carcinoma, Ureteral cancer, Ureteral cancer, Urethral cancer, Uterine adenocarcinoma, Uterine cancer, Uterine sarcoma, Vaginal cancer, and Vulvar cancer.

Infectious diseases

[0459] In some embodiments, CA2 biocircuits described herein may be used for the treatment of infectious diseases. CA2 biocircuits of the present disclosure may be introduced in cells suitable for adoptive cell transfer such as macrophages, dendritic cells, natural killer cells, and or T cells. Infectious diseases treated by the CA2 biocircuits of the present disclosure may be diseases caused by viruses, bacteria, fungi, and/or parasites. IL15-IL15Ra payloads of the present disclosure may be used to increase immune cell proliferation and/or persistence of the immune cells useful in treating infectious diseases.

[0460] “Infectious diseases” herein refer to diseases caused by any pathogen or agent that infects mammalian cells, preferably human cells and causes a disease condition. Examples thereof include bacteria, yeast, fungi, protozoans, mycoplasma, viruses, prions, and parasites. Examples include those involved in (a) viral diseases such as, for example, diseases resulting from infection by an adenovirus, a herpesvirus (e.g., HSV-I, HSV-II, CMV, or VZV), a poxvirus (e.g., an orthopoxvirus such as variola or vaccinia, or molluscum contagiosum), a picornavirus (e.g., rhinovirus or enterovirus), an orthomyxovirus (e.g., influenzavirus), a paramyxovirus (e.g., parainfluenza virus, mumps virus, measles virus, and respiratory syncytial virus (RSV)), a coronavirus (e.g., SARS), a papovavirus (e.g., papillomaviruses, such as those that cause genital warts, common warts, or plantar warts), a hepadnavirus (e.g., hepatitis B virus), a flavivirus (e.g., hepatitis C virus or Dengue virus), or a retrovirus (e.g., a lentivirus such as HIV); (b) bacterial diseases such as, for example, diseases resulting from infection by bacteria of, for example, the genus *Escherichia*, *Enterobacter*, *Salmonella*, *Staphylococcus*, *Shigella*, *Listeria*, *Aerobacter*, *Helicobacter*, *Klebsiella*, *Proteus*, *Pseudomonas*, *Streptococcus*, *Chlamydia*, *Mycoplasma*, *Pneumococcus*, *Neisseria*, *Clostridium*, *Bacillus*, *Corynebacterium*, *Mycobacterium*, *Campylobacter*, *Vibrio*, *Serratia*, *Providencia*, *Chromobacterium*, *Brucella*, *Yersinia*, *Haemophilus*, or *Bordetella*; (c) other infectious diseases, such chlamydia, fungal diseases including but not limited to candidiasis, aspergillosis, histoplasmosis, cryptococcal meningitis, parasitic diseases including but not limited to malaria, *Pneumocystis carinii* pneumonia, leishmaniasis, cryptosporidiosis, toxoplasmosis, and trypanosome infection and prions that cause human disease such as Creutzfeldt-Jakob Disease (CJD), variant Creutzfeldt-Jakob Disease (vCJD), Gerstmann-Sträussler-Scheinker syndrome, Fatal Familial Insomnia and kuru.

Combination treatments

[0461] The present disclosure further relates to the use of pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads of the present disclosure for treating one or more forms of cancer, in combination with other pharmaceuticals and/or other therapeutic methods, e.g., with known pharmaceuticals and/or known therapeutic methods, such as, for example, those which are currently employed for treating these disorders. For example, the pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads of the present disclosure can also be administered in conjunction with one or more additional anti-cancer treatments, such as biological, chemotherapy and radiotherapy. Accordingly, a treatment can include, for example, imatinib (Gleevec), all-trans-retinoic acid, a monoclonal antibody treatment (gemtuzumab, ozogamicin), chemotherapy (for example, chlorambucil, prednisone, prednisolone, vincristine, cytarabine, clofarabine, farnesyl transferase inhibitors, decitabine, inhibitors of MDR1), rituximab, interferon- α , anthracycline drugs (such as daunorubicin or idarubicin), L-asparaginase, doxorubicin, cyclophosphamide, doxorubicin, bleomycin, fludarabine, etoposide, pentostatin, or cladribine), bone marrow transplant, stem cell transplant, radiation therapy, anti-metabolite drugs (methotrexate and 6-mercaptopurine), or any of the antibodies taught in Table 5 of International Publication No. WO2017/180587 (the contents of which are herein incorporated by reference in their entirety) or combinations thereof.

Combinations with radiation

[0462] Radiation therapy (also called radiotherapy, X-ray therapy, or irradiation) is the use of ionizing radiation to kill cancer cells and shrink tumors. Radiation therapy can be administered externally via external beam radiotherapy (EBRT) or internally via brachytherapy. The effects of radiation therapy are localized and confined to the region being treated. Radiation therapy may be used to treat almost every type of solid tumor, including cancers of the brain, breast, cervix, larynx, lung, pancreas, prostate, skin, stomach, uterus, or soft tissue sarcomas. Radiation is also used to treat leukemia and lymphoma.

Combination with chemotherapy

[0463] Chemotherapy is the treatment of cancer with drugs that can destroy cancer cells. In current usage, the term "chemotherapy" usually refers to cytotoxic drugs which affect rapidly dividing cells in general, in contrast with targeted therapy. Chemotherapy drugs interfere with cell division in various possible ways, e.g. with the duplication of DNA or the separation of newly formed chromosomes. Most forms of chemotherapy target all rapidly dividing cells and are not specific to cancer cells, although some degree of specificity may come from the inability of many cancer cells to repair DNA damage, while normal cells generally can.

[0464] Most chemotherapy regimens are given in combination. Exemplary chemotherapeutic agents include, but are not limited to, 5-FU Enhancer, 9-AC, AG2037, AG3340, Aggrecanase Inhibitor, Aminoglutethimide, Amsacrine (m-AMSA), Asparaginase, Azacitidine, Batimastat (BB94), BAY 12-9566, BCH-4556, Bis-Naphtalimide, Busulfan, Capecitabine, Carboplatin, Carmustaine+Polifepyr Osan, cdk4/cdk2 inhibitors, Chlorombucil, CI-994, Cisplatin, Cladribine, CS-682, Cytarabine HCl, D2163, Dactinomycin, Daunorubicin HCl, DepoCyt, Dexifosamide, Docetaxel, Dolastain, Doxifluridine, Doxorubicin, DX8951f, E 7070, EGFR, Epirubicin, Erythropoietin, Estramustine phosphate sodium, Etoposide (VP16-213), Farnesyl Transferase Inhibitor, FK 317, Flavopiridol, Floxuridine, Fludarabine, Fluorouracil (5-FU), Flutamide, Fragyl, Gemcitabine, Hexamethylmelamine (HMM), Hydroxyurea (hydroxycarbamide), Ifosfamide, Interferon Alfa-2a, Interferon Alfa-2b, Interleukin-2, Irinotecan, ISI 641, Krestin, Lemonal DP 2202, Leuprolide acetate (LHRH-releasing factor analogue), Levamisole, LiGLA (lithium-gamma linolenate), Lodine Seeds, Lometexol, Lomustine (CCNU), Marimistat, Mechlorethamine HCl (nitrogen mustard), Megestrol acetate, Meglamine GLA, Mercaptopurine, Mesna, Mitoguazone (methyl-GAG; methyl glyoxal bis-guanyldihydrazone; MGBG), Mitotane (o.p'-DDD), Mitoxantrone, Mitoxantrone HCl, MMI 270, MMP, MTA/LY 231514, Octreotide, ODN 698, OK-432, Oral Platinum, Oral Taxoid, Paclitaxel (TAXOL.RTM.), PARP Inhibitors, PD 183805, Pentostatin (2' deoxycoformycin), PKC 412, Plicamycin, Procarbazine HCl, PSC 833, Ralitrexed, RAS Farnesyl Transferase Inhibitor, RAS Oncogene Inhibitor, Semustine (methyl-CCNU), Streptozocin, Suramin, Tamoxifen citrate, Taxane Analog, Temozolomide, Teniposide (VM-26), Thioguanine, Thiotepa, Topotecan, Tyrosine Kinase, UFT (Tegafur/Uracil), Valrubicin, Vinblastine sulfate, Vindesine sulfate, VX-710, VX-853, YM 116, ZD 0101, ZD 0473/Anormed, ZD 1839, ZD 9331.

Immuno-oncology and Cell therapies

[0465] Recent progress in the field of cancer immunology has allowed the development of several approaches to help the immune system keep the cancer at bay. Such immunotherapy approaches include the targeting of cancer antigens through monoclonal antibodies or through adoptive transfer of ex vivo engineered T cells (e.g., which contain chimeric antigen receptors or engineered T cell receptors).

[0466] In some embodiments, pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads of the present disclosure may be used in the modulation or alteration or exploitation of the immune system to target one or more cancers. This approach may also be considered with other such biological approaches, e.g., immune response modifying therapies such as the administration of interferons, interleukins, colony-stimulating factors, other monoclonal antibodies, vaccines, gene therapy, and nonspecific immunomodulating agents are also envisioned as anti-cancer therapies to be combined with the pharmaceutical

compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads of the present disclosure.

[0467] Cancer immunotherapy refers to a diverse set of therapeutic strategies designed to induce the patient's own immune system to fight the cancer. In some embodiments, pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads of the present disclosure are designed as immunology therapeutics.

Cell Therapies

[0468] There are several types of cellular immunotherapies, including tumor infiltrating lymphocyte (TIL) therapy, genetically engineered T cells bearing chimeric antigen receptors (CARs), and recombinant TCR technology.

[0469] According to the present disclosure, the CA2 biocircuits and systems may be used in the development and implementation of cell therapies such as adoptive cell therapy. Certain effector modules useful in cell therapy are given in FIGs. 8-13 of the International Publication WO2017/180587 (the contents each of which are herein incorporated by reference in their entirety). The CA2 biocircuits, CA2 effector modules and their SREs and payloads may be used in cell therapies to effect TCR removal-TCR gene disruption, TCR engineering, to regulate epitope tagged receptors, in APC platforms for stimulating T cells, as a tool to enhance ex vivo APC stimulation, to improve methods of T cell expansion, in ex vivo stimulation with antigen, in TCR/CAR combinations, in the manipulation or regulation of TILs, in allogeneic cell therapy, in combination T cell therapy with other treatment lines (e.g. radiation, cytokines), to encode engineered TCRs, or modified TCRs, or to enhance T cells other than TCRs (e.g. by introducing cytokine genes, genes for the checkpoint inhibitors PD1, CTLA4).

[0470] In some embodiments, improved response rates are obtained in support of cell therapies.

[0471] Expansion and persistence of cell populations may be achieved through regulation or fine tuning of the payloads, e.g., the receptors or pathway components in T cells, NK cells or other immune-related cells. In some embodiments, CA2 biocircuits, their components, SREs or CA2 effector modules are designed to spatially and/or temporally control the expression of proteins which enhance T-cell or NK cell response. In some embodiments, CA2 biocircuits, SREs or CA2 effector modules are designed to spatially and/or temporally control the expression of proteins which inhibit T-cell or NK cell response.

[0472] In some embodiments, CA2 biocircuits, SREs or CA2 effector modules are designed to reshape the tumor microenvironment to extend utility of the biocircuit or a pharmaceutical composition beyond direct cell killing.

[0473] In some embodiments, CA2 biocircuits, SREs or CA2 effector modules are designed to reduce, mitigate or eliminate the CAR cytokine storm. In some embodiments, such reduction, mitigation and/or elimination occurs in solid tumors or tumor microenvironments.

[0474] In some embodiments, the CA2 effector modules may encode one or more cytokines.

[0475] In one aspect, the CA2 effector module of the present disclosure may be a CA2 DD-CD40L polypeptide. Regulatable DD-CD40L polypeptide may be directly used as an immunotherapeutic agent or be transduced into an effector immune cell (T cells and TIL cells) to generate modified T cells with greater *in vivo* expansion and survival capabilities for adoptive cell transfer. The need for harsh preconditioning regimens in current adoptive cell therapies may be minimized using regulated CA2 DD-CD40L to modify tumor microenvironment and increase persistence in solid tumors that are currently refractory to tumor antigen targeted therapy. In some embodiments, CAR expressing T cells may be armored with DD regulated CD40L to relieve immunosuppression without systemic toxicity.

[0476] The immune system can be harnessed for the treatment of diseases beyond cancer. CA2 biocircuits, their components, SREs or CA2 effector modules may be utilized in immunotherapy for the treatment of diseases including, but not limited to, autoimmune diseases, allergies, graft versus host disease, and diseases and disorders that may result in immunodeficiency such as acquired immune deficiency syndrome (AIDS).

[0477] In some embodiments, payloads of the present disclosure may be a chimeric antigen receptor (CAR), which when transduced into immune cells (e.g., T cells and NK cells), can re-direct the immune cells against the target (e.g., a tumor cell) which expresses a molecule recognized by the extracellular target moiety of the CAR.

[0478] As used herein, the term “chimeric antigen receptor (CAR)” refers to a synthetic receptor that mimics the TCR on the surface of T cells. In general, a CAR is composed of an extracellular targeting domain, a transmembrane domain/region and an intracellular signaling/activation domain. In a standard CAR receptor, the components: the extracellular targeting domain, transmembrane domain and intracellular signaling/activation domain, are linearly constructed as a single fusion protein. The extracellular region comprises a targeting domain/moiety (e.g., a scFv) that recognizes a specific tumor antigen or other tumor cell-surface molecules. The intracellular region may contain a signaling domain of TCR complex (e.g., the signal region of CD3 ζ), and/or one or more costimulatory signaling domains, such as those from CD28, 4-1BB (CD137) and OX-40 (CD134). For example, a “first-generation CAR” only has the CD3 ζ signaling domain, whereas, a second-generation CARs has a CD3 ζ signal domain plus one costimulatory signaling domain, and a third-generation CARs having CD3 ζ signal domain plus two or more costimulatory signaling domains. A CAR, when expressed by a T cell, endows the T cell with antigen specificity determined by the extracellular targeting

moiety of the CAR. It is also desirable to add one or more elements such as homing and suicide genes to develop a more competent and safer architecture of CAR, which has given rise to the so called the fourth-generation CAR.

[0479] In some embodiments, the extracellular targeting domain is joined through the hinge (also called space domain or spacer) and transmembrane regions to an intracellular signaling domain. The hinge may need to be varied to optimize the potency of CAR expressing cells towards the cancer cells due to the size of the target protein where the targeting moiety binds, and the size and affinity of the targeting domain itself. Upon recognition and binding of the targeting moiety to the target cell, the intracellular signaling domain leads to an activation signal for the CAR T cell, which is further amplified by the “second signal” from one or more intracellular costimulatory domains. The CAR T cell, once activated, can destroy the target cell.

[0480] According to the present disclosure, the payload of the present disclosure may be a first-generation CAR, or a second-generation CAR, or a third-generation CAR, or a fourth-generation CAR. Representative effector module embodiments comprising CAR constructs are illustrated in FIG. 13-18 of International Publication No. WO2017/180587 (the contents of which are herein incorporated by reference in their entirety).

[0481] In accordance with the present disclosure, the extracellular target moiety of a CAR may be any agent that recognizes and binds to a given target molecule, for example, a neoantigen on tumor cells, with high specificity and affinity. The target moiety may be an antibody and variants thereof that specifically bind to a target molecule on tumor cells, or a peptide aptamer selected from a random sequence pool based on its ability to bind to the target molecule on tumor cells, or a variant or fragment thereof that can bind to the target molecule on tumor cells, or an antigen recognition domain from native T-cell receptor (TCR) (e.g. CD4 extracellular domain to recognize HIV infected cells), or exotic recognition components such as a linked cytokine that leads to recognition of target cells bearing the cytokine receptor, or a natural ligand of a receptor.

[0482] In some embodiments, the targeting moiety of a CAR construct may be a natural ligand of the target molecule, or a variant and/or fragment thereof capable of binding the target molecule. In some aspects, the targeting moiety of a CAR may be a receptor of the target molecule.

[0483] In some embodiments, the targeting moiety of a CAR may recognize a tumor specific antigen (TSA), for example a cancer neoantigen whose expression is restricted to tumor cells.

[0484] As non-limiting examples, the CAR of the present disclosure may comprise the extracellular targeting domain capable of binding to a tumor specific antigen selected from 5T4, 707-AP, A33, AFP (α -fetoprotein), AKAP-4 (A kinase anchor protein 4), ALK, α 5 β 1-integrin, androgen receptor, annexin II, alpha-actinin-4, ART-4, B1, B7H3, B7H4, BAGE (B melanoma antigen), BCMA, BCR-ABL fusion protein, beta-catenin, BKT-antigen, BTAA, CA-I

(carbonic anhydrase I), CA50 (cancer antigen 50), CA125, CA15-3, CA195, CA242, calretinin, CAIX (carbonic anhydrase), CAMEL (cytotoxic T-lymphocyte recognized antigen on melanoma), CAM43, CAP-1, Caspase-8/m, CD4, CD5, CD7, CD19, CD20, CD22, CD23, CD25, CD27, CD27/m, CD28, CD30, CD33, CD34, CD36, CD38, CD40/CD154, CD41, CD44v6, CD44v7/8, CD45, CD49f, CD56, CD68/KP1, CD74, CD79a/CD79b, CD103, CD123, CD133, CD138, CD171, cdc27/m, CDK4 (cyclin dependent kinase 4), CDKN2A, CDS, CEA (carcinoembryonic antigen), CEACAM5, CEACAM6, chromogranin, c-Met, c-Myc, coa-1, CSAP, CT7, CT10, cyclophilin B, cyclin B1, cytoplasmic tyrosine kinases, cytokeratin, DAM-10, DAM-6, dek-can fusion protein, desmin, DEPDC1 (DEP domain containing 1), E2A-PRL, EBNA, EGF-R (epidermal growth factor receptor), EGP-1 (epithelial glycoprotein-1) (TROP-2), EGP-2, EGP-40, EGFR (epidermal growth factor receptor), EGFRvIII, EF-2, ELF2M, EMMPRIN, EpCAM (epithelial cell adhesion molecule), EphA2, Epstein Barr virus antigens, Erb (ErbB1; ErbB3; ErbB4), ETA (epithelial tumor antigen), ETV6-AML1 fusion protein, FAP (fibroblast activation protein), FBP (folate-binding protein), FGF-5, folate receptor α , FOS related antigen 1, fucosyl GM1, G250, GAGE (GAGE-1; GAGE-2), galactin, GD2 (ganglioside), GD3, GFAP (glial fibrillary acidic protein), GM2 (oncofetal antigen-immunogenic-1; OFA-I-1), GnT-V, Gp100, H4-RET, HAGE (helicase antigen), HER-2/neu, HIFs (hypoxia inducible factors), HIF-1 α , HIF-2 α , HLA-A2, HLA-A*0201-R1701, HLA-A11, HMWMAA, Hom/Mel-40, HSP70-2M (Heat shock protein 70), HST-2, HTgp-175, hTERT (or hTERT), human papillomavirus-E6/human papillomavirus-E7 and E6, iCE (immune-capture EIA), IGF-1R, IGH-IGK, IL2R, IL5, ILK (integrin-linked kinase), IMP3 (insulin-like growth factor II mRNA-binding protein 3), IRF4 (interferon regulatory factor 4), KDR (kinase insert domain receptor), KIAA0205, KRAB-zinc finger protein (KID)-3; KID31, KSA (17-1A), K-ras, LAGE, LCK, LDLR/FUT (LDLR-fucosyltransferaseAS fusion protein), LeY (Lewis Y), MAD-CT-1, MAGE (tyrosinase, melanoma-associated antigen) (MAGE-1; MAGE-3), melan-A tumor antigen (MART), MART-2/Ski, MC1R (melanocortin 1 receptor), MDM2, mesothelin, MPHOSPH1, MSA (muscle-specific actin), mTOR (mammalian targets of rapamycin), MUC-1, MUC-2, MUM-1 (melanoma associated antigen (mutated) 1), MUM-2, MUM-3, Myosin/m, MYL-RAR, NA88-A, N-acetylglucosaminyltransferase, neo-PAP, NF-KB (nuclear factor-kappa B), neurofilament, NSE (neuron-specific enolase), Notch receptors, NuMa, N-Ras, NY-BR-1, NY-CO-1, NY-ESO-1, Oncostatin M, OS-9, OY-TES1, p53 mutants, p190 minor bcr-abl, pl5(58), pl85erbB2, pl80erbB-3, PAGE (prostate associated gene), PAP (prostatic acid phosphatase), PAX3, PAX5, PDGFR (platelet derived growth factor receptor), cytochrome P450 involved in piperidine and pyrrolidine utilization (PIPA), Pml-RAR alpha fusion protein, PR-3 (proteinase 3), PSA (prostate specific antigen), PSM, PSMA (Prostate stem cell antigen), PRAME (preferentially expressed antigen of melanoma), PTPRK, RAGE (renal tumor antigen), Raf (A-Raf, B-Raf and C-Raf), Ras, receptor tyrosine kinases, RCAS1, RGSS, ROR1 (receptor tyrosine kinase-like orphan receptor 1), RU1, RU2, SAGE, SART-1,

SART-3, SCP-1, SDCCAG16, SP-17 (sperm protein 17), src-family, SSX (synovial sarcoma X breakpoint)-1, SSX-2(HOM-MEL-40), SSX-3, SSX-4, SSX-5, STAT-3, STAT-5, STAT-6, STEAD, STn, survivin, syk-ZAP70, TA-90 (Mac-2 binding protein/cyclophilin C-associated protein), TAAL6, TACSTD1 (tumor associated calcium signal transducer 1), TACSTD2, TAG-72-4, TAGE, TARP (T cell receptor gamma alternate reading frame protein), TEL/AML1 fusion protein, TEM1, TEM8 (endosialin or CD248), TGF β , TIE2, TLP, TMPRSS2 ETS fusion gene, TNF-receptor (TNF- α receptor, TNF- β receptor, or TNF- γ receptor), transferrin receptor, TPS, TRP-1 (tyrosine related protein 1), TRP-2, TRP-2/INT2, TSP-180, VEGF receptor, WNT, WT-1 (Wilm's tumor antigen) and XAGE.

[0485] As non-limiting examples, the targeting moiety of the present disclosure may be a scFv antibody that recognizes a tumor specific antigen (TSA), for example scFvs of antibodies SS, SS1 and HN1 that specifically recognize and bind to human mesothelin (US Pat. NO.: 9,359, 447), scFv of antibody of GD2 (US Pat. NO.: 9, 315, 585), a CD19 antigen binding domain (U.S. Pat. NO.: 9, 328, 156); a NKG2D ligand binding domain (U.S. Pat. NO.: 9, 273,283; US patent publication NO.: US20160311906A1); human anti-mesothelin scFvs comprising the amino acid sequences of SEQ ID NO.: 11 and 12 of US Pat. 9,272,002; an anti-CS1 binding agent (US patent publication NO.: US20160075784); an anti-BCMA binding domain (International Patent Publication NO.: WO2016/014565); anti-CD19 scFv antibody of SEQ ID NO.: 20 in US Pat. NO.: 9,102,761; GFR alpha 4 antigen binding fragments having the amino acid sequences of SEQ ID NOs.: 59 and 79 of International patent publication NO.: 2016/025880; anti-CLL-1 (C-type lectin-like molecule 1) binding domains having the amino acid sequences of SEQ ID NOs.:47, 44, 48, 49, 50, 39, 40, 41, 42, 43, 45, 46, 51, 73, 70, 74, 75, 76, 65, 66, 67, 68, 69, 71, 72, 77, 195, 86, 83, 87, 88, 89, 78, 79, 80, 81, 82, 84, 85, 90 and 196 of International Patent Publication NO.: WO2016014535); CD33 binding domains having the amino acid sequences of SEQ ID NOs.: 39-46 of International patent publication NO.: WO2016014576; a GPC3 (glypican-3) binding domain (SEQ ID NO.: 2 and SEQ ID NO.: 4 of International patent publication NO.: WO2016036973); a GFR alpha4 (Glycosyl-phosphatidylinositol (GPI)-linked GDNF family α -receptor 4 cell-surface receptor) binding domain (International Patent Publication NO.: WO2016025880); CD123 binding domains having the amino acid sequences of SEQ ID NOs.: 480, 483, 485, 478, 158, 159, 160, 157, 217, 218, 219, 216, 276, 277, 278, and 275 of International patent publication NO.: WO20160258896; an anti-ROR1 antibody or fragments thereof (International patent publication NO.: WO2016016344); scFvs specific to GPC-3 (SEQ ID NOs.: 1 and 24 of International patent publication NO.: WO2016049459); scFv for CSPG4 (SEQ ID NO.: 2 of International patent publication NO.: WO2015080981; scFv for folate receptor alpha (US Patent Publication NO.: US20170002072A1); the contents of each of which are incorporated herein by reference in their entirety.

[0486] The intracellular domain of a CAR fusion polypeptide, after binding to its target molecule, transmits a signal to the effector immune cell, activating at least one of the normal effector functions of effector immune cells, including cytolytic activity (e.g., cytokine secretion) or helper activity. Therefore, the intracellular domain comprises an "intracellular signaling domain" of a T cell receptor (TCR). In some embodiments, the intracellular signaling domain of the present disclosure may contain signaling motifs which are known as immunoreceptor tyrosine-based activation motifs (ITAMs). In some embodiments, the intracellular region of the present disclosure further comprises one or more costimulatory signaling domains which provide additional signals to the effector immune cells. These costimulatory signaling domains, in combination with the signaling domain can further improve expansion, activation, memory, persistence, and tumor-eradicating efficiency of CAR engineered immune cells (e.g., CAR T cells). In some cases, the costimulatory signaling region contains 1, 2, 3, or 4 cytoplasmic domains of one or more intracellular signaling and /or costimulatory molecules.

[0487] In one embodiment of the present disclosure, the CAR of the present disclosure is a CD19 specific CAR. In the context of the present disclosure, a CA2 effector module may comprise an CA2 DD operably linked to a CD19 CAR fusion construct.

[0488] In some embodiments, pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads of the present disclosure may be used in the modulation or alteration or exploitation of the immune system to target one or more self-reactive immune components such as auto antibodies and self-reactive immune cells to attenuate autoimmune diseases. In some embodiments, the SREs of the present disclosure may be utilized in regulating or tuning the Chimeric Auto Antibody Receptor (CAAR) based T cell therapy in order to optimize its utility in the treatment of autoimmune diseases (Ellebrecht C.T. et al., Science. 2016. Jul 8;353(6295):179-84; the contents of which are incorporated herein by reference in their entirety). In some embodiments, CA2 biocircuits, SREs or CA2 effector modules are designed to modulate Tregs to attenuate autoimmune disorders.

[0489] In some embodiments, CA2 biocircuits, SREs or CA2 effector modules may be utilized in immunotherapy-based treatments to attenuate or mitigate Graft vs. Host disease (GVHD). GVHD refers to a condition following stem cell or bone marrow transplant where in the allogeneic donor immune cells react against host tissue. In some embodiments, CA2 biocircuits, SREs or CA2 effector modules are designed to modulate Tregs for the treatment of GVHD. In one embodiment, CA2 biocircuits containing a CA2 effector module encoding TNF-alpha may be used to modulate Tregs to minimize GVHD (Pierini, A. et al., Blood. 2016. Aug 11; 128(6):866-71; the contents of which are incorporated herein by reference in their entirety).

[0490] In some embodiments, CA2 biocircuits, SREs or CA2 effector modules are designed to be significantly less immunogenic than other biocircuits or switches in the art.

[0491] As used herein, “significantly less immunogenic” refers to a detectable decrease in immunogenicity. In another embodiment, the term refers to a fold decrease in immunogenicity. In another embodiment, the term refers to a decrease such that an effective amount of the CA2 biocircuits, SREs or CA2 effector modules which can be administered without triggering a detectable immune response. In another embodiment, the term refers to a decrease such that the CA2 biocircuits, SREs or CA2 effector modules can be repeatedly administered without eliciting an immune response. In another embodiment, the decrease is such that the CA2 biocircuits, SREs or CA2 effector modules can be repeatedly administered without eliciting an immune response.

[0492] In another embodiment, the CA2 biocircuits, SREs or CA2 effector modules is 2-fold less immunogenic than its unmodified counterpart or reference compound. In another embodiment, immunogenicity is reduced by a 3-fold factor. In another embodiment, immunogenicity is reduced by a 5-fold factor. In another embodiment, immunogenicity is reduced by a 7-fold factor. In another embodiment, immunogenicity is reduced by a 10-fold factor. In another embodiment, immunogenicity is reduced by a 15-fold factor. In another embodiment, immunogenicity is reduced by a 50-fold factor. In another embodiment, immunogenicity is reduced by a 100-fold factor. In another embodiment, immunogenicity is reduced by a 200-fold factor. In another embodiment, immunogenicity is reduced by a 500-fold factor. In another embodiment, immunogenicity is reduced by a 1000-fold factor. In another embodiment, immunogenicity is reduced by a 2000-fold factor. In another embodiment, immunogenicity is reduced by another fold difference.

[0493] Methods of determining immunogenicity are well known in the art, and include, e.g. measuring secretion of cytokines (e.g. IL12, IFN alpha, TNF-alpha, RANTES, MIP-1alpha or beta, IL6, IFN-beta, or IL8), measuring expression of DC activation markers (e.g. CD83, HLA-DR, CD80 and CD86), or measuring ability to act as an adjuvant for an adaptive immune response.

Diseases and toxins

[0494] Various infectious diseases may be treated with pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads of the present disclosure. As used herein, the term “infectious disease” refers to any disorders caused by organisms such as bacteria, viruses, fungi or parasites.

[0495] Various toxins may be treated with pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads of the present disclosure. Non-limited examples of toxins include Ricin, Bacillus anthracis, Shiga toxin and Shiga-like toxin, Botulinum toxins.

[0496] Various tropical diseases may be treated with pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads of the present disclosure. Non-limited examples of tropical diseases include Chikungunya fever, Dengue fever, Chagas disease, Rabies, Malaria, Ebola virus, Marburg virus, West Nile Virus, Yellow Fever, Japanese encephalitis virus, St. Louis encephalitis virus.

[0497] Various foodborne illnesses and gastroenteritis may be treated with pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads of the present disclosure. Non-limited examples of foodborne illnesses and gastroenteritis include Rotavirus, Norwalk virus (Norovirus), *Campylobacter jejuni*, *Clostridium difficile*, *Entamoeba histolytica*, *Helicobacter pylori*, Enterotoxin B of *Staphylococcus aureus*, Hepatitis A virus (HAV), Hepatitis E, *Listeria monocytogenes*, *Salmonella*, *Clostridium perfringens*, and *Salmonella*.

[0498] Various infectious agents may be treated with pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads of the present disclosure. Non-limited examples of infectious agents include adenoviruses, *Anaplasma phagocytophilum*, *Ascaris lumbricoides*, *Bacillus anthracis*, *Bacillus cereus*, *Bacteriodes* sp., Barmah Forest virus, *Bartonella bacilliformis*, *Bartonella henselae*, *Bartonella quintana*, beta-toxin of *Clostridium perfringens*, *Bordetella pertussis*, *Bordetella parapertussis*, *Borrelia burgdorferi*, *Borrelia miyamotoi*, *Borrelia recurrentis*, *Borrelia* sp., *Botulinum* toxin, *Brucella* sp., *Burkholderia pseudomallei*, California encephalitis virus, *Campylobacter*, *Candida albicans*, chikungunya virus, *Chlamydia psittaci*, *Chlamydia trachomatis*, *Clonorchis sinensis*, *Clostridium difficile* bacteria, *Clostridium tetani*, Colorado tick fever virus, *Corynebacterium diphtheriae*, *Corynebacterium minutissimum*, *Coxiella burnetii*, coxsackie A, coxsackie B, Crimean-Congo hemorrhagic fever virus, cytomegalovirus, dengue virus, Eastern Equine encephalitis virus, Ebola viruses, echovirus, *Ehrlichia chaffeensis*, *Ehrlichia equi*, *Ehrlichia* sp., *Entamoeba histolytica*, *Enterobacter* sp., *Enterococcus faecalis*, Enterovirus 71, Epstein-Barr virus (EBV), *Erysipelothrix rhusiopathiae*, *Escherichia coli*, Flavivirus, *Fusobacterium necrophorum*, *Gardnerella vaginalis*, Group B streptococcus, *Haemophilus aegyptius*, *Haemophilus ducreyi*, *Haemophilus influenzae*, hantavirus, *Helicobacter pylori*, Hepatitis A, Hepatitis B, Hepatitis C, Hepatitis D, Hepatitis E, herpes simplex virus 1 and 2, human herpes virus 6, human herpes Virus 8, human immunodeficiency virus 1 and 2, human T-cell leukemia viruses I and II, influenza viruses (A, B, C), Jamestown Canyon virus, Japanese encephalitis antigenic, Japanese encephalitis virus, John Cunningham virus, juninivirus, Kaposi's Sarcoma-associated Herpes Virus (KSHV), *Klebsiella granulomatis*, *Klebsiella* sp., Kyasanur Forest Disease virus, La Crosse virus, Lassavirus, *Legionella pneumophila*, *Leptospira interrogans*, *Listeria monocytogenes*, lymphocytic choriomeningitis virus, lyssavirus, Machupovirus, Marburg virus, measles virus, MERS coronavirus (MERS-CoV), *Micrococcus*

sedentarius, *Mobiluncus* sp., *Molluscipoxvirus*, *Moraxella catarrhalis*, *Morbilli-Rubeola virus*, *Mumpsvirus*, *Mycobacterium leprae*, *Mycobacterium tuberculosis*, *Mycobacterium ulcerans*, *Mycoplasma genitalium*, *Mycoplasma* sp., *Nairovirus*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Nocardia*, *Norwalk virus*, *norovirus*, *Omsk hemorrhagic fever virus*, *papilloma virus*, *parainfluenza viruses 1-3*, *parapoxvirus*, *parvovirus B19*, *Peptostreptococcus* sp., *Plasmodium* sp., *polioviruses types I, II, and III*, *Proteus* sp., *Pseudomonas aeruginosa*, *Pseudomonas pseudomallei*, *Pseudomonas* sp., *rabies virus*, *respiratory syncytial virus*, *ricin toxin*, *Rickettsia australis*, *Rickettsia conori*, *Rickettsia honei*, *Rickettsia prowazekii*, *Ross River Virus*, *rotavirus*, *rubellavirus*, *Saint Louis encephalitis*, *Salmonella Typhi*, *Sarcoptes scabiei*, *SARS-associated coronavirus (SARS-CoV)*, *Serratia* sp., *Shiga toxin and Shiga-like toxin*, *Shigella* sp., *Sin Nombre Virus*, *Snowshoe hare virus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptobacillus moniliformis*, *Streptococcus pneumoniae*, *Streptococcus agalactiae*, *Streptococcus agalactiae*, *Streptococcus group A-H*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Treponema pallidum subsp. Pallidum*, *Treponema pallidum var. carateum*, *Treponema pallidum var. endemicum*, *Tropheryma whippelii*, *Ureaplasma urealyticum*, *Varicella-Zoster virus*, *variola virus*, *Vibrio cholerae*, *West Nile virus*, *yellow fever virus*, *Yersinia enterocolitica*, *Yersinia pestis*, and *Zika virus*.

[0499] Various rare diseases may be treated with pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads of the present disclosure. As used herein, the term “rare disease” refers to any disease that affects a small percentage of the population.

[0500] Various autoimmune diseases and autoimmune-related diseases may be treated with pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads of the present disclosure. As used herein, the term “autoimmune disease” refers to a disease in which the body produces antibodies that attack its own tissues. As a non-limiting example, the autoimmune disease may be Acute Disseminated Encephalomyelitis (ADEM), Acute necrotizing hemorrhagic leukoencephalitis, Addison’s disease, Agammaglobulinemia, Alopecia areata, Amyloidosis, Ankylosing spondylitis, Anti-GBM/Anti-TBM nephritis, Antiphospholipid syndrome (APS), Autoimmune angioedema, Autoimmune aplastic anemia, Autoimmune dysautonomia, Autoimmune hepatitis, Autoimmune hyperlipidemia, Autoimmune immunodeficiency, Autoimmune inner ear disease (AIED), Autoimmune myocarditis, Autoimmune oophoritis, Autoimmune pancreatitis, Autoimmune retinopathy, Autoimmune thrombocytopenic purpura (ATP), Autoimmune thyroid disease, Autoimmune urticaria, Axonal & neuronal neuropathies, Balo disease, Behcet’s disease, Bullous pemphigoid, Cardiomyopathy, Castleman disease, Celiac disease, Chagas disease, Chronic fatigue syndrome, Chronic inflammatory demyelinating polyneuropathy (CIDP), Chronic recurrent multifocal osteomyelitis (CRMO), Churg-Strauss syndrome, Cicatricial pemphigoid/benign

mucosal pemphigoid, Crohn's disease, Cogans syndrome, Cold agglutinin disease, Congenital heart block, Coxsackie myocarditis, CREST disease, Essential mixed cryoglobulinemia, Demyelinating neuropathies, Dermatitis herpetiformis, Dermatomyositis, Devic's disease (neuromyelitis optica), Discoid lupus, Dressler's syndrome, Endometriosis, Eosinophilic esophagitis, Eosinophilic fasciitis, Erythema nodosum, Experimental allergic encephalomyelitis, Evans syndrome, Fibromyalgia, Fibrosing alveolitis, Giant cell arteritis (temporal arteritis), Giant cell myocarditis, Glomerulonephritis, Goodpasture's syndrome, Granulomatosis with Polyangiitis (GPA) (formerly called Wegener's Granulomatosis), Graves' disease, Guillain-Barre syndrome, Hashimoto's encephalitis, Hashimoto's thyroiditis, Hemolytic anemia, Henoch-Schonlein purpura, Herpes gestationis, Hypogammaglobulinemia, Idiopathic thrombocytopenic purpura (ITP), IgA nephropathy, IgG4-related sclerosing disease, Immunoregulatory lipoproteins, Inclusion body myositis, Interstitial cystitis, Juvenile arthritis, Juvenile diabetes (Type 1 diabetes), Juvenile myositis, Kawasaki syndrome, Lambert-Eaton syndrome, Leukocytoclastic vasculitis, Lichen planus, Lichen sclerosis, Ligneous conjunctivitis, Linear IgA disease (LAD), Lupus (SLE), Lyme disease, chronic, Meniere's disease, Microscopic polyangiitis, Mixed connective tissue disease (MCTD), Mooren's ulcer, Mucha-Habermann disease, Multiple sclerosis, Myasthenia gravis, Myositis, Narcolepsy, Neuromyelitis optica (Devic's), Neutropenia, Ocular cicatricial pemphigoid, Optic neuritis, Palindromic rheumatism, PANDAS (Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcus), Paraneoplastic cerebellar degeneration, Paroxysmal nocturnal hemoglobinuria (PNH), Parry Romberg syndrome, Parsonnage-Turner syndrome, Pars planitis (peripheral uveitis), Pemphigus, Peripheral neuropathy, Perivenous encephalomyelitis, Pernicious anemia, POEMS syndrome, Polyarteritis nodosa, Type I, II, & III autoimmune polyglandular syndromes, Polymyalgia rheumatica, Polymyositis, Postmyocardial infarction syndrome, Postpericardiotomy syndrome, Progesterone dermatitis, Primary biliary cirrhosis, Primary sclerosing cholangitis, Psoriasis, Psoriatic arthritis, Idiopathic pulmonary fibrosis, Pyoderma gangrenosum, Pure red cell aplasia, Raynauds phenomenon, Reactive Arthritis, Reflex sympathetic dystrophy, Reiter's syndrome, Relapsing polychondritis, Restless legs syndrome, Retroperitoneal fibrosis, Rheumatic fever, Rheumatoid arthritis, Sarcoidosis, Schmidt syndrome, Scleritis, Scleroderma, Sjogren's syndrome, Sperm & testicular autoimmunity, Stiff person syndrome, Subacute bacterial endocarditis (SBE), Susac's syndrome, Sympathetic ophthalmia, Takayasu's arteritis, Temporal arteritis/Giant cell arteritis, Thrombocytopenic purpura (TTP), Tolosa-Hunt syndrome, Transverse myelitis, Ulcerative colitis, Undifferentiated connective tissue disease (UCTD), Uveitis, Vasculitis, Vesiculobullous dermatosis, Vitiligo, and Wegener's granulomatosis (now termed Granulomatosis with Polyangiitis (GPA)).

[0501] Various kidney diseases may be treated with pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads of the present disclosure

[0502] Various cardiovascular diseases may be treated with pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads of the present disclosure. As a non-limiting example, the cardiovascular disease may be Ischemic heart disease also known as coronary artery disease, Cerebrovascular disease (Stroke), Peripheral vascular disease, Heart failure, Rheumatic heart disease, and Congenital heart disease.

[0503] Various antibody deficiencies may be treated with pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads of the present disclosure. As a non-limiting example, the antibody deficiencies may be X-Linked Agammaglobulinemia (XLA), Autosomal Recessive Agammaglobulinemia (ARA), Common Variable Immune Deficiency (CVID), IgG (IgG1, IgG2, IgG3 and IgG4) Subclass Deficiency, Selective IgA Deficiency, Specific Antibody Deficiency (SAD), Transient Hypogammaglobulinemia of Infancy, Antibody Deficiency with Normal or Elevated Immunoglobulins, Selective IgM Deficiency, Immunodeficiency with Thymoma (Good's Syndrome), Transcobalamin II Deficiency, Warts, Hypogammaglobulinemia, Infection, Myelokathexis (WHIM) Syndrome, Drug-Induced Antibody Deficiency, Kappa Chain Deficiency, Heavy Chain Deficiencies, Post-Meiotic Segregation (PMS2) Disorder, and Unspecified Hypogammaglobulinemia.

[0504] Various ocular diseases may be treated with pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads of the present disclosure. As a non-limiting example, the ocular disease may be thyroid eye disease (TED), Graves' disease (GD) and orbitopathy, Retina Degeneration, Cataract, optic atrophy, macular degeneration, Leber congenital amaurosis, retinal degeneration, cone-rod dystrophy, Usher syndrome, leopard syndrome, photophobia, and photoaversion.

[0505] Various neurological diseases may be treated with pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads of the present disclosure.

[0506] Various psychological disorders may be treated with pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads of the present disclosure.

[0507] Various lung diseases may be treated with pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads of the present disclosure. As a non-limiting example, the lung diseases may be Asbestosis, Asthma, Bronchiectasis, Bronchitis, Chronic Cough, Chronic Obstructive Pulmonary Disease (COPD), Croup, Cystic Fibrosis, Hantavirus, Idiopathic Pulmonary Fibrosis, Pertussis, Pleurisy, Pneumonia, Pulmonary Embolism, Pulmonary Hypertension, Sarcoidosis, Sleep Apnea, Spirometry, Sudden Infant Death Syndrome (SIDS), Tuberculosis, Alagille Syndrome, Autoimmune Hepatitis, Biliary Atresia, Cirrhosis, ERCP

(Endoscopic Retrograde Cholangiopancreatography), and Hemochromatosis. Nonalcoholic Steatohepatitis, Porphyria, Primary Biliary Cirrhosis, Primary Sclerosing Cholangitis.

[0508] Various bone diseases may be treated with pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads of the present disclosure. As a non-limiting example, the bone diseases may be osteoporosis, neurofibromatosis, osteogenesis imperfecta (OI), rickets, osteosarcoma, achondroplasia, fracture, osteomyelitis, Ewing tumor of bone, osteomalacia, hip dysplasia, Paget disease of bone, marble bone disease, osteochondroma, bone cancer, bone disease, osteochondrosis, osteoma, fibrous dysplasia, cleidocranial dysostosis, osteoclastoma, bone cyst, metabolic bone disease, melorheostosis, callus, Caffey syndrome, and mandibulofacial dysostosis.

[0509] Various blood diseases may be treated with pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads of the present disclosure. As a non-limiting example, the blood diseases may be Anemia and CKD (for health care professionals), Aplastic Anemia and Myelodysplastic Syndromes, Deep Vein Thrombosis, Hemochromatosis, Hemophilia, Henoch-Schönlein Purpura, Idiopathic Thrombocytopenic Purpura, Iron-Deficiency Anemia, Pernicious Anemia, Pulmonary Embolism, Sickle Cell Anemia, Sickle Cell Trait and Other Hemoglobinopathies, Thalassemia, Thrombotic Thrombocytopenic Purpura, and Von Willebrand Disease.

Central Nervous System (CNS)

[0510] In some embodiments, pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads of the present disclosure may be used in the modulation or alteration or exploitation of proteins in the central nervous system including cerebrospinal (CSF) proteins.

[0511] In some examples, pharmaceutical compositions, CA2 biocircuits, CA2 biocircuit components, CA2 effector modules including their SREs or payloads of the present disclosure may be used to provide tunable ERT (enzyme replacement therapy) products to the central nervous system. Many lysosomal storage diseases (LSD) involve the CNS symptoms, such as mental retardation, seizures, profound neurodegeneration, behavioral abnormalities, and psycho-motor defects. ERT for LSDs is one of the true success stories in modern molecular medicine. The successful application of ERT relies on controlled lysosomal proteins (e.g., enzymes) and delivery to CNS cells.

Metabolic peptides and hormones

[0512] In some embodiments, the CA2 biocircuits of the present disclosure and/or any of their components may be used to regulate peptides, natural or synthetic. Naturally occurring peptides may include but are not limited to, peptide hormones, natriuretic peptides, food peptides, and derivatives and precursors.

[0513] The CA2 biocircuits of the present disclosure and/or any of their components may also be utilized for pulsatile release of hormones or other peptide drugs.

Patient Stratification

[0514] In one embodiment, patients may also be stratified according to the immunogenic peptides presented by their immune cells and may be utilized as a parameter to determine suitable patient cohorts that may therapeutically benefit for the compositions of the present disclosure.

Transgenic Organisms

[0515] In some embodiments, the present disclosure provides transgenic organisms that expresses nucleic acids that encode polypeptides of the present disclosure. As used herein the term "transgenic organism" refers to any non-human entity that contains artificially transferred, exogenous genetic material. This approach provides the ability to temporally regulate payloads within defined cells, tissues or in the entire organism. Such methods may be useful in creating transgenic models for certain disease states, or for studying embryonic development.

[0516] Transgenic organisms described herein may include rodents, fish, reptiles, as well as invertebrates. In a preferred embodiment, such transgenic organisms may be selected from the rodent family including mouse, and rat.

Tunable regulations

[0517] The CA2 biocircuits of the present disclosure and/or any of their components may also be utilized to regulate the expression of another CA2 effector module such as a recombinant construct comprising a POI. In some embodiments, the CA2 biocircuits and/or CA2 effector modules may comprise a protease (also called peptidase or proteinase). The tunable protease could cleave an inactive construct to an active construct when the two components are co-introduced into a cell, a tissue or an organism.

[0518] In other examples, the CA2 biocircuits and/or CA2 effector modules comprising a protease may also be utilized to regulate protein processing including cleavage of the initial protein product to produce a smaller active protein or peptide.

[0519] In some embodiments, the CA2 biocircuits of the present disclosure and/or any of their components may comprise any of factors that play a role in protein processing and modification. Protein post-translational modification may include, but are not limited to, addition of hydrophobic groups by an enzyme (e.g., myristoylation, palmitoylation, isoprenylation, prenylation, farnesylation, geranylgeranylation, glypiation, and glycosylphosphatidylinositol (GPI) anchor); attachment of cofactors for enhanced function (e.g., lipoylation, flavin, phosphopantetheinylation, and heme C); addition of small chemical groups (e.g., acylation, formylation, alkylation, phosphorylation, methylation, arginylation, polyglutamylation, polyglycylation, butyrylation, glycosylation, propionylation, S-glutathionylation, S-nitrosylation, S-

sulfenylation, succinylation, sulfation, and acetylation); linkage of other proteins and/or peptides such as ISGylation, SUMOylation, ubiquitination, neddylation, and pupylation; chemical modification of amino acids; and structural changes.

Biofactories

[0520] The CA2 biocircuits of the present disclosure and/or any of their components may be utilized to regulate the levels of protein production in a biofactory. As used herein, the term “biofactory” refers to a cell, a tissue, an organ or an organism genetically modified or not, which can produce proteins with a number of applications including therapeutic purposes (inhibitors, enzymes, antibodies, antigens, etc.) or primary or secondary products of industrial interest. In some examples, the cell may be a prokaryotic cell, a eukaryotic cell, a mammalian cell, a plant cell, etc.

[0521] In some embodiments, the CA2 biocircuits of the present disclosure may be used to regulate medicament proteins produced in a target tissue, for example, the liver and the kidney. The liver is an organ that produces secreted proteins including major plasma proteins, factors in hemostasis and fibrinolysis, carrier proteins, hormones, prohormones and apolipoproteins, or a variety of short-lived metabolic peptides and enzymes which are usually tightly regulated, or other non-hepatic proteins. In the context, the liver fills a role of gene expression factory (biofactory), supplying a protein for treatment of a disease for example a metabolic disease.

[0522] In other embodiments, the CA2 biocircuits of the present disclosure may be used to regulate proteins for industrial processes.

Liver targeting

[0523] The liver is an important organ that produces proteins and involves blood clotting and a number of metabolic functions. A variety of diseases can affect liver and targeting the liver for disease treatment has been a promising approach, especially liver-targeted gene therapy. The CA2 biocircuits of the present disclosure and/or any of their components may be utilized to regulate liver targeted gene therapy and gene transfer.

[0524] Proteins that can be targeted to the liver and constructed to the present CA2 biocircuits for regulation may include those in liver cancers such as hepatocellular carcinoma (HCC), Fibrolamellar HCC, Cholangiocarcinoma, Angiosarcoma and secondary liver cancer; inherited disorders caused by defective genes such as hemochromatosis, Wilson disease, tyrosinemia, alpha 1 antitrypsin deficiency, glycogen storage disease; metabolic disorders due to enzyme deficiency such as Gilbert's syndrome, lysosomal acid lipase deficiency (LALD) and Gaucher disease; autoimmune hepatitis; fatty liver diseases; and viral hepatitis (A, B and C). In some examples, the present CA2 biocircuits may be used to direct IL12 for hepatocellular carcinoma (HCC), and IL10 for diabetic neuropathy.

Microfluidics

[0525] In some embodiments, cells containing CA2 biocircuits of the present disclosure and/or any of their components may be utilized in microfluidics devices. As used herein a "microfluidics device" refers to the manipulation of picoliter to nanoliter-scale volumes of fluids within artificially fabricated microsystems. Microfluidic devices comprising CA2 biocircuits of the present disclosure may be utilized to study cell culture models, cellular microenvironment, cell secretions, chemotaxis, apoptosis, vascular function, neuron cell growth, embryonic development, single cell metabolomics, gene expression, drug research, cellular separation, stem cell biology, bioreactors, three-dimensional cell culture, and tissue engineering.

Tools and agents for making therapeutics

[0526] Provided in the present disclosure are tools and agents that may be used in generating therapeutics such as, but not limited to, immunotherapeutics for reducing a tumor volume or burden in a subject in need. A considerable number of variables are involved in producing a therapeutic agent, such as structure of the payload, type of cells, method of gene transfers, method and time of ex vivo expansion, pre-conditioning and the amount and type of tumor burden in the subject. Such parameters may be optimized using tools and agents described herein.

Cell Lines

[0527] The present disclosure provides a mammalian cell that has been genetically modified with the compositions of the present disclosure. Suitable mammalian cells include primary cells and immortalized cell lines. Suitable mammalian cell lines include, but are not limited to Human embryonic kidney cell line 293, fibroblast cell line NIH 3T3, human colorectal carcinoma cell line HCT116, ovarian carcinoma cell line SKOV-3, immortalized T cell lines (e.g. Jurkat cells and SupT1 cells), lymphoma cell line Raji cells, NALM-6 cells, K562 cells, HeLa cells, PC12 cells, HL-60 cells, NK cell lines (e.g. NKL, NK92, NK962, and YTS), and the like. In some instances, the cell is not an immortalized cell line, but instead a cell obtained from an individual and is herein referred to as a primary cell. For example, the cell is a T lymphocyte obtained from an individual. Other examples include, but are not limited to cytotoxic cells, stem cells, peripheral blood mononuclear cells or progenitor cells obtained from an individual.

Tracking SREs, biocircuits and cell lines

[0528] In some embodiments, it may be desirable to track the compositions of the present disclosure or the cells modified by the compositions described herein. Tracking may be achieved by using payloads such as reporter moieties, which, as used herein, refers to any protein capable of creating a detectable signal, in response to an input. Examples include alkaline phosphatase, β -galactosidase, chloramphenicol acetyltransferase, β -glucuronidase, peroxidase, β -

lactamase, catalytic antibodies, bioluminescent proteins e.g. luciferase, and fluorescent proteins such as Green fluorescent protein (GFP).

[0529] Reporter moieties may be used to monitor the response of the SREs upon addition of the ligand corresponding to the SRE. In other instances, reporter moieties may be used to track cell survival, persistence, cell growth, and/or localization *in vitro*, *in vivo*, or *ex vivo*.

[0530] In some embodiments, the preferred reporter moiety may be luciferase proteins.

Chaperones

[0531] In some embodiments, CA2 effector modules of the present disclosure may include one or more chaperones to regulate the expression of the payload. Chaperones useful in the present disclosure may be cellular chaperones or small molecules referred to as pharmacological chaperones. Cellular chaperones refer to a large group of unrelated protein families whose role is to stabilize unfolded client proteins, or to unfold client proteins for translocation across membranes or for degradation, and/or to assist in their correct folding and assembly. Chaperones also cooperate with other components of the proteostasis network such as the proteasome system and autophagy to promote protein clearance. Examples of molecular chaperone families include small heat shock proteins such as hsp25; Heat shock protein 60 family proteins such as cpn60 and GroEL; Heat shock protein 70 family proteins such as DnaK and BiP; Heat shock protein 90 family proteins; Heat shock protein 100 family proteins such as Clp; lectin chaperones such as calnexin and calreticulin; and folding chaperones such as Protein disulfide isomerases (PDI), peptidyl prolyl ci-trans isomerase (PPI) and ERp57. In some embodiments, the payload of the present disclosure may be a cellular chaperone. In the absence of a stimulus which stabilizes the SRE, the cellular chaperone may bind to the SRE and is therefore unavailable to interact with its client proteins. In the presence of the stimulus specific to the SRE, the SRE is stabilized and the chaperone is available to interact with client proteins. In some embodiments, payloads of the present disclosure may be appended to chaperones such that the stability or instability of the payload may be enhanced. In other embodiments, the SREs of the present disclosure may consist of one or more molecular chaperones.

[0532] Chaperones useful in the present disclosure may also include pharmacological chaperones which utilizes small molecules to facilitate the correct folding and stabilization of cellular proteins. Mutations in cellular proteins can result in protein misfolding and/or aggregation which ultimately results in their degradation. Pharmacological chaperones have been designed to bind to misfolded target proteins, facilitate their correct folding and thereby prevent their degradation. In some embodiments, SREs of the present disclosure may comprise one or more misfolded proteins and the stimulus specific to the SRE may include one or pharmacological chaperones such that the CA2 effector module is stabilized only in the presence of the pharmacological chaperone.

Animal Models

[0533] The utility and efficacy of the compositions of the present disclosure may be tested in *in vivo* animal models, preferably mouse models. Mouse models used may be syngeneic mouse models wherein mouse cells are modified with compositions of the present disclosure and tested in mice of the same genetic background. Examples include pMEL-1 and 4T1 mouse models. Alternatively, xenograft models where human cells such as tumor cells and immune cells are introduced into immunodeficient mice may also be utilized in such studies. Immunodeficient mice used may be CByJ.Cg-Foxn1nu/J, B6.129S7-Rag1tm1Mom/J, B6.129S7-Rag1tm1Mom/J, B6. CB17-Prkdcscid/SzJ, NOD.129S7(B6)-Rag1tm1Mom/J, NOD.Cg-Rag1tm1MomPrf1tm1Sz/Sz, NOD.CB17-Prkdcscid/SzJ, NOD.Cg-PrkdcscidB2mtm1Unc/J, NOD-scid IL2Rgnull, Nude (nu) mice, SCID mice, NOD mice, RAG1/RAG2 mice, NOD-Scid mice, IL2rgnull mice, b2mnull mice, NOD-scid IL2r□null mice, NOD-scid-B2mnull mice, beige mouse, and HLA transgenic mice.

Cellular Assays

[0534] In some embodiments, the effectiveness of the compositions of the disclosure as immunotherapeutic agents may be evaluated using cellular assays. Levels of expression and/or identity of the compositions described herein may be determined according to any methods known in the art for identifying proteins and/or quantitating proteins levels. In some embodiments, such methods may include Western Blotting, flow cytometry, and immunoassays.

[0535] Provided herein are methods for functionally characterizing cells expressing SRE, CA2 biocircuits and compositions of the invention. In some embodiments, functional characterization is carried out in primary immune cells or immortalized immune cell lines and may be determined by expression of cell surface markers. Examples of cell surface markers for T cells include, but are not limited to, CD3, CD4, CD8, CD 14, CD20, CD11b, CD16, CD45 and HLA-DR, CD 69, CD28, CD44, IFN γ gamma. Markers for T cell exhaustion include PD1, TIM3, BTLA, CD160, 2B4, CD39, and LAG3. Examples of cell surface markers for antigen presenting cells include, but are not limited to, MHC class I, MHC Class II, CD40, CD45, B7-1, B7-2, IFN γ receptor and IL2 receptor, ICAM-1 and/or Fc γ receptor. Examples of cell surface markers for dendritic cells include, but are not limited to, MHC class I, MHC Class II, B7-2, CD18, CD29, CD31, CD43, CD44, CD45, CD54, CD58, CD83, CD86, CMRF-44, CMRF-56, DCIR and/or Dectin-1 and the like; while in some cases also having the absence of CD2, CD3, CD4, CD8, CD14, CD15, CD16, CD 19, CD20, CD56, and/or CD57. Examples of cell surface markers for NK cells include, but are not limited to, CCL3, CCL4, CCL5, CCR4, CXCR4, CXCR3, NKG2D, CD71, CD69, CCR5, Phospho JAK/STAT, phospho ERK, phospho p38/ MAPK, phospho AKT, phospho STAT3, Granulysin, Granzyme B, Granzyme K, IL10, IL22, IFN γ , LAP, Perforin, and TNFa.

T cell exhaustion

[0536] In some embodiments, CA2 biocircuits, SREs or CA2 effector modules may be utilized to prevent T cell exhaustion. As used herein, "T cell exhaustion" refers to the stepwise and progressive loss of T cell function caused by chronic T cell activation. T cell exhaustion is a major factor limiting the efficacy of antiviral and antitumor immunotherapies. Exhausted T cells have low proliferative and cytokine producing capabilities concurrent with high rates of apoptosis and high surface expression of multiple inhibitory receptors. T cell activation leading to exhaustion may occur either in the presence or absence of the antigen.

Cells

[0537] In accordance with the present disclosure, cells genetically modified to express at least one CA2 biocircuit, SRE (e.g., CA2 DD), CA2 effector module and immunotherapeutic agent of the present disclosure, are provided. Cells of the present disclosure may include, without limitation, immune cells, stem cells and tumor cells. In some embodiments, immune cells are effector immune cells, including, but not limiting to, T cells such as CD8⁺ T cells and CD4⁺ T cells (e.g., Th1, Th2, Th17, Foxp3⁺ cells), memory T cells such as T memory stem cells, central T memory cells, and effector memory T cells, terminally differentiated effector T cells, natural killer (NK) cells, NK T cells, tumor infiltrating lymphocytes (TILs), cytotoxic T lymphocytes (CTLs), regulatory T cells (Tregs), and dendritic cells (DCs), other immune cells that can elicit an effector function, or the mixture thereof. T cells may be T $\alpha\beta$ cells and T $\gamma\delta$ cells. In some embodiments, stem cells may be from human embryonic stem cells, mesenchymal stem cells, and neural stem cells. In some embodiments, T cells may be depleted endogenous T cell receptors (See US Pat. NOs. 9,273,283; 9,181,527; and 9,028,812; the contents of each of which are incorporated herein by reference in their entirety).

[0538] In some embodiments, cells of the present disclosure may be autologous, allogeneic, syngeneic, or xenogeneic in relation to a particular individual subject.

[0539] In some embodiments, cells of the present disclosure may be mammalian cells, particularly human cells. Cells described herein may be primary cells or immortalized cell lines.

[0540] In some embodiments, cells of the present disclosure may include expansion factors as payload to trigger proliferation and expansion of the cells. Exemplary payloads include members of the RAS superfamily.

[0541] Engineered immune cells can be accomplished by transducing a cell composition with a polypeptide of a CA2 biocircuit, a CA2 effector module, a SRE and/or a payload of interest (e.g., immunotherapeutic agent), or a polynucleotide encoding said polypeptide, or a vector comprising said polynucleotide. The vector may be a viral vector such as a lentiviral vector, a gamma-retroviral vector, a recombinant AAV, an adenoviral vector and an oncolytic viral vector. In other aspects, non-viral vectors for example, nanoparticles and liposomes may also be used. In some

embodiments, immune cells of the present disclosure are genetically modified to express at least one immunotherapeutic agent described herein which is tunable using a stimulus. In some examples, two, three or more immunotherapeutic agents constructed in the same CA2 biocircuit and CA2 effector module are introduced into a cell. In other examples, two, three, or more biocircuits, effector modules, each of which comprises an immunotherapeutic agent, may be introduced into a cell.

[0542] In some embodiments, immune cells of the present disclosure may be T cells modified to express an antigen-specific T cell receptor (TCR), or an antigen specific chimeric antigen receptor (CAR) taught herein (known as CAR T cells). Accordingly, at least one polynucleotide encoding a CAR system (or a TCR) described herein, or a vector comprising the polynucleotide is introduced into a T cell. The T cell expressing the CAR or TCR binds to a specific antigen via the extracellular targeting moiety of the CAR or TCR, thereby a signal via the intracellular signaling domain (s) is transmitted into the T cell, and as a result, the T cell is activated. The activated CAR T cell changes its behavior including release of a cytotoxic cytokine (e.g., a tumor necrosis factor, and lymphotoxin, etc.), improvement of a cell proliferation rate, change in a cell surface molecule, or the like. Such changes cause destruction of a target cell expressing the antigen recognized by the CAR or TCR. In addition, release of a cytokine or change in a cell surface molecule stimulates other immune cells, for example, a B cell, a dendritic cell, a NK cell, and a macrophage.

[0543] In some embodiments, CAR T cells of the present disclosure may be further modified to express another one, two, three or more immunotherapeutic agents. The immunotherapeutic agents may be another CAR or TCR specific to a different target molecule; a cytokine such as IL2, IL12, IL15 and IL18, or a cytokine receptor such as IL15Ra; a chimeric switch receptor that converts an inhibitory signal to a stimulatory signal; a homing receptor that guides adoptively transferred cells to a target site such as the tumor tissue; an agent that optimizes the metabolism of the immune cell; or a safety switch gene (e.g., a suicide gene) that kills activated T cells when a severe event is observed after adoptive cell transfer or when the transferred immune cells are no-longer needed. These molecules may be included in the same effector module or in separate effector modules.

[0544] In related embodiments of the above, an engineered cell, for example, an immune cell as described herein can be genetically manipulated to express one or more immunotherapeutic agents, wherein one or more of the immunotherapeutic agents are regulated using the effector modules described herein. In an exemplary embodiment, an engineered cell comprises: i) a first polynucleotide which encodes a first polypeptide, said first polypeptide comprising: a. a first stimulus response element (SRE), wherein the first SRE comprises a drug responsive domain (DRD), said DRD comprising a human carbonic anhydrase 2 (CA2; SEQ ID NO. 5810) or a region thereof, and further comprising one or more mutations relative to the amino acid sequence of SEQ ID NO. 5810; and b. a first payload which is operably linked

to the first SRE, wherein (I) the first payload comprises CD40L (SEQ ID NO. 6) or a portion thereof; or (II) the first payload comprises CD40L (SEQ ID NO. 6) or a portion thereof comprising one or more mutations relative to the amino acid sequence of SEQ ID NO. 6; and ii) a second polynucleotide which encodes one or more additional polypeptides, said one or more additional polypeptides comprising an immunotherapeutic agent selected from the group consisting of: a T cell receptor (TCR) and variants thereof or a chimeric antigen receptor (CAR); wherein at least one mutation of the one or more mutations in the DRD of the first SRE destabilizes the DRD and the first payload in the absence of a first stimulus and wherein the DRD and the first payload are stabilized in the presence of the first stimulus, and the second polypeptide is expressed independently of the first payload.

[0545] In various embodiments, the payloads of the engineered cells can include: i) CD40L (SEQ ID NO. 6) or a portion thereof comprising at least one mutation; or ii) CD40L (SEQ ID NO. 6) or a portion thereof comprising at least two mutations, optionally, wherein the at least two mutations are selected from: (i) H224G and G226F; (ii) H224G and G226H; (iii) Y172G and G226F; (iv) H125 and G227; (v) Y120G, H224G and G226W; and vi) S110G, F111G, E112S, M113G, Q114G, and K115S. Illustrative engineered cells as provided above having a second immunotherapeutic agent, along with a regulated first immunotherapeutic agent, for example, CD40L, the one or more additional polypeptides comprising the second immunotherapeutic agent may be linked to a second SRE comprising a second DRD, and in some examples, the second DRD is the same or different as the DRD in the first SRE linked to the first payload, and the second DRD and the second polypeptide are both destabilized in the absence of the first or a second stimulus and wherein the second DRD and the second polypeptide are stabilized in the presence of the first or the second stimulus. In some embodiments, the engineered cell comprises a first payload e.g., CD40L operably linked to a first DRD and a second immunotherapeutic agent, for example, a CAR or TCR is introduced into the engineered cell such that the second immunotherapeutic agent, for example, a CAR or TCR is expressed independently from the first payload, and the polynucleotide encoding the second immunotherapeutic agent may be stably integrated into the engineered cell's genome such that the second immunotherapeutic agent is constitutively expressed or expressed in an inducible manner using an inducible promoter in the engineered cell. In various embodiments, the engineered cell is selected from a CD8⁺ T cell, a CD4⁺ T cell, a helper T cell, a natural killer (NK) cell, a NKT cell, a cytotoxic T lymphocyte (CTL), a tumor infiltrating lymphocyte (TIL), a memory T cell, a regulatory T (Treg) cell, a cytokine-induced killer (CIK) cell, a dendritic cell, lymphokine activated killer (LAK) cells, a human embryonic stem cell, a mesenchymal stem cell, a hematopoietic stem cell, or a mixture thereof.

[0546] In one embodiment, the CAR T cell (including TCR T cell) of the present disclosure may be an "armed" CAR T cell which is transformed with a CA2 effector module comprising a CAR and a CA2 effector module comprising

a cytokine. The inducible or constitutively secreted active cytokines further armor CAR T cells to improve efficacy and persistence. In this context, such CAR T cell is also referred to as “armored CAR T cell”. The “armor” molecule may be selected based on the tumor microenvironment and other elements of the innate and adaptive immune systems. In some embodiments, the molecule may be a stimulatory factor such as IL2, IL12, IL15, IL18, type I IFN, CD40L and 4-1BBL which have been shown to further enhance CAR T cell efficacy and persistence in the face of a hostile tumor microenvironment via different mechanisms (Yeku et al., Biochem Soc Trans., 2016, 44(2): 412-418).

[0547] In some embodiments, immune cells of the present disclosure may be NK cells modified to express an antigen-specific T cell receptor (TCR), or an antigen specific chimeric antigen receptor (CAR) taught herein.

[0548] NK cells may be isolated from peripheral blood mononuclear cells (PBMCs) or derived from human embryonic stem (ES) cells and induced pluripotent stem cells (iPSCs). The primary NK cells isolated from PBMCs may be further expanded for adoptive immunotherapy. Strategies and protocols useful for the expansion of NK cells may include interleukin 2 (IL2) stimulation and the use of autologous feeder cells, or the use of genetically modified allogeneic feeder cells. In some aspects, NK cells can be selectively expanded with a combination of stimulating ligands including IL15, IL21, IL2, 4-1BBL, IL12, IL18, MICA, 2B4, LFA-1, and BCM1/SLAMF2 (e.g., US patent publication NO. US20150190471).

[0549] Immune cells expressing CA2 effector modules comprising a CAR and/or other immunotherapeutic agents can be used as cancer immunotherapy. The immunotherapy comprises the cells expressing a CAR and/or other immunotherapeutic agents as an active ingredient and may further comprise a suitable excipient. Examples of the excipient may include the aforementioned pharmaceutically acceptable excipients, including various cell culture media, and isotonic sodium chloride.

[0550] In some embodiments, cells of the present disclosure may be dendritic cells that are genetically modified to express the compositions of the present disclosure. Such cells may be used as cancer vaccines.

V. DEFINITIONS

[0551] At various places in the present specification, features or functions of the compositions of the present disclosure are disclosed in groups or in ranges. It is specifically intended that the present disclosure include each and every individual sub combination of the members of such groups and ranges. The following is a non-limiting list of term definitions.

[0552] *Activity*: As used herein, the term “activity” refers to the condition in which things are happening or being done. Compositions described herein may have activity and this activity may involve one or more biological events. In some embodiments, biological events may include cell signaling events. In some embodiments, biological events may

include cell signaling events associated protein interactions with one or more corresponding proteins, receptors, small molecules or any of the biocircuit components described herein.

[0553] *Adoptive cell therapy (ACT)*: The terms “Adoptive cell therapy” or “Adoptive cell transfer”, as used herein, refer to a cell therapy involving in the transfer of cells into a patient, wherein cells may have originated from the patient, or from another individual, and are engineered (altered) before being transferred back into the patient. The therapeutic cells may be derived from the immune system, such as effector immune cells: CD4+ T cell; CD8+ T cell, Natural Killer cell (NK cell); and B cells and tumor infiltrating lymphocytes (TILs) derived from the resected tumors. Most commonly transferred cells are autologous anti-tumor T cells after *ex vivo* expansion or manipulation. For example, autologous peripheral blood lymphocytes can be genetically engineered to recognize specific tumor antigens by expressing T-cell receptors (TCR) or chimeric antigen receptor (CAR).

[0554] *Agent*: As used herein, the term “agent” refers to a biological, pharmaceutical, or chemical compound. Non-limiting examples include simple or complex organic or inorganic molecule, a peptide, a protein, an oligonucleotide, an antibody, an antibody derivative, antibody fragment, a receptor, and soluble factor.

[0555] *Agonist*: the term “agonist” as used herein, refers to a compound that, in combination with a receptor, can produce a cellular response. An agonist may be a ligand that directly binds to the receptor. Alternatively, an agonist may combine with a receptor indirectly by, for example, (a) forming a complex with another molecule that directly binds to the receptor, or (b) otherwise resulting in the modification of another compound so that the other compound directly binds to the receptor. An agonist may be referred to as an agonist of a particular receptor or family of receptors, e.g., agonist of a co-stimulatory receptor.

[0556] *Antagonist*: the term “antagonist” as used herein refers to any agent that inhibits or reduces the biological activity of the target(s) it binds.

[0557] *Antigen*: the term “antigen” as used herein is defined as a molecule that provokes an immune response when it is introduced into a subject or produced by a subject such as tumor antigens which arise by the cancer development itself. This immune response may involve either antibody production, or the activation of specific immunologically-competent cells such as cytotoxic T lymphocytes and T helper cells, or both. An antigen can be derived from organisms, subunits of proteins/antigens, killed or inactivated whole cells or lysates. In the context of the present disclosure, the terms “antigens of interest” or “desired antigens” refers to those proteins and/or other biomolecules provided herein that are immunospecifically bound or interact with antibodies of the present disclosure and/or fragments, mutants, variants, and/or alterations thereof described herein. In some embodiments, antigens of interest may comprise any of the polypeptides or payloads or proteins described herein, or fragments or portions thereof.

[0558] *Approximately*: As used herein, the term “approximately” or “about,” as applied to one or more values of interest, refers to a value that is similar to a stated reference value. In certain embodiments, the term “approximately” or “about” refers to a range of values that fall within 25, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, or less in either direction (greater than or less than) of the stated reference value unless otherwise stated or otherwise evident from the context (except where such number would exceed 100 of a possible value).

[0559] *Associated with*: As used herein, the terms “associated with,” “conjugated,” “linked,” “attached,” and “tethered,” when used with respect to two or more moieties, mean that the moieties are physically associated or connected with one another, either directly or via one or more additional moieties that serve as linking agents, to form a structure that is sufficiently stable so that the moieties remain physically associated under the conditions in which the structure is used, e.g., physiological conditions. An “association” need not be strictly through direct covalent chemical bonding. It may also suggest ionic or hydrogen bonding or a hybridization-based connectivity sufficiently stable such that the “associated” entities remain physically associated.

[0560] *Autologous*: the term “autologous” as used herein is meant to refer to any material derived from the same individual to which it is later to be re-introduced into the individual.

[0561] *Barcode*: the term “barcode” as used herein refers to polynucleotide or amino acid sequence that distinguishes one polynucleotide or amino acid from another.

[0562] *Cancer*: the term “cancer” as used herein refers a broad group of various diseases characterized by the uncontrolled growth of abnormal cells in the body. Unregulated cell division and growth results in the formation of malignant tumors that invade neighboring tissues ultimately metastasize to distant parts of the body through the lymphatic system or bloodstream.

[0563] *Co-stimulatory molecule*: As used herein, in accordance with its meaning in immune T cell activation, refers to a group of immune cell surface receptor/ligands which engage between T cells and APCs and generate a stimulatory signal in T cells which combines with the stimulatory signal in T cells that results from T cell receptor (TCR) recognition of antigen/MHC complex (pMHC) on APCs

[0564] *Cytokines*: the term “cytokines”, as used herein, refers to a family of small soluble factors with pleiotropic functions that are produced by many cell types that can influence and regulate the function of the immune system.

[0565] *Delivery*: the term “delivery” as used herein refers to the act or manner of delivering a compound, substance, entity, moiety, cargo or payload. A “delivery agent” refers to any agent which facilitates, at least in part, the in vivo delivery of one or more substances (including, but not limited to a compound and/or composition of the present disclosure) to a cell, subject or other biological system cells.

[0566] *Destabilized*: As used herein, the term “destable,” “destabilize,” “destabilizing region” or “destabilizing domain” means a region or molecule that is less stable than a starting, reference, wild-type or native form of the same region or molecule.

[0567] *Destabilizing domain (DD)*: As used herein, the term “destabilizing domain” refers to a protein or region or domain thereof that can be operably linked to a payload of interest (POI). In the absence of a DD-binding ligand, the DD renders the operably linked POI unstable, such that the POI is rapidly degraded within the cell. In the presence of a DD-binding ligand, the operably linked POI is stabilized, and protein function restored. As used herein, the terms “destabilizing domain” and “drug responsive domain” (DRD) are interchangeable.

[0568] *Engineered*: As used herein, embodiments of the present disclosure are “engineered” when they are designed to have a feature or property, whether structural or chemical, that varies from a starting point, wild type or native molecule.

[0569] *Expression*: As used herein, “expression” of a nucleic acid sequence refers to one or more of the following events: (1) production of an RNA template from a DNA sequence (e.g., by transcription); (2) processing of an RNA transcript (e.g., by splicing, editing, 5' cap formation, and/or 3' end processing); (3) translation of an RNA into a polypeptide or protein; (4) folding of a polypeptide or protein; and (5) post-translational modification of a polypeptide or protein.

[0570] *Feature*: As used herein, a “feature” refers to a characteristic, a property, or a distinctive element.

[0571] *Formulation*: As used herein, a “formulation” includes at least a compound and/or composition of the present disclosure and a delivery agent.

[0572] *Fragment*: A “fragment,” as used herein, refers to a portion. For example, fragments of proteins may comprise polypeptides obtained by digesting full-length protein. In some embodiments, a fragment of a protein includes at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, 250 or more amino acids. In some embodiments, fragments of an antibody include portions of an antibody.

[0573] *Functional*: As used herein, a “functional” biological molecule is a biological entity with a structure and in a form in which it exhibits a property and/or activity by which it is characterized.

[0574] *Immune cells*: the term “an immune cell”, as used herein, refers to any cell of the immune system that originates from a hematopoietic stem cell in the bone marrow, which gives rise to two major lineages, a myeloid progenitor cell (which give rise to myeloid cells such as monocytes, macrophages, dendritic cells, megakaryocytes and granulocytes) and a lymphoid progenitor cell (which give rise to lymphoid cells such as T cells, B cells and natural killer (NK) cells). Exemplary immune system cells include a CD4+ T cell, a CD8+ T cell, a CD4- CD8- double negative T

cell, a T $\gamma\delta$ cell, a T $\alpha\beta$ cell, a regulatory T cell, a natural killer cell, and a dendritic cell. Macrophages and dendritic cells may be referred to as “antigen presenting cells” or “APCs,” which are specialized cells that can activate T cells when a major histocompatibility complex (MHC) receptor on the surface of the APC complexed with a peptide interacts with a TCR on the surface of a T cell.

[0575] *Immunotherapy*: the term “immunotherapy” as used herein, refers to a type of treatment of a disease by the induction or restoration of the reactivity of the immune system towards the disease.

[0576] *Immunotherapeutic agent*: The term “immunotherapeutic agent” as used herein, refers to a biological, pharmaceutical, or chemical compound capable of being used for the treatment of disease by the induction or restoration of the reactivity of the immune system towards the disease.

[0577] *In vitro*: As used herein, the term “in vitro” refers to events that occur in an artificial environment, e.g., in a test tube or reaction vessel, in cell culture, in a Petri dish, etc., rather than within an organism (e.g., animal, plant, or microbe).

[0578] *In vivo*: As used herein, the term “in vivo” refers to events that occur within an organism (e.g., animal, plant, or microbe or cell or tissue thereof).

[0579] *Linker*: As used herein, a linker refers to a moiety that connects two or more domains, moieties or entities. In one embodiment, a linker may comprise 10 or more atoms. In a further embodiment, a linker may comprise a group of atoms, e.g., 10-1,000 atoms, and can be comprised of the atoms or groups such as, but not limited to, carbon, amino, alkylamino, oxygen, sulfur, sulfoxide, sulfonyl, carbonyl, and imine. In some embodiments, a linker may comprise one or more nucleic acids comprising one or more nucleotides. In some embodiments, the linker may comprise an amino acid, peptide, polypeptide or protein. In some embodiments, a moiety bound by a linker may include, but is not limited to an atom, a chemical group, a nucleoside, a nucleotide, a nucleobase, a sugar, a nucleic acid, an amino acid, a peptide, a polypeptide, a protein, a protein complex, a payload (e.g., a therapeutic agent), or a marker (including, but not limited to a chemical, fluorescent, radioactive or bioluminescent marker). The linker can be used for any useful purpose, such as to form multimers or conjugates, as well as to administer a payload, as described herein. Examples of chemical groups that can be incorporated into the linker include, but are not limited to, alkyl, alkenyl, alkynyl, amido, amino, ether, thioether, ester, alkylene, heteroalkylene, aryl, or heterocyclyl, each of which can be optionally substituted, as described herein. Examples of linkers include, but are not limited to, unsaturated alkanes, polyethylene glycols (e.g., ethylene or propylene glycol monomeric units, e.g., diethylene glycol, dipropylene glycol, triethylene glycol, tripropylene glycol, tetraethylene glycol, or tetraethylene glycol), and dextran polymers. Other examples include, but are not limited to, cleavable moieties within the linker, such as, for example, a disulfide bond (-S-S-) or an azo bond (-N=N-), which can be cleaved using a

reducing agent or photolysis. Non-limiting examples of a selectively cleavable bonds include an amido bond which may be cleaved for example by the use of tris(2-carboxyethyl) phosphine (TCEP), or other reducing agents, and/or photolysis, as well as an ester bond which may be cleaved for example by acidic or basic hydrolysis.

[0580] *Checkpoint/factor*: As used herein, a checkpoint factor is any moiety or molecule whose function acts at the junction of a process. For example, a checkpoint protein, ligand or receptor may function to stall or accelerate the cell cycle.

[0581] *Metabolite*: Metabolites are the intermediate products of metabolic reactions catalyzed by enzymes that naturally occur within cells. This term is usually used to describe small molecules, fragments of larger biomolecules or processed products.

[0582] *Modified*: As used herein, the term “modified” refers to a changed state or structure of a molecule or entity as compared with a parent or reference molecule or entity. Molecules may be modified in many ways including chemically, structurally, and functionally. In some embodiments, compounds and/or compositions of the present disclosure are modified by the introduction of non-natural amino acids.

[0583] *Mutation*: As used herein, the term “mutation” refers to a change and/or alteration. In some embodiments, mutations may be changes and/or alterations to proteins (including peptides and polypeptides) and/or nucleic acids (including polynucleic acids). In some embodiments, mutations comprise changes and/or alterations to a protein and/or nucleic acid sequence. Such changes and/or alterations may comprise the addition, substitution and or deletion of one or more amino acids (in the case of proteins and/or peptides) and/or nucleotides (in the case of nucleic acids and or polynucleic acids e.g., polynucleotides). In some embodiments, wherein mutations comprise the addition and/or substitution of amino acids and/or nucleotides, such additions and/or substitutions may comprise 1 or more amino acid and/or nucleotide residues and may include modified amino acids and/or nucleotides. The resulting construct, molecule or sequence of a mutation, change or alteration may be referred to herein as a mutant.

[0584] *Neoantigen*: the term “neoantigen”, as used herein, refers to a tumor antigen that is present in tumor cells but not normal cells and do not induce deletion of their cognate antigen specific T cells in thymus (i.e., central tolerance). These tumor neoantigens may provide a “foreign” signal, similar to pathogens, to induce an effective immune response needed for cancer immunotherapy. A neoantigen may be restricted to a specific tumor. A neoantigen be a peptide/protein with a missense mutation (missense neoantigen), or a new peptide with long, completely novel stretches of amino acids from novel open reading frames (neoORFs). The neoORFs can be generated in some tumors by out-of-frame insertions or deletions (due to defects in DNA mismatch repair causing microsatellite instability), gene-fusion, read-through

mutations in stop codons, or translation of improperly spliced RNA (e.g., Saeterdal et al., *Proc Natl Acad Sci USA*, 2001, 98: 13255-13260).

[0585] *Off-target*: As used herein, “off target” refers to any unintended effect on any one or more target, gene, cellular transcript, cell, and/or tissue.

[0586] *Operably linked*: As used herein, the phrase “operably linked” refers to a functional connection between two or more molecules, constructs, transcripts, entities, moieties or the like.

[0587] *Payload or payload of interest (POI)*: The terms “payload” and “payload of interest (POI)”, as used herein, are used interchangeable. A payload of interest (POI) refers to any polypeptide or protein that is operably linked to a destabilizing domain (DD). In the context of the present disclosure, the POI is a component in the immune system, including both innate and adaptive immune systems. . A payload of interest may be referred to as a protein of interest.

[0588] *Pharmaceutically acceptable excipients*: the term “pharmaceutically acceptable excipient,” as used herein, refers to any ingredient other than active agents (e.g., as described herein) present in pharmaceutical compositions and having the properties of being substantially nontoxic and non-inflammatory in subjects. In some embodiments, pharmaceutically acceptable excipients are vehicles capable of suspending and/or dissolving active agents. Excipients may include, for example: antiadherents, antioxidants, binders, coatings, compression aids, disintegrants, dyes (colors), emollients, emulsifiers, fillers (diluents), film formers or coatings, flavors, fragrances, glidants (flow enhancers), lubricants, preservatives, printing inks, sorbents, suspending or dispersing agents, sweeteners, and waters of hydration. Exemplary excipients include, but are not limited to: butylated hydroxytoluene (BHT), calcium carbonate, calcium phosphate (dibasic), calcium stearate, croscarmellose, crosslinked polyvinyl pyrrolidone, citric acid, crospovidone, cysteine, ethylcellulose, gelatin, hydroxypropyl cellulose, hydroxypropyl methylcellulose, lactose, magnesium stearate, maltitol, mannitol, methionine, methylcellulose, methyl paraben, microcrystalline cellulose, polyethylene glycol, polyvinyl pyrrolidone, povidone, pregelatinized starch, propyl paraben, retinyl palmitate, shellac, silicon dioxide, sodium carboxymethyl cellulose, sodium citrate, sodium starch glycolate, sorbitol, starch (corn), stearic acid, sucrose, talc, titanium dioxide, vitamin A, vitamin E, vitamin C, and xylitol.

[0589] *Pharmaceutically acceptable salts*: Pharmaceutically acceptable salts of the compounds described herein are forms of the disclosed compounds wherein the acid or base moiety is in its salt form (e.g., as generated by reacting a free base group with a suitable organic acid). Examples of pharmaceutically acceptable salts include, but are not limited to, mineral or organic acid salts of basic residues such as amines; alkali or organic salts of acidic residues such as carboxylic acids; and the like. Representative acid addition salts include acetate, adipate, alginate, ascorbate, aspartate, benzenesulfonate, benzoate, bisulfate, borate, butyrate, camphorate, camphorsulfonate, citrate, cyclopentanepropionate,

digluconate, dodecylsulfate, ethanesulfonate, fumarate, glucoheptonate, glycerophosphate, hemisulfate, heptonate, hexanoate, hydrobromide, hydrochloride, hydroiodide, 2-hydroxy-ethanesulfonate, lactobionate, lactate, laurate, lauryl sulfate, malate, maleate, malonate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, nitrate, oleate, oxalate, palmitate, pamoate, pectinate, persulfate, 3-phenylpropionate, phosphate, picrate, pivalate, propionate, stearate, succinate, sulfate, tartrate, thiocyanate, toluenesulfonate, undecanoate, valerate salts, and the like. Representative alkali or alkaline earth metal salts include sodium, lithium, potassium, calcium, magnesium, and the like, as well as nontoxic ammonium, quaternary ammonium, and amine cations, including, but not limited to ammonium, tetramethylammonium, tetraethylammonium, methylamine, dimethylamine, trimethylamine, triethylamine, ethylamine, and the like. Pharmaceutically acceptable salts include the conventional non-toxic salts, for example, from non-toxic inorganic or organic acids. In some embodiments, a pharmaceutically acceptable salt is prepared from a parent compound which contains a basic or acidic moiety by conventional chemical methods. Generally, such salts can be prepared by reacting the free acid or base forms of these compounds with a stoichiometric amount of the appropriate base or acid in water or in an organic solvent, or in a mixture of the two; generally, nonaqueous media like ether, ethyl acetate, ethanol, isopropanol, or acetonitrile are preferred. Lists of suitable salts are found in Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Company, Easton, Pa., 1985, p. 1418, Pharmaceutical Salts: Properties, Selection, and Use, P.H. Stahl and C.G. Wermuth (eds.), Wiley-VCH, 2008, and Berge et al., Journal of Pharmaceutical Science, 66, 1-19 (1977), each of which is incorporated herein by reference in its entirety. Pharmaceutically acceptable solvate: The term "pharmaceutically acceptable solvate," as used herein, refers to a crystalline form of a compound wherein molecules of a suitable solvent are incorporated in the crystal lattice. For example, solvates may be prepared by crystallization, recrystallization, or precipitation from a solution that includes organic solvents, water, or a mixture thereof. Examples of suitable solvents are ethanol, water (for example, mono-, di-, and tri-hydrates), N-methylpyrrolidinone (NMP), dimethyl sulfoxide (DMSO), N, N'-dimethylformamide (DMF), N, N'-dimethylacetamide (DMAC), 1,3-dimethyl-2-imidazolidinone (DMEU), 1,3-dimethyl-3,4,5,6-tetrahydro-2-(1H)-pyrimidinone (DMPU), acetonitrile (ACN), propylene glycol, ethyl acetate, benzyl alcohol, 2-pyrrolidone, benzyl benzoate, and the like. When water is the solvent, the solvate is referred to as a "hydrate." In some embodiments, the solvent incorporated into a solvate is of a type or at a level that is physiologically tolerable to an organism to which the solvate is administered (e.g., in a unit dosage form of a pharmaceutical composition).

[0590] *Stable:* As used herein "stable" refers to a compound or entity that is sufficiently robust to survive isolation to a useful degree of purity from a reaction mixture, and preferably capable of formulation into an efficacious therapeutic agent.

[0591] *Stabilized*: As used herein, the term “stabilize”, “stabilized,” “stabilized region” means to make or become stable. In some embodiments, stability is measured relative to an absolute value. In some embodiments, stability is measured relative to a secondary status or state or to a reference compound or entity.

[0592] *Standard CAR*: As used herein, the term “standard CAR” refers to the standard design of a chimeric antigen receptor. The components of a CAR fusion protein including the extracellular scFv fragment, transmembrane domain and one or more intracellular domains are linearly constructed as a single fusion protein.

[0593] *Stimulus response element (SRE)*: the term “stimulus response element (SRE), as used herein, is a component of an effector module which is joined, attached, linked to or associated with one or more payloads of the effector module and in some instances, is responsible for the responsive nature of the effector module to one or more stimuli. As used herein, the “responsive” nature of an SRE to a stimulus may be characterized by a covalent or non-covalent interaction, a direct or indirect association or a structural or chemical reaction to the stimulus. Further, the response of any SRE to a stimulus may be a matter of degree or kind. The response may be a partial response. The response may be a reversible response. The response may ultimately lead to a regulated signal or output. Such output signal may be of a relative nature to the stimulus, e.g., producing a modulatory effect of between 1 and 100 or a factored increase or decrease such as 2-fold, 3-fold, 4-fold, 5-fold, 10-fold or more. One non-limiting example of an SRE is a destabilizing domain (DD).

[0594] *Subject*: As used herein, the term “subject” or “patient” refers to any organism to which a composition in accordance with the present disclosure may be administered, e.g., for experimental, diagnostic, prophylactic, and/or therapeutic purposes. Typical subjects include animals (e.g., mammals such as mice, rats, rabbits, non-human primates, and humans) and/or plants.

[0595] *T cell*: A T cell is an immune cell that produces T cell receptors (TCRs). T cells can be naïve (not exposed to antigen; increased expression of CD62L, CCR7, CD28, CD3, CD127, and CD45RA, and decreased expression of CD45RO as compared to T_{CM}), memory T cells (T_M) (antigen-experienced and long-lived), and effector cells (antigen-experienced, cytotoxic). T_M can be further divided into subsets of central memory T cells (T_{CM}, increased expression of CD62L, CCR7, CD28, CD127, CD45RO, and CD95, and decreased expression of CD54RA as compared to naïve T cell and effector memory T cells (T_{EM}, decreased expression of CD62L, CCR7, CD28, CD45RA, and increased expression of CD127 as compared to naïve T cells or T_{CM}). Effector T cells (T_E) refers to antigen-experienced CD8⁺ cytotoxic T lymphocytes that have decreased expression of CD62L, CCR7, CD28, and are positive for granzyme and perforin as compared to T_{CM}. Other exemplary T cells include regulatory T cells, such as CD4⁺ CD25⁺ (Foxp3⁺) regulatory T cells and Treg17 cells, as well as Tr1, Th3, CD8⁺CD28⁻, and Qa-1 restricted T cells.

[0596] *T cell receptor*: T cell receptor (TCR) refers to an immunoglobulin superfamily member having a variable antigen binding domain, a constant domain, a transmembrane region, and a short cytoplasmic tail, which is capable of specifically binding to an antigen peptide bound to a MHC receptor. A TCR can be found on the surface of a cell or in soluble form and generally is comprised of a heterodimer having α and β chains (also known as TCR α and TCR β , respectively), or γ and δ chains (also known as TCR γ and TCR δ , respectively). The extracellular portion of TCR chains (e.g., α -chain, β -chain) contains two immunoglobulin domains, a variable domain (e.g., α -chain variable domain or V α , β -chain variable domain or V β) at the N terminus, and one constant domain (e.g., α -chain constant domain or C α and β -chain constant domain or C β ,) adjacent to the cell membrane. Similar to immunoglobulin, the variable domains contain complementary determining regions (CDRs) separated by framework regions (FRs). A TCR is usually associated with the CD3 complex to form a TCR complex. As used herein, the term “TCR complex” refers to a complex formed by the association of CD3 with TCR. For example, a TCR complex can be composed of a CD3 γ chain, a CD3 δ chain, two CD3 ϵ chains, a homodimer of CD3 ζ chains, a TCR α chain, and a TCR β chain. Alternatively, a TCR complex can be composed of a CD3 γ chain, a CD3 δ chain, two CD3 ϵ chains, a homodimer of CD3 ζ chains, a TCR γ chain, and a TCR δ chain. A “component of a TCR complex,” as used herein, refers to a TCR chain (i.e., TCR α , TCR β , TCR γ or TCR δ), a CD3 chain (i.e., CD3 γ , CD3 δ , CD3 ϵ or CD3 ζ), or a complex formed by two or more TCR chains or CD3 chains (e.g., a complex of TCR α and TCR β , a complex of TCR γ and TCR δ , a complex of CD3 ϵ and CD3 δ , a complex of CD3 γ and CD3 ϵ , or a sub-TCR complex of TCR α , TCR β , CD3 γ , CD3 δ , and two CD3 ϵ chains).

[0597] *Therapeutically effective amount*: As used herein, the term “therapeutically effective amount” means an amount of an agent to be delivered (e.g., nucleic acid, drug, therapeutic agent, diagnostic agent, prophylactic agent, etc.) that is sufficient, when administered to a subject suffering from or susceptible to an infection, disease, disorder, and/or condition, to treat, improve symptoms of, diagnose, prevent, and/or delay the onset of the infection, disease, disorder, and/or condition. In some embodiments, a therapeutically effective amount is provided in a single dose. In some embodiments, a therapeutically effective amount is administered in a dosage regimen comprising a plurality of doses. Those skilled in the art will appreciate that in some embodiments, a unit dosage form may be considered to comprise a therapeutically effective amount of a particular agent or entity if it comprises an amount that is effective when administered as part of such a dosage regimen.

[0598] *Treatment or treating*: As used herein, the terms “treatment” or “treating” denote an approach for obtaining a beneficial or desired result including and preferably a beneficial or desired clinical result. Such beneficial or desired clinical results include, but are not limited to, one or more of the following: reducing the proliferation of (or destroying) cancerous cells or other diseased, reducing metastasis of cancerous cells found in cancers, shrinking the size of the tumor,

decreasing symptoms resulting from the disease, increasing the quality of life of those suffering from the disease, decreasing the dose of other medications required to treat the disease, delaying the progression of the disease, and/or prolonging survival of individuals.

[0599] *Tune:* As used herein, the term “tune” means to adjust, balance or adapt one thing in response to a stimulus or toward a particular outcome. In one non-limiting example, the SREs and/or DDs of the present disclosure adjust, balance or adapt the function or structure of compositions to which they are appended, attached or associated with in response to particular stimuli and/or environments.

EQUIVALENTS AND SCOPE

[0600] Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments in accordance with the present disclosure described herein. The scope of the present disclosure is not intended to be limited to the above Description, but rather is as set forth in the appended claims.

[0601] In the claims, articles such as “a,” “an,” and “the” may mean one or more than one unless indicated to the contrary or otherwise evident from the context. Claims or descriptions that include “or” between one or more members of a group are considered satisfied if one, more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process unless indicated to the contrary or otherwise evident from the context. The present disclosure includes embodiments in which exactly one member of the group is present in, employed in, or otherwise relevant to a given product or process. The present disclosure includes embodiments in which more than one, or the entire group members are present in, employed in or otherwise relevant to a given product or process.

[0602] It is also noted that the term “comprising” is intended to be open and permits but does not require the inclusion of additional elements or steps. When the term “comprising” is used herein, the term “consisting of” is thus also encompassed and disclosed.

[0603] Where ranges are given, endpoints are included. Furthermore, it is to be understood that unless otherwise indicated or otherwise evident from the context and understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value or subrange within the stated ranges in different embodiments of the present disclosure, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise.

[0604] In addition, it is to be understood that any particular embodiment of the present disclosure that falls within the prior art may be explicitly excluded from any one or more of the claims. Since such embodiments are deemed to be known to one of ordinary skill in the art, they may be excluded even if the exclusion is not set forth explicitly herein. Any particular embodiment of the compositions of the present disclosure (e.g., any antibiotic, therapeutic or active ingredient;

any method of production; any method of use; etc.) can be excluded from any one or more claims, for any reason, whether or not related to the existence of prior art.

[0605] It is to be understood that the words which have been used are words of description rather than limitation, and that changes may be made within the purview of the appended claims without departing from the true scope and spirit of the present disclosure in its broader aspects.

[0606] While the present disclosure has been described at some length and with some particularity with respect to the several described embodiments, it is not intended that it should be limited to any such particulars or embodiments or any particular embodiment, but it is to be construed with references to the appended claims so as to provide the broadest possible interpretation of such claims in view of the prior art and, therefore, to effectively encompass the intended scope of the present disclosure. The present disclosure is further illustrated by the following nonlimiting examples.

EXAMPLES

Example 1. Regulation of CD40L *in vitro* in T cells

[0607] CD40L constructs regulated by Carbonic anhydrase DDs were generated and cloned into lentiviral vectors. Purified T cells were thawed and cultured with aCD3 aCD28 Dynabeads (in the ratio of 3 beads to 1 T cell).

[0608] The constructs were transduced in T cells the next day. 48 hours after addition of virus, ligand dependent regulation was tested using 50 μ M of Acetazolamide, or vehicle control (DMSO). 24 hours after the addition of ligand T cells were stained for CD40L expression and analyzed using FACS. In the presence of Acetazolamide, OT-001990 demonstrated an increase in CD40L expression compared to vehicle control and T cells expressing the empty vector without the insert. CD40L expression in response to increasing doses of Acetazolamide were measured in CD4+ and CD8+ T cells. Cells were treated with Acetazolamide for 24 hours and CD40L expression was measured using FACS. The results are shown as median fluorescence intensity in Table 10 and as percentage CD40L positive cells in Table 11.

Table 10. CD40L MFI

Acetazolamide (μ M)	CD4+	CD8+
0.01	112	105
0.05	123	104
0.2	185	136
0.8	481	307
3.1	840	380
12.5	2783	1599
50	3728	2184

Table 11. Percentage CD40L positive cells

Acetazolamide (μ M)	CD4+	CD8+
0.01	4.13	0.59
0.05	5.44	0.44
0.2	10.3	1.48
0.8	45.6	25.2
3.1	69.3	41.2
12.5	90.3	77.2
50	91.1	80.7

[0609] As shown in Table 10 and Table 11, ligand dependent regulation was observed both in CD4+ T cells as well as CD8+ T cells. however, the absolute MFI values and the percentage CD40L positive cells were higher in the CD4+ cells. The amount of Acetazolamide required to achieve stabilization of CD40L within the level of the ligand that can be achieved in humans.

Example 2. Time Course Regulation of CD40L *in vitro* in T cells

[0610] CD40L constructs regulated by Carbonic anhydrase DDs were generated and cloned into lentiviral vectors. Purified T cells were thawed and cultured with aCD3 aCD28 Dynabeads (in the ratio of 3 beads to 1 T cell).

[0611] The constructs were transduced in T cells and dosed with 50 μ M of Acetazolamide or vehicle control (DMSO) for 48 hours. A control of empty vector (EV) and constitutive CD40L (OT-001661; amino acid SEQ ID NO. 6067 and nucleic acid SEQ ID NO. 6068) was also evaluated. Cells were fixed at 2, 4, 6, 8, 24, and 48 hours and then T cells were stained for CD40L expression and analyzed using FACS. The results are shown as median fluorescence intensity in Table 12 and as percentage CD40L positive cells in Table 13.

Table 12. CD40L MFI

Time (hours)	EV		Constitutive CD40L (OT-001661)		CA2 regulated CD40L (OT-001990) DMSO		CA2 regulated CD40L (OT-001990) 50 μ M of Acetazolamide	
	CD4+	CD8+	CD4+	CD8+	CD4+	CD8+	CD4+	CD8+
2	1030	482	4042	2212	406	305	620	351
4	1371	457	4651	2994	381	270	1731	540
6	1239	360	3156	1773	302	224	2410	742
24	1533	321	9491	3563	400	221	10982	2376
48	1282	283	5639	2313	396	210	10384	3050

Table 13. Percentage CD40L positive cells

Time (hours)	EV		Constitutive CD40L (OT-001661)		CA2 regulated CD40L (OT-001990) DMSO		CA2 regulated CD40L (OT-001990) 50 μ M of Acetazolamide	
	CD4+	CD8+	CD4+	CD8+	CD4+	CD8+	CD4+	CD8+
2	52	28.6	72.8	56.9	12.1	4.9	34.7	10.8

4	67.6	17.8	77	63.2	11.6	5.9	72.2	28
6	65	9.6	67.2	48.5	6.6	1	81.2	42.7
24	77.5	10.7	93.3	71.2	10.5	1.1	96.3	73.7
48	69.3	6.8	88.5	71.1	11.2	2.2	97.4	79.2

[0612] In the presence of Acetazolamide, OT-001990 demonstrated an increase in CD40L expression compared to vehicle control and T cells expressing the empty vector without the insert. CD40L expression in response to increasing doses of Acetazolamide were measured in CD4+ and CD8+ T cells. As shown in Table 12 and Table 13, ligand dependent regulation was observed both in CD4+ T cells as well as CD8+ T cells. The absolute MFI values and the percentage CD40L positive cells were higher in the CD4+ cells. Expression reached its peak at 24 hours with highest doses expressing higher than constitutive levels.

Example 3. Regulation of CD40L in T cells by CA2

[0613] To test regulation, activated T cells were lentivirally transduced with CA2 regulated CD40L (OT-001990) and a control of CD40L (OT-001661). Two days later, cells were treated with vehicle or 50 mM ligand for 24h as described in Table 14 after which they were analyzed for CD40L surface expression. The results for CD4+ and CD8+ cells and total cells are shown below. In the table, “Acz” is Acetazolamide.

Table 14: Percent of Cells expressing CD40L

Construct (DD)	Ligand	CD4+		CD8+		All Cells	
		CD40L positive	MFI	CD40L positive	MFI	CD40L positive	MFI
OT-001661 (CD40L control)	N/A	98.7%	24489	97.8%	14352	98%	20451
OT-001990 (CA2)	DMSO	4.9%	112	0.1%	97.9	4%	110
OT-001990 (CA2)	50 μ M Acz	91.1%	3728	80.7%	2184	88.8%	3304

[0614] Regulated expression with destabilizing domains significantly enhanced CD40L expression beyond endogenous levels. CA2 destabilizing domains show levels close to constitutive expression with ligand doses that are near clinically relevant levels.

Example 4. Regulation of CD40L by CA2 derived destabilizing domains

Regulation of CD40L by multiple carbonic anhydrase DDs was tested using the following constructs: OT-001990, OT-002072, OT-002073, OT-001968, OT-001969, OT-001970, OT-001971, OT-002074, OT-002075, OT-002076, OT-002077. HEK 293T cells were transiently transfected with the constructs. Cells were treated with 10 μ M Acetazolamide for 24 hours and cell surface expression of CD40L was measured using flow cytometry. The results are shown in Table

15, where SR indicates stabilization ratio.

Table 15: Percent of Cells expressing CD40L

Construct	DMSO	Acetazolamide	SR
OT-001990	12.4	41.8	3.37
OT-001968	13.4	47.6	3.55
OT-002077	12.2	23.6	1.93
OT-002074	15.5	33.5	2.16
OT-002075	14	35	2.50
OT-002076	14	32.3	2.31

[0615] All constructs tested showed ligand dependent stabilization with stabilization ratios greater than 1. OT-001990 and OT-001968 demonstrated the highest stabilization ratio values. Similar ligand dependent stabilization of CD40L was observed when the following constructs were tested in HEK293T cells: OT-002072, OT-002073, OT-001968, OT-001969, OT-001970, and OT-001971.

[0616] Dose response studies were conducted in T cells. Cells were transduced with constructs indicated in Table 16. Acetazolamide dose response studies were performed at 4 and 9 days after transduction. The experiments were performed in duplicates (herein referred to as “repeat 1” and “repeat 2”). The results are provided in Table 16 and Table 17 as the Median fluorescence intensity of CD40L analyzed by flow cytometry.

Table 16: Dose response studies- Day 4

Construct	Cell type	Acetazolamide (μM)						
		0.01	0.05	0.2	0.8	3.1	12.5	50
OT-002073 (repeat 1)	CD4+	122	170	129	346	714	1667	2228
	CD8+	93.4	110	94.6	188	335	871	1143
OT-002073 (repeat 2)	CD4+	5.57	9.19	6.04	30.1	66.2	81.6	84
	CD8+	0.64	0.35	0.54	6.4	34.4	63.4	65.9
OT-001990 (repeat 1)	CD4+	112	123	185	481	840	2783	3728
	CD8+	105	104	136	307	380	1599	2184
OT-001990 (repeat 2)	CD4+	4.13	5.44	10.3	45.6	69.3	90.3	91.1
	CD8+	0.59	0.44	1.48	25.2	41.2	77.2	80.7
OT-001968 (repeat 1)	CD4+	127	132	162	242	420	1414	2851
	CD8+	110	112	135	174	376	803	1448
OT-001968 (repeat 2)	CD4+	5.49	5.85	8.22	17.3	39.7	78.7	84.8
	CD8+	0.87	0.61	1.89	4.9	30	62.1	69.9
OT-001971 (repeat 1)	CD4+	137	140	169	251	593	1342	1841
	CD8+	101	109	116	155	224	563	888
OT-001971 (repeat 2)	CD4+	7.63	6.64	9.41	17.1	57.6	80.5	82.5
	CD8+	0.87	0.75	0.72	3.17	16.7	49.4	62.1

Table 17: Dose response studies- Day 9

Construct	Cell type	Acetazolamide (μ M)						
		0.01	0.05	0.2	0.8	3.1	12.5	50
OT-002073 (repeat 1)	CD4+	120	124	147	211	432	635	712
	CD8+	85.9	86.9	91	105	196	297	337
OT-002073 (repeat 2)	CD4+	6.42	6.68	8.9	16.3	40.1	56	59.9
	CD8+	0.47	0.52	0.8	1.17	8.26	24	30.1
OT-001990 (repeat 1)	CD4+	81.8	90.5	118	202	415	632	772
	CD8+	78.2	78.9	85	115	230	367	461
OT-001990 (repeat 2)	CD4+	2.53	3.48	6.1	15.1	38	55.3	62.8
	CD8+	0.11	0.17	0.3	1.33	12.1	32.9	43.5
OT-001968 (repeat 1)	CD4+	110	117	127	168	270	555	787
	CD8+	84.1	85.3	89	102	141	291	464
OT-001968 (repeat 2)	CD4+	5.15	6.01	6.6	10.9	22.4	51	62.8
	CD8+	0.22	0.42	0.3	0.96	3.41	23	44.6
OT-001971 (repeat 1)	CD4+	110	117	135	172	289	482	609
	CD8+	87.2	85.2	91	95.8	136	220	288
OT-001971 (repeat 2)	CD4+	5.27	6.26	7.9	11.6	25	44.9	53.9
	CD8+	0.32	0.47	0.6	0.53	2.48	12.7	22.7

[0617] The results in Table 16 and Table 17 suggest that carbonic anhydrase DD based regulation of CD40L is effective in regulating CD40L expression when treated with Acetazolamide. CD4 positive cells showed a greater increase in CD40L expression when compared to CD8 positive cells, likely due to the endogenous CD40L expression in CD4 cells.

[0618] Healthy donor human T cells were activated, transduced with CD40L-expressing lentivirus vector (LV) for OT-001990 and expanded using aCD3 aCD28 Dynabeads. T cells were frozen on day 9 post expansion. Acetazolamide was added to cells on day 3, day 8, post thaw, and post thaw after 24 hours of re-stimulation with aCD3 and aCD28. T cells were stained for CD40L expression 24 hours after the addition of ligand. The results are shown in Table 18.

Table 18. Expression of CD40L in OT-001990 T cells

Acetazolamide (dose μ M)	Day 4	Day 9	Post Thaw	Post Thaw + Stim
0.01		282	178	995
0.05	310	319	175	954
0.2	381	339	180	999
0.8	1119	641	198	1406
3.1	2381	1486	273	2314
12.5	5517	2970	525	4355

50	5427	3143	688	4860
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[0619] As shown in Table 18, regulation was observed in re-stimulated cells post thaw but not in cells without restimulation. These data show that regulation of CD40L is sensitive to T cell activation status.

[0620] Healthy donor human T cells were activated, transduced with CD40L-expressing lentivirus vector (LV), expanded, and frozen. Allogeneic human monocytes were differentiated into monocyte derived dendritic cells (moDCs) and also frozen. In vitro cultures were set up with freshly thawed cells which were placed in culture for 48 hours. Supernatant was collected and analyzed by meso scale discovery (MSD) for interleukin 12 (IL12). The results are shown in Table 19. In Table 19, Acetazolamide is denoted as "ACZ".

Table 19: IL 12 expression

Description	pg/mL IL-12
moDC + Empty Vector	0.18
moDC + Empty Vector (Stim)	5.84
moDC + OT-001990 DMSO	0.52
moDC + OT-001990 + Stim	0.84
moDC + OT-001990 50μM ACZ	0.40
moDC + OT-001990 50μM ACZ + Stim	472.28

[0621] Similar to the CD40L regulation, IL12 expression by CD40L expressing T cells was sensitive to the T cell activation status.

Example 5. Regulation of CA2 CD40L- CAR T cells

[0622] To measure the effect of CD40L expression that is tuned by the biocircuits of the present disclosure in the context of CARs, T cells expressing any of the CA2 CD40L-CAR constructs described herein were co-cultured with monocyte derived dendritic cells (DCs).

[0623] In vitro cultures were set up by adding 1×10^4 DCs into culture with 1×10^5 Nalm6 and 1×10^5 CAR+ T cells. 50μM of Acetazolamide was added at the same time as the cells in culture. Supernatants were collected 24 hours later and analyzed for IFNγ and IL-12 by MSD assay. OT-001661 (SEQ ID NO. 6067; encoded by SEQ ID NO. 6068), that expressed CD40L payloads constitutively was used as control. The results are provided in Table 20.

Table 20: IFN and IL12 levels

Description	IFNγ (pg/mL)	IL12 (pg/mL)
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DC + EV + Nalm6	2042.76	-
DC + OT-001661 + Nalm6	1579.21	58.88
DC + OT-001990 Vehicle + Nalm6	152.84	-
DC + OT-001990 +50 μ M of Acetazolamide + Nalm6	135.50	-
DC + OT-001407 + Nalm6	33363.09	21.289
DC + OT-001605 + Nalm6	58239.22	366.83
DC + OT-002156 Vehicle + Nalm6	70686.46	19.83
DC + OT-002156 +50 μ M of Acetazolamide + Nalm6	76679.21	129.18

[0624] IL12 secretion was increased in the supernatants of all groups except DC + OT-001990. Detectable IFN gamma levels were observed in DC + OT-001407 + Nalm6 ; DC + OT-001605 + Nalm6; DC + OT-002156 Vehicle + Nalm6; and DC + OT-002156 +50 μ M of Acetazolamide + Nalm6 supernatants. Ligand dependent regulation of IFN gamma was observed with OT-002156. These data show that CAR driven activation of T cells induces CD40L dependent IL-12 production by autologous DCs in vitro.

Example 6. Reduced Shedding with CD40L

[0625] To measure the effect of CD40L expression and shedding as a result of the replacement of a cleavage site with a linker sequence, HEK293T cells were transiently transfected with a CD40L control (OT-001661), CA2 regulated CD40L (OT-001990) or CA2 regulated CD40L-shed (OT-002172) and treated with vehicle (DMSO) or 10 μ M Acetazolamide (ACZ) for 24 hours before cell culture medium was analyzed by ELISA for soluble CD40L. As shown in Table 21, reduced shedding was seen when the cleavage site was replaced with a linker sequence.

Table 21: Soluble CD40L (sCD40L)

Description	sCD40L (pg/mL)	
	Repeat 1	Repeat 2
OT-001661	2900	-
OT-001990 + DMSO	1694.2	1534.5
OT-001990 + 10 μ M ACZ	1728.8	1760.5
OT-002172 + DMSO	160.8	144.3
OT-002172 + 10 μ M ACZ	178.9	177.4

[0626] Additionally, OT-001990 and OT-002172 were transfected into stably transduced Jurkat cells and then the cells were treated with the doses of Acetazolamide (ACZ) for 24 hours and the percent positive CD40L+ cells and the MFI are shown in Table 22.

Table 22: Percent positive CD40L cells and MFI after 24 hours

Percent CD40L+	MFI
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μ M Dose of ACZ	OT-001990		OT-002172		μ M Dose of ACZ	OT-001990		OT-002172	
	Repeat 1	Repeat 2	Repeat 1	Repeat 2		Repeat 1	Repeat 2	Repeat 1	Repeat 2
200	98.1	-	95.2	-	200	-	-	-	-
175	97.9	97.6	93.2	93.3	175	3101	3172	2248	2219
100	97.6	-	93.8	93.8	100	3101	3208	2102	2074
50	97.1	97.5	92.5	92.6	50	2885	2971	1974	2008
25	96.7	97.3	91.9	91.4	25	2570	2622	1796	1796
12.5	95.9	96	89.5	89.3	12.5	2176	2180	1475	1510
6.25	93.4	93.9	84.6	84.1	6.25	1586	1593	1110	1086
3.125	85.4	84.5	72.9	72.4	3.125	988	956	703	706
1.5625	63.2	62	48.1	48	1.5625	526	516	380	383
0.78125	22.4	22.9	17.8	18.1	0.78125	214	214	178	183
0.390625	4.1	4.4	4.7	4.8	0.390625	80.9	78	75.1	75.1
0.195313	1.9	1.6	1.5	1.4	0.195313	31.7	27.4	26	34.6
0.097656	0.9	1.0	0.8	0.9	0.097656	18.8	18.8	13	18.8
0.048828	0.8	1	0.6	0.5	0.048828	7.21	5.77	5.77	13

[0627] While the present disclosure has been described at some length and with some particularity with respect to the several described embodiments, it is not intended that it should be limited to any such particulars or embodiments or any particular embodiment, but it is to be construed with references to the appended claims so as to provide the broadest possible interpretation of such claims in view of the prior art and, therefore, to effectively encompass the intended scope of the present disclosure.

[0628] All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, section headings, the materials, methods, and examples are illustrative only and not intended to be limiting.