CLAIMS

WHAT IS CLAIMED IS:

1. A system comprising:

a deoxyribonucleic acid (DNA) endonuclease or nucleic acid encoding the DNA endonuclease;

a guide RNA (gRNA) comprising a spacer sequence complementary to a target sequence within a target genomic locus in a cell, or nucleic acid encoding the gRNA; and

a donor template comprising a donor cassette comprising a nucleic acid sequence encoding a naked FKBP-rapamycin binding (FRB) domain polypeptide,

wherein the DNA endonuclease, gRNA, and donor template are configured such that a complex formed by association of the DNA endonuclease with the gRNA is capable of promoting targeted integration of the donor cassette into the target genomic locus in a cell to generate a genetically modified cell capable of expressing the naked FRB domain polypeptide.

2. The system of claim 1, wherein the DNA endonuclease is a Cas9 endonuclease.

3. The system of any one of claims 1 to 2, wherein

the nucleic acid encoding the DNA endonuclease is codon-optimized for expression in the genetically modified cell;

the nucleic acid encoding the gRNA is codon-optimized for expression in the genetically modified cell, and/or

one or more coding sequences in the donor cassette is codon-optimized for expression in the genetically modified cell.

4. The system of any one of claims 1 to 3, wherein the donor template is configured such that the donor cassette is capable of being integrated into the target genomic locus by homology directed repair (HDR).

5. The system of any one of claims 1 to 3, wherein the donor template is configured such that the donor cassette is capable of being integrated into the target genomic locus by non-homologous end joining (NHEJ).

6. The system of any one of claims 1 to 5, wherein the DNA endonuclease or nucleic acid encoding the DNA endonuclease is formulated in a liposome or lipid nanoparticle.

7. The system of claim 6, wherein the liposome or lipid nanoparticle further comprises the gRNA or nucleic acid encoding the gRNA.

8. The system of any one of claims 1 to 7, comprising the DNA endonuclease associated with the gRNA in a ribonucleoprotein (RNP) complex.

9. The system of any one of claims 1 to 8, wherein the naked FRB polypeptide comprises the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2.

10. The system of any one of claim 1 to 9, wherein the donor cassette further comprises one or more nucleic acid sequences encoding polypeptide components of a dimerization activatable chemical-induced signaling complex (CISC), wherein the polypeptide components of the CISC comprise

i) a first CISC component comprising a first extracellular binding domain or functional derivative thereof, a hinge domain, a transmembrane domain, and a signaling domain or functional derivative thereof; and

ii) a second CISC component comprising a second extracellular binding domain or functional derivative thereof, a hinge domain, a transmembrane domain, and a signaling domain or functional derivative thereof;

wherein the first CISC component and the second CISC component are configured such that when expressed in a cell, they are capable of dimerizing in the presence of rapamycin or a rapalog to generate a signaling-competent CISC.

11. The system of claim 10, wherein the first or second extracellular binding domain or functional derivative thereof comprises an FK506 binding protein (FKBP) domain or a functional derivative thereof and/or the other extracellular binding domain or functional derivative thereof comprises an FRB domain or a functional derivative thereof.

12. The system of any one of claims 10 to 11, wherein the transmembrane domain of the first CISC component comprises an IL-2 receptor transmembrane domain and/or the transmembrane domain of the second CISC components comprises an IL-2 receptor transmembrane domain.

13. The system of any one of claims 10 to 12, wherein the signaling domain or functional derivative thereof of the first or second CISC component comprises an IL-2 receptor subunit gamma (IL2Rγ) domain or a functional derivative thereof and/or the signaling domain or functional derivative thereof of the other CISC component comprises an IL-2 receptor subunit beta (IL2Rβ) domain or a functional derivative thereof.

14. The system of claim 13, wherein the IL2Rβ domain polypeptide is truncated.

15. The system of claim 13, wherein the nucleic acid encoding the IL2Rβ domain comprises the nucleotide sequence of SEQ ID NO: 4.

16. The system of claim 13, wherein the IL2Rβ domain comprises the amino acid sequence of SEQ ID NO: 5.

17. The system of any one of claims 10 to 16, wherein the rapalog is selected from the group consisting of everolimus, CCI-779, C20-methallylrapamycin, C16-(S)-3-methylindolerapamycin, C16-iRap, AP21967, sodium mycophenolic acid, benidipine hydrochloride, AP1903, and AP23573, and metabolites and derivatives thereof.

18. The system of any one of claims 10 to 17, wherein the nucleic acid encoding the naked FRB domain is downstream of the one or more nucleic acid sequences encoding polypeptide components of the CISC.

19. The system of any one of claims 1 to 18, wherein the donor cassette further comprises a nucleic acid sequences encoding a self-cleaving polypeptide between (i) each of the one or more nucleic acid sequences encoding polypeptide components of the CISC; and/or (ii) the nucleic acid encoding the naked FRB domain and an adjacent nucleic acid sequence encoding a polypeptide component of the CISC.

20. The system of claim 19, wherein each of the self-cleaving polypeptides encoded in the donor cassette is independently selected from the group consisting of P2A, T2A, E2A, and F2A.

21. The system of any one of claims 1 to 20, wherein the donor cassette further comprises a promoter operably linked to one or more coding sequences in the donor cassette.

22. The system of claim 21, wherein the promoter is an inducible promoter or a constitutive promoter.

23. The system of claim 21 or claim 22, wherein the promoter is an MND promoter.

24. The system of any one of claims 1 to 23, wherein the donor cassette further comprises a nucleic acid encoding a detectable marker.

25. The system of claim 24, wherein the detectable marker is a green fluorescent protein (GFP) polypeptide, an mCherry polypeptide, or a low affinity nerve growth factor receptor (LNGFR).

26. The system of any one of claims 1 to 25, wherein the nucleic acid encoding the naked FRB domain polypeptide lacks a nucleic acid encoding an endoplasmic reticulum localization signal polypeptide.

27. The system of any one of claims 1 to 26, wherein the donor cassette comprises a nucleic acid sequence from the nucleotide sequence of SEQ ID NO: 3.

28. The system of any one of claims 1 to 27, wherein the donor template is a viral vector.

29. The system of claim 28, wherein the viral vector is a lentiviral, adenoviral, or adeno-associated viral (AAV) vector.

30. A method of editing a cell genome, comprising providing to the cell:

a deoxyribonucleic acid (DNA) endonuclease or nucleic acid encoding the DNA endonuclease;

a guide RNA (gRNA) comprising a spacer sequence complementary to a target sequence within a target genomic locus in the cell, or nucleic acid encoding the gRNA; and

a donor template comprising a donor cassette comprising a nucleic acid sequence encoding a naked FKBP-rapamycin binding (FRB) domain polypeptide,

wherein the DNA endonuclease, gRNA, and donor template are configured such that a complex formed by association of the DNA endonuclease with the gRNA is capable of promoting targeted integration of the donor cassette into the target genomic locus in the cell to generate a genetically modified cell capable of expressing the naked FRB domain polypeptide.

31. A genetically modified cell prepared according to the method of claim 30.

32. The cell of claim 31, wherein the donor cassette is configured such that naked FRB domain polypeptide is expressed intracellularly.

33. The cell of any one of claims 31 to 32, wherein the cell is a eukaryotic cell.

34. The cell of claim 33, wherein the cell is a mammalian cell.

35. The cell of claim 34, wherein the cell is a hematopoietic stem cell.

36. The cell of claim 35, wherein the cell is a lymphocyte.

37. The cell of claim 36, wherein the cell is a precursor T cell or a T regulatory (T_{reg}) cell.

38. The cell of any one of claims 36 to 37, wherein the cell is a CD34+, CD8+, or a CD4+ cell.

39. The cell of claim 38, wherein the cell is a CD8+ T cytotoxic lymphocyte cell selected from the group consisting of naïve CD8+ T cells, central memory CD8+ T cells, effector memory CD8+ T cells, and bulk CD8+ T cells.

40. The cell of claim 38, wherein the cell is a CD4+ T helper lymphocyte cell selected from the group consisting of naïve CD4+ T cells, central memory CD4+ T cells, effector memory CD4+ T cells, and bulk CD4+ T cells.

41. The cell of any one of claims 31 to 40, wherein the cell proliferates to a greater extent in the presence of rapamycin or a rapalog as compared to a corresponding cell where the donor cassette lacks a nucleic acid encoding a naked FRB domain polypeptide.

42. The cell of claim 41, wherein the rapamycin or rapalog is present in a concentration of from or from about 0.1 nM to or to about 100 nM.

43. A method of activating the genetically modified cell of any one of claims 31 to 42, comprising contacting the cell with rapamycin or a rapalog.

44. The method of claim 43, wherein the rapalog is selected from the group consisting of everolimus, CCI-779, C20-methallylrapamycin, C16-(S)-3-methylindolerapamycin, C16-iRap, AP21967, sodium mycophenolic acid, benidipine hydrochloride, AP1903, or AP23573, or metabolites, derivatives, and/or combinations of any thereof.

45. The method of any one claims 43 to 44, wherein the rapamycin or rapalog contacting the cell is at a concentration of from or from about 0.1 nM to or to about 100 nM.

46. The method of any one of claims 43 to 45, wherein following contact with rapamycin or the rapalog, cells expressing the CISC components are selectively expanded.

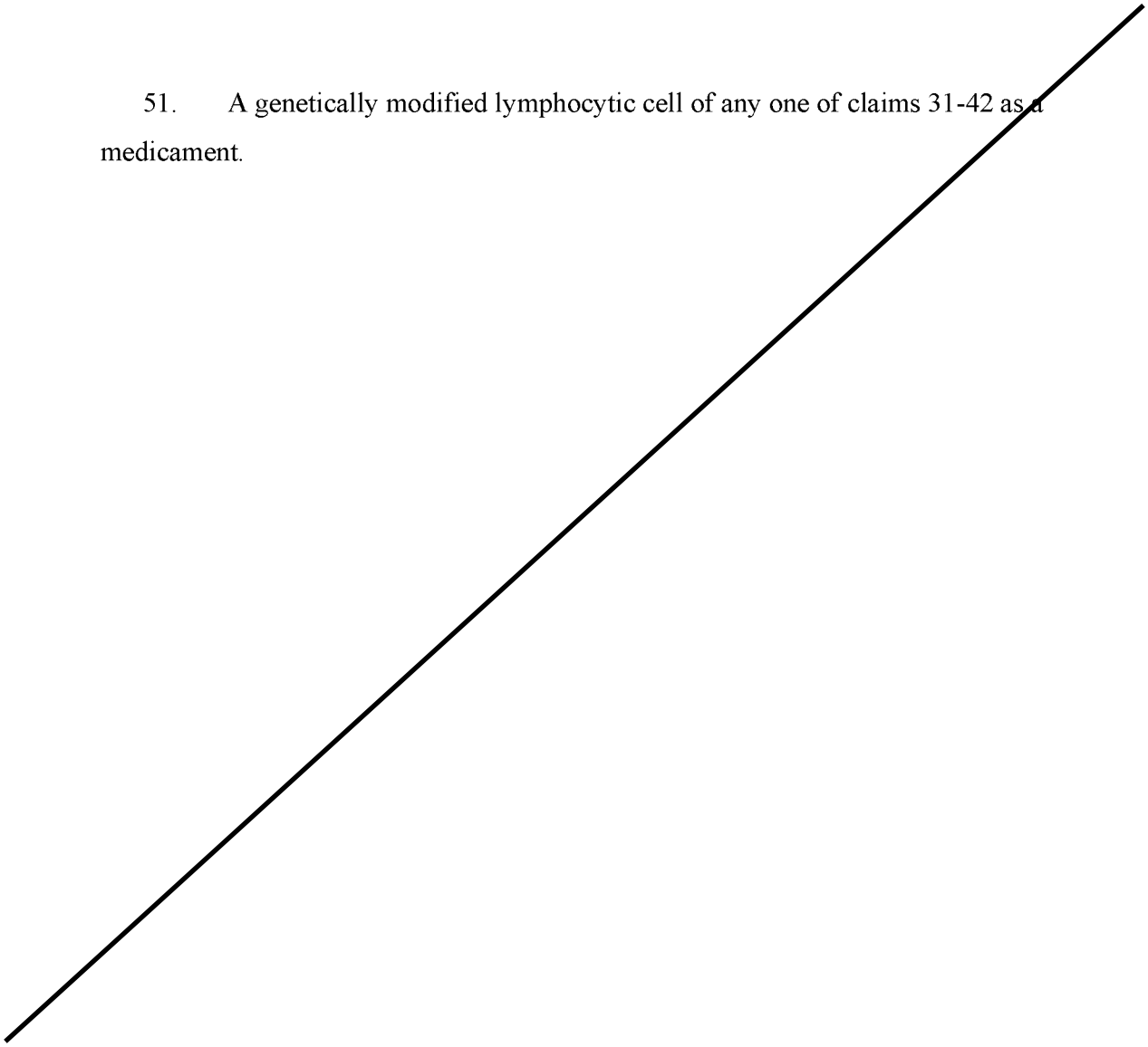
47. A method of selectively expanding a population of genetically modified cells according to any one of claims 31 to 42 contained in a mixed population of cells, comprising contacting the mixed population of cells with rapamycin or a rapalog, wherein the genetically modified cells expressing the CISC components and naked FRB domain are activated and expanded *in vitro* or *in vivo* to a greater extent than the other cells in the mixed population of cells.

48. The method of claim 47, wherein the rapalog is selected from the group consisting of everolimus, CCI-779, C20-methallylrapamycin, C16-(S)-3-methylindolerapamycin, C16-iRap, AP21967, sodium mycophenolic acid, benidipine hydrochloride, AP1903, or AP23573, or metabolites, derivatives, and/or combinations of any thereof.

49. The method of any one of claims 47 to 48, wherein the rapamycin or rapalog contacting the mixed population of cells is at a concentration of from or from about 0.1 nM to or to about 100 nM.

50. A genetically modified lymphocytic cell of any one of claims 31-42 for use in inhibiting or treating a disease or condition such as an autoimmune disease (e.g., IPEX syndrome) or a disorder that results from organ transplant (e.g., GVHD).

51. A genetically modified lymphocytic cell of any one of claims 31-42 as a medicament.



CLAIMS

1. A system comprising:

a first polynucleotide encoding a first chemically induced signaling complex (CISC) component comprising a first extracellular binding domain comprising a FK506 binding protein (FKBP) domain, a first transmembrane domain, and a first signaling domain;

a second polynucleotide encoding a second CISC component comprising a second extracellular binding domain comprising a FKBP-rapamycin binding (FRB) domain, a second transmembrane domain, and a second signaling domain; and

a third polynucleotide encoding a naked FKBP-rapamycin binding (FRB) domain polypeptide, wherein the naked FRB domain polypeptide comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 1;

wherein the first CISC component and the second CISC component dimerize in the presence of rapamycin, a rapalog, a metabolite of rapamycin, or an IMID-class drug, to create a signaling-competent CISC.

2. The system of claim 1, wherein a vector comprises the first polynucleotide, the second polynucleotide, the third polynucleotide, or a combination thereof.

3. The system of claim 2, wherein the vector comprises the first polynucleotide and the third polynucleotide.

4. The system of claim 2, wherein the vector comprises the second polynucleotide and the third polynucleotide.

5. The system of any one of claims 2-4, wherein the vector is a non-viral vector.

6. The system of any one of claims 2-4, wherein the vector is a viral vector.

7. The system of claim 6, wherein the vector is a lentiviral vector.

8. The system of claim 6, wherein the vector is an adeno-associated viral (AAV) vector.

9. The system of any one of claims 1-8, wherein the naked FRB domain polypeptide comprises an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 1.

10. The system of any one of claims 1-9, wherein the naked FRB domain polypeptide comprises the amino acid sequence of SEQ ID NO: 1.

11. The system of any one of claims 1-8, wherein the naked FRB domain polypeptide comprises a T2098L substitution.

12. The system of any one of claims 1-8, wherein the naked FRB domain polypeptide comprises the amino acid sequence of SEQ ID NO: 2.

13. The system of any one of claims 1-12, wherein the rapalog is everolimus, CCI-779, C20-methallylrapamycin, C16-(S)-3-methylindolerapamycin, C16-iRap, AP21967, sodium mycophenolic acid, benidipine hydrochloride, AP1903, or AP23573; or wherein the IMID-class drug is thalidomide, pomalidomide, lenalidomide or an IMID-class drug analogue.

14. The system of any one of claims 1-13, wherein the first CISC component comprises a first hinge domain, and the second CISC component comprises a second hinge domain.

15. The system of any one of claims 1-14, wherein the first signaling domain comprises an IL-2 receptor subunit gamma (IL2Rγ) domain and the second signaling domain comprises an IL-2 receptor subunit beta (IL2Rβ) domain.

16. The system of any one of claims 1-14, wherein the second signaling domain comprises an IL2Rγ domain and the first signaling domain comprises an IL2Rβ domain.

17. The system of claim 15 or 16, wherein the IL2Rγ domain comprises an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 11 or SED ID NO: 12.

18. The system of any one of claims 15-17, wherein the IL2Rβ domain comprises an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 7 or SEQ ID NO: 13.

19. The system of any one of claims 1-18, wherein the FKBP domain of the first extracellular binding domain comprises an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 6.

20. The system of any one of claims 1-19, wherein the FRB domain of the second extracellular binding domain comprises an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 1.

21. The system of claim 14, wherein the first CISC component comprises a sequence that is at least 95% sequence identity to the CISC component of SEQ ID NO: 12.

22. The system of claim 14, wherein the second CISC component comprises a sequence that is at least 95% sequence identity to CISC component of SEQ ID NO: 13.

23. A cell comprising the system of any one of claims 1-22.

24. The cell of claim 23, wherein the cell is a T lymphocyte or a precursor T cell.

25. The cell of claim 23 or 24, wherein the cell is a regulatory T cell.

26. The cell of any one of claims 23-25, wherein the cell is a FOXP3+ T regulatory cell.

27. The cell of claim 26, wherein endogenous FOXP3 expression is controlled by an upstream MND promoter.

28. The cell of any one of claims 23-27, wherein the cell is a CD4+ T cell or a CD8+ T cell.

29. The cell of any one of claims 23-28, wherein the first CISC component comprises an FKBP domain, a first transmembrane domain, and an IL2Rγ domain, and wherein the second CISC component comprises an FRB domain, a second transmembrane domain, and an IL2Rβ domain.

30. The cell of claim 29, wherein the first transmembrane domain comprises an IL2Rγ transmembrane domain, and wherein the second transmembrane domain comprises an IL2Rβ transmembrane domain.

31. The cell of any one of claims 23-30, wherein the protein sequence of the first CISC component comprises an amino acid sequence that comprises at least 95% sequence identity to the sequence set forth in any one of SEQ ID NOs: 9-12.

32. The cell of claim 31, wherein the first CISC component comprises amino acid sequence that comprises at least 95% sequence identity to SEQ ID NO: 12.

33. The cell of any one of claims 23-32, wherein the protein sequence of the second CISC component comprises an amino acid sequence that comprises at least 95% sequence identity to the sequence set forth in any one of SEQ ID NOs: 13-15 or 24.

34. The cell of claim 33, wherein the second CISC component comprises an amino acid sequence that comprises at least 95% sequence identity to the sequence set forth in SEQ ID NO: 13.

35. The cell of any one of claims 23-34, wherein the protein sequence of the first CISC component comprises an amino acid sequence that comprises at least 98% sequence identity to the sequence set forth in any one of SEQ ID NOs: 9–12.

36. The cell of any one of claims 23-35, wherein the protein sequence of the second CISC component comprises amino acid sequence that comprises at least 98% sequence identity to the sequence set forth in any one of SEQ ID NOs: 13–15 or 24.

37. A method for making a cell, comprising introducing into the cell the system of any one of claims 1-22.

38. The method of claim 37, wherein the cell is a T lymphocyte or a precursor T cell.

39. The method of claim 37 or 38, wherein the cell is a CD4+ T cell or a CD8+ T cell.

40. The method of any one of claims 37-39, wherein the cell is a regulatory T cell.

41. The method of any one of claims 37-40, wherein the cell is a FOXP3+ T regulatory cell.

42. The method of any one of claims 37-41, further comprising introducing into the cell:

the first polynucleotide encoding the first CISC component;

the second polynucleotide encoding the second CISC component; and

a guide RNA (gRNA) or a nucleic acid encoding the gRNA, wherein the gRNA comprises a spacer complementary to a target sequence within a target genomic locus,

wherein one or more of the first polynucleotide encoding the first CISC component, the second polynucleotide encoding the second CISC component, and the third polynucleotide are comprised in a donor template comprising a donor cassette.

43. The method of claim 42, wherein the gRNA comprises a nucleic acid sequence of any one of SEQ ID NOs: 40–45.

44. The method of claim 42 or 43, further comprising a DNA endonuclease or a nucleic acid encoding the DNA endonuclease, wherein the DNA endonuclease, gRNA, and donor template are configured such that a complex formed by association of the DNA endonuclease with the gRNA is capable of promoting targeted integration of the donor cassette into the target genomic locus in a cell to generate a genetically modified cell.

45. A system comprising:

a deoxyribonucleic acid (DNA) endonuclease or nucleic acid encoding the DNA endonuclease;

a guide RNA (gRNA) comprising a spacer sequence complementary to a target sequence within a target genomic locus in a cell, or nucleic acid encoding the gRNA; and

a donor template comprising a donor cassette comprising a nucleic acid sequence encoding a naked FKBP-rapamycin binding (FRB) domain polypeptide,

wherein the DNA endonuclease, gRNA, and donor template are configured such that a complex formed by association of the DNA endonuclease with the gRNA is capable of promoting targeted integration of the donor cassette into the target genomic locus in a cell to generate a genetically modified cell capable of expressing the naked FRB domain polypeptide, and

wherein the donor cassette further comprises one or more nucleic acid sequences encoding polypeptide components of a dimerization activatable chemical-induced signaling complex (CISC), wherein the polypeptide components of the CISC comprises

i) a first CISC component comprising a first extracellular binding domain or functional derivative thereof, a transmembrane domain, and a signaling domain or functional derivative thereof; and

ii) a second CISC component comprising a second extracellular binding domain or functional derivative thereof, a transmembrane domain, and a signaling domain or functional derivative thereof;

wherein the first CISC component and the second CISC component are configured such that when expressed in a cell, they are capable of dimerizing in the presence of rapamycin or a rapalog to generate a signaling-competent CISC.

46. The system of claim 45, wherein the first CISC component further comprises a first hinge domain, and/or the second CISC component further comprises a second hinge domain.

47. The system of claim 45 or 46, wherein the DNA endonuclease is a Cas9 endonuclease.

48. The system of any one of claims 45-47, wherein

the nucleic acid encoding the DNA endonuclease is codon-optimized for expression in the genetically modified cell;

the nucleic acid encoding the gRNA is codon-optimized for expression in the genetically modified cell; and/or

one or more coding sequences in the donor cassette is codon-optimized for expression in the genetically modified cell.

49. The system of any one of claims 45-48, wherein the donor template is configured such that the donor cassette is capable of being integrated into the target genomic locus by homology directed repair (HDR).

50. The system of any one of claims 45-49, wherein the donor template is configured such that the donor cassette is capable of being integrated into the target genomic locus by non-homologous end joining (NHEJ).

51. The system of any one of claims 45-50, wherein the DNA endonuclease or nucleic acid encoding the DNA endonuclease is formulated in a liposome or lipid nanoparticle.

52. The system of claim 51, wherein the liposome or lipid nanoparticle further comprises the gRNA or nucleic acid encoding the gRNA.

53. The system of any one of claims 45-52, comprising the DNA endonuclease associated with the gRNA in a ribonucleoprotein (RNP) complex.

54. The system of any one of claims 45-53, wherein the naked FRB polypeptide comprises the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2.

55. The system of any one of claims 45-54, wherein the first or second extracellular binding domain or functional derivative thereof comprises an FK506 binding protein (FKBP) domain or a functional derivative thereof and/or the other extracellular binding domain or functional derivative thereof comprises an FRB domain or a functional derivative thereof.

56. The system of any one of claims 45-55, wherein the transmembrane domain of the first CISC component comprises an IL-2 receptor transmembrane domain and/or the transmembrane domain of the second CISC components comprises an IL-2 receptor transmembrane domain.

57. The system of any one of claims 45-56, wherein the signaling domain or functional derivative thereof of the first or second CISC component comprises an IL-2 receptor

subunit gamma (IL2Rγ) domain or a functional derivative thereof and/or the signaling domain or functional derivative thereof of the other CISC component comprises an IL-2 receptor subunit beta (IL2Rβ) domain or a functional derivative thereof.

58. The system of claim 57, wherein the IL2Rβ domain polypeptide is truncated.

59. The system of claim 57, wherein the nucleic acid encoding the IL2Rβ domain comprises the nucleotide sequence of SEQ ID NO: 4.

60. The system of claim 57, wherein the IL2Rβ domain comprises the amino acid sequence of SEQ ID NO: 5.

61. The system of any one of claims 45-60, wherein the rapalog is selected from the group consisting of everolimus, CCI-779, C20-methallylrapamycin, C16-(S)-3-methylindolerapamycin, C16-iRap, AP21967, sodium mycophenolic acid, benidipine hydrochloride, AP1903, and AP23573, and metabolites and derivatives thereof.

62. The system of any one of claims 45-61, wherein the nucleic acid encoding the naked FRB domain is downstream of the one or more nucleic acid sequences encoding polypeptide components of the CISC.

63. The system of any one of claims 45-62, wherein the donor cassette further comprises a nucleic acid sequences encoding a self-cleaving polypeptide between (i) each of the one or more nucleic acid sequences encoding polypeptide components of the CISC; and/or (ii) the nucleic acid encoding the naked FRB domain and an adjacent nucleic acid sequence encoding a polypeptide component of the CISC.

64. The system of claim 63, wherein each of the self-cleaving polypeptides encoded in the donor cassette is independently selected from the group consisting of P2A, T2A, E2A, and F2A.

65. The system of any one of claims 45-64, wherein the donor cassette further comprises a promoter operably linked to one or more coding sequences in the donor cassette.

66. The system of claim 65, wherein the promoter is an inducible promoter or a constitutive promoter.

67. The system of claim 65 or 66, wherein the promoter is an MND promoter.

68. The system of any one of claims 45-67, wherein the donor cassette further comprises a nucleic acid encoding a detectable marker.

69. The system of claim 68, wherein the detectable marker is a green fluorescent protein (GFP) polypeptide, an mCherry polypeptide, or a low affinity nerve growth factor receptor (LNGFR).

70. The system of any one of claims 45-69, wherein the nucleic acid encoding the naked FRB domain polypeptide lacks a nucleic acid encoding an endoplasmic reticulum localization signal polypeptide.

71. The system of any one of claims 45-70, wherein the donor cassette comprises a nucleic acid sequence from the nucleotide sequence of SEQ ID NO: 3.

72. The system of any one of claims 45-71, wherein the donor template is a viral vector.

73. The system of claim 72, wherein the viral vector is a lentiviral, adenoviral, or adeno-associated viral (AAV) vector.

74. A vector comprising: (a) a first polynucleotide or a second polynucleotide, and (b) a third polynucleotide; wherein:

the first polynucleotide encodes a first chemically induced signaling complex (CISC) component comprising a first extracellular binding domain comprising a FK506 binding protein (FKBP) domain, a first transmembrane domain, and a first signaling domain;

the second polynucleotide encodes a second CISC component comprising a second extracellular binding domain comprising a FKBP-rapamycin binding (FRB) domain, a second transmembrane domain, and a second signaling domain, wherein the first CISC component and the second CISC component are capable of dimerizing in the presence of rapamycin, a rapalog, a metabolite of rapamycin, or an IMID-class drug, to create a signaling-competent CISC; and

the third polynucleotide encodes a naked FKBP-rapamycin binding (FRB) domain polypeptide, wherein the naked FRB domain polypeptide comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 1.

75. The vector of claim 74 comprising the first polynucleotide.

76. The vector of claim 74 comprising the second polynucleotide.