PROTEIN PAYLOAD RELEASE

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application Nos. 63/193,004 filed May 25, 2021 and 63/109,812 filed November 4, 2020, each of which is hereby incorporated in their entirety by reference for all purposes.

~~SEQUENCE LISTING~~

~~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 copy, created on XXXX, 20XX, is named XXX-XXX_sequencelisting.txt and is ###,### bytes in size.~~

BACKGROUND

Cell-based therapy platforms provide promising avenues for treating a variety of diseases. One such promising platform is CAR-T based therapies in the treatment of cancer. Given their promise, improvements in cell-based therapies are needed. An active area of exploration is engineering cell-based therapies to produce and/or secrete effector molecules such as cytokines, a process referred to as armoring, that enhance the cell-based therapy. For example, unarmored CAR-T therapies have poor efficacy in solid tumors and armoring can impact the entire cancer immunity cycle and boost the activity of CAR-T. However, uncontrolled or unregulated armoring strategies can have negative impacts on treatment, such as off-target effects and toxicity in subjects. Thus, additional methods of controlling and regulating the armoring of cell-based therapies, such as regulating production and/or secretion of payload effector molecules, are required.

SUMMARY

Provided herein, in an aspect, is a membrane-cleavable chimeric protein, oriented from N-terminal to C-terminal, having the formula S – C – MT or MT – C – S, wherein S comprises a secretable effector molecule, C comprises a protease cleavage site, and MT comprises a cell membrane tethering domain, wherein S – C – MT or MT – C – S is configured to be expressed as a single polypeptide.

In another aspect, provided herein is an engineered nucleic acid comprising an expression cassette comprising a promoter and an exogenous polynucleotide sequence encoding the membrane-cleavable chimeric protein described herein, optionally wherein the promoter is selected from the group consisting of a constitutive promoter, an inducible promoter, a tissue-specific promoter, and a synthetic promoter.

In another aspect, provided herein is an expression vector comprising the engineered nucleic acid described herein.

In another aspect, provided herein is an isolated cell comprising the membrane-cleavable chimeric protein described herein, the engineered nucleic acid described herein, or the expression vector described herein.

In another aspect, provided herein is a composition comprising the membrane-cleavable chimeric protein described herein, the engineered nucleic acid described herein, the expression vector described herein, or the isolated cell described herein and a pharmaceutically acceptable carrier, pharmaceutically acceptable excipient, or a combination thereof.

In another aspect, provided herein is the isolated cells described herein or the composition described herein for use in therapy.

In another aspect, provided herein method of inducing release of a membrane-tethered effector molecule, comprising a) providing a cell described herein; and b) culturing the cell under conditions suitable for expression of the membrane-bound protease and the membrane-cleavable chimeric protein, wherein upon expression, the membrane-cleavable chimeric protein is tethered to the cell membrane of the cell, and wherein, upon expression, the membrane-bound protease cleaves the cognate membrane-bound protease cleavage site of the membrane-cleavable chimeric protein, thereby releasing the secretable effector molecule from the cell membrane.

Provided herein, in some embodiments, is a cell-based therapy platform involving regulated armoring of the cell-based therapy, such as regulated secretion of payload effector molecules. Also provided herein, in some embodiments, is a combinatorial cell-based immunotherapy involving regulated armoring for the targeted treatment of cancer, such as ovarian cancer, breast cancer, colon cancer, lung cancer, and pancreatic cancer.

In some aspects, the promoter is a synthetic promoter. In some aspects, the synthetic promoter comprises an activation-conditional control polypeptide- (ACP-) binding domain sequence and a promoter sequence. In some aspects, the promoter sequence is derived from a promoter selected from the group consisting of: minP, NFkB response element, CREB response element, NFAT response element, SRF response element 1, SRF response element 2, AP1 response element, TCF-LEF response element promoter fusion, Hypoxia responsive element, SMAD binding element, STAT3 binding site, minCMV, YB_TATA, minTK, inducer molecule responsive promoters, and tandem repeats thereof. In some aspects, the ACP-binding domain comprises one or more zinc finger binding sites. In some aspects, the synthetic promoter is regulatable by an activation-conditional control polypeptide (ACP) that binds to the ACP-binding domain of the synthetic promoter.

In some aspects, the ACP is a transcriptional modulator. In some aspects, the ACP is a transcriptional repressor. In some aspects, the ACP is a transcriptional activator. In some aspects, the ACP further comprises a repressible protease and one or more cognate cleavage sites of the repressible protease. In some aspects, the ACP further comprises a hormone-binding domain of estrogen receptor (ERT2 domain).

In some aspects, the ACP is a transcription factor. In some aspects, the transcription factor is a zinc-finger-containing transcription factor. In some aspects, the ACP comprises a DNA-binding zinc finger protein domain (ZF protein domain) and a transcriptional effector domain. In some aspects, the ZF protein domain is modular in design and is composed of zinc finger arrays (ZFA). In some aspects, the ZF protein domain comprises one to ten ZFA.

In some aspects, the effector domain is selected from the group consisting of: a Herpes Simplex Virus Protein 16 (VP16) activation domain; an activation domain comprising four tandem copies of VP16, a VP64 activation domain; a p65 activation domain of NFκB; an Epstein-Barr virus R transactivator (Rta) activation domain; a tripartite activator comprising the VP64, the p65, and the Rta activation domains (VPR activation domain); a histone acetyltransferase (HAT) core domain of the human E1A-associated protein p300 (p300 HAT core activation domain); a Krüppel associated box (KRAB) repression domain; a Repressor Element Silencing Transcription Factor (REST) repression domain; a WRPW (SEQ ID NO: 224) motif of the hairy-related basic helix-loop-helix repressor proteins, the motif is known as a WRPW (SEQ ID NO: 224) repression domain; a DNA (cytosine-5)-methyltransferase 3B (DNMT3B) repression domain; and an HP1 alpha chromoshadow repression domain.

In some aspects, the one or more cognate cleavage sites of the repressible protease are localized between the ZF protein domain and the effector domain. In some aspects, the

SIP protease cleavage site, an MT1-MMP protease cleavage site, an MT3-MMP protease cleavage site, an MT5-MMP protease cleavage site, a furin protease cleavage site, a PCSK7 protease cleavage site, a matriptase protease cleavage site, a matriptase-2 protease cleavage site, an MMP9 protease cleavage site, and an NS3 protease cleavage site. In some aspects, the protease cleavage site is cleavable by a protease selected from the group consisting of: a Type 1 transmembrane protease, a Type II transmembrane protease, a GPI anchored protease, an ADAM8 protease, an ADAM9 protease, an ADAM10 protease, an ADAM12 protease, an ADAM15 protease, an ADAM17 protease, an ADAM19 protease, an ADAM20 protease, an ADAM21 protease, an ADAM28 protease, an ADAM30 protease, an ADAM33 protease, a BACE1 protease, a BACE2 protease, a SIP protease, an MT1-MMP protease, an MT3-MMP protease, an MT5-MMP protease, a furin protease, a PCSK7 protease, a matriptase protease, a matriptase-2 protease, an MMP9 protease, and an NS3 protease.

In some aspects, the protease cleavage site is cleavable by an ADAM17 protease. In some aspects, the protease cleavage site comprises a first region having the amino acid sequence of PRAE (SEQ ID NO: 176). In some aspects, the protease cleavage site comprises a second region having the amino acid sequence of KGG (SEQ ID NO: 177). In some aspects, the first region is located N-terminal to the second region. In some aspects, the protease cleavage site comprises the amino acid sequence of PRAEX1X2KGG (SEQ ID NO: ~~178~~219), wherein X1 is A, Y, P, S, or F, and wherein X2 is V, L, S, I, Y, T, or A. In some aspects, the protease cleavage site comprises the amino acid sequence of PRAEX1X2KGG (SEQ ID NO: 178), wherein X1 is A, Y, P, S, or F, and wherein X2 is V, L, S, I, Y, or T. In some aspects, the protease cleavage site comprises the amino acid sequence of PRAEAVKGG (SEQ ID NO: 179). In some aspects, the protease cleavage site comprises the amino acid sequence of PRAEALKGG (SEQ ID NO: 180). In some aspects, the protease cleavage site comprises the amino acid sequence of PRAEYSKGG (SEQ ID NO: 181). In some aspects, the protease cleavage site comprises the amino acid sequence of PRAEPIKGG (SEQ ID NO: 182). In some aspects, the protease cleavage site comprises the amino acid sequence of PRAEAYKGG (SEQ ID NO: 183). In some aspects, the protease cleavage site comprises the amino acid sequence of PRAESSKGG (SEQ ID NO: 184). In some aspects, the protease cleavage site comprises the amino acid sequence of PRAEFTKGG (SEQ ID NO: 185). In some aspects, the protease cleavage site comprises the amino acid sequence of PRAEAAKGG (SEQ ID NO: 186). In some aspects, the protease cleavage site comprises the amino acid sequence of DEPHYSQRR (SEQ ID NO: 187). In some aspects, the protease cleavage site comprises the amino acid sequence of PPLGPIFNPG (SEQ ID NO: 188). In

ADAM21 protease, an ADAM28 protease, an ADAM30 protease, an ADAM33 protease, a BACE1 protease, a BACE2 protease, a SIP protease, an MT1-MMP protease, an MT3-MMP protease, an MT5-MMP protease, a furin protease, a PCSK7 protease, a matriptase protease, a matriptase-2 protease, and an MMP9 protease. In some aspects, the endogenous protease is an ADAM17 protease. In some aspects, the protease is a heterologous protease. In some aspects, the heterologous protease is hepatitis C virus (HCV) nonstructural protein 3 (NS3). In some aspects, the protease is expressed on the cell membrane of the isolated cell. In some aspects, the protease is capable of cleaving the protease cleavage site. In some aspects, cleavage of the protease cleavage site releases the secretable effector molecule from the cell membrane of the isolated cell.

In some aspects, the protease cleavage site comprises a first region having the amino acid sequence of PRAE (SEQ ID NO: 176). In some aspects, the protease cleavage site comprises a second region having the amino acid sequence of KGG (SEQ ID NO: 177). In some aspects, the first region is located N-terminal to the second region. In some aspects, the protease cleavage site comprises the amino acid sequence of PRAEX1X2KGG (SEQ ID NO: ~~178~~219), wherein X1 is A, Y, P, S, or F, and wherein X2 is V, L, S, I, Y, T, or A. In some aspects, the protease cleavage site comprises the amino acid sequence of PRAEX1X2KGG (SEQ ID NO: 178), wherein X1 is A, Y, P, S, or F, and wherein X2 is V, L, S, I, Y, or T. In some aspects, the protease cleavage site comprises the amino acid sequence of PRAEAVKGG (SEQ ID NO: 179). In some aspects, the protease cleavage site comprises the amino acid sequence of PRAEALKGG (SEQ ID NO: 180). In some aspects, the protease cleavage site comprises the amino acid sequence of PRAEYSKGG (SEQ ID NO: 181). In some aspects, the protease cleavage site comprises the amino acid sequence of PRAEPIKGG (SEQ ID NO: 182). In some aspects, the protease cleavage site comprises the amino acid sequence of PRAEAYKGG (SEQ ID NO: 183). In some aspects, the protease cleavage site comprises the amino acid sequence of PRAESSKGG (SEQ ID NO: 184). In some aspects, the protease cleavage site comprises the amino acid sequence of PRAEFTKGG (SEQ ID NO: 185). In some aspects, the protease cleavage site comprises the amino acid sequence of PRAEAAKGG (SEQ ID NO: 186). In some aspects, the protease cleavage site comprises the amino acid sequence of DEPHYSQRR (SEQ ID NO: 187). In some aspects, the protease cleavage site comprises the amino acid sequence of PPLGPIFNPG (SEQ ID NO: 188). In some aspects, the protease cleavage site comprises the amino acid sequence of PLAQAYRSS (SEQ ID NO: 189). In some aspects, the protease cleavage site comprises the amino acid sequence of TPIDSSFNPD (SEQ ID NO: 190). In some aspects, the protease cleavage site

In some embodiments, the protease cleavage site comprises a first region having the amino acid sequence of PRAE (SEQ ID NO: 176). In some embodiments, the protease cleavage site comprises a second region having the amino acid sequence of KGG (SEQ ID NO: 177). In some embodiments, the first region is located N-terminal to the second region. In some embodiments, the protease cleavage site comprises the amino acid sequence of PRAEX_1X_2KGG (SEQ ID NO: ~~178~~219), wherein X_1 is A, Y, P, S, or F, and wherein X_2 is V, L, S, I, Y, T, or A. In some embodiments, the protease cleavage site comprises the amino acid sequence of PRAEX_1X_2KGG (SEQ ID NO: 178), wherein X_1 is A, Y, P, S, or F, and wherein X_2 is V, L, S, I, Y, or T. In some embodiments, the protease cleavage site comprises the amino acid sequence of PRAEAVKGG (SEQ ID NO: 179). In some embodiments, the protease cleavage site comprises the amino acid sequence of PRAEALKGG (SEQ ID NO: 180). In some embodiments, the protease cleavage site comprises the amino acid sequence of PRAEYSKGG (SEQ ID NO: 181). In some embodiments, the protease cleavage site comprises the amino acid sequence of PRAEPIKGG (SEQ ID NO: 182). In some embodiments, the protease cleavage site comprises the amino acid sequence of PRAEAYKGG (SEQ ID NO: 183). In some embodiments, the protease cleavage site comprises the amino acid sequence of PRAESSKGG (SEQ ID NO: 184). In some embodiments, the protease cleavage site comprises the amino acid sequence of PRAEFTKGG (SEQ ID NO: 185). In some embodiments, the protease cleavage site comprises the amino acid sequence of PRAEAAKGG (SEQ ID NO: 186).

In some embodiments, the protease cleavage site comprises the amino acid sequence of DEPHYSQRR (SEQ ID NO: 187). In some embodiments, the protease cleavage site comprises the amino acid sequence of PPLGPIFNPG (SEQ ID NO: 188). In some embodiments, the protease cleavage site comprises the amino acid sequence of PLAQAYRSS (SEQ ID NO: 189). In some embodiments, the protease cleavage site comprises the amino acid sequence of TPIDSSFNPD (SEQ ID NO: 190). In some embodiments, the protease cleavage site comprises the amino acid sequence of VTPEPIFSLI (SEQ ID NO: 191). The protease cleavage sites of SEQ ID NOs: 187, 189, and 191 are cleavable by ADAM17.

In some embodiments, the protease cleavage site comprises the amino acid sequence of ITQGLAVSTISSFF (SEQ ID NO: 198), which is a cleavage site that is native to CD16 and is cleavable by ADAM17.

The protease cleavage site can be C-terminal of the secretable effector molecule. The protease cleavage site can be N-terminal of the secretable effector molecule. In general, for all membrane-cleavable chimeric proteins described herein, the protease cleavage site is

either: (1) C-terminal of the secretable effector molecule and N-terminal of the cell membrane tethering domain (in other words, the protease cleavage site is in between the secretable effector molecule and the cell membrane tethering domain); or (2) N-terminal of the secretable effector molecule and C-terminal of the cell membrane tethering domain (also between the secretable effector molecule and the cell membrane tethering domain with domain orientation inverted). The protease cleavage site can be connected to the secretable effector molecule by a polypeptide linker, *i.e.*, a polypeptide sequence not generally considered to be part of the effector molecule or protease cleavage site. The protease cleavage site can be connected to the cell membrane tethering domain by a polypeptide linker, *i.e.*, a polypeptide sequence not generally considered to be part of the cell membrane tethering domain or protease cleavage site. A polypeptide linker can be any amino acid sequence that connects a first polypeptide sequence and a second polypeptide sequence. A polypeptide linker can be a flexible linker (*e.g.*, a Gly-Ser-Gly sequence). Examples of polypeptide linkers include, but are not limited to, GSG linkers (*e.g.*, $[GS]_4GG$ [SEQ ID NO: 220~~182~~]), $A(EAAAK)_3A$ (SEQ ID NO: 221~~183~~), and Whitlow linkers (*e.g.*, a "KEGS" linker such as the amino acid sequence KESGSVSSEQLAQFRSLD (SEQ ID NO: 222~~184~~), an eGK linker such as the amino acid sequence EGKSSGSGSESKST (SEQ ID NO: 223~~185~~), and linkers described in more detail in Issued U.S. Pat. No. 5,990,275 herein incorporated by reference). Additional exemplary polypeptide linkers include SEQ ID NO: 194, SEQ ID NO: 195, SEQ ID NO: 196, and SEQ ID NO: 197. Other polypeptide linkers may be selected based on desired properties (*e.g.*, length, flexibility, amino acid composition etc.) and are known to those skilled in the art.

In the Membrane-Cleavable system, following expression and localization of the chimeric protein into the cell membrane, the protease cleavage site directs cleavage of the chimeric protein such that the effector molecule is released ("secreted") into the extracellular space of a cell.

In general, a protease that cleaves the protease cleavage site is a protease specific for that specific protease cleavage site. For example, in the case of a disintegrin and metalloproteinase ("ADAM") family protease, the protease that cleaves a specific ADAM protease cleavage site is generally limited to the ADAM protease(s) that specifically recognize the specific ADAM protease cleavage site motif. A protease cleavage site can be selected and/or engineered such that cleavage by undesired proteases is reduced or eliminated. Proteases can be membrane-bound or membrane-associated. Proteases can be

Protease (UniProt Accession No.)	Cognate cleavage site	Protease inhibitors
	hydrophobic amino-acid residue at P2 and a hydrophilic amino-acid residue at P3, although Val or Ala are also accepted at this position.	
caspase 4 (P70343, P49662)	Strict requirement for Asp at the P1 position. It has a preferred cleavage sequence of Tyr-Val-Ala-Asp-|- (YVAD; SEQ ID NO: 146) but also cleaves at Asp-Glu-Val-Asp-|-(DEVD; SEQ ID NO: 148).	
caspase 5 (P51878)	Strict requirement for Asp at the P1 position. It has a preferred cleavage sequence of Tyr-Val-Ala-Asp-|-(YVAD; SEQ ID NO: 146) but also cleaves at Asp-Glu-Val-Asp-|- -|-(DEVD; SEQ ID NO: 148).	
caspase 6 (P55212)	Strict requirement for Asp at position P1 and has a preferred cleavage sequence of Val-Glu-His-Asp-|-(VEHD; SEQ ID NO: 149).	
caspase 7 (P97864, P55210)	Strict requirement for an Asp residue at position P1 and has a preferred cleavage sequence of Asp-Glu-Val-Asp-|- (DEVD; SEQ ID NO: 148).	
caspase 8 (Q8IRY7, O89110, Q14790)	Strict requirement for Asp at position P1 and has a preferred cleavage sequence of (Leu/Asp/Val)-Glu-Thr-Asp-|-(Gly/Ser/Ala).	
caspase 9 (P55211, Q8C3Q9, Q5IS54)	Strict requirement for an Asp residue at position P1 and with a marked preference for His at position P2. It has a preferred cleavage sequence of Leu-Gly-His-Asp-|-Xaa (LGHDX; SEQ ID NO: 150).	
caspase 10 (Q92851)	Strict requirement for Asp at position P1 and has a preferred cleavage sequence of Leu-Gln-Thr-Asp-|-Gly (LQTDG; SEQ ID NO: 151).	
puromycin sensitive aminopeptidase (P55786, Q11011)	Release of an N-terminal amino acid, preferentially alanine, from a wide range of peptides, amides and arylamides.	

Protease (UniProt Accession No.)	Cognate cleavage site	Protease inhibitors
	776 in the alpha-1(I) chain. Cleaves synthetic substrates and alpha-macroglobulins at bonds where P1' is a hydrophobic residue.	
MMP 2 (P08253, P33434)	Cleavage of gelatin type I and collagen types IV, V, VII, X. Cleaves the collagen-like sequence Pro-Gln-Gly-|-Ile-Ala-Gly-Gln (PQGIAGQ; SEQ ID NO: 153).	SB-3CT *p*-OH SB-3CT *O*-phosphate SB-3CT RXP470.1
MMP 3 (P08254, P28862)	Preferential cleavage where P1', P2' and P3' are hydrophobic residues.	SB-3CT *p*-OH SB-3CT *O*-phosphate SB-3CT RXP470.1
MMP 7 (P09237, Q10738)	Cleavage of 14-Ala-|-Leu-15 and 16-Tyr-|-Leu-17 in B chain of insulin. No action on collagen types I, II, IV, V. Cleaves gelatin chain alpha-2(I) > alpha-1(I).	SB-3CT *p*-OH SB-3CT *O*-phosphate SB-3CT RXP470.1
MMP 8 (P22894, O70138)	Can degrade fibrillar type I, II, and III collagens. Cleavage of interstitial collagens in the triple helical domain. Unlike EC 3.4.24.7, this enzyme cleaves type III collagen more slowly than type I.	SB-3CT *p*-OH SB-3CT *O*-phosphate SB-3CT RXP470.1
MMP 9 (P14780, P41245)	Cleavage of gelatin types I and V and collagen types IV and V. Cleaves KiSS1 at a Gly-|-Leu bond. Cleaves type IV and type V collagen into large C-terminal three quarter fragments and shorter N-terminal one quarter fragments. Degrades fibronectin but not laminin or Pz-peptide.	SB-3CT *p*-OH SB-3CT *O*-phosphate SB-3CT RXP470.1
MMP 10 (P09238, O55123)	Can degrade fibronectin, gelatins of type I, III, IV, and V; weakly collagens III, IV, and V.	SB-3CT *p*-OH SB-3CT *O*-phosphate SB-3CT RXP470.1
MMP 11 (P24347, Q02853)	A(A/Q)(N/A)↓(L/Y)(T/V/M/R)(R/K) G(G/A)E↓LR (SEQ ID NO: 225)	SB-3CT *p*-OH SB-3CT *O*-phosphate SB-3CT RXP470.1

Protease (UniProt Accession No.)	Cognate cleavage site	Protease inhibitors
threonine proteases (Q9UI38, Q16512, Q9H6P5, Q8IWU2)		
Mast cell (MC) chymase (CMA1) (NM_001836)	Abz-HPFHL(SEQ ID NO: 155)-Lys(Dnp)-NH2 (SEQ ID NO: 226)	BAY 1142524 SUN13834
Rat mast cell protease-1, -2, -3, -4, -5 (NM_017145, NM_172044, NM_001170466, NM_019321, NM_013092)	Abz-HPFHL(SEQ ID NO: 155)-Lys(Dnp)-NH2 (SEQ ID NO: 226)	TY-51469
Rat vascular chymase (RVCH) (O70500)	Abz-HPFHL(SEQ ID NO: 155)-Lys(Dnp)-NH2 (SEQ ID NO: 226)	
DENV NS3pro (NS2B/NS3) SEQ ID NOs: 157, 158, 159, 160	A strong preference for basic amino acid residues (Arg/Lys) at the P1 positions was observed, whereas the preferences for the P2– 4 sites were in the order of Arg > Thr > Gln/Asn/Lys for P2, Lys > Arg > Asn for P3, and Nle > Leu > Lys > Xaa for P4. The prime site substrate specificity was for small and polar amino acids in P1 and P3.	Anthraquinone BP13944 ZINC04321905 MB21 Policresulen SK-12 NSC135618 Biliverdin

A protease can be any of the following human proteases (MEROPS peptidase database number provided in parentheses; Rawlings N. D., Morton F. R., Kok, C. Y., Kong, J. & Barrett A. J. (2008) MEROPS: the peptidase database. Nucleic Acids Res. 36 Database issue, D320-325; herein incorporated by reference for all purposes): pepsin A (MER000885), gastricsin (MER000894), memapsin-2 (MER005870), renin (MER000917), cathepsin D (MER000911), cathepsin E (MER000944), memapsin-1 (MER005534), napsin A (MER004981), Mername-AA034 peptidase (MER014038), pepsin A4 (MER037290), pepsin A5 (Homo sapiens) (MER037291), hCG1733572 (Homo sapiens)-type putative peptidase (MER107386), napsin B pseudogene (MER004982), CYMP g.p. (Homo sapiens) (MER002929), subfamily A1A unassigned peptidases (MER181559), mouse mammary tumor virus retropepsin (MER048030), rabbit endogenous retrovirus endopeptidase (MER043650), S71-related human endogenous retropepsin (MER001812), RTVL-H-type putative peptidase (MER047117), RTVL-H-type putative peptidase (MER047133), RTVL-H-type putative peptidase (MER047160), RTVL-H-type putative peptidase (MER047206), RTVL-H-type putative peptidase (MER047253), RTVL-H-type putative peptidase

connects a first polypeptide sequence and a second polypeptide sequence. A polypeptide linker can be a flexible linker (*e.g.*, a Gly-Ser-Gly sequence). Examples of polypeptide linkers include, but are not limited to, GSG linkers (*e.g.*, $[GS]_4GG$ [SEQ ID NO: 220~~182~~]), $A(EAAAK)_3A$ (SEQ ID NO: 221~~183~~), and Whitlow linkers (*e.g.*, a "KEGS" linker such as the amino acid sequence KESGSVSSEQLAQFRSLD (SEQ ID NO: 222~~184~~), an eGK linker such as the amino acid sequence EGKSSGSGSESKST (SEQ ID NO: 223~~185~~), and linkers described in more detail in Issued U.S. Pat. No. 5,990,275 herein incorporated by reference). Additional polypeptide linkers include SEQ ID NO: 194, SEQ ID NO: 195, SEQ ID NO: 196, and SEQ ID NO: 197. Other polypeptide linkers may be selected based on desired properties (*e.g.*, length, flexibility, amino acid composition etc.) and are known to those skilled in the art.

In general, the cell-membrane tethering domain is oriented such that the secreted effector molecule and the protease cleavage site are extracellularly exposed following insertion into, or association with, the cell membrane, such that the protease cleavage site is capable of being cleaved by its respective protease and releasing ("secreting") the effector molecule into the extracellular space.

Degron Systems and Domains

In some embodiments, any of the proteins described herein can include a degron domain including, but not limited to, a protease, a transcription factor, a promoter or constituent of a promoter system (*e.g.*, an ACP), and/or any of the membrane-cleavable chimeric protein described herein. In general, the degron domain can be any amino acid sequence motif capable of directing regulated degradation, such as regulated degradation through a ubiquitin-mediated pathway. In the presence of an immunomodulatory drug (IMiD), the degron domain directs ubiquitin-mediated degradation of a degron-fusion protein.

The degron domain can be a cereblon (CRBN) polypeptide substrate domain capable of binding CRBN in response to an immunomodulatory drug (IMiD) including, but not limited to, IKZF1, IKZF3, CKla, ZFP91, GSPT1, MEIS2, GSS E4F1, ZN276, ZN517, ZN582, ZN653, ZN654, ZN692, ZN787, and ZN827, or a fragment thereof that is capable of drug-inducible binding of CRBN. The CRBN polypeptide substrate domain can be a chimeric fusion product of native CRBN polypeptide sequences, such as a IKZF3/ZFP91/IKZF3 chimeric fusion product having the amino acid sequence of FNVLMVHKRSHTGERPLQCEICGFTCRQKGNLLRHIKLHTGEKPFKCHLCNYACQRRDAL (SEQ ID NO: 175). Degron domains, and in particular CRBN degron systems, are

protein such that the effector molecule is released ("secreted") into the extracellular space. In the presence of an immunomodulatory drug (IMiD), the degron domain directs ubiquitin-mediated degradation of the chimeric protein such that secretion of the effector molecule is reduced or eliminated. In general, for membrane-cleavable chimeric proteins fused to a degron domain, the degron domain is the terminal cytoplasmic-oriented domain, specifically relative to the cell membrane tethering domain, *e.g.*, the most C-terminal domain in the formula **S – C – MT – D** or the most N-terminal domain in the formula **D – MT – C – S**. The degron domain can be connected to the cell membrane tethering domain by a polypeptide linker, *i.e.*, a polypeptide sequence not generally considered to be part of the cell membrane tethering domain or the degron domain. A polypeptide linker can be any amino acid sequence that connects a first polypeptide sequence and a second polypeptide sequence. A polypeptide linker can be a flexible linker (*e.g.*, a Gly-Ser-Gly sequence). Examples of polypeptide linkers include, but are not limited to, GSG linkers (*e.g.*, $[GS]_4GG$ [SEQ ID NO: 220~~182~~]), $A(EAAAK)_3A$ (SEQ ID NO: 221~~183~~), and Whitlow linkers (*e.g.*, a "KEGS" linker such as the amino acid sequence KESGSVSSEQLAQFRSLD (SEQ ID NO: 222~~184~~), an eGK linker such as the amino acid sequence EGKSSGSGSESKST (SEQ ID NO: 223~~185~~), and linkers described in more detail in Issued U.S. Pat. No. 5,990,275 herein incorporated by reference). Additional polypeptide linkers include SEQ ID NO: 194, SEQ ID NO: 195, SEQ ID NO: 196, and SEQ ID NO: 197. Other polypeptide linkers may be selected based on desired properties (*e.g.*, length, flexibility, amino acid composition etc.) and are known to those skilled in the art. In general, the degron is oriented in relation to the cell membrane tethering domain such that the degron is exposed to the cytosol following localization to the cell membrane such that the degron domain is capable of mediating degradation (*e.g.*, exposure to the cytosol and cytosol) and is capable of mediating ubiquitin-mediated degradation.

For degron-fusion proteins, the degron domain can be N-terminal or C-terminal of the protein of interest, *e.g.*, the effector molecule. The degron domain can be connected to the protein of interest by a polypeptide linker, *i.e.*, a polypeptide sequence not generally considered to be part of the protein of interest or the degron domain. A polypeptide linker can be any amino acid sequence that connects a first polypeptide sequence and a second polypeptide sequence. A polypeptide linker can be a flexible linker (*e.g.*, a Gly-Ser-Gly sequence). Examples of polypeptide linkers include, but are not limited to, GSG linkers (*e.g.*, $[GS]_4GG$ [SEQ ID NO: 220~~182~~]), $A(EAAAK)_3A$ (SEQ ID NO: 221~~183~~), and Whitlow linkers (*e.g.*, a "KEGS" linker such as the amino acid sequence KESGSVSSEQLAQFRSLD

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223~~185~~), and linkers described in more detail in Issued U.S. Pat. No. 5,990,275 herein incorporated by reference). Additional polypeptide linkers include SEQ ID NO: 194, SEQ ID NO: 195, SEQ ID NO: 196, and SEQ ID NO: 197. Other polypeptide linkers may be selected based on desired properties (*e.g.*, length, flexibility, amino acid composition etc.) and are known to those skilled in the art. A polypeptide linker can be cleavable, *e.g.*, any of the protease cleavage sites described herein.

Homing Molecules

A "tumor microenvironment" is the cellular environment in which a tumor exists, including surrounding blood vessels, immune cells, fibroblasts, bone marrow-derived inflammatory cells, lymphocytes, signaling molecules and the extracellular matrix (ECM) (see, *e.g.*, Pattabiraman, D.R. & Weinberg, R.A. *Nature Reviews Drug Discovery* 13, 497–512 (2014); Balkwill, F.R. *et al. J Cell Sci* 125, 5591-5596, 2012; and Li, H. *et al. J Cell Biochem* 101(4), 805-15, 2007).

In some embodiments, engineered nucleic acids are configured to produce at least one homing molecule. For example, in membrane-cleavable chimeric proteins described herein containing a secreted effector molecule, the secreted effector molecule can be a homing molecule. "Homing," refers to active navigation (migration) of a cell to a target site (*e.g.*, a cell, tissue (*e.g.*, tumor), or organ). A "homing molecule" refers to a molecule that directs cells to a target site. In some embodiments, a homing molecule functions to recognize and/or initiate interaction of an engineered cell to a target site. Non-limiting examples of homing molecules include CXCR1, CCR9, CXCR2, CXCR3, CXCR4, CCR2, CCR4, FPR2, VEGFR, IL6R, CXCR1, CSCR7, and PDGFR.

In some embodiments, a homing molecule is a chemokine receptor (cell surface molecule that binds to a chemokine). Chemokines are small cytokines or signaling proteins secreted by cells that can induce directed chemotaxis in cells. Chemokines can be classified into four main subfamilies: CXC, CC, CX3C and XC, all of which exert biological effects by binding selectively to chemokine receptors located on the surface of target cells. In some embodiments, engineered nucleic acids are configured to produce CXCR4, a chemokine receptor which allows engineered cells to home along a chemokine gradient towards a stromal cell-derived factor 1 (also known as SDF1, C-X-C motif chemokine 12, and CXCL12)-expressing cell, tissue, or tumor. Non-limiting examples of chemokine receptors that may be encoded by the engineered nucleic acids of the present disclosure include: CXC

In some embodiments, the ACP is a transcriptional modulator. In some embodiments, the ACP is a transcriptional repressor. In some embodiments, the ACP is a transcriptional activator. In some embodiments, the ACP is a transcription factor. In some embodiments, the ACP comprises a DNA-binding domain and a transcriptional effector domain. In some embodiments, the DNA-binding domain comprises a tetracycline (or derivative thereof) repressor (TetR) domain. In some embodiments, the ACP is an antigen recognizing receptor of the present disclosure.

The ACP can also further include an effector domain, such as a transcriptional effector domain. For instance, a transcriptional effector domain can be the effector or activator domain of a transcription factor. Transcription factor activation domains are also known as transactivation domains, and act as scaffold domains for proteins such as transcription coregulators that act to activate or repress transcription of genes. Any suitable transcriptional effector domains can be used in the ACP including, but not limited to, a Herpes Simplex Virus Protein 16 (VP16) activation domain; an activation domain consisting of four tandem copies of VP16, a VP64 activation domain; a p65 activation domain of NFκB; an Epstein-Barr virus R transactivator (Rta) activation domain; a tripartite activator comprising the VP64, the p65, and the Rta activation domains, the tripartite activator is known as a VPR activation domain; a histone acetyltransferase (HAT) core domain of the human E1A-associated protein p300, known as a p300 HAT core activation domain; a Krüppel associated box (KRAB) repression domain; a Repressor Element Silencing Transcription Factor (REST) repression domain; a WRPW (SEQ ID NO: 224) motif of the hairy-related basic helix-loop-helix repressor proteins, the motif is known as a WRPW (SEQ ID NO: 224) repression domain; a DNA (cytosine-5)-methyltransferase 3B (DNMT3B) repression domain; and an HP1 alpha chromoshadow repression domain, or any combination thereof.

In some embodiments, the effector domain is s transcription effector domain selected from: a Herpes Simplex Virus Protein 16 (VP16) activation domain; an activation domain consisting of four tandem copies of VP16, a VP64 activation domain; a p65 activation domain of NFκB; an Epstein-Barr virus R transactivator (Rta) activation domain; a tripartite activator comprising the VP64, the p65, and the Rta activation domains, the tripartite activator is known as a VPR activation domain; a histone acetyltransferase (HAT) core domain of the human E1A-associated protein p300, known as a p300 HAT core activation domain; a Krüppel associated box (KRAB) repression domain; a Repressor Element Silencing Transcription Factor (REST) repression domain; a WRPW (SEQ ID NO: 224)

motif of the hairy-related basic helix-loop-helix repressor proteins, the motif is known as a WRPW (SEQ ID NO: 224) repression

domain; a Repressor Element Silencing Transcription Factor (REST) repression domain; a WRPW (SEQ ID NO: 224) motif of the hairy-related basic helix-loop-helix repressor proteins, the motif is known as a WRPW (SEQ ID NO: 224) repression domain; a DNA (cytosine-5)-methyltransferase 3B (DNMT3B) repression domain; and an HP1 alpha chromoshadow repression domain.

22. The engineered nucleic acid of any one of embodiments 14-21, wherein the one or more cognate cleavage sites of the repressible protease are localized between the ZF protein domain and the effector domain.

23. The engineered nucleic acid of any one of embodiments 14-22, wherein the repressible protease is hepatitis C virus (HCV) nonstructural protein 3 (NS3).

24. The engineered nucleic acid of embodiment 23, wherein the cognate cleavage site comprises an NS3 protease cleavage site.

25. The engineered nucleic acid of embodiment 24, wherein the NS3 protease cleavage site comprises a NS3/NS4A, a NS4A/NS4B, a NS4B/NS5A, or a NS5A/NS5B junction cleavage site.

26. The engineered nucleic acid of any one of embodiments 23-25, wherein the NS3 protease can be repressed by a protease inhibitor.

27. The engineered nucleic acid of embodiment 26, wherein the protease inhibitor is selected from the group consisting of: simeprevir, danoprevir, asunaprevir, ciluprevir, boceprevir, sovaprevir, paritaprevir, telaprevir, grazoprevir, glecaprevir, and voxiloprevir.

28. The engineered nucleic acid of any one of embodiments 15-27, wherein the ACP is capable of undergoing nuclear localization upon binding of the ERT2 domain to tamoxifen or a metabolite thereof.

29. The engineered nucleic acid of embodiment 28, wherein the tamoxifen metabolite is selected from the group consisting of: 4-hydroxytamoxifen, N-desmethyltamoxifen, tamoxifen-N-oxide, and endoxifen.

62. The engineered nucleic acid of any one of embodiments 1-61, wherein the protease cleavage site is cleavable by a protease selected from the group consisting of: a Type 1 transmembrane protease, a Type II transmembrane protease, a GPI anchored protease, an ADAM8 protease, an ADAM9 protease, an ADAM10 protease, an ADAM12 protease, an ADAM15 protease, an ADAM17 protease, an ADAM19 protease, an ADAM20 protease, an ADAM21 protease, an ADAM28 protease, an ADAM30 protease, an ADAM33 protease, a BACE1 protease, a BACE2 protease, a SIP protease, an MT1-MMP protease, an MT3-MMP protease, an MT5-MMP protease, a furin protease, a PCSK7 protease, a matriptase protease, a matriptase-2 protease, an MMP9 protease, and an NS3 protease.

63. The engineered nucleic acid of any one of embodiments 1-62, wherein the protease cleavage site is cleavable by an ADAM17 protease.

64. The engineered nucleic acid of any one of embodiments 1-63, wherein the protease cleavage site comprises a first region having the amino acid sequence of PRAE (SEQ ID NO: 176).

65. The engineered nucleic acid of embodiment 64, wherein the protease cleavage site comprises a second region having the amino acid sequence of KGG (SEQ ID NO: 177).

66. The engineered nucleic acid of embodiment 65, wherein the first region is located N-terminal to the second region.

67. The engineered nucleic acid of any one of embodiments 1-66, wherein the protease cleavage site comprises the amino acid sequence of PRAEX_1X_2KGG (SEQ ID NO: ~~178~~219),
wherein X_1 is A, Y, P, S, or F, and
wherein X_2 is V, L, S, I, Y, T, or A.

68. The engineered nucleic acid of any one of embodiments 1-66, wherein the protease cleavage site comprises the amino acid sequence of PRAEX_1X_2KGG (SEQ ID NO: 178),
wherein X_1 is A, Y, P, S, or F, and
wherein X_2 is V, L, S, I, Y, or T.

130. The chimeric protein of any one of embodiments 105-129, wherein the protease cleavage site comprises a first region having the amino acid sequence of PRAE (SEQ ID NO: 176).

131. The chimeric protein of embodiment 130, wherein the protease cleavage site comprises a second region having the amino acid sequence of KGG (SEQ ID NO: 177).

132. The chimeric protein of embodiment 131, wherein the first region is located N-terminal to the second region.

133. The chimeric protein of any one of embodiments 105-132, wherein the protease cleavage site comprises the amino acid sequence of PRAEX_1X_2KGG (SEQ ID NO: ~~178~~219),
wherein X_1 is A, Y, P, S, or F, and
wherein X_2 is V, L, S, I, Y, T, or A.

134. The chimeric protein of any one of embodiments 105-132, wherein the protease cleavage site comprises the amino acid sequence of PRAEX_1X_2KGG (SEQ ID NO: 178),
wherein X_1 is A, Y, P, S, or F, and
wherein X_2 is V, L, S, I, Y, or T.

135. The chimeric protein of any one of embodiments 105-134, wherein the protease cleavage site comprises the amino acid sequence of PRAEAVKGG (SEQ ID NO: 179).

136. The chimeric protein of any one of embodiments 105-134, wherein the protease cleavage site comprises the amino acid sequence of PRAEALKGG (SEQ ID NO: 180).

137. The chimeric protein of any one of embodiments 105-134, wherein the protease cleavage site comprises the amino acid sequence of PRAEYSKGG (SEQ ID NO: 181).

138. The chimeric protein of any one of embodiments 105-134, wherein the protease cleavage site comprises the amino acid sequence of PRAEPIKGG (SEQ ID NO: 182).

190. The isolated cell of embodiment 189, wherein the protease cleavage site comprises a second region having the amino acid sequence of KGG (SEQ ID NO: 177).

191. The isolated cell of embodiment 190, wherein the first region is located N-terminal to the second region.

192. The isolated cell of any one of embodiments 169-191, wherein the protease cleavage site comprises the amino acid sequence of PRAEX_1X_2KGG (SEQ ID NO: ~~178~~219),
wherein X_1 is A, Y, P, S, or F, and
wherein X_2 is V, L, S, I, Y, T, or A.

193. The isolated cell of any one of embodiments 169-191, wherein the protease cleavage site comprises the amino acid sequence of PRAEX_1X_2KGG (SEQ ID NO: 178),
wherein X_1 is A, Y, P, S, or F, and
wherein X_2 is V, L, S, I, Y, or T.

194. The isolated cell of any one of embodiments 169-193, wherein the protease cleavage site comprises the amino acid sequence of PRAEAVKGG (SEQ ID NO: 179).

195. The isolated cell of any one of embodiments 169-193, wherein the protease cleavage site comprises the amino acid sequence of PRAEALKGG (SEQ ID NO: 180).

196. The isolated cell of any one of embodiments 169-193, wherein the protease cleavage site comprises the amino acid sequence of PRAEYSKGG (SEQ ID NO: 181).

197. The isolated cell of any one of embodiments 169-193, wherein the protease cleavage site comprises the amino acid sequence of PRAEPIKGG (SEQ ID NO: 182).

198. The isolated cell of any one of embodiments 169-193, wherein the protease cleavage site comprises the amino acid sequence of PRAEAYKGG (SEQ ID NO: 183).

199. The isolated cell of any one of embodiments 169-193, wherein the protease cleavage site comprises the amino acid sequence of PRAESSKGG (SEQ ID NO: 184).

200. The isolated cell of any one of embodiments 169-193, wherein the protease cleavage site comprises the amino acid sequence of PRAEFTKGG (SEQ ID NO: 185).

273. The method of embodiment 272, wherein the protease cleavage site comprises a second region having the amino acid sequence of KGG (SEQ ID NO: 177).

274. The method of embodiment 273, wherein the first region is located N-terminal to the second region.

275. The method of any one of embodiments 243-274, wherein the protease cleavage site comprises the amino acid sequence of PRAEX_1X_2KGG (SEQ ID NO: ~~178~~219),
wherein X_1 is A, Y, P, S, or F, and
wherein X_2 is V, L, S, I, Y, T, or A.

276. The method of any one of embodiments 243-275, wherein the protease cleavage site comprises the amino acid sequence of PRAEAVKGG (SEQ ID NO: 179).

277. The method of any one of embodiments 243-275, wherein the protease cleavage site comprises the amino acid sequence of PRAEALKGG (SEQ ID NO: 180).

278. The method of any one of embodiments 243-275, wherein the protease cleavage site comprises the amino acid sequence of PRAEYSKGG (SEQ ID NO: 181).

279. The method of any one of embodiments 243-275, wherein the protease cleavage site comprises the amino acid sequence of PRAEPIKGG (SEQ ID NO: 182).

280. The method of any one of embodiments 243-275, wherein the protease cleavage site comprises the amino acid sequence of PRAEAYKGG (SEQ ID NO: 183).

281. The method of any one of embodiments 243-275, wherein the protease cleavage site comprises the amino acid sequence of PRAESSKGG (SEQ ID NO: 184).

282. The method of any one of embodiments 243-275, wherein the protease cleavage site comprises the amino acid sequence of PRAEFTKGG (SEQ ID NO: 185).

283. The method of any one of embodiments 243-275, wherein the protease cleavage site comprises the amino acid sequence of PRAEAAKGG (SEQ ID NO: 186).

284. The method of any one of embodiments 243-274, wherein the protease cleavage site comprises the amino acid sequence of DEPHYSQRR (SEQ ID NO: 187).