

COMPOSITIONS AND METHODS FOR CD123 MODIFICATION

RELATED APPLICATIONS

- 5 This application claims priority to U.S. Serial No.: 62/892,888 filed August 28, 2019 and U.S. Serial No.: 62/962,135 filed January 16, 2020, the entire contents of each of which are incorporated herein by reference.

SEQUENCE LISTING

- 10 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 August 26, 2020, is named V0291_70006WO00_SL.txt and is 77,025 bytes in size.

15 BACKGROUND

- When a cancer patient is administered an anti-CD123 cancer therapy, the therapy can deplete not only CD123+ cancer cells, but also noncancerous CD123+ cells in an “on-target, off-tumor” effect. Since certain hematopoietic cells typically express CD123, the loss of the noncancerous CD123+ cells can deplete the hematopoietic system of the patient. To address
20 this depletion, the subject can be administered rescue cells (e.g., HSCs and/or HPCs) comprising a modification in the CD123 gene. These CD123-modified cells can be resistant to the anti-CD123 cancer therapy, and can therefore repopulate the hematopoietic system during or after anti-CD123 therapy.

25 SUMMARY OF THE INVENTION

- Some aspects of this disclosure provide, e.g., novel cells having a modification (*e.g.*, substitution, insertion or deletion) in the endogenous CD123 gene. Some aspects of this disclosure also provide compositions, *e.g.*, gRNAs, that can be used to make such a modification. Some aspects of this disclosure provide methods of using the compositions
30 provided herein, *e.g.*, methods of using certain gRNAs provided to create genetically engineered cells, *e.g.*, cells having a modification in the endogenous CD123 gene. Some aspects of this disclosure provide methods of administering genetically engineered cells provided herein, *e.g.*, cells having a modification in the endogenous CD123 gene, to a subject in need thereof. Some aspects of this disclosure provide strategies, compositions, methods,

and treatment modalities for the treatment of patients having cancer and receiving or in need of receiving an anti-CD123 cancer therapy.

Enumerated Embodiments

- 5 1. A gRNA comprising a targeting domain which binds a target domain of Table 1 (e.g., a target domain of any of SEQ ID NOS: 1-20 or 40-47).
2. A gRNA comprising a targeting domain capable of directing cleavage or editing of a target domain of Table 1 (e.g., a target domain of any of SEQ ID NOS: 1-20 or 40-47).
- 10 3. A gRNA comprising a targeting domain which binds a target domain of any of SEQ ID NOS: 1-8 or 10, or SEQ ID NOS: 11-18 or 20.
4. A gRNA comprising a targeting domain which binds a target domain of SEQ ID NO:
- 15 9.
5. A gRNA comprising a targeting domain which binds a target domain SEQ ID NO: 19, wherein the targeting domain does not comprise SEQ ID NO: 9.
- 20 6. A gRNA comprising a targeting domain which binds a target domain SEQ ID NO: 19, wherein the targeting domain is at least 21 nucleotides in length.
7. A gRNA comprising a targeting domain which binds a target domain of SEQ ID NO:
- 25 20.
8. The guide RNA of embodiment 5a, wherein the targeting domain base pairs or is complementary with at least 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides of the target domain.
- 30 9. The gRNA of any of the preceding embodiments, wherein the targeting domain base pairs or is complementary with at least 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides of the target domain, or wherein the targeting domain comprises 0, 1, 2, or 3 mismatches with the target domain.

10. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises at least 16 (e.g., 17, 18, 19, 20, 21, 22, 23, 24, 25, or 26) consecutive nucleotides of SEQ ID NO: 31.
- 5 11. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises at least 16 (e.g., 17, 18, 19, 20, 21, 22, 23, 24, 25, or 26) consecutive nucleotides of SEQ ID NO: 31, and base pairs or is complementary with at least 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides of the target domain.
- 10 12. The gRNA of any of the preceding embodiments, wherein said targeting domain is configured to provide a cleavage event (e.g., a single strand break or double strand break) within the target domain, e.g., immediately after nucleotide position 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 of the target domain.
- 15 13. A gRNA comprising a targeting domain, wherein the targeting domain comprises a sequence of SEQ ID NO: 21.
14. A gRNA comprising a targeting domain, wherein the targeting domain comprises a sequence of SEQ ID NO: 22.
- 20 15. A gRNA comprising a targeting domain, wherein the targeting domain comprises a sequence of SEQ ID NO: 23.
16. A gRNA comprising a targeting domain, wherein the targeting domain comprises a sequence of SEQ ID NO: 24.
- 25 17. A gRNA comprising a targeting domain, wherein the targeting domain comprises a sequence of SEQ ID NO: 25.
- 30 18. A gRNA comprising a targeting domain, wherein the targeting domain comprises a sequence of SEQ ID NO: 26.
19. A gRNA comprising a targeting domain, wherein the targeting domain comprises a sequence of SEQ ID NO: 27.

20. A gRNA comprising a targeting domain, wherein the targeting domain comprises a sequence of SEQ ID NO: 28.
- 5 21. A gRNA comprising a targeting domain, wherein the targeting domain comprises a sequence of SEQ ID NO: 29.
22. A gRNA comprising a targeting domain, wherein the targeting domain comprises a sequence of SEQ ID NO: 30.
- 10 23. A gRNA comprising a targeting domain, wherein the targeting domain comprises a sequence of SEQ ID NO: 48.
24. A gRNA comprising a targeting domain, wherein the targeting domain comprises a sequence of SEQ ID NO: 49.
- 15 25. A gRNA comprising a targeting domain, wherein the targeting domain comprises a sequence of SEQ ID NO: 50.
- 20 26. A gRNA comprising a targeting domain, wherein the targeting domain comprises a sequence of SEQ ID NO: 51.
27. A gRNA comprising a targeting domain, wherein the targeting domain comprises a sequence of Table 2 or 6.
- 25 28. A gRNA comprising a targeting domain, wherein the targeting domain comprises a sequence of Table 8 (e.g., a targeting domain of any of SEQ ID NOs: 1-10, 40, 42, 44, 46, 66-71, 73, 76, 77, 79-82, 85, 87, 88, 122, 133, 134, 135, 141-144, 153, 157, or 158).

29. A gRNA comprising a targeting domain capable of directing cleavage or editing of a target domain of Table 2 (e.g., a target domain of any of SEQ ID NOS: 1-10, 40, 42, 44, 46, 48).
- 5 30. A gRNA comprising a targeting domain capable of directing cleavage or editing of a target domain of Table 6 (e.g., a target domain of any of SEQ ID NOS: 8, 11, 14, or 66-258).
31. A gRNA comprising a targeting domain capable of directing cleavage or editing of a target domain of Table 8 (e.g., a target domain any of SEQ ID NOS: 1-10, 40, 42, 44, 46, 66-10 71, 73, 76, 77, 79-82, 85, 87, 88, 122, 133, 134, 135, 141-144, 153, 157, or 158).
32. The gRNA of any of the preceding embodiments, wherein the target domain is in exon 1, exon 2, exon 3, exon 4, exon 5, exon 6, exon 7, exon 8, exon 9, or exon 10 of the CD123 sequence of SEQ ID NO: 31.
- 15 33. The gRNA of any of the preceding embodiments, wherein the target domain is in exon 1, exon 2, exon 3, exon 4, exon 5, exon 6, exon 7, exon 8, exon 9, exon 10, exon 11, or exon 12 of the CD123 sequence of SEQ ID NO: 52.
- 20 34. The gRNA of any of the preceding embodiments, which is a single guide RNA (sgRNA).
35. The gRNA of any of the preceding embodiments, wherein the targeting domain is 16 nucleotides or more in length.
- 25 36. The gRNA of any of the preceding embodiments, wherein the targeting domain is 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or 26 nucleotides in length.
37. The gRNA of any of the preceding embodiments, wherein the targeting domain 30 comprises a sequence of any of SEQ ID NOS: 1-10, 21-30, 40, 42, 44, 46, or 48-51 or the reverse complement thereof, or a sequence having at least 90% or 95% identity to any of the foregoing, or a sequence having no more than 1, 2, or 3 mutations relative to any of the foregoing.

38. The gRNA of embodiment 37, wherein the 2 mutations are not adjacent to each other.
39. The gRNA of embodiment 37, wherein none of the 3 mutations are adjacent to each other.
- 5 40. The gRNA of any of embodiments 37-39, wherein the 1, 2, or 3 mutations are substitutions.
- 10 41. The gRNA of any of embodiments 37-39, wherein one or more of the mutations is an insertion or deletion.
42. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of any of SEQ ID NOS: 1-10, 40, 42, 44, or 46.
- 15 43. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of SEQ ID NO: 1.
44. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of SEQ ID NO: 2.
- 20 45. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of SEQ ID NO: 3.
46. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of SEQ ID NO: 4.
- 25 47. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of SEQ ID NO: 5.
- 30 48. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of SEQ ID NO: 6.
49. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of SEQ ID NO: 7.

50. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of SEQ ID NO: 8.
- 5 51. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of SEQ ID NO: 9.
52. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of SEQ ID NO: 10.
- 10 53. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of SEQ ID NO: 40.
54. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of SEQ ID NO: 42.
- 15 55. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of SEQ ID NO: 44.
- 20 56. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of SEQ ID NO: 46.
57. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of any of SEQ ID NOS: 1-10, 40, 42, 44, 46, 66-71, 73, 76, 77, 79-82,
- 25 85, 87, 88, 122, 133, 134, 135, 141-144, 153, 157, or 158.
58. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of any of SEQ ID NOS: 1-10, 40, 42, 44, or 46.
- 30 59. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of any of SEQ ID NOS: 8, 11, 14, or 66-258.
60. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of any of SEQ ID NOS: 21-30 or 48-51.

61. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of SEQ ID NO: 21.
- 5 62. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of SEQ ID NO: 22.
63. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of SEQ ID NO: 23.
- 10 64. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of SEQ ID NO: 24.
65. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of SEQ ID NO: 25.
- 15 66. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of SEQ ID NO: 26.
- 20 67. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of SEQ ID NO: 27.
68. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of SEQ ID NO: 28.
- 25 69. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of SEQ ID NO: 29.
70. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of SEQ ID NO: 30.
- 30 71. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of SEQ ID NO: 48.

72. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of SEQ ID NO: 49.
73. The gRNA of any of the preceding embodiments, wherein the targeting domain
5 comprises a sequence of SEQ ID NO: 50.
74. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of SEQ ID NO: 51.
- 10 75. The gRNA of any of the preceding embodiments, wherein the targeting domain comprises a sequence of any of SEQ ID NOS: 247 or 297-461.
76. The gRNA of any of the preceding embodiments, which comprises one or more chemical modifications (e.g., a chemical modification to a nucleobase, sugar, or backbone
15 portion).
77. The gRNA of any of the preceding embodiments, which comprises one or more 2'-methyl nucleotide, e.g., at a position described herein.
- 20 78. The gRNA of any of the preceding embodiments, which comprises one or more phosphorothioate or thioPACE linkage, e.g., at a position described herein.
79. The gRNA of any of the preceding embodiments, which binds a Cas9 molecule.
- 25 80. The gRNA of any one of the preceding embodiments, wherein the targeting domain is about 18-23, e.g., 20 nucleotides in length.
81. The gRNA of any of embodiments 1-80, which binds to a tracrRNA.
- 30 82. The gRNA of any of embodiments 1-80, which comprises a scaffold sequence.
83. The gRNA of any of the preceding embodiments, which comprises one or more of (e.g., all of):
a first complementarity domain;

- a linking domain;
a second complementarity domain which is complementary to the first complementarity domain;
a proximal domain; and
5 a tail domain.
84. The gRNA of any of the preceding embodiments, which comprises a first complementarity domain.
- 10 85. The gRNA of any of the preceding embodiments, which comprises a linking domain.
86. The gRNA of embodiment 84 or 85, which comprises a second complementarity domain which is complementary to the first complementarity domain
87. The gRNA of any of the preceding embodiments, which comprises a proximal
15 domain.
88. The gRNA of any of the preceding embodiments, which comprises a tail domain.
89. The gRNA of any of embodiments 83-88, wherein the targeting domain is
20 heterologous to one or more of (e.g., all of):
the first complementarity domain;
the linking domain;
the second complementarity domain which is complementary to the first complementarity domain;
25 the proximal domain; and
the tail domain.
90. The gRNA of any of the preceding embodiments, wherein the gRNA has editing frequency as measured by an ICE of 70-100, e.g., 75-100, 80-100, 85-100, 90-100, 95-100, or
30 at least 70, at least 75, at least 80, at least 85, at least 90, at least 95, at least 99, or at least 100.
91. The gRNA of any of embodiments 1-90, wherein the gRNA has an editing frequency as measured by ICE of 20-70, e.g., at least 25-70, at least 30-70, at least 35-70, at least 40-70, at

least 45-70, at least 50-70, at least 55-70, at least 60-70, at least 65-70, at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, at least 50, at least 55, at least 60, at least 65, or at least 70.

- 5 92. The gRNA of any of the preceding embodiments, wherein the gRNA has an editing frequency as measured by an ICE of at least 80.

93. The gRNA of any of the preceding embodiments, wherein the gRNA has an R^2 value of the editing frequency as measured by ICE of 0.8-1, e.g., 0.85-1, 0.9-1, 0.95-1, or at least 0.8,
10 at least 0.85, at least 0.9, at least 0.95, at least 0.98, at least 0.99, or at least 1.

94. The gRNA of any of the preceding embodiments, wherein the gRNA has an R^2 value of the editing frequency as measured by ICE of at least 0.85.

- 15 95. The gRNA of any of the preceding embodiments, wherein the gRNA has an editing frequency as measured by an ICE of at least 80 and an R^2 value of the editing frequency as measured by ICE of at least 0.85.

96. The gRNA of any of the preceding embodiments, wherein the gRNA has an editing
20 frequency, e.g., as measured by Sanger sequencing followed by ICE or TIDE analysis, of 70-100, e.g., 75-100, 80-100, 85-100, 90-100, 95-100, or at least 70, at least 75, at least 80, at least 85, at least 90, at least 95, at least 99, or at least 100.

97. The gRNA of any of the preceding embodiments, wherein the gRNA has an editing
25 frequency, e.g., as measured by Next Generation-Targeted Amplicon Sequencing (Amplicon sequencing), of 70-100, e.g., 75-100, 80-100, 85-100, 90-100, 95-100, or at least 70, at least 75, at least 80, at least 85, at least 90, at least 95, at least 99, or at least 100.

98. A kit or composition comprising:
30 a) a gRNA of any of embodiments 1-97, or a nucleic acid encoding the gRNA, and
b) a second gRNA, or a nucleic acid encoding the second gRNA.

99. The kit or composition of embodiment 98, wherein the first gRNA comprises a targeting domain that comprises a sequence of TTTCTTGAGCTGCAGCTGGG (SEQ ID NO: 7).
- 5 100. The kit or composition of embodiment 98, wherein the first gRNA comprises a targeting domain that comprises a sequence of AGTTCCCACATCCTGGTGCG (SEQ ID NO: 9).
- 10 101. The kit or composition of embodiment 98-100, wherein the second gRNA targets a lineage-specific cell-surface antigen.
102. The kit or composition of any of embodiments 98-101, wherein the second gRNA targets a lineage-specific cell-surface antigen other than CD123.
- 15 103. The kit or composition of any of embodiments 98-102, wherein the second gRNA targets CD33, e.g., wherein the second gRNA comprises a targeting domain that comprises a sequence of CCCCAGGACTACTCACTCCT (SEQ ID NO: 64).
- 20 104. The kit or composition of any of embodiments 98-102, wherein the second gRNA targets CLL-1 (e.g., wherein the second gRNA comprises a targeting domain that comprises a sequence of GGTGGCTATTGTTTGCAGTG (SEQ ID NO: 65).
105. The kit or composition of any of embodiments 98-104, wherein the second gRNA comprises a targeting domain that comprises a sequence of Table A.
- 25 106. The kit or composition of any of embodiments 98-105, wherein the gRNA of (a) comprises a targeting domain that comprises a sequence of TTTCTTGAGCTGCAGCTGGG (SEQ ID NO: 7) and the second gRNA comprises a targeting domain that comprises a sequence of AGTTCCCACATCCTGGTGCG (SEQ ID NO: 9).
- 30 107. The kit or composition of any of embodiments 98-105, wherein the gRNA of (a) comprises a targeting domain that comprises a sequence of TTTCTTGAGCTGCAGCTGGG (SEQ ID NO: 7) and the second gRNA comprises a targeting domain that comprises a sequence of CCCCAGGACTACTCACTCCT (SEQ ID NO: 64).

108. The kit or composition of any of embodiments 98-105, wherein the gRNA of (a) comprises a targeting domain that comprises a sequence of TTTCTTGAGCTGCAGCTGGG (SEQ ID NO: 7) and the second gRNA comprises a targeting domain that comprises a
5 sequence of GGTGGCTATTGTTTGCAGTG (SEQ ID NO: 65).

109. The kit or composition of any of embodiments 98-105, wherein the gRNA of (a) comprises a targeting domain that comprises a sequence of AGTTCACACATCCTGGTGCG (SEQ ID NO: 9) and the second gRNA comprises a targeting domain that comprises a
10 sequence of CCCAGGACTACTCACTCCT (SEQ ID NO: 64).

110. The kit or composition of any of embodiments 98-105, wherein the gRNA of (a) comprises a targeting domain that comprises a sequence of AGTTCACACATCCTGGTGCG (SEQ ID NO: 9) and the second gRNA comprises a targeting domain that comprises a
15 sequence of GGTGGCTATTGTTTGCAGTG (SEQ ID NO: 65).

111. The kit or composition of any of embodiments 98-110, which further comprises a third gRNA, or a nucleic acid encoding the third gRNA.

20 112. The kit or composition of embodiment 111, wherein the third gRNA targets a lineage-specific cell-surface antigen.

113. The kit or composition of embodiment 111, wherein the third gRNA targets CD33, CLL-1, or CD123.

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114. The kit or composition of any of embodiments 111-113, wherein the gRNA of (a) comprises a targeting domain that comprises a sequence of TTTCTTGAGCTGCAGCTGGG (SEQ ID NO: 7), the second gRNA comprises a targeting domain that comprises a sequence of CCCAGGACTACTCACTCCT (SEQ ID NO: 64), and the third gRNA comprises a
30 targeting domain that comprises a sequence of GGTGGCTATTGTTTGCAGTG (SEQ ID NO: 65).

115. The kit or composition of any of embodiments 111-113, wherein the gRNA of (a) comprises a targeting domain that comprises a sequence of AGTTCACACATCCTGGTGCG

(SEQ ID NO: 9), the second gRNA comprises a targeting domain that comprises a sequence of CCCAGGACTACTCACTCCT (SEQ ID NO: 64), and the third gRNA comprises a targeting domain that comprises a sequence of GGTGGCTATTGTTTGCAGTG (SEQ ID NO: 65).

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116. The kit or composition of any of embodiments 111-113, wherein the gRNA of (a) comprises a targeting domain that comprises a sequence of TTTCTTGAGCTGCAGCTGGG (SEQ ID NO: 7), the second gRNA comprises a targeting domain that comprises a sequence of AGTTCCCACATCCTGGTGCG (SEQ ID NO: 9), and the third gRNA comprises a targeting domain that comprises a sequence of GGTGGCTATTGTTTGCAGTG (SEQ ID NO: 65).

117. The kit or composition of any of embodiments 111-113, wherein the gRNA of (a) comprises a targeting domain that comprises a sequence of TTTCTTGAGCTGCAGCTGGG (SEQ ID NO: 7), the second gRNA comprises a targeting domain that comprises a sequence of AGTTCCCACATCCTGGTGCG (SEQ ID NO: 9), and the third gRNA comprises a targeting domain that comprises a sequence of CCCAGGACTACTCACTCCT (SEQ ID NO: 64).

118. The kit or composition of any of embodiments 111-117, which further comprises a fourth gRNA, or a nucleic acid encoding the fourth gRNA.

119. The kit or composition of embodiment 118, wherein the fourth gRNA targets a lineage-specific cell-surface antigen.

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120. The kit or composition of embodiment 118, wherein the fourth gRNA targets CD33, CLL-1, or CD123.

121. The kit or composition of any of embodiments 118-120, wherein the gRNA of (a) comprises a targeting domain that comprises a sequence of TTTCTTGAGCTGCAGCTGGG (SEQ ID NO: 7), the second gRNA comprises a targeting domain that comprises a sequence of AGTTCCCACATCCTGGTGCG (SEQ ID NO: 9), the third gRNA comprises a targeting domain that comprises a sequence of CCCAGGACTACTCACTCCT (SEQ ID NO: 64),

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and the fourth gRNA comprises a targeting domain that comprises a sequence of GGTGGCTATTGTTTGCAGTG (SEQ ID NO: 65).

122. The kit or composition of any of embodiments 118-121, wherein the gRNA of (a), the second gRNA, the third gRNA, and the fourth gRNA are admixed.

123. The kit or composition of any of embodiments 118-121, wherein the gRNA of (a), the second gRNA, the third gRNA, and the fourth gRNA are in separate containers.

124. The kit or composition of any of embodiments 98-121, wherein (a) and (b) are admixed.

125. The kit or composition of any of embodiments 98-121, wherein (a) and (b) are in separate containers.

126. The kit or composition of any of embodiments 98-125, wherein the nucleic acid of (a) and the nucleic acid of (b) are part of the same nucleic acid.

127. The kit or composition of any of embodiments 98-125, wherein the nucleic acid of (a) and the nucleic acid of (b) are separate nucleic acids.

128. A genetically engineered hematopoietic cell (e.g., hematopoietic stem or progenitor cell), which comprises:

(a) a mutation at a target domain of Table 1 (e.g., a target domain of any of SEQ ID NOS: 1-10, 40, 42, 44, or 46); and

(b) a second mutation at a gene encoding a lineage-specific cell surface antigen other than CD123.

129. The genetically engineered hematopoietic cell of embodiment 128, wherein the mutation of (a) is at a target domain of SEQ ID NO: 1.

130. The genetically engineered hematopoietic cell of embodiment 128, wherein the mutation of (a) is at a target domain of SEQ ID NO: 2.

131. The genetically engineered hematopoietic cell of embodiment 128, wherein the mutation of (a) is at a target domain of SEQ ID NO: 3.

5 132. The genetically engineered hematopoietic cell of embodiment 128, wherein the mutation of (a) is at a target domain of SEQ ID NO: 4.

133. The genetically engineered hematopoietic cell of embodiment 128, wherein the mutation of (a) is at a target domain of SEQ ID NO: 5.

10 134. The genetically engineered hematopoietic cell of embodiment 128, wherein the mutation of (a) is at a target domain of SEQ ID NO: 6.

135. The genetically engineered hematopoietic cell of embodiment 128, wherein the mutation of (a) is at a target domain of SEQ ID NO: 7.
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136. The genetically engineered hematopoietic cell of embodiment 128, wherein the mutation of (a) is at a target domain of SEQ ID NO: 8.

137. The genetically engineered hematopoietic cell of embodiment 128, wherein the mutation of (a) is at a target domain of SEQ ID NO: 9.
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138. The genetically engineered hematopoietic cell of embodiment 128, wherein the mutation of (a) is at a target domain of SEQ ID NO: 10.

25 139. The genetically engineered hematopoietic cell of embodiment 128, wherein the mutation of (a) is at a target domain of SEQ ID NO: 40.

140. The genetically engineered hematopoietic cell of embodiment 128, wherein the mutation of (a) is at a target domain of SEQ ID NO: 42.
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141. The genetically engineered hematopoietic cell of embodiment 128, wherein the mutation of (a) is at a target domain of SEQ ID NO: 44.

142. The genetically engineered hematopoietic cell of embodiment 128, wherein the mutation of (a) is at a target domain of SEQ ID NO: 46.

143. The genetically engineered hematopoietic cell of embodiment 128, wherein the mutation of (a) is at a target domain of any of Tables 2 or 6.

144. The genetically engineered hematopoietic cell of embodiment 128, wherein the mutation of (a) is at a target domain of Table 8 (e.g., a target domain any of SEQ ID NOS: 1-10, 40, 42, 44, 46, 66-71, 73, 76, 77, 79-82, 85, 87, 88, 122, 133, 134, 135, 141-144, 153, 157, or 158).

145. The genetically engineered hematopoietic cell of any of embodiments 128-144, wherein the mutation of (a) comprises an insertion, a deletion, or a substitution (e.g., a single nucleotide variant).

146. The genetically engineered cell of embodiment 145, wherein the deletion is fully within the target domain of any of SEQ ID NOS: 1-20 or 40-47.

147. The genetically engineered cell of embodiment 100, wherein the deletion is 1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16, or 17 nucleotides in length.

148. The genetically engineered cell of embodiment 145, wherein the deletion has one or both endpoints outside of the target domain of any of SEQ ID NOS: 1-20 or 40-47.

149. The genetically engineered cell of any of embodiments 145-148, wherein the mutation results in a frameshift.

150. The genetically engineered hematopoietic cell of any of embodiments 145-148, wherein the second mutation comprises an insertion, a deletion, or a substitution (e.g., a single nucleotide variant).

151. The genetically engineered hematopoietic cell of any of embodiments 145-148, which comprises an insertion of 1 nt or 2 nt, or a deletion of 1 nt, 2 nt, 3 nt, or 4 nt in CD123.

152. The genetically engineered hematopoietic cell of any of embodiments 145-148, which comprises an indel as described herein, e.g., an indel produced by or producible by a gRNA described herein (e.g., any of gRNA A, gRNA G, gRNA I, gRNA N3, gRNA P3, gRNA S3, or gRNA D1).

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153. The genetically engineered hematopoietic cell of any of embodiments 145-148, which comprises an indel produced by or producible by a CRISPR system described herein, e.g., a method of Example 1, 2, 3, or 4.

10 154. Use of a gRNA of any of embodiments 1-97 or a composition or kit of any of embodiments 98-127 for reducing expression of CD123 in a sample of hematopoietic cells stem or progenitor cells using a CRISPR/Cas9 system.

15 155. Use of a CRISPR/Cas9 system for reducing expression of CD123 in a sample of hematopoietic cells stem or progenitor cells, wherein the gRNA of the CRISPR/Cas9 system is a gRNA of any of embodiments 1-98, or gRNAs of a composition or kit of any of embodiments 98-127.

156. A method of producing a genetically engineered cell, comprising:
20 (i) providing a cell (e.g., a hematopoietic stem or progenitor cell, e.g., a wild-type hematopoietic stem or progenitor cell), and
(ii) introducing into the cell (a) a guide RNA (gRNA) of any of the preceding embodiments 1-98 or gRNAs of a composition or kit of any of embodiments 98-127; and (b) an endonuclease that binds the gRNA (e.g., a Cas9 molecule),
25 thereby producing the genetically engineered cell.

157. A method of producing a genetically engineered cell, comprising:
(i) providing a cell (e.g., a hematopoietic stem or progenitor cell, e.g., a wild-type hematopoietic stem or progenitor cell), and
30 (ii) introducing into the cell (a) a gRNA of any of embodiments 1-97 or gRNAs of a composition or kit of any of embodiments 98-127; and (b) a Cas9 molecule that binds the gRNA,
thereby producing the genetically engineered cell.

158. The method or use of any of embodiments 154-157 which results in the genetically engineered hematopoietic stem or progenitor cell having a reduced expression level of CD123 as compared with a wild-type counterpart cell.
- 5 159. The method or use of any of embodiments 154-158, which results in the genetically engineered hematopoietic stem or progenitor cell having a reduced expression level of CD123 that is less than 20% of the level of CD123 in a wild-type counterpart cell.
- 10 160. The method or use of any of embodiments 154-159, which is performed on a plurality of hematopoietic stem or progenitor cells.
161. The method or use of any of embodiments 154-160, which is performed on a cell population comprising a plurality of hematopoietic stem cells and a plurality of hematopoietic progenitor cells.
- 15 162. The method or use of any of embodiments 154-161, which produces a cell population according to any of embodiments 284-386 or 389-391.
- 20 163. The method of any of embodiments 156-162, wherein the nucleic acids of (a) and (b) are encoded on one vector, which is introduced into the cell.
164. The method of embodiment 163, wherein the vector is a viral vector.
- 25 165. The method of any of embodiments 156-163, wherein (a) and (b) are introduced into the cell as a pre-formed ribonucleoprotein complex.
166. The method of embodiment 165, wherein the ribonucleoprotein complex is introduced into the cell via electroporation.
- 30 167. The method of any of embodiments 156-166, wherein the endonuclease (e.g., a Cas9 molecule) is introduced into the cell by delivering into the cell a nucleic acid molecule (e.g., an mRNA molecule or a viral vector, e.g., AAV) encoding the endonuclease.

168. The method of any of embodiments 162-167, wherein the cell (e.g., the hematopoietic stem or progenitor cell) is CD34+.

169. The method of any of embodiments 162-168, wherein cell viability of a population of the cells is at least 80%, 90%, 95%, or 98% of the cell viability of control cells (e.g., mock electroporated cells) with 48 hours after introduction of the gRNA into the cells.

170. The method of any of embodiments 162-169, wherein at least 80%, 85%, 90%, 95%, or 98% of cells in the population are viable 48 hours after introduction of the gRNA into the cells.

171. The method of any of embodiments 162-170, wherein the hematopoietic stem or progenitor cell is from bone marrow cells or peripheral blood mononuclear cells (PBMCs) of a subject.

172. The method of any of embodiments 162-171, wherein the subject has a hematopoietic disorder, e.g., a hematopoietic malignancy, e.g., a leukemia (e.g., AML), blastic plasmacytoid dendritic cell neoplasm (BPDCN), acute lymphoblastic leukemia (ALL), or hairy cell leukemia.

173. The method of any of embodiments 162-172, wherein the subject has a hematopoietic disorder, e.g., a hematopoietic malignancy, e.g., a leukemia, e.g., AML.

174. The method of any of embodiments 162-172, wherein the subject has a hematological disorder, e.g., a precancerous condition, e.g., a myelodysplasia, a myelodysplastic syndrome (MDS), or a preleukemia.

175. The method of any of embodiments 162-174, wherein the subject has a cancer, wherein cells of the cancer express CD123 (e.g., wherein at least a plurality of the cancer cells express CD123).

176. The method or use of any of embodiments 154-175, which results in a mutation that causes a reduced expression level of CD123 as compared with a wild-type counterpart cell.

177. The method or use of any of embodiments 154-176, which results in a mutation that causes a reduced expression level of wild-type CD123 as compared with a wild-type counterpart cell.
- 5 178. The method or use of any of embodiments 154-177, which produces a genetically engineered hematopoietic stem or progenitor cell having a reduced expression level of CD123 as compared with a wild-type counterpart cell.
- 10 179. The method or use of any of embodiments 154-178, which produces a genetically engineered hematopoietic stem or progenitor cell having a reduced expression level of wild-type CD123 as compared with a wild-type counterpart cell.
- 15 180. A genetically engineered hematopoietic stem or progenitor cell, which is produced by a method of any of embodiments 154-179.
181. A nucleic acid (e.g., DNA) encoding the gRNA of any of embodiments 1-97.
- 20 182. A genetically engineered cell (e.g., a hematopoietic stem or progenitor cell), which comprises a mutation at a target domain of Table 1 (e.g., a target domain of any of SEQ ID NOS: 1-20), e.g., wherein the mutation is a result of the genetic engineering.
- 25 183. A genetically engineered cell (e.g., a hematopoietic stem or progenitor cell), which comprises a mutation at a target domain of Table 6 (e.g., a target domain of any of SEQ ID NOS: 8, 11, 14, or 66-258), e.g., wherein the mutation is a result of the genetic engineering.
- 30 184. A genetically engineered cell (e.g., a hematopoietic stem or progenitor cell), which comprises a mutation at a target domain of Table 8 (e.g., a target domain of any of SEQ ID NOS: 1-10, 40, 42, 44, 46, 66-71, 73, 76, 77, 79-82, 85, 87, 88, 122, 133, 134, 135, 141-144, 153, 157, or 158), e.g., wherein the mutation is a result of the genetic engineering.
185. A genetically engineered cell (e.g., a hematopoietic stem or progenitor cell), which comprises a mutation within 100, 90, 80, 70, 60, 50, 40, 30, 20, or 10 nucleotides (upstream or downstream) of a target domain of Table 1 (e.g., a target domain of any of SEQ ID NOS: 1-20 or 40-47).

186. The genetically engineered cell of embodiment 185, wherein the mutation is within 100, 90, 80, 70, 60, 50, 40, 30, 20, or 10 nucleotides (upstream or downstream) of any of SEQ ID NOS: 1, 7, or 9.

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187. The genetically engineered cell of embodiment 185, wherein the mutation is within 100, 90, 80, 70, 60, 50, 40, 30, 20, or 10 nucleotides downstream of SEQ ID NO: 9.

188. The genetically engineered cell of embodiment 185, wherein the mutation is within 100, 90, 80, 70, 60, 50, 40, 30, 20, or 10 nucleotides upstream of SEQ ID NO: 9.

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189. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 1.

190. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 1, wherein the mutation results in a reduced expression level of CD123 as compared with a wild-type counterpart cell.

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191. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 1, wherein the mutation results in a reduced expression level of CD123 that is less than 20% of the level of CD123 in a wild-type counterpart cell.

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192. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 2.

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193. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 2, wherein the mutation results in a reduced expression level of CD123 as compared with a wild-type counterpart cell.

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194. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 2, wherein the mutation results in a reduced expression level of CD123 that is less than 20% of the level of CD123 in a wild-type counterpart cell.

195. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 3.
- 5 196. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 3, wherein the mutation results in a reduced expression level of CD123 as compared with a wild-type counterpart cell.
- 10 197. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 3, wherein the mutation results in a reduced expression level of CD123 that is less than 20% of the level of CD123 in a wild-type counterpart cell.
- 15 198. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 4.
- 20 199. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 4, wherein the mutation results in a reduced expression level of CD123 as compared with a wild-type counterpart cell.
- 25 200. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 4, wherein the mutation results in a reduced expression level of CD123 that is less than 20% of the level of CD123 in a wild-type counterpart cell.
- 30 201. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 5.
202. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 5, wherein the mutation results in a reduced expression level of CD123 as compared with a wild-type counterpart cell.
203. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 5, wherein the mutation results in a reduced

expression level of CD123 that is less than 20% of the level of CD123 in a wild-type counterpart cell.

204. A genetically engineered hematopoietic stem or progenitor cell, which comprises a
5 mutation at a target domain of SEQ ID NO: 6.

205. A genetically engineered hematopoietic stem or progenitor cell, which comprises a
mutation at a target domain of SEQ ID NO: 6, wherein the mutation results in a reduced
expression level of CD123 as compared with a wild-type counterpart cell.
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206. A genetically engineered hematopoietic stem or progenitor cell, which comprises a
mutation at a target domain of SEQ ID NO: 6, wherein the mutation results in a reduced
expression level of CD123 that is less than 20% of the level of CD123 in a wild-type
counterpart cell.
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207. A genetically engineered hematopoietic stem or progenitor cell, which comprises a
mutation at a target domain of SEQ ID NO: 7.

208. A genetically engineered hematopoietic stem or progenitor cell, which comprises a
20 mutation at a target domain of SEQ ID NO: 7, wherein the mutation results in a reduced
expression level of CD123 as compared with a wild-type counterpart cell.

209. A genetically engineered hematopoietic stem or progenitor cell, which comprises a
mutation at a target domain of SEQ ID NO: 7, wherein the mutation results in a reduced
25 expression level of CD123 that is less than 20% of the level of CD123 in a wild-type
counterpart cell.

210. A genetically engineered hematopoietic stem or progenitor cell, which comprises a
mutation at a target domain of SEQ ID NO: 8.
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211. A genetically engineered hematopoietic stem or progenitor cell, which comprises a
mutation at a target domain of SEQ ID NO: 8, wherein the mutation results in a reduced
expression level of CD123 as compared with a wild-type counterpart cell.

212. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 8, wherein the mutation results in a reduced expression level of CD123 that is less than 20% of the level of CD123 in a wild-type counterpart cell.

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213. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 9.

214. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 9, wherein the mutation results in a reduced expression level of CD123 as compared with a wild-type counterpart cell.

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215. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 9, wherein the mutation results in a reduced expression level of CD123 that is less than 20% of the level of CD123 in a wild-type counterpart cell.

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216. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 10.

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217. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 10, wherein the mutation results in a reduced expression level of CD123 as compared with a wild-type counterpart cell.

218. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 10, wherein the mutation results in a reduced expression level of CD123 that is less than 20% of the level of CD123 in a wild-type counterpart cell.

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219. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 40.

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220. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 40, wherein the mutation results in a reduced expression level of CD123 as compared with a wild-type counterpart cell.
- 5 221. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 40, wherein the mutation results in a reduced expression level of CD123 that is less than 20% of the level of CD123 in a wild-type counterpart cell.
- 10 222. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 42.
223. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 42, wherein the mutation results in a reduced expression level of CD123 as compared with a wild-type counterpart cell.
- 15 224. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 42, wherein the mutation results in a reduced expression level of CD123 that is less than 20% of the level of CD123 in a wild-type counterpart cell.
- 20 225. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 44.
226. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 44, wherein the mutation results in a reduced expression level of CD123 as compared with a wild-type counterpart cell.
- 25 227. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 44, wherein the mutation results in a reduced expression level of CD123 that is less than 20% of the level of CD123 in a wild-type counterpart cell.
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228. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 46.

229. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 46, wherein the mutation results in a reduced expression level of CD123 as compared with a wild-type counterpart cell.

230. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of SEQ ID NO: 46, wherein the mutation results in a reduced expression level of CD123 that is less than 20% of the level of CD123 in a wild-type counterpart cell.

231. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of any of SEQ ID NO: 66-71, 73, 76, 77, 79-82, 85, 87, 88, 122, 133, 134, 135, 141-144, 153, 157, or 158.

232. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of any of SEQ ID NOs: 66-71, 73, 76, 77, 79-82, 85, 87, 88, 122, 133, 134, 135, 141-144, 153, 157, or 158, wherein the mutation results in a reduced expression level of CD123 as compared with a wild-type counterpart cell.

233. A genetically engineered hematopoietic stem or progenitor cell, which comprises a mutation at a target domain of any of SEQ ID NOs: 66-71, 73, 76, 77, 79-82, 85, 87, 88, 122, 133, 134, 135, 141-144, 153, 157, or 158, wherein the mutation results in a reduced expression level of CD123 that is less than 20% of the level of CD123 in a wild-type counterpart cell.

234. A genetically engineered cell (e.g., a hematopoietic stem or progenitor cell), which comprises a mutation at a target domain of SEQ ID NO: 20.

235. A genetically engineered cell (e.g., a hematopoietic stem or progenitor cell), which comprises a mutation within 20 nucleotides (upstream or downstream) of a target domain of SEQ ID NO: 20.

236. The genetically engineered cell of any of embodiments 182-235, comprising a predicted off target site which does not comprise a mutation or sequence change relative to the sequence of the site prior to gene editing of CD123.
- 5 237. The genetically engineered cell of any of embodiments 182-236, comprising two predicted off target sites which do not comprise a mutation or sequence change relative to the sequence of the site prior to gene editing of CD123.
238. The genetically engineered cell of any of embodiments 182-237, comprising at least
10 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 predicted off target sites which do not comprise a mutation or sequence change relative to the sequence of the site prior to gene editing of CD123.
239. The genetically engineered cell of any of embodiments 128-153, 180, or 182-238, which
15 does not comprise a mutation in any predicted off-target site, e.g., in any site in the human genome having 1, 2, 3, or 4 mismatches relative to the target domain.
240. The genetically engineered cell of any of embodiments 128-153, 180, or 182-239, which
20 does not comprise a mutation in any site in the human genome having 1 mismatch relative to the target domain.
241. The genetically engineered cell of any of embodiments 128-153, 180, or 182-240, which
25 does not comprise a mutation in any site in the human genome having 1 or 2 mismatches relative to the target domain.
242. The genetically engineered cell of any of embodiments 128-153, 180, or 182-241, which
does not comprise a mutation in any site in the human genome having 1, 2, or 3 mismatches relative to the target domain.
- 30 243. The genetically engineered cell of any of embodiments 128-153, 180, or 182-242, which does not comprise a mutation in any site in the human genome having 1, 2, 3, or 4 mismatches relative to the target domain.

244. The genetically engineered cell of any of embodiments 239-243, wherein the mutation comprises an insertion, a deletion, or a substitution (e.g., a single nucleotide variant).

245. The genetically engineered cell of embodiment 244, wherein the deletion is fully
5 within the target domain of any of SEQ ID NOS: 1-20, 40-47, 66-71, 73, 76, 77, 79-82, 85, 87, 88, 122, 133, 134, 135, 141-144, 153, 157, or 158.

246. The genetically engineered cell of embodiment 244-245, wherein the deletion is 1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16, or 17 nucleotides in length.

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247. The genetically engineered cell of embodiment 244, wherein the deletion has one or both endpoints outside of the target domain of any of SEQ ID NOS: 1-20, 40-47, 66-71, 73, 76, 77, 79-82, 85, 87, 88, 122, 133, 134, 135, 141-144, 153, 157, or 158.

15 248. The genetically engineered cell of any of embodiments 128-153, 180, or 182-247, wherein the mutation results in a frameshift.

249. The genetically engineered cell (e.g., hematopoietic stem or progenitor cell) of any of embodiments 128-153, 180, or 182-248, wherein the mutation results in a reduced expression
20 level of wild-type CD123 as compared with a wild-type counterpart cell (e.g., less than 50%, 40%, 30%, 20%, 15%, 10%, or 5% of the level in the wild-type counterpart cell).

250. The genetically engineered cell of any of embodiments 128-153, 180, or 182-249, wherein the cell has a reduced level of wild-type CD123 protein as compared with a wild-
25 type counterpart cell (e.g., less than 50%, 40%, 30%, 20%, 15%, 10%, or 5% of the level in the wild-type counterpart cell).

251. The genetically engineered cell of any of embodiments 128-153, 180, or 182-250, which does not express CD123.

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252. The genetically engineered cell (e.g., hematopoietic stem or progenitor cell) of any of embodiments 128-153, 180, or 182-251, wherein the mutation results in a lack of expression of CD123.

253. The genetically engineered cell (e.g., hematopoietic stem or progenitor cell) of any of embodiments 128-153, 180, or 182-252, which expresses less than 20% of the CD123 expressed by a wild-type counterpart cell.

- 5 254. The genetically engineered cell (e.g., hematopoietic stem or progenitor cell) of any of embodiments 128-153, 180, or 182-253, wherein the reduced expression level of CD123 is in a cell differentiated from (e.g., terminally differentiated from) the hematopoietic stem or progenitor cell, and the wild-type counterpart cell is a cell differentiated from (e.g., terminally differentiated from) a wild-type hematopoietic stem or progenitor cell.

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255. The genetically engineered cell (e.g., hematopoietic stem or progenitor cell) of embodiment 254, wherein the cell differentiated from the hematopoietic stem or progenitor cell is a myeloblast, monocyte, or myeloid dendritic cell.

- 15 256. The genetically engineered cell of any of embodiments 128-153, 180, or 182-253, which is CD34+.

257. The genetically engineered cell of any of embodiments 128-153, 180, or 182-256, which is from bone marrow cells or peripheral blood mononuclear cells of a subject.

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258. The genetically engineered cell of embodiment 257, wherein the subject is a human patient having a hematopoietic malignancy, e.g., AML.

- 25 259. The genetically engineered cell of embodiment 257, wherein the subject is a human patient having a hematological disorder, e.g., a precancerous condition, e.g., a myelodysplasia, a myelodysplastic syndrome (MDS), or a preleukemia.

- 30 260. The genetically engineered cell of any of embodiments 257-259, wherein the subject has a cancer, wherein cells of the cancer express CD123 (e.g., wherein at least a plurality of the cancer cells express CD123).

261. The genetically engineered cell of embodiment 257, wherein the subject is a healthy human donor (e.g., an HLA-matched donor).

262. The genetically engineered cell of any of embodiments 128-153, 180, or 182-261, which further comprises a nuclease chosen from a CRISPR endonuclease, a zinc finger nuclease (ZFN), a transcription activator-like effector-based nuclease (TALEN), or a meganuclease, or a nucleic acid (e.g., DNA or RNA) encoding the nuclease, wherein
5 optionally the nuclease is specific for CD123.

263. The genetically engineered cell of any of embodiments 128-153, 180, or 182-262, which further comprises a gRNA (e.g., a single guide RNA) specific for CD123, or a nucleic acid encoding the gRNA.
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264. The genetically engineered cell of embodiment 263, wherein the gRNA is a gRNA described herein, e.g., a gRNA of any of embodiments 1-98.

265. The genetically engineered cell of any of embodiments 128-153, 180, or 182-264, which was made by a process comprising contacting the cell with a nuclease chosen from a CRISPR endonuclease, a zinc finger nuclease (ZFN), a transcription activator-like effector-based nuclease (TALEN), or a meganuclease (e.g., by contacting the cell with the nuclease or a nucleic acid encoding the nuclease).
15

266. The genetically engineered cell of any of embodiments 128-153, 180, or 182-264, which was made by a process comprising contacting the cell with a nickase or a catalytically inactive Cas9 molecule (dCas9), e.g., fused to a function domain (e.g., by contacting the cell with the nuclease or a nucleic acid encoding the nuclease).
20

267. The genetically engineered cell of any of embodiments 128-153, 180, or 182-266, in which both copies of CD123 are mutant.
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268. The genetically engineered cell of embodiment 267, wherein both copies of CD123 have the same mutation.
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269. The genetically engineered cell of embodiment 267, wherein the copies of CD123 have different mutations.

270. The genetically engineered cell of any of embodiments 128-153, 180, or 182-269, comprising a first copy of CD123 having a first mutation and a second copy of CD123 having a second mutation, wherein the first and second mutations are different.

5 271. The genetically engineered cell of embodiment 270, wherein the first copy of CD123 comprises a first deletion.

272. The genetically engineered cell of embodiment 270 or 271, wherein the second copy of CD123 comprises a second deletion.

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273. The genetically engineered cell of any of embodiments 270-272, wherein the first and second deletions overlap.

15 274. The genetically engineered cell of any of embodiments 270-273, wherein an endpoint of the first deletion is within the second deletion.

275. The genetically engineered cell of any of embodiments 270-274, wherein both endpoints of the first deletion are within the second deletion.

20 276. The genetically engineered cell of any of embodiments 270-272, wherein the first and second deletion share an endpoint.

277. The genetically engineered cell of any of embodiments 128-153, 180, or 182-276, wherein the first and second mutations are each independently selected from: an insertion of
25 1 nt or 2 nt, or a deletion of 1 nt, 3, 2 nt, or 4 nt.

278. The genetically engineered cell of any of embodiments 128-153, 180, or 182-277, which is capable of forming a BFU-E colony, a CFU-G colony, a CFU-M colony, a CFU-GM colony, or a CFU-GEMM colony.

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279. The genetically engineered cell of any of embodiments 128-153, 180, or 182-278, which is capable of producing a cytokine, e.g., an inflammatory cytokine, e.g., IL-6, TNF- α , IL-1 β , or MIP-1 α .

280. The genetically engineered cell of any of embodiments 128-153, 180, or 182-279, which is capable of producing a cytokine, e.g., an inflammatory cytokine, e.g., IL-6, TNF- α , IL-1 β , or MIP-1 α , at a level comparable to an otherwise similar cell that is CD123 wildtype.
- 5 281. The genetically engineered cell of any of embodiments 128-153, 180, or 182-280, which is capable of producing a cytokine, e.g., an inflammatory cytokine, e.g., IL-6, TNF- α , IL-1 β , or MIP-1 α , at a level that is at least 70%, 80%, 85%, 90%, or 95% of the levels produced by an otherwise similar cell that is CD123 wildtype.
- 10 282. The genetically engineered cell of any of embodiments 279-281, which is capable of producing the cytokine when simulated with a TLR agonist, e.g., LPS or R848, e.g., as described in Example 5.
- 15 283. The genetically engineered cell of any of embodiments 279-281, which is capable of phagocytosis.
284. A cell population, comprising a plurality of the genetically engineered hematopoietic stem or progenitor cells of any embodiments 128-153, 180, or 182-283(e.g., comprising hematopoietic stem cells, hematopoietic progenitor cells, or a combination thereof).
- 20 285. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 1.
286. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 1, wherein the mutation results in a reduced expression level of CD123 as compared with a wild-type counterpart cell population.
- 25 287. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 1, wherein the mutation results in a reduced expression level of CD123 that is less than 20% of the level of CD123 in a wild-type counterpart cell population.
- 30

288. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 2.

289. A cell population, comprising a plurality of genetically engineered hematopoietic stem
5 or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 2, wherein the mutation results in a reduced expression level of CD123 as compared with a wild-type counterpart cell population.

290. A cell population, comprising a plurality of genetically engineered hematopoietic stem
10 or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 2, wherein the mutation results in a reduced expression level of CD123 that is less than 20% of the level of CD123 in a wild-type counterpart cell population.

291. A cell population, comprising a plurality of genetically engineered hematopoietic stem
15 or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 3.

292. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 3, wherein the mutation results in a reduced expression level of CD123 as compared with a wild-type
20 counterpart cell population.

293. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 3, wherein the mutation results in a reduced expression level of CD123 that is less than 20% of the level
25 of CD123 in a wild-type counterpart cell population.

294. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 4.

30 295. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 4, wherein the mutation results in a reduced expression level of CD123 as compared with a wild-type counterpart cell population.

296. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 4, wherein the mutation results in a reduced expression level of CD123 that is less than 20% of the level of CD123 in a wild-type counterpart cell population.

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297. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 5.

298. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 5, wherein the mutation results in a reduced expression level of CD123 as compared with a wild-type counterpart cell population.

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299. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 5, wherein the mutation results in a reduced expression level of CD123 that is less than 20% of the level of CD123 in a wild-type counterpart cell population.

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300. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 6.

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301. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 6, wherein the mutation results in a reduced expression level of CD123 as compared with a wild-type counterpart cell population.

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302. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 6, wherein the mutation results in a reduced expression level of CD123 that is less than 20% of the level of CD123 in a wild-type counterpart cell population.

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303. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 7.

304. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 7, wherein the mutation results in a reduced expression level of CD123 as compared with a wild-type counterpart cell population.

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305. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 7, wherein the mutation results in a reduced expression level of CD123 that is less than 20% of the level of CD123 in a wild-type counterpart cell population.

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306. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 8.

307. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 8, wherein the mutation results in a reduced expression level of CD123 as compared with a wild-type counterpart cell population.

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308. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 8, wherein the mutation results in a reduced expression level of CD123 that is less than 20% of the level of CD123 in a wild-type counterpart cell population.

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309. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 9.

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310. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 9, wherein the mutation results in a reduced expression level of CD123 as compared with a wild-type counterpart cell population.

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311. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 9, wherein

the mutation results in a reduced expression level of CD123 that is less than 20% of the level of CD123 in a wild-type counterpart cell population.

5 312. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 10.

10 313. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 10, wherein the mutation results in a reduced expression level of CD123 as compared with a wild-type counterpart cell population.

15 314. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 10, wherein the mutation results in a reduced expression level of CD123 that is less than 20% of the level of CD123 in a wild-type counterpart cell population.

315. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 40.

20 316. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 40, wherein the mutation results in a reduced expression level of CD123 as compared with a wild-type counterpart cell population.

25 317. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 40, wherein the mutation results in a reduced expression level of CD123 that is less than 20% of the level of CD123 in a wild-type counterpart cell population.

30 318. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 42.

319. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 42, wherein

the mutation results in a reduced expression level of CD123 as compared with a wild-type counterpart cell population.

5 320. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 42, wherein the mutation results in a reduced expression level of CD123 that is less than 20% of the level of CD123 in a wild-type counterpart cell population.

10 321. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 44.

15 322. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 44, wherein the mutation results in a reduced expression level of CD123 as compared with a wild-type counterpart cell population.

20 323. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 44, wherein the mutation results in a reduced expression level of CD123 that is less than 20% of the level of CD123 in a wild-type counterpart cell population.

324. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 46.

25 325. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 46, wherein the mutation results in a reduced expression level of CD123 as compared with a wild-type counterpart cell population.

30 326. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of SEQ ID NO: 46, wherein the mutation results in a reduced expression level of CD123 that is less than 20% of the level of CD123 in a wild-type counterpart cell population.

327. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of any of SEQ ID NOs: 66-71, 73, 76, 77, 79-82, 85, 87, 88, 122, 133, 134, 135, 141-144, 153, 157, or 158.

- 5 328. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of any of SEQ ID NOs: 66-71, 73, 76, 77, 79-82, 85, 87, 88, 122, 133, 134, 135, 141-144, 153, 157, or 158, wherein the mutation results in a reduced expression level of CD123 as compared with a wild-type counterpart cell population.

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329. A cell population, comprising a plurality of genetically engineered hematopoietic stem or progenitor cells which comprise a mutation at a target domain of any of SEQ ID NOs: 66-71, 73, 76, 77, 79-82, 85, 87, 88, 122, 133, 134, 135, 141-144, 153, 157, or 158, wherein the mutation results in a reduced expression level of CD123 that is less than 20% of the level of
15 CD123 in a wild-type counterpart cell population.

330. The cell population of any of embodiments 284-220, wherein the cell population can differentiate into a cell type which expresses CD123 at a level that is reduced with regard to the level of CD123 expressed by the same differentiated cell type which is derived from a
20 CD123-wildtype hematopoietic stem or progenitor cell.

331. The cell population of any of embodiments 284-330, wherein the hematopoietic stem or progenitor cells are engineered such that a myeloid progenitor cell descended therefrom is deficient in CD123 levels as compared with a myeloid progenitor cell descended from a
25 CD123-wildtype hematopoietic stem or progenitor cell.

332. The cell population of any of embodiments 284-330, wherein the hematopoietic stem or progenitor cells are engineered such that a myeloid cell (e.g., a terminally differentiated myeloid cell) descended therefrom is deficient in CD123 levels as compared with a myeloid
30 cell (e.g., a terminally differentiated myeloid cell) descended from a CD123-wildtype hematopoietic stem or progenitor cell.

333. The cell population of any of embodiments 284-332, which further comprises one or more cells that comprise one or more non-engineered CD123 genes.

334. The cell population of any of embodiments 284-333, which further comprises one or more cells that are homozygous wild-type for CD123.

- 5 335. The cell population of any of embodiments 284-334, wherein about 0-1%, 1-2%, 2-5%, 5-10%, 10-15%, or 15-20% of cells in the population are homozygous wild-type for CD123, e.g., are hematopoietic stem or progenitor cells that are homozygous wild-type for CD123.

336. The cell population of any of embodiments 284-334 which further comprises one or
10 more cells that are heterozygous for CD123, e.g., comprise one wild-type copy of CD123 and one mutant copy of CD123.

337. The cell population of any of embodiments 284-336, wherein about 0-1%, 1-2%, 2-5%, 5-10%, 10-15%, or 15-20% of cells in the population are heterozygous wild-type for
15 CD123, e.g., are hematopoietic stem or progenitor cells that comprise one wild-type copy of CD123 and one mutant copy of CD123.

338. The cell population of any of embodiments 284-337, wherein at least 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, or 95% of the copies of CD123 in the population are
20 mutant.

339. The cell population of any of embodiments 284-338, which comprises a plurality of different CD123 mutations, e.g., which comprises at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 different mutations.

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340. The cell population of any of embodiments 284-339, which comprises at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 different mutations.

341. The cell population of any of embodiments 284-340, which comprises at 2, 3, 4, 5, 6,
30 7, 8, 9, or 10 different insertions.

342. The cell population of any of embodiments 284-341, which comprises a plurality of insertions and a plurality of deletions.

343. The cell population of any of embodiments 284-342, which expresses less than 20% of the CD123 expressed by a wild-type counterpart cell population.

5 344. The cell population of any of embodiments 284-343, wherein the reduced expression level of CD123 is in a cell differentiated from (e.g., terminally differentiated from) the hematopoietic stem or progenitor cell, and the wild-type counterpart cell is a cell differentiated from (e.g., terminally differentiated from) a wild-type hematopoietic stem or progenitor cell.

10 345. The cell population of embodiment 344, wherein the cell differentiated from the hematopoietic stem or progenitor cell is a myeloblast, monocyte, or myeloid dendritic cell.

15 346. The cell population of any of embodiments 284-345, which, when administered to a subject, produces hCD45+ cells in the subject, e.g., when assayed at 16 weeks after administration.

20 347. The cell population of embodiment 346, which produces levels of hCD45+ cells comparable to the levels of hCD45+ cells produced with an otherwise similar cell population that is CD123 wildtype.

348. The cell population of embodiments 346 or 347, which produces levels of hCD45+ cells that is at least 70%, 80%, 85%, 90%, or 95% the levels of hCD45+ cells produced by an otherwise similar cell population that is CD123 wildtype.

25 349. The cell population of any of embodiments 284-348, which, when administered to a subject, produces CD34+ cells in the subject, e.g., when assayed at 16 weeks after administration.

30 350. The cell population of embodiment 349, which produces levels of hCD34+ cells comparable to the levels of hCD34+ cells produced with an otherwise similar cell population that is CD123 wildtype.

351. The cell population of embodiments 349 or 350, which produces levels of hCD34+ cells that is at least 70%, 80%, 85%, 90%, or 95% the levels of hCD34+ cells produced by an otherwise similar cell population that is CD123 wildtype.

5 352. The cell population of any of embodiments 284-351, which, when administered to a subject, produces mast cells, basophils, eosinophils, common dendritic cells (cDCs), plasmacytoid dendritic cells (pDCs), neutrophils, monocytes, T cells, B cells or any combination thereof, in the subject, e.g., when assayed at 16 weeks after administration.

10 353. The cell population of embodiment 352, which produces levels of mast cells, basophils, eosinophils, common dendritic cells (cDCs), plasmacytoid dendritic cells (pDCs), neutrophils, monocytes, T cells, B cells or any combination thereof comparable to the levels of said cell type produced with an otherwise similar cell population that is CD123 wildtype.

15 354. The cell population of embodiments 352 or 353, which produces levels of mast cells, basophils, eosinophils, common dendritic cells (cDCs), plasmacytoid dendritic cells (pDCs), neutrophils, monocytes, T cells, B cells or any combination thereof that is at least 70%, 80%, 85%, 90%, or 95% the levels of said cell type produced by an otherwise similar cell population that is CD123 wildtype.

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355. The cell population of any of embodiments 352-354, wherein the produced cells are detected in a blood sample, a bone marrow sample, or a spleen sample obtained from the subject.

25 356. The cell population of any of embodiments 284-355, which, when administered to a subject, persists for at least 8, 12, or 16 weeks in the subject.

357. The cell population of any of embodiments 284-356, which, when administered to a subject, provides multilineage hematopoietic reconstitution.

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358. The cell population of any of embodiments 284-357, which, produces CD14+ cells, e.g., when assayed at 7 or 14 days after genetic engineering.

359. The cell population of embodiment 358, which produces levels of CD14+ cells comparable to the levels of CD14+ cells produced with an otherwise similar cell population that is CD123 wildtype.
- 5 360. The cell population of embodiments 358 or 359, which produces levels of CD14+ cells that is at least 70%, 80%, 85%, 90%, or 95% the levels of CD14+ cells produced by an otherwise similar cell population that is CD123 wildtype.
361. The cell population of embodiments any of 358-360, wherein CD14+ levels are assayed
10 after culturing in vitro in myeloid differentiation media.
362. The cell population of any of embodiments 284-361, which, produces CD11b+ cells, e.g., when assayed at 7 or 14 days after genetic engineering.
- 15 363. The cell population of embodiment 362, which produces levels of CD11b+ cells comparable to the levels of CD11b+ cells produced with an otherwise similar cell population that is CD123 wildtype.
364. The cell population of embodiments 362 or 363, which produces levels of CD11b+ cells
20 that is at least 70%, 80%, 85%, 90%, or 95% the levels of CD11b+ cells produced by an otherwise similar cell population that is CD123 wildtype.
365. The cell population of embodiments any of 358-364, wherein CD11b+ levels are assayed after culturing in vitro in myeloid differentiation media.
25
366. The cell population of any of embodiments 284-365, which, produces CD15+ cells, e.g., when assayed at 7 or 14 days after genetic engineering.
367. The cell population of embodiment 366, which produces levels of CD15+ cells
30 comparable to the levels of CD11b+ cells produced with an otherwise similar cell population that is CD123 wildtype.

368. The cell population of embodiments 366 or 367, which produces levels of CD15+ cells that is at least 70%, 80%, 85%, 90%, or 95% the levels of CD15+ cells produced by an otherwise similar cell population that is CD123 wildtype.

- 5 369. The cell population of embodiments any of 358-368, wherein CD15+ levels are assayed after culturing in vitro in myeloid differentiation media.

370. The cell population of any of embodiments 284-369, wherein the most abundant mutation in CD123 in the cell population is an insertion, e.g., an insertion of 1 nt, 2 nt, or 3
10 nt.

371. The cell population of any of embodiments 284-370, wherein the most abundant mutation in CD123 in the cell population is an insertion of 1 nt.

- 15 372. The cell population of any of embodiments 284-370, wherein the most abundant mutation in the cell population within the sequence of SEQ ID NO: 11 in CD123 is an insertion, e.g., an insertion of 1 nt.

373. The cell population of embodiment 284-370 or 372, which further comprises a 1 nt
20 deletion within the sequence of SEQ ID NO: 11 in a copy of CD123.

374. The cell population of any of embodiments 284-370, wherein the most abundant mutation in the cell population within the sequence of SEQ ID NO: 7 in CD123 is an insertion, e.g., an insertion of 1 nt.

- 25 375. The cell population of embodiment 284-370 or 374, which further comprises a 1 nt deletion within the sequence of SEQ ID NO: 7 in a copy of CD123.

376. The cell population of any of embodiments 284-370, wherein the most abundant
30 mutation in the cell population within the sequence of SEQ ID NO: 9 in CD123 is an insertion, e.g., an insertion of 1 nt.

377. The cell population of embodiment 284-370 or 376, which further comprises a 1 nt deletion within the sequence of SEQ ID NO: 9 in a copy of CD123.

378. The cell population of any of embodiments 284-370, wherein the most abundant mutation in the cell population within the sequence of SEQ ID NO: 41 in CD123 is an insertion, e.g., an insertion of 1 nt.

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379. The cell population of embodiment 284-370 or 378, which further comprises a 2 nt deletion within the sequence of SEQ ID NO: 41 in a copy of CD123.

10 380. The cell population of any of embodiments 284-370, wherein the most abundant mutation in the cell population within the sequence of SEQ ID NO: 43 in CD123 is an insertion, e.g., an insertion of 1 nt.

15 381. The cell population of embodiment 284-370 or 380, which further comprises a 7 nt deletion within the sequence of SEQ ID NO: 43 in a copy of CD123.

382. The cell population of any of embodiments 284-370, wherein the most abundant mutation in the cell population within the sequence of SEQ ID NO: 44 in CD123 is an insertion, e.g., an insertion of 1 nt.

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383. The cell population of embodiment 284-370 or 382, which further comprises a 2 nt deletion within the sequence of SEQ ID NO: 44 in a copy of CD123.

25 384. The cell population of any of embodiments 284-370, wherein the most abundant mutation in the cell population within the sequence of SEQ ID NO: 46 in CD123 is an insertion, e.g., an insertion of 1 nt.

385. The cell population of embodiment 284-370 or 384, which further comprises a 5 nt deletion within the sequence of SEQ ID NO: 46 in a copy of CD123.

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386. The cell population of any of embodiments 284-385, which comprises hematopoietic stem cells and hematopoietic progenitor cells.

387. A pharmaceutical composition comprising the genetically engineered hematopoietic stem or progenitor cell of any of embodiments 128-153, 180, or 182-283.

388. A pharmaceutical composition comprising the cell population of any of embodiments
5 284-386.

389. The cell population of any of embodiments 284-386, wherein at least 80%, 85%, 90%, 95%, or 98% of cells in the population are viable.

10 390. The cell population of any of embodiments 284-386 or 389, wherein at least 50%, 60%, 70%, 80%, or 90% of copies of CD123 comprise a mutation.

391. The cell population of any of embodiments 284-386, 389, or 390, wherein at least 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% of cells in the population are negative
15 for cell surface expression of CD123, e.g., using a flow cytometry assay for CD123 cell surface expression, e.g., as described in Example 1.

392. A mixture, e.g., a reaction mixture comprising:
20 a) a gRNA of any of embodiments 1-98 or gRNAs of a composition or kit of any of embodiments 99-127; and
b) a cell, e.g., a hematopoietic cell, e.g., an HSC or HPC, e.g., a genetically engineered cell of any of embodiments 128-153, 180, or 182-283.

393. The mixture of embodiment 392, wherein the cell is a wild-type cell or a cell having a
25 mutation in CD123.

394. A kit comprising any two or more (e.g., three or all) of:
30 a) a gRNA of any of embodiments 1-97;
b) a cell, e.g., a hematopoietic cell, e.g., an HSC or HPC, e.g., a genetically engineered cell of any of embodiments 128-153, 180, or 182-283;
c) a Cas9 molecule; and
d) agent that targets CD123, e.g., an agent as described herein.

395. The kit of embodiment 394, which comprises (a) and (b), (a) and (c), (a) and d), (b) and (c), (b) and (d), or (c) and (d).

396. A method of making the genetically engineered cell (e.g., hematopoietic stem or progenitor cell) of any of embodiments 126 or 128-153, 180, or 182-283, or the cell population of any of embodiments 284-386, 389-391, which comprises:

(i) providing a cell (e.g., a hematopoietic stem or progenitor cell, e.g., a wild-type hematopoietic stem or progenitor cell), and

(ii) introducing into the cell a nuclease (e.g., an endonuclease) that cleaves the target domain,

thereby producing a genetically engineered hematopoietic stem or progenitor cell.

397. The method of embodiment 396, wherein (ii) comprises introducing into the cell a gRNA that binds the target domain (e.g., a gRNA of any of embodiments 1-97 and an endonuclease that binds the gRNA.

398. The method of embodiment 397, wherein the endonuclease is a ZFN, TALEN, or meganuclease.

399. A method of supplying HSCs, HPCs, or HSPCs to a subject, comprising administering to the subject a plurality of cells of any of embodiments 126 or 128-153, 180, or 182-283, or the cell population of any of embodiments 284-386 or 389-391.

400. A method, comprising administering to a subject a subject in need thereof a plurality of cells of any of embodiments 126 or 128-153, 180, or 182-283, or the cell population of any of embodiments 284-386 or 389-391.

401. The method of embodiment 399 or 400, wherein the subject has a cancer, wherein cells of the cancer express CD123 (e.g., wherein at least a plurality of the cancer cells express CD123).

402. The method of any of embodiments 399-401, which further comprises administering to the subject an effective amount of an agent that targets CD123, and wherein the agent comprises an antigen-binding fragment that binds CD123.

403. The method of embodiment 402, wherein the agent that targets CD123 is an immune cell expressing a chimeric antigen receptor (CAR), which comprises the antigen-binding fragment that binds CD123.

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404. A genetically engineered hematopoietic stem or progenitor cell of any of embodiments 128-153, 180, or 182-283 or a cell population of any of embodiments 284-386 or 389-391 for use in treating a hematopoietic disorder, wherein the treating comprises administering to a subject in need thereof an effective amount of the genetically engineered hematopoietic stem or progenitor cell or the cell population, and further comprises administering to the subject an effective amount of an agent that targets CD123, wherein the agent comprises an antigen-binding fragment that binds CD123.

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405. An agent that targets CD123, wherein the agent comprises an antigen-binding fragment that binds CD123, for use in treating a hematopoietic disorder, wherein the treating comprises administering to a subject in need thereof an effective amount of the agent that targets CD123, and further comprises administering to the subject an effective amount of a genetically engineered hematopoietic stem or progenitor cell of any of embodiments 128-153, 180, or 182-283 or a cell population of any of embodiments 284-386 or 389-391.

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406. A combination of a genetically engineered hematopoietic stem or progenitor cell of any of embodiments 128-153, 180, or 182-283 or a cell population of any of embodiments 284-386 or 389-391, and an agent that targets CD123, wherein the agent comprises an antigen-binding fragment that binds CD123, for use in treating a hematopoietic disorder, wherein the treating comprises administering to a subject in need thereof an effective amount of the genetically engineered hematopoietic stem or progenitor cell or the cell population, and the agent that binds CD123.

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407. A genetically engineered hematopoietic stem or progenitor cell of any of embodiments 128-153, 180, or 182-283 or a cell population of any of embodiments 284-386 or 389-391 for use in cancer immunotherapy.

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408. A genetically engineered hematopoietic stem or progenitor cell of any of embodiments 128-153, 180, or 182-283 or a cell population of any of embodiments 284-386 or 389-391 for use in cancer immunotherapy, wherein the subject has a hematopoietic disorder.
- 5 409. A genetically engineered hematopoietic stem or progenitor cell of any of embodiments 128-153, 180, or 182-283 or a cell population of any of embodiments 284-386 or 389-391 for use in hematopoietic repopulation of a subject having a hematopoietic disorder.
- 10 410. A genetically engineered hematopoietic stem or progenitor cell of any of embodiments 128-153, 180, or 182-283 or a cell population of any of embodiments 284-386 or 389-391 for use in a method of treating a hematopoietic disorder, whereby the genetically engineered hematopoietic stem or progenitor cell described herein or a cell population described herein repopulate the subject.
- 15 411. A genetically engineered hematopoietic stem or progenitor cell of any of embodiments 128-153, 180, or 182-283 or a cell population of any of embodiments 284-386 or 389-391 for use in reducing cytotoxic effects of an agent that targets CD123 in immunotherapy.
- 20 412. A genetically engineered hematopoietic stem or progenitor cell of any of embodiments 128-153, 180, or 182-283 or a cell population of any of embodiments 284-386 or 389-391 for use in an immunotherapy method using an agent that targets CD123, whereby the genetically engineered hematopoietic stem or progenitor cell described herein or a cell population described herein reduces cytotoxic effects of the agent that targets CD123.
- 25 413. The method, cell, agent, or combination of any of embodiments 399-412, wherein the genetically engineered hematopoietic stem or progenitor cell or the cell population is administered concomitantly with the agent that targets CD123.
- 30 414. The method, cell, agent, or combination of any of embodiments 399-413, wherein the genetically engineered hematopoietic stem or progenitor cell or the cell population is administered prior to the agent that targets CD123.

415. The method, cell, agent, or combination of any of embodiments 399-414, wherein the agent that targets CD123 is administered prior to the genetically engineered hematopoietic stem or progenitor cell or the cell population.

5 416. The method, cell, agent, or combination of any of embodiments 399-415 , wherein the immune cell is a T cell.

417. The method, cell, agent, or combination of any of embodiments 399-416, wherein the immune cell, the genetically engineered hematopoietic stem and/or progenitor cell, or both,
10 are allogeneic.

418. The method, cell, agent, or combination of any of embodiments 399-417 , wherein the immune cell, the genetically engineered hematopoietic stem and/or progenitor cell, or both,
15 are autologous.

419. The method, cell, agent, or combination of any of embodiments 399-418, wherein the antigen-binding fragment in the chimeric receptor is a single-chain antibody fragment (scFv) that specifically binds human CD123.

20 420. The method, cell, agent, or combination of any of embodiments 399-419, wherein hematopoietic disorder is a cancer, and wherein at least a plurality of cancer cells in the cancer express CD123.

421. The method, cell, agent, or combination of any of embodiments 399-420, wherein the
25 subject has a hematopoietic malignancy, e.g., a hematopoietic malignancy chosen from Hodgkin's lymphoma, non-Hodgkin's lymphoma, leukemia (e.g., acute myeloid leukemia, acute lymphoid leukemia, chronic myelogenous leukemia, acute lymphoblastic leukemia or chronic lymphoblastic leukemia, and chronic lymphoid leukemia), or multiple myeloma.

30 422. The method, cell, agent, or combination of any of embodiments 399-420, wherein the subject has a hematological disorder, e.g., a precancerous condition, e.g., a myelodysplasia, a myelodysplastic syndrome (MDS), or a preleukemia.

The summary above is meant to illustrate, in a non-limiting manner, some of the embodiments, advantages, features, and uses of the technology disclosed herein. Other embodiments, advantages, features, and uses of the technology disclosed herein will be apparent from the Detailed Description, the Drawings, the Examples, and the Claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing CD123 gRNA screening on CD34⁺ cells. Human CD34⁺ cells were electroporated with Cas9 protein and CD123-targeting gRNAs (listed on the y-axis). Editing efficiency of *IL3RA* locus, shown on the x-axis, was determined by Sanger sequencing and TIDE analysis.

Figures 2A-2C are a series of graphs showing gene-editing efficiency of CD123 gRNAs on THP-1 cells. **(A)** Human THP-1 cells were electroporated with Cas9 protein and CD123-targeting gRNAs. Editing efficiency of *IL3RA* locus was determined by Sanger sequencing and TIDE analysis. The expression of CD123 was assessed by flow cytometry **(B)**, and the percentages of CD123-negative cells were plotted **(C)**.

Figures 3A-3D are a series of diagrams showing survival and differentiation of CD123-edited CD34⁺ cells. **(A)** Schematic showing the workflow of the experiment. Human CD34⁺ cells were electroporated with Cas9 protein and CD123-targeting gRNA I, followed by analysis of editing efficiency by TIDE and a CFU assay to assess *in vitro* differentiation. **(B)** Cell viability was measured 48 hours post electroporation. **(C)** Editing efficiency of *IL3RA* locus was determined by Sanger sequencing and TIDE analysis. No Cas9 RNP group was used as control. **(D)** Control or CD123-edited CD34⁺ cells were plated in Methocult 2 days after electroporation and scored for colony formation after 14 days. BFU-E: burst forming unit-erythroid; CFU-GM: colony forming unit-granulocyte/macrophage; CFU-GEMM: colony forming unit of multipotential myeloid progenitor cells (generate granulocytes, erythrocytes, monocytes, and megakaryocytes). Student's t-test was used.

Figure 4 shows target expression on AML cell lines. The expression of CD33, CD123 and CLL1 in MOLM-13 and THP-1 cells and an unstained control was determined by flow cytometric analysis. The X-axis indicates the intensity of antibody staining and the Y-axis corresponds to number of cells.

Figure 5 shows CD33- and CD123-modified MOLM-13 cells. The expression of CD33 and CD123 in wild-type (WT), CD33^{-/-}, CD123^{-/-} and CD33^{-/-} CD123^{-/-} MOLM-13 cells was assessed by flow cytometry. For the generation of CD33^{-/-} or CD123^{-/-} MOLM-13 cells, WT MOLM-13 cells were electroporated with CD33- or CD123-targeting RNP, followed by flow cytometric sorting of CD33- or CD123-negative cells. CD33^{-/-}CD123^{-/-} MOLM-13 cells were generated by electroporating CD33^{-/-} cells with CD123-targeting RNP and sorted for CD123-negative population. The X-axis indicates the intensity of antibody staining and the Y-axis corresponds to number of cells.

Figure 6 shows an in vitro cytotoxicity assay of CD33 and CD123 CAR-Ts. Anti-

CD33 CAR-T and anti-CD123 CAR-T were incubated with wild-type (WT), CD33^{-/-}, CD123^{-/-} and CD33^{-/-} CD123^{-/-} MOLM-13 cells, and cytotoxicity was assessed by flow cytometry.

Non-transduced T cells were used as mock CAR-T control. The CARpool group was composed of 1:1 pooled combination of anti-CD33 and anti-CD123 CAR-T cells. Student's t test was used. ns = not significant; *P < 0.05; **P < 0.01. The Y-axis indicates the percentage of specific killing.

Figure 7 shows gene-editing efficiency of CD34+ cells. Human CD34+ cells were electroporated with Cas9 protein and CD33-, CD123- or CLL1- targeting gRNAs, either alone or in combination. Editing efficiency of CD33, CD123 or CLL1 locus was determined by Sanger sequencing and TIDE analysis. The Y-axis indicates the editing efficiency (% by TIDE).

Figures 8A-8C shows in vitro colony formation of gene-edited CD34+ cells. Control or CD33, CD123, CLL-1-modified CD34+ cells were plated in Methocult 2 days after electroporation and scored for colony formation after 14 days. BFU-E: burst forming unit-erythroid; CFU-GM: colony forming unit-granulocyte/macrophage; CFU-GEMM: colony forming unit of multipotential myeloid progenitor cells (generate granulocytes, erythrocytes, monocytes, and megakaryocytes). Student's t test was used.

Figure 9 shows gene editing frequency of CD34+ cells. Human CD34+ cells were electroporated with ribonucleoprotein (RNP) complexes composed of Cas9 protein and the CD123- targeting gRNAs indicated on the X-axis, the sequences of which are found in Table 8. Editing frequency of the CD123 locus was determined by Sanger sequencing. The Y-axis indicates the editing frequency.

Figure 10 shows gene editing frequency of CD34+ cells. Human CD34+ cells were electroporated with Cas9 protein and the CD123- targeting gRNAs indicated on the X-axis, specifically from left to right, gRNA A, G, I, N3, P3, and S3. Editing frequency of the CD123 locus was determined by Sanger sequencing. The Y-axis indicates the editing frequency. All gRNAs in **Figure 10** led to an editing frequency $\geq 80\%$.

Figure 11 shows the INDEL (insertion/deletion) distribution for human CD34+ cells edited with the CD123-targeting gRNAs, specifically gRNA A (top left), gRNA G (middle left), gRNA I (bottom left), gRNA N3 (top right), gRNA P3 (middle right), and gRNA S3 (bottom right). The X-axis indicates the size of the INDEL and the Y-axis indicates the percentage of the specific INDEL in the mixture.

Figure 12 shows the INDEL (insertion/deletion) distribution for human CD34+ cells edited with the CD123-targeting gRNA D1. The X-axis indicates the size of the INDEL and the Y-axis indicates the percentage of the specific INDEL in the mixture.

Figure 13 is a schematic and overview of the protocol and experimental procedure/timeline used for *in vivo* characterization of CD123-edited HSPCs in NBSGW mice.

Figures 14A-14C depict long-term lineage engraftment of CD123-edited cells in the bone marrow of mice 16 weeks post-engraftment of non-edited control cells or CD123KO cells. **Figure 14A** shows the rates of human leukocyte chimerism calculated as percentage of human CD45+ (hCD45+) cells in the total CD45+ cell population (the sum of human and mouse CD45+ cells) in the bone marrow at week 16 post-engraftment of control cells (EP ctrl) or CD123KO cells edited with the gRNA indicated (from left to right on X-axis, gRNA I or gRNA D1). **Figure 14B** shows the percentage of hCD45+ cells that were also positive for human CD34 (hCD34+) in the bone marrow at week 16 post-engraftment of control cells (EP ctrl) or CD123KO cells edited with the gRNA indicated (from left to right on X-axis, gRNA I or gRNA D1). **Figure 14C** shows the percentage of hCD45+ cells that were B-cells, T cells, monocytes, neutrophils, conventional dendritic cells (cDCs), plasmacytoid dendritic cells (pDCs), eosinophils, basophils, and mast cells) in the bone marrow at week 16 post-engraftment of control cells (EP ctrl) or CD123KO cells edited with the gRNA indicated (from left to right on X-axis, gRNA I or gRNA D1).

Figure 15 shows the percentages of hCD45+ that were also CD123+ quantified in the bone marrow at week 16 post-engraftment of control cells (EP ctrl) or CD123KO cells edited with the gRNA indicated (from left to right on X-axis, gRNA I or gRNA D1).

Figure 16A shows cell-surface expression of CD123 *in vitro* as measured by FACs in, from top to bottom, non-edited control cells, CD123KO cells edited by gRNA I (editing frequency of 75.8% as measured by TIDE), CD123KO cells edited by gRNA D1 (editing frequency of 71.1% as measured by amplicon sequencing), and a FMO (fluorescence minus one) control. **Figure 16B** shows the quantification granulocytes produced over time from *in vitro* culturing of non-edited control cells (EP ctrl) or CD123KO cells edited by gRNA I or gRNA D1. **Figure 16C** shows the quantification monocytes produced over time from *in vitro* culture of non-edited control cells (EP ctrl) or CD123KO cells edited by gRNA I or gRNA D1.

Figure 17 shows the percentage of CD132+ granulocytes (top) or monocytes (bottom) produced over time from *in vitro* culturing non-edited control cells (EP ctrl) or CD123KO cells edited by gRNA I or gRNA D1.

Figure 18 shows the percentage of CD15+ (top left) or CD11b+ positive granulocytes (top right) or the percentage of CD14+ (bottom left) or CD11b+ positive monocytes (bottom right) quantified at day 0, 7, and 14 following editing and culture of non-edited control cells or CD123KO cells edited by gRNA I or gRNA D1.

Figure 19A shows the percentage of phagocytosis measured in granulocytes (top) or monocytes (bottom) produced from non-edited control cells (EP ctrl) or CD123KO cells edited by the gRNA indicated (from left to right on X-axis, gRNA I or gRNA D1). **Figure 19B** shows the production of IL-6 in pg/mL (right) or TNF- α in pg/mL (left) by granulocytes produced from non-edited control cells (EP ctrl) or CD123KO cells edited by the gRNA I or gRNA D1, that were unstimulated, stimulated by LPS, or stimulated by R848. **Figure 19C** shows the production of IL-6 in pg/mL (right) or TNF- α in pg/mL (left) by monocytes produced from non-edited control cells (EP ctrl) or CD123KO cells edited by the gRNA I or gRNA D1 that were unstimulated, stimulated by LPS, or stimulated by R848.

Figure 20A-20B shows *in vitro* colony formation of gene-edited CD34+ cells. Control or CD123-modified CD34+ cells were plated in after electroporation and scored for colony formation after 14 days. BFU-E: burst forming unit-erythroid; CFU-GM: colony forming unit-granulocyte/macrophage; CFU-GEMM: colony forming unit of multipotential myeloid progenitor cells (generate granulocytes, erythrocytes, monocytes, and megakaryocytes). **Figure 20A** shows colony count of BFU-E, CFU-G/M/GM, or CFU-GEMM that resulted from non-edited cells (EP ctrl) or CD123KO cells edited by gRNA I (editing frequency of 77.9%) or gRNA D1 (editing frequency of 72.5%). **Figure 20B** shows percent colony distribution of BFU-E, CFU-G/M/GM, or CFU-GEMM that resulted from non-edited cells (EP ctrl) or CD123KO cells edited by gRNA I or gRNA D1.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

The term “binds”, as used herein with reference to a gRNA interaction with a target domain, refers to the gRNA molecule and the target domain forming a complex. The complex may comprise two strands forming a duplex structure, or three or more strands forming a multi-stranded complex. The binding may constitute a step in a more extensive process, such as the cleavage of the target domain by a Cas endonuclease. In some

embodiments, the gRNA binds to the target domain with perfect complementarity, and in other embodiments, the gRNA binds to the target domain with partial complementarity, e.g., with one or more mismatches. In some embodiments, when a gRNA binds to a target domain, the full targeting domain of the gRNA base pairs with the targeting domain. In other
5 embodiments, only a portion of the target domain and/or only a portion of the targeting domain base pairs with the other. In an embodiment, the interaction is sufficient to mediate a target domain-mediated cleavage event.

A “Cas9 molecule” as that term is used herein, refers to a molecule or polypeptide that can interact with a gRNA and, in concert with the gRNA, home or localize to a site
10 which comprises a target domain. Cas9 molecules include naturally occurring Cas9 molecules and engineered, altered, or modified Cas9 molecules that differ, e.g., by at least one amino acid residue, from a naturally occurring Cas9 molecule.

The terms “gRNA” and “guide RNA” are used interchangeably throughout and refer to a nucleic acid that promotes the specific targeting or homing of a gRNA/Cas9 molecule
15 complex to a target nucleic acid. A gRNA can be unimolecular (having a single RNA molecule), sometimes referred to herein as sgRNAs, or modular (comprising more than one, and typically two, separate RNA molecules). A gRNA may bind to a target domain in the genome of a host cell. The gRNA may comprise a targeting domain that may be partially or completely complementary to the target domain. The gRNA may also comprise a “scaffold
20 sequence,” (e.g., a tracrRNA sequence), that recruits a Cas9 molecule to a target domain bound to a gRNA sequence (e.g., by the targeting domain of the gRNA sequence). The scaffold sequence may comprise at least one stem loop structure and recruits an endonuclease. Exemplary scaffold sequences can be found, for example, in Jinek, et al. Science (2012) 337(6096):816-821, Ran, et al. Nature Protocols (2013) 8:2281-2308, PCT
25 Application No. WO2014/093694, and PCT Application No. WO2013/176772.

The term “mutation” is used herein to refer to a genetic change (e.g., insertion, deletion, or substitution) in a nucleic acid compared to a reference sequence, e.g., the corresponding wild-type nucleic acid. In some embodiments, a mutation to a gene
30 detargetizes the protein produced by the gene. In some embodiments, a detargetized CD123 protein is not bound by, or is bound at a lower level by, an agent that targets CD123.

The “targeting domain” of the gRNA is complementary to the “target domain” on the target nucleic acid. The strand of the target nucleic acid comprising the nucleotide sequence complementary to the core domain of the gRNA is referred to herein as the “complementary strand” of the target nucleic acid. Guidance on the selection of targeting domains can be

found, e.g., in Fu Y et al, Nat Biotechnol 2014 (doi: 10.1038/nbt.2808) and Sternberg SH et al., Nature 2014 (doi: 10.1038/nature13011).

Nucleases

5 In some embodiments, a cell (e.g., HSC or HPC) described herein is made using a nuclease described herein. Exemplary nucleases include Cas molecules (e.g., Cas9, TALENs, ZFNs, and meganucleases. In some embodiments, a nuclease is used in combination with a CD123 gRNA described herein (e.g., according to Table 2, 6, or 8).

10 Cas9 molecules

In some embodiments, a CD123 gRNA described herein is complexed with a Cas9 molecule. Various Cas9 molecules can be used. In some embodiments, a Cas9 molecule is selected that has the desired PAM specificity to target the gRNA/Cas9 molecule complex to the target domain in CD123. In some embodiments, genetically engineering a cell also
15 comprises introducing one or more (e.g., 1, 2, 3 or more) Cas9 molecules into the cell.

Cas9 molecules of a variety of species can be used in the methods and compositions described herein. In embodiments, the Cas9 molecule is of, or derived from, *S. pyogenes* (SpCas9), *S. aureus* (SaCas9), or *S. thermophilus*. Additional suitable Cas9 molecules
20 include those of, or derived from, *Staphylococcus aureus*, *Neisseria meningitidis* (NmCas9), *Acidovorax avenae*, *Actinobacillus pleuropneumoniae*, *Actinobacillus succinogenes*, *Actinobacillus suis*, *Actinomyces* sp., *Cyclophilus denitrificans*, *Aminomonas paucivorans*, *Bacillus cereus*, *Bacillus smithii*, *Bacillus thuringiensis*, *Bacteroides* sp., *Blastopirellula marina*, *Bradyrhizobium* sp., *Brevibacillus laterosporus*, *Campylobacter coli*, *Campylobacter*
25 *jejuni* (CjCas9), *Campylobacter lari*, *Candidatus Puniceispirillum*, *Clostridium cellulolyticum*, *Clostridium perfringens*, *Corynebacterium accolens*, *Corynebacterium diphtheria*, *Corynebacterium matruchotii*, *Dinoroseobacter shibae*, *Eubacterium dolichum*, *gamma proteobacterium*, *Gluconacetobacter diazotrophicus*, *Haemophilus parainfluenzae*, *Haemophilus sputorum*, *Helicobacter canadensis*, *Helicobacter cinaedi*, *Helicobacter*
30 *mustelae*, *Ilyobacter polytropus*, *Kingella kingae*, *Lactobacillus crispatus*, *Listeria ivanovii*, *Listeria monocytogenes*, *Listeriaceae bacterium*, *Methylocystis* sp., *Methylosinus trichosporium*, *Mobiluncus mulieris*, *Neisseria bacilliformis*, *Neisseria cinerea*, *Neisseria flavescens*, *Neisseria lactamica*, *Neisseria meningitidis*, *Neisseria* sp., *Neisseria wadsworthii*, *Nitrosomonas* sp., *Parvibaculum lavamentivorans*, *Pasteurella multocida*,

Phascolarctobacterium succinatutens, *Ralstonia syzygii*, *Rhodopseudomonas palustris*, *Rhodovulum* sp., *Simonsiella muelleri*, *Sphingomonas* sp., *Sporolactobacillus vineae*, *Staphylococcus lugdunensis*, *Streptococcus* sp., *Subdoligranulum* sp., *Tistrella mobilis*, *Treponema* sp., or *Verminephrobacter eiseniae*.

5 In some embodiments, the Cas9 molecule is a naturally occurring Cas9 molecule. In some embodiments, the Cas9 molecule is an engineered, altered, or modified Cas9 molecule that differs, e.g., by at least one amino acid residue, from a reference sequence, e.g., the most similar naturally occurring Cas9 molecule or a sequence of Table 50 of WO2015157070, which is herein incorporated by reference in its entirety. In some embodiments, the Cas9
10 molecule comprises Cpf1 or a fragment or variant thereof.

A naturally occurring Cas9 molecule typically comprises two lobes: a recognition (REC) lobe and a nuclease (NUC) lobe; each of which further comprises domains described, e.g., in WO2015157070, e.g., in Figs. 9A-9B therein (which application is incorporated herein by reference in its entirety).

15 The REC lobe comprises the arginine-rich bridge helix (BH), the REC1 domain, and the REC2 domain. The REC lobe appears to be a Cas9-specific functional domain. The BH domain is a long alpha helix and arginine rich region and comprises amino acids 60-93 of the sequence of *S. pyogenes* Cas9. The REC1 domain is involved in recognition of the repeat:anti-repeat duplex, e.g., of a gRNA or a tracrRNA. The REC1 domain comprises two
20 REC1 motifs at amino acids 94 to 179 and 308 to 717 of the sequence of *S. pyogenes* Cas9. These two REC1 domains, though separated by the REC2 domain in the linear primary structure, assemble in the tertiary structure to form the REC1 domain. The REC2 domain, or parts thereof, may also play a role in the recognition of the repeat: anti-repeat duplex. The REC2 domain comprises amino acids 180-307 of the sequence of *S. pyogenes* Cas9.

25 The NUC lobe comprises the RuvC domain (also referred to herein as RuvC-like domain), the HNH domain (also referred to herein as HNH-like domain), and the PAM-interacting (PI) domain. The RuvC domain shares structural similarity to retroviral integrase superfamily members and cleaves a single strand, e.g., the non-complementary strand of the target nucleic acid molecule. The RuvC domain is assembled from the three split RuvC
30 motifs (RuvC I, RuvCII, and RuvCIII, which are often commonly referred to in the art as RuvCI domain, or N-terminal RuvC domain, RuvCII domain, and RuvCIII domain) at amino acids 1-59, 718-769, and 909-1098, respectively, of the sequence of *S. pyogenes* Cas9. Similar to the REC1 domain, the three RuvC motifs are linearly separated by other domains in the primary structure, however in the tertiary structure, the three RuvC motifs assemble

and form the RuvC domain. The HNH domain shares structural similarity with HNH endonucleases, and cleaves a single strand, e.g., the complementary strand of the target nucleic acid molecule. The HNH domain lies between the RuvC II-III motifs and comprises amino acids 775-908 of the sequence of *S. pyogenes* Cas9. The PI domain interacts with the PAM of the target nucleic acid molecule, and comprises amino acids 1099-1368 of the sequence of *S. pyogenes* Cas9.

Crystal structures have been determined for naturally occurring bacterial Cas9 molecules (Jinek et al., *Science*, 343(6176): 1247997, 2014) and for *S. pyogenes* Cas9 with a guide RNA (e.g., a synthetic fusion of crRNA and tracrRNA) (Nishimasu et al., *Cell*, 156:935-949, 2014; and Anders et al., *Nature*, 2014, doi: 10.1038/nature13579).

In some embodiments, a Cas9 molecule described herein has nuclease activity, e.g., double strand break activity. In some embodiments, the Cas9 molecule has been modified to inactivate one of the catalytic residues of the endonuclease. In some embodiments, the Cas9 molecule is a nickase and produces a single stranded break. See, e.g., Dabrowska et al. *Frontiers in Neuroscience* (2018) 12(75). It has been shown that one or more mutations in the RuvC and HNH catalytic domains of the enzyme may improve Cas9 efficiency. See, e.g., Sarai et al. *Currently Pharma. Biotechnol.* (2017) 18(13). In some embodiments, the Cas9 molecule is fused to a second domain, e.g., a domain that modifies DNA or chromatin, e.g., a deaminase or demethylase domain. In some such embodiments, the Cas9 molecule is modified to eliminate its endonuclease activity.

In some embodiments, a Cas9 molecule described herein is administered together with a template for homology directed repair (HDR). In some embodiments, a Cas9 molecule described herein is administered without a HDR template.

In some embodiments, the Cas9 molecule is modified to enhance specificity of the enzyme (e.g., reduce off-target effects, maintain robust on-target cleavage). In some embodiments, the Cas9 molecule is an enhanced specificity Cas9 variant (e.g., eSPCas9). See, e.g., Slaymaker et al. *Science* (2016) 351 (6268): 84-88. In some embodiments, the Cas9 molecule is a high fidelity Cas9 variant (e.g., SpCas9-HF1). See, e.g., Kleinstiver et al. *Nature* (2016) 529: 490-495.

Various Cas9 molecules are known in the art and may be obtained from various sources and/or engineered/modified to modulate one or more activities or specificities of the enzymes. In some embodiments, the Cas9 molecule has been engineered/modified to recognize one or more PAM sequence. In some embodiments, the Cas9 molecule has been engineered/modified to recognize one or more PAM sequence that is different than the PAM

sequence the Cas9 molecule recognizes without engineering/modification. In some embodiments, the Cas9 molecule has been engineered/modified to reduce off-target activity of the enzyme.

In some embodiments, the nucleotide sequence encoding the Cas9 molecule is modified further to alter the specificity of the endonuclease activity (*e.g.*, reduce off-target cleavage, decrease the endonuclease activity or lifetime in cells, increase homology-directed recombination and reduce non-homologous end joining). See, *e.g.*, Komor et al. *Cell* (2017) 168: 20-36. In some embodiments, the nucleotide sequence encoding the Cas9 molecule is modified to alter the PAM recognition of the endonuclease. For example, the Cas9 molecule SpCas9 recognizes PAM sequence NGG, whereas relaxed variants of the SpCas9 comprising one or more modifications of the endonuclease (*e.g.*, VQR SpCas9, EQR SpCas9, VRER SpCas9) may recognize the PAM sequences NGA, NGAG, NGCG. PAM recognition of a modified Cas9 molecule is considered “relaxed” if the Cas9 molecule recognizes more potential PAM sequences as compared to the Cas9 molecule that has not been modified. For example, the Cas9 molecule SaCas9 recognizes PAM sequence NNGRRT, whereas a relaxed variant of the SaCas9 comprising one or more modifications (*e.g.*, KKH SaCas9) may recognize the PAM sequence NNNRRT. In one example, the Cas9 molecule FnCas9 recognizes PAM sequence NNG, whereas a relaxed variant of the FnCas9 comprising one or more modifications of the endonuclease (*e.g.*, RHA FnCas9) may recognize the PAM sequence YG. In one example, the Cas9 molecule is a Cpf1 endonuclease comprising substitution mutations S542R and K607R and recognize the PAM sequence TYCV. In one example, the Cas9 molecule is a Cpf1 endonuclease comprising substitution mutations S542R, K607R, and N552R and recognize the PAM sequence TATV. See, *e.g.*, Gao et al. *Nat. Biotechnol.* (2017) 35(8): 789-792.

In some embodiments, more than one (*e.g.*, 2, 3, or more) Cas9 molecules are used. In some embodiments, at least one of the Cas9 molecule is a Cas9 enzyme. In some embodiments, at least one of the Cas molecules is a Cpf1 enzyme. In some embodiments, at least one of the Cas9 molecule is derived from *Streptococcus pyogenes*. In some embodiments, at least one of the Cas9 molecule is derived from *Streptococcus pyogenes* and at least one Cas9 molecule is derived from an organism that is not *Streptococcus pyogenes*. In some embodiments, the Cas9 molecule is a base editor. Base editor endonuclease generally comprises a catalytically inactive Cas9 molecule fused to a function domain. See, *e.g.*, Eid et al. *Biochem. J.* (2018) 475(11): 1955-1964; Rees et al. *Nature Reviews Genetics* (2018) 19:770-788. In some embodiments, the catalytically inactive Cas9 molecule is dCas9.

In some embodiments, the endonuclease comprises a dCas9 fused to one or more uracil glycosylase inhibitor (UGI) domains. In some embodiments, the endonuclease comprises a dCas9 fused to an adenine base editor (ABE), for example an ABE evolved from the RNA adenine deaminase TadA. In some embodiments, the endonuclease comprises a dCas9 fused to cytidine deaminase enzyme (*e.g.*, APOBEC deaminase, pmCDA1, activation-induced cytidine deaminase (AID)). In some embodiments, the catalytically inactive Cas9 molecule has reduced activity and is nCas9. In some embodiments, the catalytically inactive Cas9 molecule (dCas9) is fused to one or more uracil glycosylase inhibitor (UGI) domains. In some embodiments, the Cas9 molecule comprises a nCas9 fused to an adenine base editor (ABE), for example an ABE evolved from the RNA adenine deaminase TadA. In some embodiments, the Cas9 molecule comprises a nCas9 fused to cytidine deaminase enzyme (*e.g.*, APOBEC deaminase, pmCDA1, activation-induced cytidine deaminase (AID)).

Examples of base editors include, without limitation, BE1, BE2, BE3, HF-BE3, BE4, BE4max, BE4-Gam, YE1-BE3, EE-BE3, YE2-BE3, YEE-CE3, VQR-BE3, VRER-BE3, SaBE3, SaBE4, SaBE4-Gam, Sa(KKH)-BE3, Target-AID, Target-AID-NG, xBE3, eA3A-BE3, BE-PLUS, TAM, CRISPR-X, ABE7.9, ABE7.10, ABE7.10*, xABE, ABESa, VQR-ABE, VRER-ABE, Sa(KKH)-ABE, and CRISPR-SKIP. Additional examples of base editors can be found, for example, in US Publication No. 2018/0312825A1, US Publication No. 2018/0312828A1, and PCT Publication No. WO 2018/165629A1, which are incorporated by reference herein in their entireties.

In some embodiments, the base editor has been further modified to inhibit base excision repair at the target site and induce cellular mismatch repair. Any of the Cas9 molecules described herein may be fused to a Gam domain (bacteriophage Mu protein) to protect the Cas9 molecule from degradation and exonuclease activity. See, *e.g.*, Eid et al. *Biochem. J.* (2018) 475(11): 1955-1964.

In some embodiments, the Cas9 molecule belongs to class 2 type V of Cas endonuclease. Class 2 type V Cas endonucleases can be further categorized as type V-A, type V-B, type V-C, and type V-U. See, *e.g.*, Stella et al. *Nature Structural & Molecular Biology* (2017). In some embodiments, the Cas molecule is a type V-A Cas endonuclease, such as a Cpf1 nuclease. In some embodiments, the Cas9 molecule is a type V-B Cas endonuclease, such as a C2c1 endonuclease. See, *e.g.*, Shmakov et al. *Mol Cell* (2015) 60: 385-397. In some embodiments, the Cas molecule is Mad7. Alternatively or in addition, the Cas9 molecule is a Cpf1 nuclease or a variant thereof. As will be appreciated by one of skill in the art, the Cpf1 nuclease may also be referred to as Cas12a. See, *e.g.*, Strohkendl et al.

Mol. Cell (2018) 71: 1-9. In some embodiments, a composition or method described herein involves, or a host cell expresses a Cpf1 nuclease derived from *Provetella spp.* or *Francisella spp.*, *Acidaminococcus sp.* (AsCpf1), *Lachnospiraceae bacterium* (LpCpf1), or *Eubacterium rectale*. In some embodiments, the nucleotide sequence encoding the Cpf1 nuclease may be
5 codon optimized for expression in a host cell. In some embodiments, the nucleotide sequence encoding the Cpf1 endonuclease is further modified to alter the activity of the protein.

In some embodiments, catalytically inactive variants of Cas molecules (e.g., of Cas9 or Cas12a) are used according to the methods described herein. A catalytically inactive variant of Cpf1 (Cas12a) may be referred to dCas12a. As described herein, catalytically
10 inactive variants of Cpf1 maybe fused to a function domain to form a base editor. See, e.g., Rees et al. Nature Reviews Genetics (2018) 19:770-788. In some embodiments, the catalytically inactive Cas9 molecule is dCas9. In some embodiments, the endonuclease comprises a dCas12a fused to one or more uracil glycosylase inhibitor (UGI) domains. In some embodiments, the Cas9 molecule comprises a dCas12a fused to an adenine base editor
15 (ABE), for example an ABE evolved from the RNA adenine deaminase TadA. In some embodiments, the Cas molecule comprises a dCas12a fused to cytidine deaminase enzyme (e.g., APOBEC deaminase, pmCDA1, activation-induced cytidine deaminase (AID)).

Alternatively or in addition, the Cas9 molecule may be a Cas14 endonuclease or variant thereof. Cas14 endonucleases are derived from archaea and tend to be smaller in size
20 (e.g., 400–700 amino acids). Additionally Cas14 endonucleases do not require a PAM sequence. See, e.g., Harrington et al. Science (2018).

Any of the Cas9 molecules described herein may be modulated to regulate levels of expression and/or activity of the Cas9 molecule at a desired time. For example, it may be advantageous to increase levels of expression and/or activity of the Cas9 molecule during
25 particular phase(s) of the cell cycle. It has been demonstrated that levels of homology-directed repair are reduced during the G1 phase of the cell cycle, therefore increasing levels of expression and/or activity of the Cas9 molecule during the S phase, G2 phase, and/or M phase may increase homology-directed repair following the Cas endonuclease editing. In some embodiments, levels of expression and/or activity of the Cas9 molecule are increased
30 during the S phase, G2 phase, and/or M phase of the cell cycle. In one example, the Cas9 molecule fused to the N-terminal region of human Geminin. See, e.g., Gutschner et al. Cell Rep. (2016) 14(6): 1555-1566. In some embodiments, levels of expression and/or activity of the Cas9 molecule are reduced during the G1 phase. In one example, the Cas9 molecule is

modified such that it has reduced activity during the G1 phase. See, e.g., Lomova et al. Stem Cells (2018).

Alternatively or in addition, any of the Cas9 molecules described herein may be fused to an epigenetic modifier (e.g., a chromatin-modifying enzyme, e.g., DNA methylase, histone deacetylase). See, e.g., Kungulovski et al. Trends Genet. (2016) 32(2):101-113. Cas9 molecule fused to an epigenetic modifier may be referred to as “epieffectors” and may allow for temporal and/or transient endonuclease activity. In some embodiments, the Cas9 molecule is a dCas9 fused to a chromatin-modifying enzyme.

10 *Zinc Finger Nucleases*

In some embodiments, a cell or cell population described herein is produced using zinc finger (ZFN) technology. In some embodiments, the ZFN recognizes a target domain described herein, e.g., in Table 1. In general, zinc finger mediated genomic editing involves use of a zinc finger nuclease, which typically comprises a zinc finger DNA binding domain and a nuclease domain. The zinc finger binding domain may be engineered to recognize and bind to any target domain of interest, e.g., may be designed to recognize a DNA sequence ranging from about 3 nucleotides to about 21 nucleotides in length, or from about 8 to about 19 nucleotides in length. Zinc finger binding domains typically comprise at least three zinc finger recognition regions (e.g., zinc fingers).

20 Restriction endonucleases (restriction enzymes) capable of sequence-specific binding to DNA (at a recognition site) and cleaving DNA at or near the site of binding are known in the art and may be used to form ZFN for use in genomic editing. For example, Type IIS restriction endonucleases cleave DNA at sites removed from the recognition site and have separable binding and cleavage domains. In one example, the DNA cleavage domain may be derived from the FokI endonuclease.

TALENs

In some embodiments, a cell or cell population described herein is produced using TALEN technology. In some embodiments, the TALEN recognizes a target domain described herein, e.g., in Table 1. In general, TALENs are engineered restriction enzymes that can specifically bind and cleave a desired target DNA molecule. A TALEN typically contains a Transcriptional Activator-Like Effector (TALE) DNA-binding domain fused to a DNA cleavage domain. The DNA binding domain may contain a highly conserved 33-34 amino acid sequence with a divergent 2 amino acid RVD (repeat variable dipeptide motif) at

positions 12 and 13. The RVD motif determines binding specificity to a nucleic acid sequence and can be engineered to specifically bind a desired DNA sequence. In one example, the DNA cleavage domain may be derived from the FokI endonuclease. In some embodiments, the FokI domain functions as a dimer, using two constructs with unique DNA binding domains for sites in the target genome with proper orientation and spacing.

A TALEN specific to a target gene of interest can be used inside a cell to produce a double-stranded break (DSB). A mutation can be introduced at the break site if the repair mechanisms improperly repair the break via non-homologous end joining. For example, improper repair may introduce a frame shift mutation. Alternatively, a foreign DNA molecule having a desired sequence can be introduced into the cell along with the TALEN. Depending on the sequence of the foreign DNA and chromosomal sequence, this process can be used to correct a defect or introduce a DNA fragment into a target gene of interest, or introduce such a defect into the endogenous gene, thus decreasing expression of the target gene.

Some exemplary, non-limiting embodiments of endonucleases and nuclease variants suitable for use in connection with the guide RNAs and genetic engineering methods provided herein have been described above. Additional suitable nucleases and nuclease variants will be apparent to those of skill in the art based on the present disclosure and the knowledge in the art. The disclosure is not limited in this respect.

gRNA sequences and configurations

gRNA configuration generally

A gRNA can comprise a number of domains. In an embodiment, a unimolecular, sgRNA, or chimeric, gRNA comprises, e.g., from 5' to 3':

- a targeting domain (which is complementary, or partially complementary, to a target nucleic acid sequence in a target gene, e.g., in the CD123 gene;
- a first complementarity domain;
- a linking domain;
- a second complementarity domain (which is complementary to the first complementarity domain);
- a proximal domain; and
- optionally, a tail domain.

Each of these domains is now described in more detail.

The targeting domain may comprise a nucleotide sequence that is complementary, e.g., at least 80, 85, 90, or 95% complementary, e.g., fully complementary, to the target sequence on the target nucleic acid. The targeting domain is part of an RNA molecule and will therefore typically comprise the base uracil (U), while any DNA encoding the gRNA molecule will comprise the base thymine (T). While not wishing to be bound by theory, in an embodiment, it is believed that the complementarity of the targeting domain with the target sequence contributes to specificity of the interaction of the gRNA /Cas9 molecule complex with a target nucleic acid. It is understood that in a targeting domain and target sequence pair, the uracil bases in the targeting domain will pair with the adenine bases in the target sequence. In an embodiment, the target domain itself comprises in the 5' to 3' direction, an optional secondary domain, and a core domain. In an embodiment, the core domain is fully complementary with the target sequence. In an embodiment, the targeting domain is 5 to 50 nucleotides in length. The targeting domain may be between 15-25 nucleotides, 18-22 nucleotides, or 19-21 nucleotides in length. In some embodiments, the targeting domain is 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length. In some embodiments, the targeting domain is between 10-30, or between 15-25, nucleotides in length.

In some embodiments, a targeting domain comprises a core domain and a secondary targeting domain, e.g., as described in International Application WO2015157070, which is incorporated by reference in its entirety. In an embodiment, the core domain comprises about 8 to about 13 nucleotides from the 3' end of the targeting domain (e.g., the most 3' 8 to 13 nucleotides of the targeting domain). In an embodiment, the secondary domain is positioned 5' to the core domain. In many embodiments, the core domain has exact complementarity with the corresponding region of the target sequence. In other embodiments, the core domain can have 1 or more nucleotides that are not complementary with the corresponding nucleotide of the target sequence.

The first complementarity domain is complementary with the second complementarity domain, and in an embodiment, has sufficient complementarity to the second complementarity domain to form a duplexed region under at least some physiological conditions. In an embodiment, the first complementarity domain is 5 to 30 nucleotides in length. In an embodiment, the first complementarity domain comprises 3 subdomains, which, in the 5' to 3' direction are: a 5' subdomain, a central subdomain, and a 3' subdomain. In an embodiment, the 5' subdomain is 4 to 9, e.g., 4, 5, 6, 7, 8 or 9 nucleotides in length. In an embodiment, the central subdomain is 1, 2, or 3, e.g., 1, nucleotide in length. In an embodiment, the 3' subdomain is 3 to 25, e.g., 4 to 22, 4 to 18, or 4 to 10, or 3, 4, 5, 6, 7, 8, 9,

10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length. The first complementarity domain can share homology with, or be derived from, a naturally occurring first complementarity domain. In an embodiment, it has at least 50% homology with a *S. pyogenes*, *S. aureus* or *S. thermophilus*, first complementarity domain.

5 The sequence and placement of the above-mentioned domains are described in more detail in WO2015157070, which is herein incorporated by reference in its entirety, including p. 88-112 therein.

10 A linking domain serves to link the first complementarity domain with the second complementarity domain of a unimolecular gRNA. The linking domain can link the first and second complementarity domains covalently or non-covalently. In an embodiment, the linkage is covalent. In an embodiment, the linking domain is, or comprises, a covalent bond interposed between the first complementarity domain and the second complementarity domain. In some embodiments, the linking domain comprises one or more, e.g., 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides. In some embodiments, the linking domain comprises at least one
15 non-nucleotide bond, e.g., as disclosed in WO2018126176, the entire contents of which are incorporated herein by reference.

 The second complementarity domain is complementary, at least in part, with the first complementarity domain, and in an embodiment, has sufficient complementarity to the second complementarity domain to form a duplexed region under at least some physiological
20 conditions. In an embodiment, the second complementarity domain can include a sequence that lacks complementarity with the first complementarity domain, e.g., a sequence that loops out from the duplexed region. In an embodiment, the second complementarity domain is 5 to 27 nucleotides in length. In an embodiment, the second complementarity domain is longer than the first complementarity region. In an embodiment, the complementary domain is 5, 6,
25 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or 25 nucleotides in length. In an embodiment, the second complementarity domain comprises 3 subdomains, which, in the 5' to 3' direction are: a 5' subdomain, a central subdomain, and a 3' subdomain. In an embodiment, the 5' subdomain is 3 to 25, e.g., 4 to 22, 4 to 18, or 4 to 10, or 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length. In an
30 embodiment, the central subdomain is 1, 2, 3, 4 or 5, e.g., 3, nucleotides in length. In an embodiment, the 3' subdomain is 4 to 9, e.g., 4, 5, 6, 7, 8 or 9 nucleotides in length. In an embodiment, the 5' subdomain and the 3' subdomain of the first complementarity domain, are respectively, complementary, e.g., fully complementary, with the 3' subdomain and the 5' subdomain of the second complementarity domain.

In an embodiment, the proximal domain is 5 to 20 nucleotides in length. In an embodiment, the proximal domain can share homology with or be derived from a naturally occurring proximal domain. In an embodiment, it has at least 50% homology with an *S. pyogenes*, *S. aureus* or *S. thermophilus*, proximal domain.

5 A broad spectrum of tail domains are suitable for use in gRNAs. In an embodiment, the tail domain is 0 (absent), 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides in length. In some embodiments, the tail domain nucleotides are from or share homology with a sequence from the 5' end of a naturally occurring tail domain. In an embodiment, the tail domain includes sequences that are complementary to each other and which, under at least some physiological
10 conditions, form a duplexed region. In an embodiment, the tail domain is absent or is 1 to 50 nucleotides in length. In an embodiment, the tail domain can share homology with or be derived from a naturally occurring proximal tail domain. In an embodiment, it has at least 50% homology with an *S. pyogenes*, *S. aureus* or *S. thermophilus*, tail domain. In an embodiment, the tail domain includes nucleotides at the 3' end that are related to the method
15 of in vitro or in vivo transcription.

In some embodiments, modular gRNA comprises:

a first strand comprising, e.g., from 5' to 3':

a targeting domain (which is complementary to a target nucleic acid in the CD123 gene) and

20 a first complementarity domain; and

a second strand, comprising, preferably from 5' to 3':

optionally, a 5' extension domain;

a second complementarity domain;

a proximal domain; and

25 optionally, a tail domain.

In some embodiments, the gRNA is chemically modified. For instance, the gRNA may comprise one or more modification chosen from phosphorothioate backbone modification, 2'-O-Me-modified sugars (e.g., at one or both of the 3' and 5' termini), 2'-F-modified sugar, replacement of the ribose sugar with the bicyclic nucleotide-cEt, 3'thioPACE
30 (MSP), or any combination thereof. Suitable gRNA modifications are described, e.g., in Rahdar et al. PNAS December 22, 2015 112 (51) E7110-E7117 and Hendel et al., Nat Biotechnol. 2015 Sep; 33(9): 985-989, each of which is incorporated herein by reference in its entirety. In some embodiments, a gRNA described herein comprises one or more 2'-O-methyl-3'-phosphorothioate nucleotides, e.g., at least 2, 3, 4, 5, or 6 2'-O-methyl-3'-

phosphorothioate nucleotides. In some embodiments, a gRNA described herein comprises modified nucleotides (e.g., 2'-O-methyl-3'-phosphorothioate nucleotides) at the three terminal positions and the 5' end and/or at the three terminal positions and the 3' end. In some embodiments, the gRNA may comprise one or more modified nucleotides, e.g., as described
5 in International Applications WO/2017/214460, WO/2016/089433, and WO/2016/164356, which are incorporated by reference their entirety.

In some embodiments, a gRNA described herein is chemically modified. For example, the gRNA may comprise one or more 2'-O modified nucleotides, e.g., 2'-O-methyl nucleotides. In some embodiments, the gRNA comprises a 2'-O modified nucleotide, e.g.,
10 2'-O-methyl nucleotide at the 5' end of the gRNA. In some embodiments, the gRNA comprises a 2'-O modified nucleotide, e.g., 2'-O-methyl nucleotide at the 3' end of the gRNA. In some embodiments, the gRNA comprises a 2'-O-modified nucleotide, e.g., 2'-O-methyl nucleotide at both the 5' and 3' ends of the gRNA. In some embodiments, the gRNA is 2'-O-modified, e.g. 2'-O-methyl-modified at the nucleotide at the 5' end of the gRNA, the
15 second nucleotide from the 5' end of the gRNA, and the third nucleotide from the 5' end of the gRNA. In some embodiments, the gRNA is 2'-O-modified, e.g. 2'-O-methyl-modified at the nucleotide at the 3' end of the gRNA, the second nucleotide from the 3' end of the gRNA, and the third nucleotide from the 3' end of the gRNA. In some embodiments, the gRNA is 2'-O-modified, e.g. 2'-O-methyl-modified at the nucleotide at the 5' end of the gRNA, the
20 second nucleotide from the 5' end of the gRNA, the third nucleotide from the 5' end of the gRNA, the nucleotide at the 3' end of the gRNA, the second nucleotide from the 3' end of the gRNA, and the third nucleotide from the 3' end of the gRNA. In some embodiments, the gRNA is 2'-O-modified, e.g. 2'-O-methyl-modified at the second nucleotide from the 3' end of the gRNA, the third nucleotide from the 3' end of the gRNA, and at the fourth nucleotide
25 from the 3' end of the gRNA. In some embodiments, the nucleotide at the 3' end of the gRNA is not chemically modified. In some embodiments, the nucleotide at the 3' end of the gRNA does not have a chemically modified sugar. In some embodiments, the gRNA is 2'-O-modified, e.g. 2'-O-methyl-modified, at the nucleotide at the 5' end of the gRNA, the second nucleotide from the 5' end of the gRNA, the third nucleotide from the 5' end of the gRNA,
30 the second nucleotide from the 3' end of the gRNA, the third nucleotide from the 3' end of the gRNA, and the fourth nucleotide from the 3' end of the gRNA. In some embodiments, the 2'-O-methyl nucleotide comprises a phosphate linkage to an adjacent nucleotide. In some embodiments, the 2'-O-methyl nucleotide comprises a phosphorothioate linkage to an

adjacent nucleotide. In some embodiments, the 2'-O-methyl nucleotide comprises a thioPACE linkage to an adjacent nucleotide.

In some embodiments, the gRNA may comprise one or more 2'-O-modified and 3'phosphorous-modified nucleotide, e.g., a 2'-O-methyl 3'phosphorothioate nucleotide. In some embodiments, the gRNA comprises a 2'-O-modified and 3'phosphorous-modified, e.g., 2'-O-methyl 3'phosphorothioate nucleotide at the 5' end of the gRNA. In some embodiments, the gRNA comprises a 2'-O-modified and 3'phosphorous-modified, e.g., 2'-O-methyl 3'phosphorothioate nucleotide at the 3' end of the gRNA. In some embodiments, the gRNA comprises a 2'-O-modified and 3'phosphorous-modified, e.g., 2'-O-methyl 3'phosphorothioate nucleotide at the 5' and 3' ends of the gRNA. In some embodiments, the gRNA comprises a backbone in which one or more non-bridging oxygen atoms has been replaced with a sulfur atom. In some embodiments, the gRNA is 2'-O-modified and 3'phosphorous-modified, e.g. 2'-O-methyl 3'phosphorothioate-modified at the nucleotide at the 5' end of the gRNA, the second nucleotide from the 5' end of the gRNA, and the third nucleotide from the 5' end of the gRNA. In some embodiments, the gRNA is 2'-O-modified and 3'phosphorous-modified, e.g. 2'-O-methyl 3'phosphorothioate-modified at the nucleotide at the 3' end of the gRNA, the second nucleotide from the 3' end of the gRNA, and the third nucleotide from the 3' end of the gRNA. In some embodiments, the gRNA is 2'-O-modified and 3'phosphorous-modified, e.g. 2'-O-methyl 3'phosphorothioate-modified at the nucleotide at the 5' end of the gRNA, the second nucleotide from the 5' end of the gRNA, the third nucleotide from the 5' end of the gRNA, the nucleotide at the 3' end of the gRNA, the second nucleotide from the 3' end of the gRNA, and the third nucleotide from the 3' end of the gRNA. In some embodiments, the gRNA is 2'-O-modified and 3'phosphorous-modified, e.g. 2'-O-methyl 3'phosphorothioate-modified at the second nucleotide from the 3' end of the gRNA, the third nucleotide from the 3' end of the gRNA, and the fourth nucleotide from the 3' end of the gRNA. In some embodiments, the nucleotide at the 3' end of the gRNA is not chemically modified. In some embodiments, the nucleotide at the 3' end of the gRNA does not have a chemically modified sugar. In some embodiments, the gRNA is 2'-O-modified and 3'phosphorous-modified, e.g. 2'-O-methyl 3'phosphorothioate-modified at the nucleotide at the 5' end of the gRNA, the second nucleotide from the 5' end of the gRNA, the third nucleotide from the 5' end of the gRNA, the second nucleotide from the 3' end of the gRNA, the third nucleotide from the 3' end of the gRNA, and the fourth nucleotide from the 3' end of the gRNA.

In some embodiments, the gRNA may comprise one or more 2'-O-modified and 3'-phosphorous-modified, e.g., 2'-O-methyl 3'thioPACE nucleotide. In some embodiments, the gRNA comprises a 2'-O-modified and 3'phosphorous-modified, e.g., 2'-O-methyl 3'thioPACE nucleotide at the 5' end of the gRNA. In some embodiments, the gRNA
5 comprises a 2'-O-modified and 3'phosphorous-modified, e.g., 2'-O-methyl 3'thioPACE nucleotide at the 3' end of the gRNA. In some embodiments, the gRNA comprises a 2'-O-modified and 3'phosphorous-modified, e.g., 2'-O-methyl 3'thioPACE nucleotide at the 5' and 3' ends of the gRNA. In some embodiments, the gRNA comprises a backbone in which one or more non-bridging oxygen atoms have been replaced with a sulfur atom and one or
10 more non-bridging oxygen atoms have been replaced with an acetate group. In some embodiments, the gRNA is 2'-O-modified and 3'phosphorous-modified, e.g. 2'-O-methyl 3'thioPACE-modified at the nucleotide at the 5' end of the gRNA, the second nucleotide from the 5' end of the gRNA, and the third nucleotide from the 5' end of the gRNA. In some embodiments, the gRNA is 2'-O-modified and 3'phosphorous-modified, e.g. 2'-O-methyl
15 3'thioPACE-modified at the nucleotide at the 3' end of the gRNA, the second nucleotide from the 3' end of the gRNA, and the third nucleotide from the 3' end of the gRNA. In some embodiments, the gRNA is 2'-O-modified and 3'phosphorous-modified, e.g. 2'-O-methyl 3'thioPACE-modified at the nucleotide at the 5' end of the gRNA, the second nucleotide from the 5' end of the gRNA, the third nucleotide from the 5' end of the gRNA, the
20 nucleotide at the 3' end of the gRNA, the second nucleotide from the 3' end of the gRNA, and the third nucleotide from the 3' end of the gRNA. In some embodiments, the gRNA is 2'-O-modified and 3'phosphorous-modified, e.g. 2'-O-methyl 3'thioPACE-modified at the second nucleotide from the 3' end of the gRNA, the third nucleotide from the 3' end of the gRNA, and the fourth nucleotide from the 3' end of the gRNA. In some embodiments, the
25 nucleotide at the 3' end of the gRNA is not chemically modified. In some embodiments, the nucleotide at the 3' end of the gRNA does not have a chemically modified sugar. In some embodiments, the gRNA is 2'-O-modified and 3'phosphorous-modified, e.g. 2'-O-methyl 3'thioPACE-modified at the nucleotide at the 5' end of the gRNA, the second nucleotide from the 5' end of the gRNA, the third nucleotide from the 5' end of the gRNA, the second
30 nucleotide from the 3' end of the gRNA, the third nucleotide from the 3' end of the gRNA, and the fourth nucleotide from the 3' end of the gRNA.

In some embodiments, the gRNA comprises a chemically modified backbone. In some embodiments, the gRNA comprises a phosphorothioate linkage. In some embodiments, one or more non-bridging oxygen atoms have been replaced with a sulfur atom. In some

embodiments, the nucleotide at the 5' end of the gRNA, the second nucleotide from the 5' end of the gRNA, and the third nucleotide from the 5' end of the gRNA each comprise a phosphorothioate linkage. In some embodiments, the nucleotide at the 3' end of the gRNA, the second nucleotide from the 3' end of the gRNA, and the third nucleotide from the 3' end of the gRNA each comprise a phosphorothioate linkage. In some embodiments, the nucleotide at the 5' end of the gRNA, the second nucleotide from the 5' end of the gRNA, the third nucleotide from the 5' end of the gRNA, the nucleotide at the 3' end of the gRNA, the second nucleotide from the 3' end of the gRNA, and the third nucleotide from the 3' end of the gRNA each comprise a phosphorothioate linkage. In some embodiments, the second nucleotide from the 3' end of the gRNA, the third nucleotide from the 3' end of the gRNA, and at the fourth nucleotide from the 3' end of the gRNA each comprise a phosphorothioate linkage. In some embodiments, the nucleotide at the 5' end of the gRNA, the second nucleotide from the 5' end of the gRNA, the third nucleotide from the 5' end, the second nucleotide from the 3' end of the gRNA, the third nucleotide from the 3' end of the gRNA, and the fourth nucleotide from the 3' end of the gRNA each comprise a phosphorothioate linkage.

In some embodiments, the gRNA comprises a thioPACE linkage. In some embodiments, the gRNA comprises a backbone in which one or more non-bridging oxygen atoms have been replaced with a sulfur atom and one or more non-bridging oxygen atoms have been replaced with an acetate group. In some embodiments, the nucleotide at the 5' end of the gRNA, the second nucleotide from the 5' end of the gRNA, and the third nucleotide from the 5' end of the gRNA each comprise a thioPACE linkage. In some embodiments, the nucleotide at the 3' end of the gRNA, the second nucleotide from the 3' end of the gRNA, and the third nucleotide from the 3' end of the gRNA each comprise a thioPACE linkage. In some embodiments, the nucleotide at the 5' end of the gRNA, the second nucleotide from the 5' end of the gRNA, the third nucleotide from the 5' end of the gRNA, the nucleotide at the 3' end of the gRNA, the second nucleotide from the 3' end of the gRNA, and the third nucleotide from the 3' end of the gRNA each comprise a thioPACE linkage. In some embodiments, the second nucleotide from the 3' end of the gRNA, the third nucleotide from the 3' end of the gRNA, and at the fourth nucleotide from the 3' end of the gRNA each comprise a thioPACE linkage. In some embodiments, the nucleotide at the 5' end of the gRNA, the second nucleotide from the 5' end of the gRNA, the third nucleotide from the 5' end, the second nucleotide from the 3' end of the gRNA, the third nucleotide from the 3' end

of the gRNA, and the fourth nucleotide from the 3' end of the gRNA each comprise a thioPACE linkage.

Some exemplary, non-limiting embodiments of modifications, e.g., chemical modifications, suitable for use in connection with the guide RNAs and genetic engineering methods provided herein have been described above. Additional suitable modifications, e.g., chemical modifications, will be apparent to those of skill in the art based on the present disclosure and the knowledge in the art, including, but not limited to those described in Hendel, A. et al., Nature Biotech., 2015, Vol 33, No. 9; in WO/2017/214460; in WO/2016/089433; and/or in WO/2016/164356; each one of which is herein incorporated by reference in its entirety.

gRNAs targeting CD123

The present disclosure provides a number of useful gRNAs that can target an endonuclease to human CD123. Table 1 below illustrates target domains in human endogenous CD123 that can be bound by gRNAs described herein.

Table 1. Exemplary target domains of human CD123 bound by various gRNAs are described herein. For each target domain, the first sequence represents a 20-nucleotide DNA sequence corresponding to the target domain sequence that can be targeted by a suitable gRNA, which may comprise an equivalent RNA targeting domain sequence (comprising RNA nucleotides instead of DNA nucleotides), and the second sequence is the reverse complement thereof. Bolding indicates that the sequence is present in the human CD123 cDNA sequence shown below as SEQ ID NO: 31.

	Target Domain Sequences
gRNA A	GCCCTGTCTCCTGCAAACGA (SEQ ID NO: 1) TCGTTTGCAGGAGACAGGGC (SEQ ID NO: 11)
gRNA B	TGAGCCAAAGGAGGACCATC (SEQ ID NO: 2) GATGGTCCTCCTTTGGCTCA (SEQ ID NO: 12)
gRNA C	TCAGGAGCAGCGTGAGCCAA (SEQ ID NO: 3) TTGGCTCACGCTGCTCCTGA (SEQ ID NO: 13)
gRNA D	TCCTTCGTTTGCAGGAGACA (SEQ ID NO: 4) TGTCTCCTGCAAACGAAGGA (SEQ ID NO: 14)
gRNA E	ATCCACGTCATGAATCCAGC (SEQ ID NO: 5)

	GCTGGATTTCATGACGTGGAT (SEQ ID NO: 15)
gRNA F	CAGGTCGTACTGGACGTCCG (SEQ ID NO: 6) CGGACGTCCAGTACGACCTG (SEQ ID NO: 16)
gRNA G	TTTCTTGAGCTGCAGCTGGG (SEQ ID NO: 7) CCCAGCTGCAGCTCAAGAAA (SEQ ID NO: 17)
gRNA H	GGTCGTACTGGACGTCCGCG (SEQ ID NO: 8) CGCGGACGTCCAGTACGACC (SEQ ID NO: 18)
gRNA I	AGTTCCCACATCCTGGTGCG (SEQ ID NO: 9) CGCACCAGGATGTGGAACT (SEQ ID NO: 19)
gRNA J	CACTACAAAACGGATGCTCA (SEQ ID NO: 10) TGAGCATCCGTTTTGTAGTG (SEQ ID NO: 20)
gRNA D1	TTCATCCTTAGGTTTCGTGAT (SEQ ID NO: 40) ATCACGAACCTAAGGATGAA (SEQ ID NO: 41)
gRNA N3	TTGACGCCTGCTGCGGTAAG (SEQ ID NO: 42) CTTACCGCAGCAGGCGTCAA (SEQ ID NO: 43)
gRNA P3	CGAGTGTCTTCACTACAAAA (SEQ ID NO: 44) TTTTGTAGTGAAGACACTCG (SEQ ID NO: 45)
gRNA S3	ATGCTCAGGGAACACGTATC (SEQ ID NO: 46) GATACGTGTTCCCTGAGCAT (SEQ ID NO: 47)

Table 2. Exemplary target domain sequences of human CD123 bound by various gRNAs are provided herein. For each target domain, the first sequence represents a DNA target sequence adjacent to a suitable PAM in the human CD123 genomic sequence, and the second sequence represents an exemplary equivalent gRNA targeting domain sequence.

	Sequences	PAM
gRNA A	GCCCTGTCTCCTGCAAACGA (SEQ ID NO: 1) GCCCUGUCUCCUGCAAACGA (SEQ ID NO: 21)	AGG
gRNA B	TGAGCCAAAGGAGGACCATC (SEQ ID NO: 2) UGAGCCAAAGGAGGACCAUC (SEQ ID NO: 22)	GGG
gRNA C	TCAGGAGCAGCGTGAGCCAA (SEQ ID NO: 3) UCAGGAGCAGCGUGAGCCAA (SEQ ID NO: 23)	AGG
gRNA D	TCCTTCGTTTGCAGGAGACA (SEQ ID NO: 4) UCCUUCGUUUGCAGGAGACA (SEQ ID NO: 24)	GGG
gRNA E	ATCCACGTCATGAATCCAGC (SEQ ID NO: 5)	AGG

	GCUGGAUUCAUGACGUGGAU (SEQ ID NO: 25)	
gRNA F	CAGGTCGTACTGGACGTCCG (SEQ ID NO: 6) CAGGUCGUACUGGACGUCCG (SEQ ID NO: 26)	CGG
gRNA G	TTTCTTGAGCTGCAGCTGGG (SEQ ID NO: 7) UUUCUUGAGCUGCAGCUGGG (SEQ ID NO: 27)	CGG
gRNA H	GGTCGTACTGGACGTCCGCG (SEQ ID NO: 8) GGUCGUACUGGACGUCCGCG (SEQ ID NO: 28)	GGG
gRNA I	AGTTCCCACATCCTGGTGCG (SEQ ID NO: 9) AGUUCCCACAUCUGGUGCG (SEQ ID NO: 29)	GGG
gRNA J	CACTACAAAACGGATGCTCA (SEQ ID NO: 10) UGAGCAUCCGUUUUGUAGUG (SEQ ID NO: 30)	GGG
gRNA D1	TTCATCCTTAGGTTCTGTGAT (SEQ ID NO: 40) UUCAUCCUUAGGUUCGUGAU (SEQ ID NO: 48)	TGG
gRNA N3	TTGACGCCTGCTGCGGTAAG (SEQ ID NO: 42) UUGACGCCUGCUGCGGUAAG (SEQ ID NO: 49)	CGG
gRNA P3	CGAGTGTCTTCACTACAAAA (SEQ ID NO: 44) CGAGUGUCUUCACUACAAAA (SEQ ID NO: 50)	CGG
gRNA S3	ATGCTCAGGGAACACGTATC (SEQ ID NO: 46) AUGCUCAGGGAACACGUAUC (SEQ ID NO: 51)	GGG

Table 6. Exemplary target domain sequences of human CD123 bound by various gRNAs are provided herein. For each target domain, a DNA target sequence adjacent to a suitable PAM in the human CD123 genomic sequence is provided. A gRNA targeting a target domain provided herein may comprise an equivalent RNA sequence within its targeting domain.

	SEQ ID NO:	Sequence	PAM
gRNA K	66	TTCCGGAGCTGCGTTCCCGA	TGG
gRNA L	67	GACCATCGGGAACGCAGCTC	CGG
gRNA M	68	CGTTCCCGATGGTCCTCCTT	TGG
gRNA N	69	GTGAGCCAAAGGAGGACCAT	CGG
gRNA O	70	GGAGCAGCGTGAGCCAAAGG	AGG

gRNA P	71	GGAGACAGGGCAGGGCGATC	AGG
gRNA Q	72	CGTTTGCAGGAGACAGGGCA	GGG
gRNA R	11	TCGTTTGCAGGAGACAGGGC	AGG
gRNA S	14	TGTCTCCTGCAAACGAAGGA	AGG
gRNA T	73	TTCCTTCGTTTGCAGGAGAC	AGG
gRNA U	74	TCTTACCTTCCTTCGTTTGC	AGG
gRNA V	75	AAACGAAGGAAGGTAAGAAC	TGG
gRNA W	76	GATCTAAAACGGTGACAGGT	TGG
gRNA X	77	TTTGGATCTAAAACGGTGAC	AGG
gRNA Y	78	TGGTGGGTTTGGATCTAAAA	CGG
gRNA Z	79	AGGTTTCGTGATTGGTGGGTT	TGG
gRNA A1	80	ACCCACCAATCACGAACCTA	AGG
gRNA B1	81	TCCTTAGGTTTCGTGATTGGT	GGG
gRNA C1	82	ATCCTTAGGTTTCGTGATTGG	TGG
gRNA E1	83	GAACCTAAGGATGAAAGCAA	AGG
gRNA F1	84	GAGCCTTTGCTTTCATCCTT	AGG
gRNA G1	85	CAAAGGCTCAGCAGTTGACC	TGG
gRNA H1	86	AAAGGCTCAGCAGTTGACCT	GGG
gRNA I1	87	CACATTTCTGTTAAGGTCCC	AGG
gRNA J1	88	TATCGGTCACATTTCTGTTA	AGG
gRNA K1	89	GTCTTTAACACACTCGATAT	CGG
gRNA L1	90	AGACGCCGACTATTCTATGC	CGG
gRNA M1	91	ATTTACCGGCATAGAATAGT	CGG

gRNA N1	92	CAATAGAGAGTATGATTAC	CGG
gRNA O1	93	CATAGTCCTATGTCTCTCTT	AGG
gRNA P1	94	TCACTGCCTAAGAGAGACAT	AGG
gRNA Q1	95	AACAATAGCTATTGCCAGTT	TGG
gRNA R1	96	ATAAGGAAATTGCTCCAAAC	TGG
gRNA S1	97	GTAGTTGGTCACTTCACATA	AGG
gRNA T1	98	GACCAACTACACCGTCCGAG	TGG
gRNA U1	99	GGCCACTCGGACGGTGTAGT	TGG
gRNA V1	100	TGGTGGGTTGGCCACTCGGA	CGG
gRNA W1	101	AGAATGGTGGGTTGGCCACT	CGG
gRNA X1	102	CCAACCCACCATTCTCCACG	TGG
gRNA Y1	103	CCACGTGGAGAATGGTGGGT	TGG
gRNA Z1	104	GGATCCACGTGGAGAATGGT	GGG
gRNA A2	105	AGGATCCACGTGGAGAATGG	TGG
gRNA B2	106	AAGAGGATCCACGTGGAGAA	TGG
gRNA C2	107	CTCAGGGAAGAGGATCCACG	TGG
gRNA D2	108	TCTCACTGTTCTCAGGGAAG	AGG
gRNA E2	109	CATTTTTCTCACTGTTCTCA	GGG
gRNA F2	110	ACATTTTTCTCACTGTTCTC	AGG
gRNA G2	111	TCTTTCATGTTTGTGAACCC	AGG
gRNA H2	112	TTCATGTTTGTGAACCCAGG	TGG
gRNA I2	113	TCATGTTTGTGAACCCAGGT	GGG
gRNA J2	114	TGAACCCAGGTGGGAAGCCT	TGG

gRNA K2	115	GAACCCAGGTGGGAAGCCTT	GGG
gRNA L2	116	CCAGGTGGGAAGCCTTGGGC	AGG
gRNA M2	117	CTGCCCCAAGGCTTCCCACCT	GGG
gRNA N2	118	CCTGCCCCAAGGCTTCCCACC	TGG
gRNA O2	119	TGGGAAGCCTTGGGCAGGTG	CGG
gRNA P2	120	AGATTCTCCGCACCTGCCCA	AGG
gRNA Q2	121	GTGCGGAGAATCTGACCTGC	TGG
gRNA R2	122	GACCTGCTGGATTCATGACG	TGG
gRNA S2	123	TGGATTTCTTGAGCTGCAGC	TGG
gRNA T2	124	GGATTTCTTGAGCTGCAGCT	GGG
gRNA U2	125	TTGAGCTGCAGCTGGGCGGT	AGG
gRNA V2	126	CTGCAGCTGGGCGGTAGGCC	CGG
gRNA W2	127	TGCAGCTGGGCGGTAGGCCC	GGG
gRNA X2	128	GCAGCTGGGCGGTAGGCCCCG	GGG
gRNA Y2	129	CAGCTGGGCGGTAGGCCCCGG	GGG
gRNA Z2	130	GGTAGGCCCCGGGGGCCCCCG	CGG
gRNA A3	131	GGACGTCCGCGGGGGCCCCC	GGG
gRNA B3	132	TGGACGTCCGCGGGGGCCCC	CGG
gRNA C3	133	GTCGTACTGGACGTCCGCGG	GGG
gRNA D3	134	AGGTCGTACTGGACGTCCGC	GGG
gRNA E3	135	CGTTCAAGTACAGGTCGTAC	TGG
gRNA F3	136	GTACTTGAACGTTGCCAAGT	AGG
gRNA G3	137	ACTTGGCAACGTTCAAGTAC	AGG

gRNA H3	138	TTGCCAAGTAGGTGTGCCCCG	TGG
gRNA I3	139	TGCCAAGTAGGTGTGCCCCGT	GGG
gRNA J3	140	TGCCCACGGGCACACCTACT	TGG
gRNA K3	141	ACCTTACCGCTTACCGCAGC	AGG
gRNA L3	142	GCTGCGGTAAGCGGTAAGGT	TGG
gRNA M3	143	GCCTGCTGCGGTAAGCGGTA	AGG
gRNA O3	144	CGTACTGTTGACGCCTGCTG	CGG
gRNA Q3	145	TCACTACAAAACGGATGCTC	AGG
gRNA R3	146	GATGCTCAGGGAACACGTAT	CGG
gRNA T3	147	GACATCTCTCGACTCTCCAG	CGG
gRNA U3	148	GTGGGAACTTTGAGAACCGC	TGG
gRNA V3	149	TTCTCAAAGTTCCCACATCC	TGG
gRNA W3	150	AAAGTTCCCACATCCTGGTG	CGG
gRNA X3	151	AAGTTCCCACATCCTGGTGC	GGG
gRNA Y3	152	CCCACATCCTGGTGCGGGGC	AGG
gRNA Z3	153	CCTGCCCCGCACCAGGATGT	GGG
gRNA A4	154	TCCTGCCCCGCACCAGGATG	TGG
gRNA B4	155	CTGCGCTCCTGCCCCGCACC	AGG
gRNA C4	156	CGGGGCAGGAGCGCAGCCTT	CGG
gRNA D4	157	ATCTGTGCAGGGGATACCGA	AGG
gRNA E4	158	CGACAAACTTATCTGTGCAG	GGG
gRNA F4	159	ACGACAAACTTATCTGTGCA	GGG
gRNA G4	160	GACGACAAACTTATCTGTGC	AGG

gRNA H4	161	TTTGTCGTCTTTTCACAGAT	TGG
gRNA I4	162	TCACAGATTGGTGAGTAGCC	CGG
gRNA J4	163	CACAGATTGGTGAGTAGCCC	GGG
gRNA K4	164	CACTTTGCAGTCATGTTGGG	TGG
gRNA L4	165	TTACACTTTGCAGTCATGTT	GGG
gRNA M4	166	ATTACACTTTGCAGTCATGT	TGG
gRNA N4	167	AGACACATTCCTTTATGCAC	TGG
gRNA O4	168	TCTCATTTTCCAGTGCATAA	AGG
gRNA P4	169	CTATGAGCTTCAGATACAAA	AGG
gRNA Q4	170	GCAGCCTGTAATCACAGAAC	AGG
gRNA R4	171	CTCACCTGTTCTGTGATTAC	AGG
gRNA S4	172	TTTATTTTCTTTCAAACCAC	AGG
gRNA T4	173	GAGGTTCTGTCTCTGACCTG	TGG
gRNA U4	174	TCCTTCCAGCTACTCAATCC	TGG
gRNA V4	175	AGGATTGAGTAGCTGGAAGG	AGG
gRNA W4	176	TCCAGGATTGAGTAGCTGGA	AGG
gRNA X4	177	ACGTTCCAGGATTGAGTAGC	TGG
gRNA Y4	178	ATTTGTACTGTGTACGTTCC	AGG
gRNA Z4	179	ACACAGTACAAATAAGAGCC	CGG
gRNA A5	180	CACAGTACAAATAAGAGCCC	GGG
gRNA B5	181	GAATTCATACACTCTTTCCC	GGG
gRNA C5	182	AGAATTCATACACTCTTTCC	CGG
gRNA D5	183	TGTATGAATTCTTGAGCGCC	TGG

gRNA E5	184	TGGAGCACCCCCCAGCGCTT	CGG
gRNA F5	185	GAAGCGCTGGGGGGTGCTCC	AGG
gRNA G5	186	CCCCCAGCGCTTCGGTGAG	TGG
gRNA H5	187	CCCCCAGCGCTTCGGTGAGT	GGG
gRNA I5	188	CCACTCACC GAAGCGCTGGG	GGG
gRNA J5	189	CCCACTCACC GAAGCGCTGG	GGG
gRNA K5	190	GCCCACTCACC GAAGCGCTG	GGG
gRNA L5	191	AGCCCACTCACC GAAGCGCT	GGG
gRNA M5	192	CAGCCCACTCACC GAAGCGC	TGG
gRNA N5	193	GCTTCGGTGAGTGGGCTGTG	CGG
gRNA O5	194	CTTCGGTGAGTGGGCTGTGC	GGG
gRNA P5	195	TTCGGTGAGTGGGCTGTGCG	GGG
gRNA Q5	196	TCTAGGGGTAAAGGGTGAGA	GGG
gRNA R5	197	CTCTAGGGGTAAAGGGTGAG	AGG
gRNA S5	198	TTTACCCCTAGAGTGCGACC	AGG
gRNA T5	199	GGTCGCACTCTAGGGGTAAA	GGG
gRNA U5	200	TGGTCGCACTCTAGGGGTAA	AGG
gRNA V5	201	ACCCCTAGAGTGCGACCAGG	AGG
gRNA W5	202	CCTAGAGTGCGACCAGGAGG	AGG
gRNA X5	203	CTAGAGTGCGACCAGGAGGA	GGG
gRNA Y5	204	TCCTCCTGGTCGCACTCTAG	GGG
gRNA Z5	205	CTCCTCCTGGTCGCACTCTA	GGG
gRNA A6	206	CCTCCTCCTGGTCGCACTCT	AGG

gRNA B6	207	GTGTGTTTGC GCCCTCCTCC	TGG
gRNA C6	208	AGGGCGCAAACACACGTGCC	TGG
gRNA D6	209	GCGCAAACACACGTGCCTGG	CGG
gRNA E6	210	GATCAGCAGCGACGTCCGCC	AGG
gRNA F6	211	GACGTCGCTGCTGATCGCGC	TGG
gRNA G6	212	ACGTCGCTGCTGATCGCGCT	GGG
gRNA H6	213	CGTCGCTGCTGATCGCGCTG	GGG
gRNA I6	214	GATCGCGCTGGGGACGCTGC	TGG
gRNA J6	215	GCTGGGGACGCTGCTGGCCC	TGG
gRNA K6	216	GATCACGAAGACACAGACCA	GGG
gRNA L6	217	AGATCACGAAGACACAGACC	AGG
gRNA M6	218	GTGTCTTCGTGATCTGCAGA	AGG
gRNA N6	219	CTGCAGAAGGTGAGCCCTCG	AGG
gRNA O6	220	TGCAGAAGGTGAGCCCTCGA	GGG
gRNA P6	221	GGCCATTTCTCTTTCTCCG	AGG
gRNA Q6	222	TACCTCGGAGGAAAGAGAAA	TGG
gRNA R6	223	TCTCTTTCCTCCGAGGTATC	TGG
gRNA S6	224	TGCATCACCAGATACCTCGG	AGG
gRNA T6	225	CTCTGCATCACCAGATACCT	CGG
gRNA U6	226	TCTTTCATGTGAGGGATGCG	GGG
gRNA V6	227	GTCTTTCATGTGAGGGATGC	GGG
gRNA W6	228	GGTCTTTCATGTGAGGGATG	CGG
gRNA X6	229	CCTCACATGAAAGACCCCAT	CGG

gRNA Y6	230	CGATGGGGTCTTTCATGTGA	GGG
gRNA Z6	231	CCGATGGGGTCTTTCATGTG	AGG
gRNA A7	232	TTTGGAAGCTGTCACCGATG	GGG
gRNA B7	233	TTTTGGAAGCTGTCACCGAT	GGG
gRNA C7	234	GTTTTGGAAGCTGTCACCGA	TGG
gRNA D7	235	CAGCTTCCAAAACGACAAGC	TGG
gRNA E7	236	AACATACCAGCTTGTCGTTT	TGG
gRNA F7	237	CTGCCTCCTCTCGTCTCTGC	AGG
gRNA G7	238	CCTCCTCTCGTCTCTGCAGG	TGG
gRNA H7	239	CCACCTGCAGAGACGAGAGG	AGG
gRNA I7	240	TCTCGTCTCTGCAGGTGGTC	TGG
gRNA J7	241	CTCGTCTCTGCAGGTGGTCT	GGG
gRNA K7	242	AGACCACCTGCAGAGACGAG	AGG
gRNA L7	243	GTCTCTGCAGGTGGTCTGGG	AGG
gRNA M7	244	TCTGCAGGTGGTCTGGGAGG	CGG
gRNA N7	245	CTGCAGGTGGTCTGGGAGGC	GGG
gRNA O7	246	GTCTGGGAGGCGGGCAAAGC	CGG
gRNA P7	247	GGAGGCGGGCAAAGCCGGCC	TGG
gRNA Q7	248	GGCGGGCAAAGCCGGCCTGG	AGG
gRNA R7	249	AGCCGGCCTGGAGGAGTGTC	TGG
gRNA S7	250	CACCAGACACTCCTCCAGGC	CGG
gRNA T7	251	CAGTCACCAGACACTCCTCC	AGG
gRNA U7	252	GTGTCTGGTGACTGAAGTAC	AGG

gRNA V7	253	TCGTGCAGAAACTTGAGAC	TGG
gRNA W7	254	CGTGCAGAAACTTGAGACT	GGG
gRNA X7	255	GTGCAGAAACTTGAGACTG	GGG
gRNA Y7	256	AAACTTGAGACTGGGGTTC	AGG
gRNA Z7	257	AAACTTGAGACTGGGGTTCA	GGG
gRNA A8	258	AGACTGGGGTTCAGGGCTTG	TGG
gRNA B8	122	GACCTGCTGGATTCATGACG	TGG
gRNA C8	133	GTCGTA CTGGACGTCCGCGG	GGG
gRNA D8	8	GGTCGTA CTGGACGTCCGCG	GGG

A CD123 (NM_001267713.1) cDNA sequence is provided below as SEQ ID NO: 31.

Underlining or bolding indicates the regions complementary to gRNA A, B, C, D, E, F, G, H, I, J, P3, or S3 (or the reverse complement thereof). Bolding is used where there is overlap

5 between two such regions.

GTCAGGTTTCATGGTTACGAAGCTGCTGACCCAGGATCCCAGCCCGTGGGAGAGAAGGGGGT
CTCTGACAGCCCCACCCCTCCCCACTGCCAGATCCTTATTGGGTCTGAGTTTCAGGGGTGG
GGCCCCAGCTGGAGGTTATAAAACAGCTCAATCGGGGAGTACAACCTTCGGTTTTCTCTTCGG
10 GGAAAGCTGCTTTCAGCGCACACGGGAAGATATCAGAAACATCCTAGGATCAGGACACCCCA
GATCTTCTCAACTGGAACCACGAAGGCTGTTTCTTCCACACAGTACTTTGATCTCCATTTAA
GCAGGCACCTCTGTCTGCGTTCGGGAGCTGCGTTCCTCGATGGTCCTCCT**TTGGCTCACGCT**
GCTCCTGATCGCCCTGCCCT**TGTCTCCTGCAAACGAAGGA**AGGTGGGAAGCCTTGGGCAGGTG
CGGAGAATCTGACCT**GCTGGATTCATGACGTGGAT**TTCTTGAGCTGCAGCTGGGCGGTAGGC
CCGGGGGCCCC**CGCGGACGTCCAGTACGACC**TGTA CTTGAACGTTGCCAACAGGCGTCAACA
15 GTAC**CGAGTGTCTTCACTACAAAA**CGGAT**GTCTCAGGGAACACGTATC**GGGTGTCGTTTCGATG
ACATCTCTCGACTCTCCAGCGGTTCTCAAAGTTCCACATCCTGGTGCGGGGCAGGAGCGCA
GCCTTCGGTATCCCCTGACAGATAAGTTTGTGCTCTTTTCACAGATTGAGATATTA ACTCC
ACCCAACATGACTGCAAAGTGTAATAAGACACATTCCTTTATGCACTGGAAAATGAGAAGTC
ATTTCAATCGCAAATTTGCTATGAGCTTCAGATACAAAAGAGAATGCAGCCTGTAATCACA
20 GAACAGGTCAGAGACAGAACCTCCTTCCAGCTACTCAATCCTGGAACGTACACAGTACAAAT
AAGAGCCCGGGAAAGAGTGTATGAATTCTTGAGCGCCTGGAGCACCCCCAGCGCTTCGAGT
GCGACCAGGAGGAGGGCGAAACACACGTGCCTGGCGGACGTGCTGCTGATCGCGCTGGGG
ACGCTGCTGGCCCTGGTCTGTGTCTTCGTGATCTGCAGAAGGTATCTGGTGATGCAGAGACT
CTTTCCCCGCATCCCTCACATGAAAGACCCCATCGGTGACAGCTTCCAAAACGACAAGCTGG
25 TGGTCTGGGAGGCGGGCAAAGCCGGCCTGGAGGAGTGTCTGGTGACTGAAGTACAGGTCGTG
CAGAAACTTGAGACTGGGGTTCAGGGCTTGTGGGGGTCTGCCTCAATCTCCCTGGCCGGGC
CAGGCGCCTGCACAGACTGGCTGCTGGACCTGCGCACGCAGCCAGGAATGGACATTCTCTAA

CGGGTGGTGGGCATGGGAGATGCCTGTGTAATTTTCGTCCGAAGCTGCCAGGAAGAAGAACAG
AACTTTGTGTGTTTATTTTCATGATAAAGTGATTTTTTTTTTTTTTAACCCAAA

(SEQ ID NO: 31)

5 An additional CD123 isoform (NM_002183.4) cDNA is provided as:

CTTCGGTTTTCTCTTCGGGGAAAGCTGCTTTCAGCGCACACGGGAAGATATCAGAAACATCCT
AGGATCAGGACACCCAGATCTTCTCAACTGGAACCACGAAGGCTGTTTCTTCCACACAGTA
CTTTGATCTCCATTTAAGCAGGCACCTCTGTCTGCGTTCCGGAGCTGCGTTCCCGATGGTC
CTCCTTTGGCTCACGCTGCTCCTGATCGCCCTGCCCTGTCTCCTGCAAACGAAGGAAGATCC
10 AAACCCACCAATCACGAACCTAAGGATGAAAGCAAAGGCTCAGCAGTTGACCTGGGACCTTA
ACAGAAATGTGACCGATATCGAGTGTGTTAAAGACGCCGACTATTCTATGCCGGCAGTGAAC
AATAGCTATTGCCAGTTTGGAGCAATTTCTTATGTGAAGTGACCAACTACACCGTCCGAGT
GGCCAACCCACCATTTCTCCACGTGGATCCTCTTCCCTGAGAACAGTGGGAAGCCTTGGGCAG
GTGCGGAGAATCTGACCTGCTGGATTCTGACGTGGATTTCTTGAGCTGCAGCTGGGCGGTA
15 GGCCCGGGGGCCCCCGCGGACGTCCAGTACGACCTGTACTTGAACGTTGCCAACAGGCGTCA
ACAGTACGAGTGTCTTCACTACAAAACGGATGCTCAGGGAACACGTATCGGGTGTCTTTTCG
ATGACATCTCTCGACTCTCCAGCGTTCTCAAAGTTCCCACATCCTGGTGCAGGGCAGGAGC
GCAGCCTTCCGTATCCCCTGCACAGATAAGTTTGTCTCTTTTTCACAGATTGAGATATTAAC
TCCACCCAACATGACTGCAAAGTGTAATAAGACACATTCCTTTATGCACTGGAAAATGAGAA
20 GTCATTTCAATCGCAAATTTTCGCTATGAGCTTACAGATACAAAAGAGAATGCAGCCTGTAATC
ACAGAACAGGTCAGAGACAGAACCTCCTTCCAGCTACTCAATCCTGGAACGTACACAGTACA
AATAAGAGCCCCGGGAAAGAGTGTATGAATTCTTGAGCGCCTGGAGCACCCCCCAGCGCTTCG
AGTGCGACCAGGAGGAGGGCGCAAACACACGTGCCTGGCGGACGTGCTGCTGATCGCGCTG
GGGACGCTGCTGGCCCTGGTCTGTGTCTTCGTGATCTGCAGAAGGTATCTGGTGATGCAGAG
25 ACTCTTTCCCCGCATCCCTCACATGAAAAGACCCCATCGGTGACAGCTTCCAAAACGACAAGC
TGGTGGTCTGGGAGGCGGGCAAAGCCGGCCTGGAGGAGTGTCTGGTGACTGAAGTACAGGTC
GTGCAGAAAACCTGAGACTGGGGTTTCAGGGCTTGTGGGGTCTGCCTCAATCTCCCTGGCCG
GGCCAGGCGCCTGCACAGACTGGCTGCTGGACCTGCGCACGCAGCCCAGGAATGGACATTCC
TAACGGGTGGTGGGCATGGGAGATGCCTGTGTAATTTTCGTCCGAAGCTGCCAGGAAGAAGAA
30 CAGAACTTTGTGTGTTTATTTTCATGATAAAGTGATTTTTTTTTTTTTTAACCCA (SEQ ID
NO: 52)

Underlining indicates the regions complementary to gRNA D1 (or the reverse complement thereof).

35

Dual gRNA compositions and uses thereof

In some embodiments, a gRNA described herein (e.g., a gRNA of Table 2, 6 or 8) can be used in combination with a second gRNA, e.g., for directing nucleases to two sites in a genome. For instance, in some embodiments it is desired to produce a hematopoietic cell that
40 is deficient for CD123 and a second lineage-specific cell surface antigen, e.g., so that the cell can be resistant to two agents: an anti-CD123 agent and an agent targeting the second lineage-specific cell surface antigen. In some embodiments, it is desirable to contact a cell with two different gRNAs that target different regions of CD123, in order to make two cuts

and create a deletion between the two cut sites. Accordingly, the disclosure provides various combinations of gRNAs.

In some embodiments, two or more (e.g., 3, 4, or more) gRNAs described herein are admixed. In some embodiments, each gRNA is in a separate container. In some
5 embodiments, a kit described herein (e.g., a kit comprising one or more gRNAs according to Table 2, 6, or 8) also comprises a Cas9 molecule, or a nucleic acid encoding the Cas9 molecule.

In some embodiments, the first and second gRNAs are gRNAs according to Table 2, Table 6, Table 8, or variants thereof.

10 In some embodiments, the first gRNA is a CD123 gRNA described herein (e.g., a gRNA of Table 2, 6, 8 or a variant thereof) and the second gRNA targets a lineage-specific cell-surface antigen chosen from: BCMA, CD19, CD20, CD30, ROR1, B7H6, B7H3, CD23, CD38, C-type lectin like molecule-1, CS1, IL-5, L1-CAM, PSCA, PSMA, CD138, CD133, CD70, CD7, CD13, NKG2D, NKG2D ligand, CLEC12A, CD11, CD123, CD56, CD34,
15 CD14, CD66b, CD41, CD61, CD62, CD235a, CD146, CD326, LMP2, CD22, CD52, CD10, CD3/TCR, CD79/BCR, and CD26.

In some embodiments, the first gRNA is a CD123 gRNA described herein (e.g., a gRNA according to Table 2, 6, 8 or a variant thereof) and the second gRNA targets a lineage-specific cell-surface antigen associated with a specific type of cancer, such as, without
20 limitation, CD20, CD22 (Non-Hodgkin's lymphoma, B-cell lymphoma, chronic lymphocytic leukemia (CLL)), CD52 (B-cell CLL), CD33 (Acute myelogenous leukemia (AML)), CD10 (gp100) (Common (pre-B) acute lymphocytic leukemia and malignant melanoma), CD3/T-cell receptor (TCR) (T-cell lymphoma and leukemia), CD79/B-cell receptor (BCR) (B-cell lymphoma and leukemia), CD26 (epithelial and lymphoid malignancies), human leukocyte
25 antigen (HLA)-DR, HLA-DP, and HLA-DQ (lymphoid malignancies), RCAS1 (gynecological carcinomas, biliary adenocarcinomas and ductal adenocarcinomas of the pancreas) as well as prostate specific membrane antigen.

In some embodiments, the first gRNA is a CD123 gRNA described herein (e.g., a gRNA according to Table 2, 6, 8 or a variant thereof) and the second gRNA targets a lineage-
30 specific cell-surface antigen chosen from: CD7, CD13, CD19, CD22, CD20, CD25, CD32, CD38, CD44, CD45, CD47, CD56, 96, CD117, CD123, CD135, CD174, CLL-1, folate receptor β , IL1RAP, MUC1, NKG2D/NKG2DL, TIM-3, or WT1.

In some embodiments, the first gRNA is a CD123 gRNA described herein (e.g., a gRNA according to Table 2, 6, 8 or a variant thereof) and the second gRNA targets a lineage-

specific cell-surface antigen chosen from: CD1a, CD1b, CD1c, CD1d, CD1e, CD2, CD3, CD3d, CD3e, CD3g, CD4, CD5, CD6, CD7, CD8a, CD8b, CD9, CD10, CD11a, CD11b, CD11c, CD11d, CDw12, CD13, CD14, CD15, CD16, CD16b, CD17, CD18, CD19, CD20, CD21, CD22, CD23, CD24, CD25, CD26, CD27, CD28, CD29, CD30, CD31, CD32a, 5 CD32b, CD32c, CD34, CD35, CD36, CD37, CD38, CD39, CD40, CD41, CD42a, CD42b, CD42c, CD42d, CD43, CD44, CD45, CD45RA, CD45RB, CD45RC, CD45RO, CD46, CD47, CD48, CD49a, CD49b, CD49c, CD49d, CD49e, CD49f, CD50, CD51, CD52, CD53, CD54, CD55, CD56, CD57, CD58, CD59, CD60a, CD61, CD62E, CD62L, CD62P, CD63, CD64a, CD65, CD65s, CD66a, CD66b, CD66c, CD66F, CD68, CD69, CD70, CD71, CD72, 10 CD73, CD74, CD75, CD75S, CD77, CD79a, CD79b, CD80, CD81, CD82, CD83, CD84, CD85A, CD85C, CD85D, CD85E, CD85F, CD85G, CD85H, CD85I, CD85J, CD85K, CD86, CD87, CD88, CD89, CD90, CD91, CD92, CD93, CD94, CD95, CD96, CD97, CD98, CD99, CD99R, CD100, CD101, CD102, CD103, CD104, CD105, CD106, CD107a, CD107b, CD108, CD109, CD110, CD111, CD112, CD113, CD114, CD115, CD116, CD117, 15 CD118, CD119, CD120a, CD120b, CD121a, CD121b, CD121a, CD121b, CD122, CD123, CD124, CD125, CD126, CD127, CD129, CD130, CD131, CD132, CD133, CD134, CD135, CD136, CD137, CD138, CD139, CD140a, CD140b, CD141, CD142, CD143, CD14, CDw145, CD146, CD147, CD148, CD150, CD152, CD152, CD153, CD154, CD155, CD156a, CD156b, CD156c, CD157, CD158b1, CD158b2, CD158d, CD158e1/e2, CD158f, 20 CD158g, CD158h, CD158i, CD158j, CD158k, CD159a, CD159c, CD160, CD161, CD163, CD164, CD165, CD166, CD167a, CD168, CD169, CD170, CD171, CD172a, CD172b, CD172g, CD173, CD174, CD175, CD175s, CD176, CD177, CD178, CD179a, CD179b, CD180, CD181, CD182, CD183, CD184, CD185, CD186, CD191, CD192, CD193, CD194, CD195, CD196, CD197, CDw198, CDw199, CD200, CD201, CD202b, CD203c, CD204, 25 CD205, CD206, CD207, CD208, CD209, CD210a, CDw210b, CD212, CD213a1, CD213a2, CD215, CD217, CD218a, CD218b, CD220, CD221, CD222, CD223, CD224, CD225, CD226, CD227, CD228, CD229, CD230, CD231, CD232, CD233, CD234, CD235a, CD235b, CD236, CD236R, CD238, CD239, CD240, CD241, CD242, CD243, CD244, CD245, CD246, CD247, CD248, CD249, CD252, CD253, CD254, CD256, CD257, CD258, 30 CD261, CD262, CD263, CD264, CD265, CD266, CD267, CD268, CD269, CD270, CD272, CD272, CD273, CD274, CD275, CD276, CD277, CD278, CD279, CD280, CD281, CD282, CD283, CD284, CD286, CD288, CD289, CD290, CD292, CDw293, CD294, CD295, CD296, CD297, CD298, CD299, CD300a, CD300c, CD300e, CD301, CD302, CD303, CD304, CD305, CD306, CD307a, CD307b, CD307c, CD307d, CD307e, CD309, CD312,

CD314, CD315, CD316, CD317, CD318, CD319, CD320, CD321, CD322, CD324, CD325, CD326, CD327, CD328, CD329, CD331, CD332, CD333, CD334, CD335, CD336, CD337, CD338, CD339, CD340, CD344, CD349, CD350, CD351, CD352, CD353, CD354, CD355, CD357, CD358, CD359, CD360, CD361, CD362 or CD363.

- 5 In some embodiments, the first gRNA is a CD123 gRNA described herein (e.g., a gRNA according to Table 2, 6, 8 or a variant thereof) and the second gRNA targets a lineage-specific cell-surface antigen chosen from: CD19; CD123; CD22; CD30; CD171; CS-1 (also referred to as CD2 subset 1, CRACC, SLAMF7, CD319, and 19A24); C-type lectin-like molecule-1 (CLECL1); epidermal growth factor receptor variant III (EGFRvIII); ganglioside
- 10 G2 (CD2); ganglioside GD3 (aNeu5Ac(2-8)aNeu5Ac(2-3)bDGalp(1-4)bDGlep(1-1)Cer); TNF receptor family member B cell maturation (BCMA), Tn antigen ((Tn Ag) or (GalNAc.alpha.-Ser/Thr)); prostate-specific membrane antigen (PSMA); Receptor tyrosine kinase-like orphan receptor 1 (ROR1); Fms-Like tyrosine Kinase 3 (FLT3); Tumor-associated glycoprotein 72 (TAG72); CD38; CD44v6; Carcinoembryonic antigen (CEA);
- 15 Epithelial cell adhesion molecule (EPCAM); B7H3 (CD276); KIT (CD117); Interleukin-13 receptor subunit alpha-2 (IL-13Ra2 or CD213A2); Mesothelin; Interleukin 11 receptor alpha (IL-11Ra); prostate stem cell antigen (PSCA); Protease Serine 21 (Testisin or PRSS21); vascular endothelial growth factor receptor 2 (VEGFR2); Lewis(Y) antigen; CD24; Platelet-derived growth factor receptor beta (PDGFR-beta); Stage-specific embryonic antigen-4
- 20 (SSEA-4); CD20; Folate receptor alpha; Receptor tyrosine-protein kinase ERBB2 (Her2/neu); Mucin 1, cell surface associated (MUC1); epidermal growth factor receptor (EGFR); neural cell adhesion molecule (NCAM); Prostase; prostatic acid phosphatase (PAP); elongation factor 2 mutated (ELF2M); Ephrin B2; fibroblast activation protein alpha (FAP); insulin-like growth factor I receptor (IGF-I receptor), carbonic anhydrase IX (CAIX),
- 25 Proteasome (Prosome, Macropain) Subunit, Beta Type 9 (LMP2); glycoprotein 100 (gp100); oncogene fusion protein consisting of breakpoint cluster region (BCR) and Abelson murine leukemia viral oncogene homolog 1 (Abl) (bcr-abl); tyrosinase; ephrin type-A receptor 2 (EphA2); Fucosyl GM1; sialyl Lewis adhesion molecule (sLe); ganglioside GM3 (aNeu5Ac(2-3)bDGalp(1-4)bDGlep(1-1)Cer); transglutaminase 5 (TGS5); high molecular
- 30 weight-melanoma-associated antigen (HMWMAA); o-acetyl-GD2 ganglioside (OAcGD2); Folate receptor beta; tumor endothelial marker 1 (TEM1/CD248); tumor endothelial marker 7-related (TEM7R); claudin 6 (CLDN6); thyroid stimulating hormone receptor (TSHR); G protein-coupled receptor class C group 5, member D (GPRC5D); chromosome X open reading frame 61 (CXORF61); CD97; CD179a; anaplastic lymphoma kinase (ALK);

Polysialic acid; placenta-specific 1 (PLAC1); hexasaccharide portion of globoH glycosphingolipid (GloboH); mammary gland differentiation antigen (NY-BR-1); uroplakin 2 (UPK2); Hepatitis A virus cellular receptor 1 (HAVCR1); adrenoceptor beta 3 (ADRB3); pannexin 3 (PANX3); G protein-coupled receptor 20 (GPR20); lymphocyte antigen 6 complex; locus K 9 (LY6K); Olfactory receptor 51E2 (OR51E2); TCR Gamma Alternate Reading Frame Protein (TARP); Wilms tumor protein (WT1); Cancer/testis antigen 1 (NY-ESO-1); Cancer/testis antigen 2 (LAGE-1a); Melanoma-associated antigen 1 (MAGE-A1), ETS translocation-variant gene 6, located on chromosome 12p (ETV6-AML); sperm protein 17 (SPA17); X Antigen Family, member 1A (XAGE1); angiopoietin-binding cell surface receptor 2 (Tie 2); melanoma cancer testis antigen-1 (MAD-CT-1); melanoma cancer testis antigen-2 (MAD-CT-2); Fos-related antigen 1; tumor protein p53 (p53); p53 mutant; prostatein; surviving; telomerase; prostate carcinoma tumor antigen-1 (PCTA-1 or Galectin 8), melanoma antigen recognized by T cells 1 (MelanA or MART1); Rat sarcoma (Ras) mutant; human Telomerase reverse transcriptase (hTERT); sarcoma translocation breakpoints; melanoma inhibitor of apoptosis (ML-1AP); ERG (transmembrane protease, serine 2 (TMPRSS2) ETS fusion gene); N-Acetyl glucosaminyl-transferase V (NA17); paired box protein Pax-3 (PAX3); Androgen receptor; Cyclin B1; v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog (MYCN); Ras Homolog Family Member C (RhoC); Tyrosinase-related protein 2 (TRP-2); Cytochrome P450 1B1 (CYP1B1); CCCTC-Binding Factor (Zinc Finger Protein)-Like (BORIS or Brother of the Regulator of Imprinted Sites), Squamous Cell Carcinoma Antigen Recognized By T Cells 3 (SART3); Paired box protein Pax-5 (PAX5); proacrosin binding protein sp32 (OY-TES1); lymphocyte-specific protein tyrosine kinase (LCK); A kinase anchor protein 4 (AKAP-4); synovial sarcoma, X breakpoint 2 (SSX2); Receptor for Advanced Glycation Endproducts (RAGE-1); renal ubiquitous 1 (RU1); renal ubiquitous 2 (RU2); legumain; human papilloma virus E6 (HPV E6); human papilloma virus E7 (HPV E7); intestinal carboxy esterase; heat shock protein 70-2 mutated (mut hsp70-2); CD79a; CD79b; CD72; Leukocyte-associated immunoglobulin-like receptor 1 (LAIR1); Fc fragment of IgA receptor (FCAR or CD89); Leukocyte immunoglobulin-like receptor subfamily A member 2 (LILRA2); CD300 molecule-like family member f (CD300LF); C-type lectin domain family 12 member A (CLEC12A); bone marrow stromal cell antigen 2 (BST2); EGF-like module-containing mucin-like hormone receptor-like 2 (EMR2), lymphocyte antigen 75 (LY75); Glypican-3 (GPC3); Fc receptor-like 5 (FCRL5); and immunoglobulin lambda-like polypeptide 1 (IGLL1).

In some embodiments, the first gRNA is a CD123 gRNA described herein (e.g., a gRNA according to Table 2, 6, 8 or a variant thereof) and the second gRNA targets a lineage-specific cell-surface antigen chosen from: CD11a, CD18, CD19, CD20, CD31, CD33, CD34, CD44, CD45, CD47, CD51, CD58, CD59, CD63, CD97, CD99, CD100, CD102, CD123, CD127, CD133, CD135, CD157, CD172b, CD217, CD300a, CD305, CD317, CD321, and CLL1.

In some embodiments, the first gRNA is a CD123 gRNA described herein (e.g., a gRNA according to Table 2, 6, 8 or a variant thereof) and the second gRNA targets a lineage-specific cell-surface antigen chosen from: CD123, CLL1, CD38, CD135 (FLT3), CD56 (NCAM1), CD117 (c-KIT), FR β (FOLR2), CD47, CD82, TNFRSF1B (CD120B), CD191, CD96, PTPRJ (CD148), CD70, LILRB2 (CD85D), CD25 (IL2R α), CD44, CD96, NKG2D Ligand, CD45, CD7, CD15, CD19, CD20, CD22, CD37, and CD82.

In some embodiments, the first gRNA is a CD123 gRNA described herein (e.g., a gRNA according to Table 2, 6, 8 or a variant thereof) and the second gRNA targets a lineage-specific cell-surface antigen chosen from: CD7, CD11a, CD15, CD18, CD19, CD20, CD22, CD25, CD31, CD34, CD37, CD38, CD44, CD45, CD47, CD51, CD56, CD58, CD59, CD63, CD70, CD82, CD85D, CD96, CD97, CD99, CD100, CD102, CD117, CD120B, CD123, CD127, CD133, CD135, CD148, CD157, CD172b, CD191, CD217, CD300a, CD305, CD317, CD321, CLL1, FR β (FOLR2), or NKG2D Ligand.

In some embodiments, the first gRNA is a CD123 gRNA described herein (e.g., a gRNA according to Table 2, 6, 8 or a variant thereof) and the second gRNA targets CD33. In some embodiments, the first gRNA is a CD123 gRNA described herein (e.g., a gRNA according to Table 2, 6, 8 or a variant thereof) and the second gRNA targets CLL1.

In some embodiments, the first gRNA is a CD123 gRNA described herein (e.g., a gRNA according to Table 2, 6, 8 or a variant thereof) and the second gRNA comprises a sequence from Table A. In some embodiments, the first gRNA is a CLL1 gRNA comprising a targeting domain, wherein the targeting domain comprises a sequence of any of SEQ ID NOs: 1-10, 40, 42, 44, 46, and the second gRNA comprises a targeting domain corresponding to a sequence of Table A. In some embodiments, the first gRNA is a CD123 gRNA comprising a targeting domain, wherein the targeting domain comprises a sequence of SEQ ID NO: 9, and the second gRNA comprises a targeting domain corresponding to a sequence of Table A. In some embodiments, the first gRNA is a CD123 gRNA comprising a targeting domain, wherein the targeting domain comprises a sequence of SEQ ID NO: 10, and the second gRNA comprises a targeting domain corresponding to a sequence of Table A. In

some embodiments, the first gRNA is a CD123 gRNA comprising a targeting domain, wherein the targeting domain comprises a sequence of SEQ ID NO: 11, and the second gRNA comprises a targeting domain corresponding to a sequence of Table A. In some embodiments, the first gRNA is a CD123 gRNA comprising a targeting domain, wherein the targeting domain comprises a sequence of SEQ ID NO: 12, and the second gRNA comprises a targeting domain corresponding to a sequence of Table A. In some embodiments, the second gRNA is a gRNA disclosed in any of WO2017/066760, WO2019/046285, WO/2018/160768, or Borot et al. PNAS June 11, 2019 116 (24) 11978-11987, each of which is incorporated herein by reference in its entirety.

10

Table A. Exemplary human CD33 target sequences. Certain target sequences are followed by a PAM sequence, indicated by a space in the text. Suitable gRNAs binding the target sequences provided will typically comprise a targeting domain comprising an RNA nucleotide sequence equivalent to the respective target sequence (and excluding the PAM).

gRNA target	Target Sequences	SEQ ID NO:
hCD33	ACCTGTCAGGTGAAGTTTCGC TGG	259
hCD33	TGGCCGGGTTCTAGAGTGCC AGG	260
hCD33	GGCCGGGTTCTAGAGTGCCA GGG	261
hCD33	CACCGAGGAGTGAGTAGTCC TGG	262
hCD33	TCCAGCGAACTTCACCTGAC AGG	263
CD33 (in intron 1)	GCTGTGGGGAGAGGGGTTGT	264
CD33 (in intron 1)	CTGTGGGGAGAGGGGTTGTC	265
CD33 (in intron 1)	TGGGGAACGAGGGTCAGCT	266
CD33 (in intron 1)	GGGCCCCGTGTGGGGAACGA	267
CD33 (in intron 1)	AGGGCCCCGTGTGGGGAACG	268
CD33 (in intron 1)	GCTGACCCTCGTTTCCCCAC	269
CD33 (in intron 1)	CTGACCCTCGTTTCCCCACA	270
CD33 (in intron 1)	TGACCCTCGTTTCCCCACAG	271
CD33 (in intron 1)	CCATAGCCAGGGCCCCTGTG	272
CD33 (in intron 2)	GCAATGTGACAGGTGAGGCAC	273
CD33 (in intron 2)	TGAGGCACAGGCTTCAGAAG	274
CD33 (in intron 2)	AGGCTTCAGAAGTGGCCGCA	275
CD33 (in intron 2)	GGCTTCAGAAGTGGCCGCAA	276
CD33 (in intron 2)	GTACCCATGAACTTCCCTTG	277
CD33 (in intron 2)	GTGGCCGCAAGGGAAGTTCA	278
CD33 (in intron 2)	TGGCCGCAAGGGAAGTTCAT	279
CD33 (in intron 2)	GGAAGTTCATGGGTACTGCA	280
CD33 (in intron 2)	TTCATGGGTACTGCAGGGCA	281
CD33 (in intron 2)	CTAAACCCCTCCAGTACCA	282
CD33 (in intron 1)	CACTCACCTGCCCACAGCAG	283
CD33 (in intron 1)	CCCTGCTGTGGGCAGGTGAG	284
CD33 (in intron 1)	TGGGCAGGTGAGTGGCTGTG	285

CD33 (in intron 1)	GGTGAGTGGCTGTGGGGAGA	286
CD33 (in intron 1)	GTGAGTGGCTGTGGGGAGAG	287
CD33 (exon 2)	ATCCATAGCCAGGGCCCCTG	288
CD33 (exon 2)	TCCATAGCCAGGGCCCCTGT	289
CD33 (exon 2)	CCATAGCCAGGGCCCCTGTG	272
CD33 (exon 2)	TCGTTTCCCCACAGGGGCCC	290
CD33 (exon 2)	TGGCTATGGATCCAAATTC	291
CD33 (exon 2)	TGGGGAAACGAGGGTCAGCT	266
CD33 (exon 2)	GGGCCCCCTGTGGGGAAACGA	267
CD33 (exon 2)	AGAAATTTGGATCCATAGCC AGG	292
CD33 (exon 3)	ATCCCTGGCACTCTAGAACC CGG	293
CD33 (exon 3)	CCTCACTAGACTTGACCCAC AGG	294

Cells comprising two or more mutations

In some embodiments, an engineered cell described herein comprises two or more mutations. In some embodiments, an engineered cell described herein comprises two mutations, the first mutation being in CD123 and the second mutation being in a second lineage-specific cell surface antigen. Such a cell can, in some embodiments, be resistant to two agents: an anti-CD123 agent and an agent targeting the second lineage-specific cell surface antigen. In some embodiments, such a cell can be produced using two or more gRNAs described herein, e.g., a gRNA of Table 2 and a second gRNA. In some embodiments, such a cell can be produced using two or more gRNAs described herein, e.g., a gRNA of Table 6 and a second gRNA. In some embodiments, such a cell can be produced using two or more gRNAs described herein, e.g., a gRNA of Table 8 and a second gRNA. In some embodiments, the cell can be produced using, e.g., a ZFN or TALEN. The disclosure also provides populations comprising cells described herein.

In some embodiments, the second mutation is at a gene encoding a lineage-specific cell-surface antigen, e.g., one listed in the preceding section. In some embodiments, the second mutation is at a site listed in Table A.

Typically, a mutation effected by the methods and compositions provided herein, e.g., a mutation in a target gene, such as, for example, CD123 and/or any other target gene mentioned in this disclosure, results in a loss of function of a gene product encoded by the target gene, e.g., in the case of a mutation in the CD123 gene, in a loss of function of a CD123 protein. In some embodiments, the loss of function is a reduction in the level of expression of the gene product, e.g., reduction to a lower level of expression, or a complete abolishment of expression of the gene product. In some embodiments, the mutation results in the expression of a non-functional variant of the gene product. For example, in the case of

the mutation generating a premature stop codon in the encoding sequence, a truncated gene product, or, in the case of the mutation generating a nonsense or missense mutation, a gene product characterized by an altered amino acid sequence, which renders the gene product non-functional. In some embodiments, the function of a gene product is binding or
5 recognition of a binding partner. In some embodiments, the reduction in expression of the gene product, e.g., of CD123, of the second lineage-specific cell-surface antigen, or both, is to less than or equal to 50%, less than or equal to 40%, less than or equal to 30%, less than or equal to 20%, less than or equal to 10%, less than or equal to 5%, less than or equal to 2%, or less than or equal to 1% of the level in a wild-type or non-engineered counterpart cell.

10 In some embodiments, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% of copies of CD123 in the population of cells generated by the methods and/or using the compositions provided herein have a mutation. In some embodiments, at least at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% of copies of
15 the second lineage-specific cell surface antigen in the population of cells have a mutation. In some embodiments, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% of copies of CD123 and of the second lineage-specific cell surface antigen in the population of cells have a mutation. In some embodiments, the population comprises one or more wild-type cells. In some embodiments,
20 the population comprises one or more cells that comprise one wild-type copy of CD123. In some embodiments, the population comprises one or more cells that comprise one wild-type copy of the second lineage-specific cell surface antigen.

Cells

25 In some embodiments, a cell (e.g., an HSC or HPC) having a modification of CD123 is made using a nuclease and/or a gRNA described herein. In some embodiments, a cell (e.g., an HSC or HPC) having a modification of CD123 and a modification of a second lineage-specific cell surface antigen is made using a nuclease and/or a gRNA described herein. It is understood that the cell can be made by contacting the cell itself with the nuclease and/or a
30 gRNA, or the cell can be the daughter cell of a cell that was contacted with the nuclease and/or a gRNA. In some embodiments, a cell described herein (e.g., an HSC) is capable of reconstituting the hematopoietic system of a subject. In some embodiments, a cell described herein (e.g., an HSC) is capable of one or more of (e.g., all of): engrafting in a human subject, producing myeloid lineage cell, and producing and lymphoid lineage cells.

In some embodiments, a cell described herein is a human cell having a mutation in exon 2 of CD123. In some embodiments, a cell described herein is a human cell having a mutation in exon 5 of CD123. In some embodiments, a cell described herein is a human cell having a mutation in exon 6 of CD123.

5 In some embodiments, a population of cells described herein comprises hematopoietic stem cells (HSCs), hematopoietic progenitor cells (HPCs), or both (HSPCs). In some embodiments, the cells are CD34+.

In some embodiments, the cell comprises only one genetic modification. In some embodiments, the cell is only genetically modified at the CD123 locus. In some
10 embodiments, the cell is genetically modified at a second locus. In some embodiments, the cell does not comprise a transgenic protein, e.g., does not comprise a CAR.

In some embodiments, a modified cell described herein comprises substantially no CD123 protein. In some embodiments, a modified cell described herein comprises substantially no wild-type CD123 protein, but comprises mutant CD123 protein. In some
15 embodiments, the mutant CD123 protein is not bound by an agent that targets CD123 for therapeutic purposes.

In some embodiments, the cells are hematopoietic cells, e.g., hematopoietic stem cells. Hematopoietic stem cells (HSCs) are typically capable of giving rise to both myeloid and lymphoid progenitor cells that further give rise to myeloid cells (e.g., monocytes,
20 macrophages, neutrophils, basophils, dendritic cells, erythrocytes, platelets, etc) and lymphoid cells (e.g., T cells, B cells, NK cells), respectively. HSCs are characterized by the expression of the cell surface marker CD34 (e.g., CD34+), which can be used for the identification and/or isolation of HSCs, and absence of cell surface markers associated with commitment to a cell lineage.

25 In some embodiments, a population of cells described herein comprises a plurality of hematopoietic stem cells; in some embodiments, a population of cells described herein comprises a plurality of hematopoietic progenitor cells; and in some embodiments, a population of cells described herein comprises a plurality of hematopoietic stem cells and a plurality of hematopoietic progenitor cells.

30 In some embodiments, the HSCs are obtained from a subject, such as a human subject. Methods of obtaining HSCs are described, e.g., in PCT/US2016/057339, which is herein incorporated by reference in its entirety. In some embodiments, the HSCs are peripheral blood HSCs. In some embodiments, the mammalian subject is a non-human primate, a rodent (e.g., mouse or rat), a bovine, a porcine, an equine, or a domestic animal. In

some embodiments, the HSCs are obtained from a human subject, such as a human subject having a hematopoietic malignancy. In some embodiments, the HSCs are obtained from a healthy donor. In some embodiments, the HSCs are obtained from the subject to whom the immune cells expressing the chimeric receptors will be subsequently administered. HSCs that are administered to the same subject from which the cells were obtained are referred to as autologous cells, whereas HSCs that are obtained from a subject who is not the subject to whom the cells will be administered are referred to as allogeneic cells.

In some embodiments, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% of copies of CD123 in the population of cells have a mutation. By way of example, a population can comprise a plurality of different CD123 mutations and each mutation of the plurality contributes to the percent of copies of CD123 in the population of cells that have a mutation.

In some embodiments, the expression of CD123 on the genetically engineered hematopoietic cell is compared to the expression of CD123 on a naturally occurring hematopoietic cell (e.g., a wild-type counterpart). In some embodiments, the genetic engineering results in a reduction in the expression level of CD123 by at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% as compared to the expression of CD123 on a naturally occurring hematopoietic cell (e.g., a wild-type counterpart). For example, in some embodiments, the genetically engineered hematopoietic cell expresses less than 20%, less than 19%, less than 18%, less than 17%, less than 16%, less than 15%, less than 14%, less than 13%, less than 12%, less than 11%, less than 10%, less than 9%, less than 8%, less than 7%, less than 6%, less than 5%, less than 4%, less than 3%, less than 2%, or less than 1% of CD123 as compared to a naturally occurring hematopoietic cell (e.g., a wild-type counterpart).

In some embodiments, the genetic engineering results in a reduction in the expression level of wild-type CD123 by at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% as compared to the expression of the level of wild-type CD123 on a naturally occurring hematopoietic cell (e.g., a wild-type counterpart). That is, in some embodiments, the genetically engineered hematopoietic cell expresses less than 20%, 19%, less than 18%, less than 17%, less than 16%, less than 15%, less than 14%, less than 13%, less than 12%, less than 11%, less than 10%, less than 9%, less than 8%, less than 7%, less than 6%, less than 5%, less than 4%, less than 3%, less than 2%, or less than 1% of

CD123 as compared to a naturally occurring hematopoietic cell (e.g., a wild-type counterpart).

In some embodiments, the genetic engineering results in a reduction in the expression level of wild-type lineage-specific cell surface antigen (e.g., CD123) by at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% as compared to a suitable control (e.g., a cell or plurality of cells). In some embodiments, the suitable control comprises the level of the wild-type lineage-specific cell surface antigen measured or expected in a plurality of non-engineered cells from the same subject. In some embodiments, the suitable control comprises the level of the wild-type lineage-specific cell surface antigen measured or expected in a plurality of cells from a healthy subject. In some embodiments, the suitable control comprises the level of the wild-type lineage-specific cell surface antigen measured or expected in a population of cells from a pool of healthy individuals (e.g., 10, 20, 50, or 100 individuals). In some embodiments, the suitable control comprises the level of the wild-type lineage-specific cell surface antigen measured or expected in a subject in need of a treatment described herein, e.g., an anti-CD123 therapy, e.g., wherein the subject has a cancer, wherein cells of the cancer express CD123

Methods of treatment and administration

In some embodiments, an effective number of CD123-modified cells described herein is administered to a subject in combination with an anti-CD123 therapy, e.g., an anti-CD123 cancer therapy. In some embodiments, an effective number of cells comprising a modified CD123 and a modified second lineage-specific cell surface antigen are administered in combination with an anti-CD123 therapy, e.g., an anti-CD123 cancer therapy. In some embodiments, the anti-CD123 therapy comprises an antibody, a bispecific T cell engager, an ADC, or an immune cell expressing a CAR.

It is understood that when agents (e.g., CD123-modified cells and an anti-CD123 therapy) are administered in combination, the agent may be administered at the same time or at different times in temporal proximity. Furthermore, the agents may be admixed or in separate volumes. For example, in some embodiments, administration in combination includes administration in the same course of treatment, e.g., in the course of treating a cancer with an anti-CD123 therapy, the subject may be administered an effective number of CD123-modified cells concurrently or sequentially, e.g., before, during, or after the treatment, with the anti-CD123 therapy.

In some embodiments, the agent that targets a CD123 as described herein is an immune cell that expresses a chimeric receptor, which comprises an antigen-binding fragment (e.g., a single-chain antibody) capable of binding to CD123. The immune cell may be, e.g., a T cell (e.g., a CD4+ or CD8+ T cell) or an NK cell.

5 A Chimeric Antigen Receptor (CAR) can comprise a recombinant polypeptide comprising at least an extracellular antigen binding domain, a transmembrane domain and a cytoplasmic signaling domain comprising a functional signaling domain, e.g., one derived from a stimulatory molecule. In one some embodiments, the cytoplasmic signaling domain further comprises one or more functional signaling domains derived from at least one
10 costimulatory molecule, such as 4-1BB (i.e., CD137), CD27 and/or CD28 or fragments of those molecules. The extracellular antigen binding domain of the CAR may comprise a CD123-binding antibody fragment. The antibody fragment can comprise one or more CDRs, the variable region (or portions thereof), the constant region (or portions thereof), or combinations of any of the foregoing.

15 Amino acid and nucleic acid sequences of an exemplary heavy chain variable region and light chain variable region of an anti-human CD123 antibody are provided below. The CDR sequences are shown in boldface in the amino acid sequences.

Amino acid sequence of anti-CD123 Heavy Chain Variable Region (SEQ ID NO: 32)

20 MADYKDIVMTQSHKFMSTSVGDRVNITCKAS**QNVDS**AVAWYQQKPGQSPKALIYS
ASYRYSQVDPDRFTGRGSGTD
FTLTISSVQAEDLAVYYC**QQYYSTPWTF**GGGGTKLEIKR

Amino acid sequence of anti-CD123 Light Chain Variable Region (SEQ ID NO: 33)

25 EVKLVESGGGLVQPGGSLSLSCAAS**GFTFTDYY**MSWVRQPPGKALEWLALIRSKAD
GYTTEYSASVKGRFTLSRDDSQSILYLQMNALRPEDSATYYCARDAAYYSYYSPEG
AMD YWGQGTSTVTVSS

Additional anti-CD123 sequences are found, e.g., in WO2015140268A1, incorporated herein by reference in its entirety.

30 The anti-CD123 antibody binding fragment for use in constructing the agent that targets CD123 as described herein may comprise the same heavy chain and/or light chain CDR regions as those in SEQ ID NO:32 and SEQ ID NO:33. Such antibodies may comprise amino acid residue variations in one or more of the framework regions. In some instances, the anti-CD123 antibody fragment may comprise a heavy chain variable region that shares at

least 70% sequence identity (e.g., 75%, 80%, 85%, 90%, 95%, or higher) with SEQ ID NO:32 and/or may comprise a light chain variable region that shares at least 70% sequence identity (e.g., 75%, 80%, 85%, 90%, 95%, or higher) with SEQ ID NO:33.

Exemplary chimeric receptor component sequences are provided in Table 3 below.

5

Table 3: Exemplary components of a chimeric receptor

Chimeric receptor component	Amino acid sequence
Antigen-binding fragment	Light chain- GSTSSGSGKPGSGEGSTKG (SEQ ID NO: 34)-Heavy chain
4-1BB costimulatory domain	KRGRKKLLYIFKQPFMRPVQTTQEEDGCSC RFPEEEEGGCE (SEQ ID NO: 295)
CD28 costimulatory domain	IEVMYPPPYLDNEKSNGTIIHVKGKHLCPSP LFGPSKPFWVLVVVGGVLACYSLLVTVA FIIFWVRSKRSRLLHSDYMNMTPRRPGPTR KHYPYAPPRDFAAYRS (SEQ ID NO: 35)
ICOS costimulatory domain (boldface), ICOS transmembrane domain (italics) and a portion of the extracellular domain of ICOS (underlined)	<u>LSIFDPPPFKVTLTGGYLHIYESQLCCQLKF</u> <i>WLPIGCAAFVVVCILGCILCWLTKKKYSSS</i> VHDPNGEYMFMRVNTAKKSRLTDVTL (SEQ ID NO: 36)
ICOS costimulatory domain	CWLTKKKYSSSVHDPNGEYMFMRVNTA KKSRLTDVTL (SEQ ID NO: 37)
CD28/ICOS chimera (the ICOS portion shown in underline) including the hinge domain (italics) and transmembrane domain (bold) from CD28	<i>IEVMYPPPYLDNEKSNGTIIHVKGKHLCPSP</i> <i>FPGPSKPFWVLVVVGGVLACYSLLVTVA</i> FIIFWVRSKRSRLLHSDYMFMRVNTAKK <u>SRLTDVTL</u> (SEQ ID NO: 38)
CD8 α transmembrane domain (italics) and a portion of the extracellular domain of CD8 α (underlined)	<u>TTTPAPRPPTPAPTASQPLSLRPEACRPAA</u> <u>GGAVHTRGLDFACDIYIWAPLAGTCGVLLLS</u> <i>LVITLYC</i> (SEQ ID NO: 296)
CD3 ζ cytoplasmic signaling domain	RVKFSRSADAPAYQQGQNQLYNELNLGRR EEYDVLDKRRGRDPEMGGKPQRRKNPQE GLYNELQKDKMAEAYSEIGMKGERRRGK GHDGLYQGLSTATKDTYDALHMQALPPR (SEQ ID NO: 39)

In some embodiments, the CAR comprises a 4-1BB costimulatory domain (e.g., as shown in Table 3), a CD8 α transmembrane domain and a portion of the extracellular domain of CD8 α (e.g., as shown in Table 3), and a CD3 ζ cytoplasmic signaling domain (e.g., as shown in Table 3).

A typical number of cells, e.g., immune cells or hematopoietic cells, administered to a mammal (e.g., a human) can be, for example, in the range of one million to 100 billion cells; however, amounts below or above this exemplary range are also within the scope of the present disclosure.

In some embodiments, the agent that targets CD123 is an antibody-drug conjugate (ADC). The ADC may be a molecule comprising an antibody or antigen-binding fragment thereof conjugated to a toxin or drug molecule. Binding of the antibody or fragment thereof to the corresponding antigen allows for delivery of the toxin or drug molecule to a cell that presents the antigen on the its cell surface (e.g., target cell), thereby resulting in death of the target cell.

In some embodiments, the antigen-bind fragment of the antibody-drug conjugate has the same heavy chain CDRs as the heavy chain variable region provided by SEQ ID NO: 32 and the same light chain CDRs as the light chain variable region provided by SEQ ID NO: 33. In some embodiments, the antigen-bind fragment of the antibody-drug conjugate has the heavy chain variable region provided by SEQ ID NO: 32 and the same light chain variable region provided by SEQ ID NO: 33.

Toxins or drugs compatible for use in antibody-drug conjugates known in the art and will be evident to one of ordinary skill in the art. See, e.g., Peters et al. *Biosci. Rep.*(2015) 35(4): e00225; Beck et al. *Nature Reviews Drug Discovery* (2017) 16:315-337; Marin-Acevedo et al. *J. Hematol. Oncol.*(2018)11: 8; Elgundi et al. *Advanced Drug Delivery Reviews* (2017) 122: 2-19.

In some embodiments, the antibody-drug conjugate may further comprise a linker (e.g., a peptide linker, such as a cleavable linker) attaching the antibody and drug molecule.

Examples of antibody-drug conjugates include, without limitation, brentuximab vedotin, glembatumumab vedotin/CDX-011, depatuxizumab mafodotin/ABT-414, PSMA ADC, polatuzumab vedotin/RG7596/DCDS4501A, denintuzumab mafodotin/SGN-CD19A, AGS-16C3F, CDX-014, RG7841/DLYE5953A, RG7882/DMUC406A, RG7986/DCDS0780A, SGN-LIV1A, enfortumab vedotin/ASG-22ME, AG-15ME, AGS67E,

telisotuzumab vedotin/ABBV-399, ABBV-221, ABBV-085, GSK-2857916, tisotumab vedotin/HuMax-TF-ADC, HuMax-Ax1-ADC, pinatuzumab vedotin/RG7593/DCDT2980S, lifastuzumab vedotin/RG7599/DNIB0600A, indusatumab vedotin/MLN-0264/TAK-264, vandortuzumab vedotin/RG7450/DSTP3086S, sofituzumab vedotin/RG7458/DMUC5754A, 5 RG7600/DMOT4039A, RG7336/DEDN6526A, ME1547, PF-06263507/ADC 5T4, trastuzumab emtansine/T-DM1, mirvetuximab soravtansine/ IMGN853, coltuximab ravtansine/SAR3419, naratuximab emtansine/IMGN529, indatuximab ravtansine/BT-062, anetumab ravtansine/BAY 94-9343, SAR408701, SAR428926, AMG 224, PCA062, HKT288, LY3076226, SAR566658, lorvotuzumab mertansine/IMGN901, cantuzumab 10 mertansine/SB-408075, cantuzumab ravtansine/IMGN242, laprituximab emtansine/IMGN289, IMGN388, bivatuzumab mertansine, AVE9633, BIIB015, MLN2704, AMG 172, AMG 595, LOP 628, vadastuximab talirine/SGN-CD123A, SGN-CD70A, SGN-CD19B, SGN-CD123A, SGN-CD352A, rovalpituzumab tesirine/SC16LD6.5, SC-002, SC-003, ADCT-301/HuMax-TAC-PBD, ADCT-402, MEDI3726/ADC-401, IMGN779, 15 IMGN632, gemtuzumab ozogamicin, inotuzumab ozogamicin/ CMC-544, PF-06647263, CMD-193, CMB-401, trastuzumab duocarmazine/SYD985, BMS-936561/MDX-1203, sacituzumab govitecan/IMMU-132, labetuzumab govitecan/IMMU-130, DS-8201a, U3-1402, milatuzumab doxorubicin/IMMU-110/hLL1-DOX, BMS-986148, RC48-ADC/hertuzumab-vc-MMAE, PF-06647020, PF-06650808, PF-06664178/RN927C, 20 lupartumab amadotin/ BAY1129980, aprutumab ixadotin/BAY1187982, ARX788, AGS62P1, XMT-1522, AbGn-107, MEDI4276, DSTA4637S/RG7861. In one example, the antibody-drug conjugate is gemtuzumab ozogamicin.

In some embodiments, binding of the antibody-drug conjugate to the epitope of the cell-surface lineage-specific protein induces internalization of the antibody-drug conjugate, 25 and the drug (or toxin) may be released intracellularly. In some embodiments, binding of the antibody-drug conjugate to the epitope of a cell-surface lineage-specific protein induces internalization of the toxin or drug, which allows the toxin or drug to kill the cells expressing the lineage-specific protein (target cells). In some embodiments, binding of the antibody-drug conjugate to the epitope of a cell-surface lineage-specific protein induces internalization 30 of the toxin or drug, which may regulate the activity of the cell expressing the lineage-specific protein (target cells). The type of toxin or drug used in the antibody-drug conjugates described herein is not limited to any specific type.

CD123 Associated Diseases and/or Disorders

The present disclosure provides, among other things, compositions and methods for treating a disease associated with expression of CD123 or a condition associated with cells expressing CD123, including, e.g., a proliferative disease such as a cancer or malignancy (e.g., a hematopoietic malignancy), or a precancerous condition such as a myelodysplasia, a myelodysplastic syndrome or a preleukemia.

In some embodiments, the hematopoietic malignancy or a hematological disorder is associated with CD123 expression. A hematopoietic malignancy has been described as a malignant abnormality involving hematopoietic cells (e.g., blood cells, including progenitor and stem cells). Examples of hematopoietic malignancies include, without limitation, Hodgkin's lymphoma, non-Hodgkin's lymphoma, leukemia, or multiple myeloma. Exemplary leukemias include, without limitation, acute myeloid leukemia, acute lymphoid leukemia, chronic myelogenous leukemia, acute lymphoblastic leukemia or chronic lymphoblastic leukemia, and chronic lymphoid leukemia.

In some embodiments, cells involved in the hematopoietic malignancy are resistant to conventional or standard therapeutics used to treat the malignancy. For example, the cells (e.g., cancer cells) may be resistant to a chemotherapeutic agent and/or CAR T cells used to treat the malignancy.

In some embodiments, the leukemia is acute myeloid leukemia (AML). AML is characterized as a heterogeneous, clonal, neoplastic disease that originates from transformed cells that have progressively acquired critical genetic changes that disrupt key differentiation and growth-regulatory pathways. (Dohner et al., *NEJM*, (2015) 373:1136). Without wishing to be bound by theory, it is believed in some embodiments, that CD123 is expressed on myeloid leukemia cells as well as on normal myeloid and monocytic precursors and is an attractive target for AML therapy.

In some cases, a subject may initially respond to a therapy (e.g., for a hematopoietic malignancy) and subsequently experience relapse. Any of the methods or populations of genetically engineered hematopoietic cells described herein may be used to reduce or prevent relapse of a hematopoietic malignancy. Alternatively or in addition, any of the methods described herein may involve administering any of the populations of genetically engineered hematopoietic cells described herein and an immunotherapeutic agent (e.g., cytotoxic agent) that targets cells associated with the hematopoietic malignancy and further administering one or more additional immunotherapeutic agents when the hematopoietic malignancy relapses. In some embodiments, the subject has or is susceptible to relapse of a hematopoietic malignancy (e.g., AML) following administration of one or more previous therapies. In some

embodiments, the methods described herein reduce the subject's risk of relapse or the severity of relapse.

In some embodiments, the hematopoietic malignancy or hematological disorder associated with CD123 is a precancerous condition such as a myelodysplasia, a myelodysplastic syndrome or a preleukemia. Myelodysplastic syndromes (MDS) are hematological medical conditions characterized by disorderly and ineffective hematopoiesis, or blood production. Thus, the number and quality of blood-forming cells decline irreversibly. Some patients with MDS can develop severe anemia, while others are asymptomatic. The classification scheme for MDS is known in the art, with criteria designating the ratio or frequency of particular blood cell types, e.g., myeloblasts, monocytes, and red cell precursors. MDS includes refractory anemia, refractory anemia with ring sideroblasts, refractory anemia with excess blasts, refractory anemia with excess blasts in transformation, chronic myelomonocytic leukemia (CMML). In some embodiments, MDS can progress to an acute myeloid leukemia (AML).

EXAMPLES

Example 1: Genetic editing of CD123 in human cells

Design of sgRNA constructs

The sgRNAs indicated in Table 4 were designed by manual inspection for the SpCas9 PAM (5'-NGG-3') with close proximity to the target region and prioritized according to predicted specificity by minimizing potential off-target sites in the human genome with an online search algorithm (e.g., the Benchling algorithm, Doench et al 2016, Hsu et al 2013). All designed synthetic sgRNAs were produced with chemically modified nucleotides at the three terminal positions at both the 5' and 3' ends. Modified nucleotides contained 2'-O-methyl-3'-phosphorothioate (abbreviated as "ms") and the ms-sgRNAs were HPLC-purified. Cas9 protein was purchased from Aldervon.

Table 4: Sequences of target domains of human CD123 that can be bound by suitable gRNAs. A corresponding gRNA will typically comprise a targeting domain that may comprise an equivalent RNA sequence.

gRNA Name	Sequence	PAM	Exon
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gRNA A	GCCCTGTCTCCTGCAAACGA (SEQ ID NO: 1)	AGG	Exon 2
gRNA B	TGAGCCAAAGGAGGACCATC (SEQ ID NO: 2)	GGG	Exon 2
gRNA C	TCAGGAGCAGCGTGAGCCAA (SEQ ID NO: 3)	AGG	Exon 2
gRNA D	TCCTTCGTTTGCAGGAGACA (SEQ ID NO: 4)	GGG	Exon 2
gRNA E	ATCCACGTCATGAATCCAGC (SEQ ID NO: 5)	AGG	Exon 5
gRNA F	CAGGTCGTA CTGGACGTCCG (SEQ ID NO: 6)	CGG	Exon 5
gRNA G	TTTCTTGAGCTGCAGCTGGG (SEQ ID NO: 7)	CGG	Exon 5
gRNA H	GGTCGTA CTGGACGTCCGCG (SEQ ID NO: 8)	GGG	Exon 5
gRNA I	AGTTCCCACATCCTGGTGCG (SEQ ID NO: 9)	GGG	Exon 6
gRNA J	CACTACAAAACGGATGCTCA (SEQ ID NO: 10)	GGG	Exon 6

Human CD34+ Cell Culture and Electroporation

Cryopreserved human CD34+ cells were purchased from Hemacare and thawed according to manufacturer's instructions. Human CD34+ cells were cultured for 2 days in GMP SCGM media (CellGenix), supplemented with human cytokines (Flt3, SCF, and TPO, all purchased from Peprotech). Cas9 protein and ms-sgRNA (at a 1:1 weight ratio) were mixed and incubated at room temperature for 10 minutes prior to electroporation. CD34+ cells were electroporated with the Cas9 ribonucleoprotein complex (RNP) using Lonza 4D-Nucleofector and P3 Primary Cell Kit (Program CA-137). Cells were cultured at 37°C until analysis. Cell viability was measured by Cellometer and ViaStain AOPI Staining (Nexcelom Biosciences).

Cell line culture and electroporation

Human AML cell line THP-1 was obtained from the American Type Culture Collection (ATCC). THP-1 cells were cultured in RPMI-1640 medium (ATCC) supplemented with 10% heat-inactivated HyClone Fetal Bovine Serum (GE Healthcare) and 0.05 mM 2-mercaptoethanol (Gibco). Cas9 protein and ms-sgRNA (at a 1:1 weight ratio) were mixed and incubated at room temperature for 10 minutes prior to electroporation. THP-

1 cells were electroporated with the Cas9 RNP using Lonza 4D-Nucleofector and SG Cell Line Nucleofector Kit (Program FF-100). Cells were incubated at 37°C for 4 days until flow cytometric analysis.

5 *Genomic DNA analysis*

Genomic DNA was extracted from cells 2 days post electroporation using the prepGEM DNA extraction kit (ZyGEM). The genomic region of interest was amplified by PCR.

PCR amplicons were analyzed by Sanger sequencing (Genewiz) and allele modification frequency was calculated using TIDE (Tracking of Indels by Decomposition).

In vitro colony-forming unit (CFU) assay

Two days after electroporation, 500 CD34+ cells were plated in 1.1 mL of methylcellulose (MethoCult H4034 Optimum, Stem Cell Technologies) on 6 well plates in duplicates and cultured for two weeks. Colonies were then counted and scored using StemVision (Stem Cell Technologies).

Flow cytometry analysis

Fluorochrome-conjugated antibody against human CD123 (9F5) was purchased from BD Biosciences and was tested with its respective isotype control. Cell surface staining was performed by incubating cells with specific antibodies for 30 minutes on ice in the presence of human TruStain FcX. For all stains, dead cells were excluded from analysis by DAPI (Biolegend) stain. All samples were acquired and analyzed on the Attune NxT flow cytometer (ThermoFisher Scientific) and FlowJo software (TreeStar).

25

Results

Human CD34+ cells were electroporated with Cas9 protein and the indicated CD123-targeting gRNA as described above.

The percentage editing was determined by % INDEL as assessed by TIDE (Figures 1, 2A, and 3C) or surface CD123 protein expression by flow cytometry (Figure 2B).

As shown in Figure 1 and Figure 2A, gRNAs A, G, and I showed a high proportion of indels, in the range of approximately 60-100% of cells. In comparison, gRNAs C, E, H, and J gave much lower proportions of indels, in the range of approximately 20-40% of cells.

gRNAs B, D, and F showed an intermediate proportion of indels, in the range of approximately 50-60% of cells.

As shown in Figures 2B-2C, gRNAs A, G, and I showed a marked reduction in CD123 expression, as detected by FACS.

5 CD123 gRNA I was further assessed for cell viability and *in vitro* differentiation (Figure 3A). As shown in Figure 3B, cells electroporated with gRNA I showed comparable viability to negative control cells 48 hours after electroporation. These cells also showed strong editing efficiency of the *CD123/IL3RA* locus, with an indel percentage of approximately 60% (Figure 3C). Furthermore, as shown in Figure 3D, cells electroporated
 10 with gRNA I were able to differentiate *in vitro*. In particular, substantial numbers of BFU-E and CFU-G/M/GM colonies formed from cells receiving gRNA I. Lower levels of CFU-GEMM colony formation was observed in gRNA I-electroporated cells as well.

Example 2: Generation and evaluation of cells edited for two cell surface antigens

15 Results

Cell surface levels of CD33, CD123 and CLL1 (CLEC12A) were measured in unedited MOLM-13 cells and THP-1 cells (both human AML cell lines) by flow cytometry. MOLM-13 cells had high levels of CD33 and CD123, and moderate-to-low levels of CLL1. HL-60 cells had high levels of CD33 and CLL1, and low levels of CD123 (FIGURE 4).

20 CD33 and CD123 were mutated in MOLM-13 cells using gRNAs and Cas9 as described herein, CD33 and CD123-modified cells were purified by flow cytometric sorting, and the cell surface levels of CD33 and CD123 were measured. CD33 and CD123 levels were high in wild-type MOLM-13 cells; editing of CD33 only resulted in low CD33 levels; editing of CD123 only resulted in low CD123 levels, and editing of both CD33 and CD123
 25 resulted in low levels of both CD33 and CD123 (FIGURE 5). The edited cells were then tested for resistance to CART effector cells using an *in vitro* cytotoxicity assay as described herein. All four cell types (wild-type, CD33^{-/-}, CD123^{-/-}, and CD33^{-/-}CD123^{-/-}) experienced low levels of specific killing in mock CAR control conditions (FIGURE 6, leftmost set of bars). CD33 CAR cells effectively killed wild-type and CD123^{-/-} cells, while CD33^{-/-} and
 30 CD33^{-/-}CD123^{-/-} cells showed a statistically significant resistance to CD33 CAR (FIGURE 35, second set of bars). CD123 CAR cells effectively killed wild-type and CD33^{-/-} cells, while CD123^{-/-} and CD33^{-/-}CD123^{-/-} cells showed a statistically significant resistance to CD123 CAR (FIGURE 6, third set of bars). A pool of CD33 CAR and CD123 CAR cells effectively killed wild-type cells, CD33^{-/-} cells, and CD123^{-/-} cells, while CD33^{-/-}CD123^{-/-}

cells showed a statistically significant resistance to the pool of CAR cells (FIGURE 6, rightmost set of bars). This experiment demonstrates that knockout of two antigens (CD33 and CD123) protected the cells against CAR cells targeting both antigens. Furthermore, the population of edited cells contained a high enough proportion of cells that were edited at both alleles of both antigens, and had sufficiently low cell surface levels of cell surface antigens, that a statistically significant resistance to both types of CAR cells was achieved.

The efficiency of gene editing in human CD34+ cells was quantified using TIDE analysis as described herein. At the endogenous CD33 locus, editing efficiency of between about 70-90% was observed when CD33 was targeted alone or in combination with CD123 or CLL1 (FIGURE 7, left graph). At the endogenous CD123 locus, editing efficiency of about 60% was observed when CD123 was targeted alone or in combination with CD33 or CLL1 (FIGURE 7, center graph). At the endogenous CLL1 locus, editing efficiency of between about 40-70% was observed when CLL1 was targeted alone or in combination with CD33 or CD123 (FIGURE 7, right graph). This experiment illustrates that human CD34+ cells can be edited at a high frequency at two cell surface antigen loci.

The differentiation potential of gene-edited human CD34+ cells as measured by colony formation assay as described herein. Cells edited for CD33, CD123, or CLL1, individually or in all pairwise combinations, produced BFU-E colonies, showing that the cells retain significant differentiation potential in this assay (FIGURE 8A). The edited cells also produced CFU-G/M/GM colonies, showing that the cells retain differentiation potential in this assay that is statistically indistinguishable from the non-edited control (FIGURE 8B). The edited cells also produced detectable CFU-GEMM colonies (FIGURE 8C). Colony forming unit (CFU)-G/M/GM colonies refer to CFU-G (granulocyte), CFU-M (macrophage), and CFU-GM (granulocyte/macrophage) colonies. CFU-GEMM (granulocyte/erythroid/macrophage/megakaryocyte) colonies arise from a less differentiated cell that is a precursor to the cells that give rise to CFU-GM colonies. Taken together, the differentiation assays indicate that human CD34+ cells edited at two loci retain the capacity to differentiate into variety of cell types.

30 *Materials and Methods*

AML cell lines

Human AML cell line HL-60 was obtained from the American Type Culture Collection (ATCC). HL-60 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM, Gibco) supplemented with 20% heat-inactivated HyClone Fetal Bovine Serum (GE

Healthcare). Human AML cell line MOLM-13 was obtained from AddexBio Technologies. MOLM-13 cells were cultured in RPMI-1640 media (ATCC) supplemented with 10% heat-inactivated HyClone Fetal Bovine Serum (GE Healthcare).

5 *Guide RNA design*

All sgRNAs were designed by manual inspection for the SpCas9 PAM (5'-NGG-3') with close proximity to the target region and prioritized according to predicted specificity by minimizing potential off-target sites in the human genome with an online search algorithm (Benchling, Doench et al 2016, Hsu et al 2013). All designed synthetic sgRNAs were purchased from Synthego with chemically modified nucleotides at the three terminal positions at both the 5' and 3' ends. Modified nucleotides contained 2'-O-methyl-3'-phosphorothioate (abbreviated as "ms") and the ms-sgRNAs were HPLC-purified. Cas9 protein was purchased from Aldervon. Typically, the gRNAs described in the Examples herein are sgRNAs comprising a 20 nucleotide (nt) targeting domain sequence, 12 nt of the crRNA repeat sequence, a 4 nt tetraloop sequence, and 64 nt of tracrRNA sequence.

Table 5: Sequences of target domains of human CD33, CD123, or CLL-1 that can be bound by suitable gRNAs. The adjacent PAM sequences are also provided. A suitable gRNA typically comprises a targeting domain that may comprise an RNA sequence equivalent to the target domain sequence

Target gene	Sequence	PAM	Target location
CD33	CCCCAGGACTACTCACTCCT (SEQ ID NO: 64)	CGG	CD33 exon 3
CD123	TTTCTTGAGCTGCAGCTGGG (SEQ ID NO: 7)	CGG	CD123 exon 5
	AGTTCACACATCCTGGTGCG (SEQ ID NO: 9)	GGG	CD123 exon 6
CLL1	GGTGGCTATTGTTTGCAGTG (SEQ ID NO: 65)	TGG	CLL1 exon 4

AML cell line electroporation

Cas9 protein and ms-sgRNA (at a 1:1 weight ratio) were mixed and incubated at room temperature for 10 minutes prior to electroporation. MOLM-13 and HL-60 cells were

electroporated with the Cas9 ribonucleoprotein complex (RNP) using the MaxCyte ATx Electroporator System with program THP-1 and Opt-3, respectively. Cells were incubated at 37°C for 5-7 days until flow cytometric sorting.

5 *Human CD34+ cell culture and electroporation*

Cryopreserved human CD34+ cells were purchased from Hemacare and thawed according to manufacturer's instructions. Human CD34+ cells were cultured for 2 days in GMP SCGM media (CellGenix) supplemented with human cytokines (Flt3, SCF, and TPO, all purchased from Peprotech). CD34+ cells were electroporated with the Cas9 RNP (Cas9
10 protein and ms-sgRNA at a 1:1 weight ratio) using Lonza 4D-Nucleofector and P3 Primary Cell Kit (Program CA-137). For electroporation with dual ms-sgRNAs, equal amount of each ms-sgRNA was added. Cells were cultured at 37°C until analysis.

Genomic DNA analysis

15 Genomic DNA was extracted from cells 2 days post electroporation using prepGEM DNA extraction kit (ZyGEM). Genomic region of interest was amplified by PCR. PCR amplicons were analyzed by Sanger sequencing (Genewiz) and allele modification frequency was calculated using TIDE (Tracking of Indels by Decomposition) software available on the World Wide Web at tide.deskgen.com.

20

In vitro colony-forming unit (CFU) assay

Two days after electroporation, 500 CD34+ cells were plated in 1.1 mL of methylcellulose (MethoCult H4034 Optimum, Stem Cell Technologies) on 6 well plates in duplicates and cultured for two weeks. Colonies were then counted and scored using
25 StemVision (Stem Cell Technologies).

Flow cytometric analysis and sorting

Fluorescence-conjugated antibodies against human CD33 (P67.6), CD123 (9F5), and CLL1 (REA431) were purchased from Biolegend, BD Biosciences and Miltenyi Biotec,
30 respectively. All antibodies were tested with their respective isotype controls. Cell surface staining was performed by incubating cells with specific antibodies for 30 min on ice in the presence of human TruStain FcX. For all stains, dead cells were excluded from analysis by

DAPI (Biolegend) stain. All samples were acquired and analyzed with Attune NxT flow cytometer (ThermoFisher Scientific) and FlowJo software (TreeStar).

For flow cytometric sorting, cells were stained with flurochrome-conjugated antibodies followed by sorting with Moflow Astrios Cell Sorter (Beckman Coulter).

5

CAR constructs and lentiviral production

Second-generation CARs were constructed to target CD33 and CD123, with the exception of the anti-CD33 CAR-T used in CD33/CLL-1 multiplex cytotoxicity experiment. Each CAR consisted of an extracellular scFv antigen-binding domain, using CD8 α signal peptide, CD8 α hinge and transmembrane regions, the 4-1BB costimulatory domain, and the CD3 ξ signaling domain. The anti-CD33 scFv sequence was obtained from clone P67.6 (Mylotarg) and the anti-CD123 scFv sequence from clone 32716. The anti-CD33 and anti-CD123 CAR constructs uses a heavy-to-light orientation of the scFv. The heavy and light chains were connected by (GGGS)₃ linker (SEQ ID NO: 63). CAR cDNA sequences for each target were sub-cloned into the multiple cloning site of the pCDH-EF1 α -MCS-T2A-GFP expression vector, and lentivirus was generated following the manufacturer's protocol (System Biosciences). Lentivirus can be generated by transient transfection of 293TN cells (System Biosciences) using Lipofectamine 3000 (ThermoFisher). The CAR construct was generated by cloning the light and heavy chain of anti-CD33 scFv (clone My96), to the CD8 α hinge domain, the ICOS transmembrane domain, the ICOS signaling domain, the 4-1BB signaling domain and the CD3 ξ signaling domain into the lentiviral plasmid pHIV-Zsgreen.

CAR transduction and expansion

Human primary T cells were isolated from Leuko Pak (Stem Cell Technologies) by magnetic bead separation using anti-CD4 and anti-CD8 microbeads according to the manufacturer's protocol (Stem Cell Technologies). Purified CD4⁺ and CD8⁺ T cells were mixed 1:1, and activated using anti-CD3/CD28 coupled Dynabeads (Thermo Fisher) at a 1:1 bead to cell ratio. T cell culture media used was CTS Optimizer T cell expansion media supplemented with immune cell serum replacement, L-Glutamine and GlutaMAX (all purchased from Thermo Fisher) and 100 IU/mL of IL-2 (Peprotech). T cell transduction was performed 24 hours post activation by spinoculation in the presence of polybrene (Sigma).

CAR-T cells were cultured for 9 days prior to cryopreservation. Prior to all experiments, T cells were thawed and rested at 37°C for 4-6 hours.

Flow Cytometry based CAR-T cytotoxicity assay

5 The cytotoxicity of target cells was measured by comparing survival of target cells relative to the survival of negative control cells. For CD33/CD123 multiplex cytotoxicity assays, wildtype and CRISPR/Cas9 edited MOLM-13 cells were used as target cells. Wildtype Raji cell lines (ATCC) were used as negative control for both experiments. Target cells and negative control cells were stained with CellTrace Violet (CTV) and CFSE (Thermo
10 Fisher), respectively, according to the manufacturer's instructions. After staining, target cells and negative control cells were mixed at 1:1.

Anti-CD33 or CD123 CAR-T cells were used as effector T cells. Non-transduced T cells (mock CAR-T) were used as control. For the CARpool groups, appropriate CAR-T cells were mixed at 1:1. The effector T cells were co-cultured with the target cell/negative control
15 cell mixture at a 1:1 effector to target ratio in duplicate. A group of target cell/negative control cell mixture alone without effector T cells was included as control. Cells were incubated at 37°C for 24 hours before flow cytometric analysis. Propidium iodide (ThermoFisher) was used as a viability dye. For the calculation of specific cell lysis, the fraction of live target cell to live negative control cell (termed target fraction) was used.
20 Specific cell lysis was calculated as $((\text{target fraction without effector cells} - \text{target fraction with effector cells}) / (\text{target fraction without effectors})) \times 100\%$.

Example 3: Design and Screening of gRNAs for editing CD123 in human cells

Design of sgRNA constructs

25 The gRNAs investigated in this Example were designed by inspection of the SpCas9 PAM (5'-NGG-3') with close proximity to the target region. All the 20bp sequences in the coding region with an SpCas9 PAM (5'-NGG-3') at the 3' end were extracted. Using these methods, 209 total gRNAs targeting the target domains of human CD123 as described in Table 2 and 6 were designed.

30

Screening of gRNAs in THP-1 cells

The 209 gRNAs were filtered according to an off-target prediction algorithm (based on number of mismatches), which identified 178 gRNAs for further investigation in THP-1

cells. Human AML cell line THP-1 was obtained from the American Type Culture Collection (ATCC). THP-1 cells were cultured and electroporated with the ribonucleoprotein RNP complexes composed of Cas9 protein and gRNA (mixed at a 1:1 weight ratio). Genomic DNA was extracted from cells and the genomic region of interest was amplified by

5 PCR. PCR amplification of the genomic region of interest was obtained for 148 of the 178 gRNAs investigated. PCR amplicons were then analyzed by Sanger sequencing to calculate editing frequency (ICE, or interference of CRISPR edits) in two replicates, which is shown in Table 7. In the first replicate, the editing frequency was obtained for 146 of the 148 gRNAs that were amplified and sequenced. In the second replicate, the editing frequency was

10 obtained for 96/146 gRNAs, and the results for each gRNA were comparable across the two replicates. As depicted in Table 7, 59 of the gRNAs investigated had an ICE value or editing frequency ≥ 80 .

Table 7. Editing frequency of gRNAs designed to target human CD123 in THP-1 cells

gRNA	SEQ ID NO:	Sequence	Replicate 1		Replicate 2	
			ICE	R ²	ICE	R ²
gRNA M	297	CGUCCCCGAUGGUCCUCCUU	88	0.92	90	0.94
gRNA A	21	GCCCUGUCUCCUGCAAACGA	91	0.95	94	0.94
gRNA S	298	UGUCUCCUGCAAACGAAGGA	96	0.96	90	0.95
gRNA V	299	AAACGAAGGAAGGUAAGAAC	49	0.95	43	0.97
gRNA U	300	UCUUACCUUCCUUCGUUUGC	0	1	0	1
gRNA T	301	UCCUUCGUUUGCAGGAGAC	46	0.96	49	0.96
gRNA D	24	UCCUUCGUUUGCAGGAGACA	94	0.97	93	0.96
gRNA R	302	UCGUUUGCAGGAGACAGGGC	84	0.97	84	0.97
gRNA Q	303	CGUUUGCAGGAGACAGGGCA	85	0.97	-	-
gRNA P	304	GGAGACAGGGCAGGGCGAUC	72	0.93	84	0.84
gRNA C	23	UCAGGAGCAGCGUGAGCCAA	80	0.93	64	0.92
gRNA N	305	GUGAGCCAAAGGAGGACCAU	84	0.93	83	0.88
gRNA B	22	UGAGCCAAAGGAGGACCAUC	90	0.95	90	0.94
gRNA L	306	GACCAUCGGGAACGCAGCUC	86	0.97	88	0.97
gRNA A1	307	ACCCACCAAUCACGAACCUA	-	-	-	-
gRNA G1	308	CAAAGGCUCAGCAGUUGACC	-	-	-	-
gRNA H1	309	AAAGGCUCAGCAGUUGACCU	82	0.92	-	-

gRNA L1	310	AGACGCCGACUAUUCUAUGC	82	0.85	-	-
gRNA M1	311	AUUUACCGGCAUAGAAUAGU	88	0.88	-	-
gRNA K1	312	GUCUUUAAACACACUCGAUUAU	85	0.91	92	0.93
gRNA J1	313	UAUCGGUCACAUUUCUGUUA	47	0.94	48	0.94
gRNA I1	314	CACAUUUCUGUUAAGGUCCC	86	0.96	73	0.92
gRNA F1	315	GAGCCUUUGCUUUCAUCCUU	88	0.94	-	-
gRNA D1	48	UUCAUCCUUAGGUUCGUGAU	65	0.93	62	0.91
gRNA C1	316	AUCCUUAGGUUCGUGAUUGG	60	0.94	60	0.93
gRNA B1	317	UCCUUAGGUUCGUGAUUGGU	72	0.85	77	0.88
gRNA Z	318	AGGUUCGUGAUUGGUGGGUU	30	0.95	30	0.96
gRNA Y	319	UGGUGGGUUUGGAUCUAAAA	74	0.93	68	0.95
gRNA X	320	UUUGGAUCUAAAACGGUGAC	74	0.95	70	0.95
gRNA Q1	321	AACAAUAGCUAUUGCCAGUU	-	-	-	-
gRNA T1	322	GACCAACUACACCGUCCGAG	58	0.89	-	-
gRNA X1	323	CCAACCCACCAUUCUCCACG	67	0.98	65	0.98
gRNA F2	324	ACAUUUUUCUCACUGUUCUC	37	0.98	-	-
gRNA E2	325	CAUUUUUCUCACUGUUCUCA	74	0.97	-	-
gRNA D2	326	UCUCACUGUUCUCAGGGAAG	75	0.96	-	-
gRNA C2	327	CUCAGGGAAGAGGAUCCACG	99	0.99	99	0.99
gRNA B2	328	AAGAGGAUCCACGUGGAGAA	86	0.98	85	0.97
gRNA A2	329	AGGAUCCACGUGGAGAAUGG	87	0.97	90	0.97
gRNA Z1	330	GGAUCCACGUGGAGAAUGGU	91	0.96	95	0.96
gRNA Y1	331	CCACGUGGAGAAUGGUGGGU	82	0.96	82	0.97
gRNA W1	332	AGAAUGGUGGGUUGGCCACU	95	0.95	90	0.93
gRNA V1	333	UGGUGGGUUGGCCACUCGGA	50	0.95	-	-
gRNA U1	334	GGCCACUCGGACGGUGUAGU	84	0.92	84	0.91
gRNA R1	335	AUAAGGAAAUUGCUCCAAAC	90	0.95	-	-
gRNA P1	336	UCACUGCCUAAGAGAGACAU	-	-	-	-
gRNA J2	337	UGAACCCAGGUGGGAAGCCU	-	-	-	-
gRNA K2	338	GAACCCAGGUGGGAAGCCUU	-	-	-	-
gRNA L2	339	CCAGGUGGGAAGCCUUGGGC	-	-	-	-
gRNA O2	340	UGGGAAGCCUUGGGCAGGUG	-	-	-	-

gRNA Q2	341	GUGCGGAGAAUCUGACCUGC	-	-	-	-
gRNA R2	342	GACCUGCUGGAUUCAUGACG	40	0.98	-	-
gRNA S2	343	UGGAUUUCUUGAGCUGCAGC	0	1	0	1
gRNA T2	344	GGAUUUUCUUGAGCUGCAGCU	73	0.94	77	0.94
gRNA G	27	UUUCUUGAGCUGCAGCUGGG	86	0.97	86	0.97
gRNA U2	345	UUGAGCUGCAGCUGGGCGGU	24	0.97	27	0.97
gRNA V2	346	CUGCAGCUGGGCGGUAGGCC	1	1	2	1
gRNA W2	347	UGCAGCUGGGCGGUAGGCC	17	0.98	18	0.98
gRNA X2	348	GCAGCUGGGCGGUAGGCCCG	0	1	0	1
gRNA Y2	349	CAGCUGGGCGGUAGGCCCGG	59	0.97	61	0.97
gRNA Z2	350	GGUAGGCCCGGGGCCCCCG	3	1	4	0.99
gRNA F3	351	GUACUUGAACGUUGCCAAGU	35	0.95	40	0.95
gRNA H3	352	UUGCCAAGUAGGUGUGCCCG	3	0.99	3	0.99
gRNA I3	353	UGCCAAGUAGGUGUGCCCGU	29	0.98	25	0.98
gRNA G3	354	ACUUGGCAACGUUCAAGUAC	62	0.96	66	0.95
gRNA E3	355	CGUUCAAGUACAGGUCGUAC	59	0.93	65	0.9
gRNA F	26	CAGGUCGUACUGGACGUCCG	47	0.92	53	0.89
gRNA D3	356	AGGUCGUACUGGACGUCCGC	4	0.99	9	0.98
gRNA H	28	GGUCGUACUGGACGUCCGCG	29	0.98	-	-
gRNA C3	357	GUCGUACUGGACGUCCGCGG	44	0.93	42	0.92
gRNA B3	358	UGGACGUCCGCGGGGCCCC	33	0.97	33	0.97
gRNA A3	359	GGACGUCCGCGGGGCCCC	36	0.98	38	0.98
gRNA E	360	AUCCACGUCAUGAAUCCAGC	-	-	-	-
gRNA P2	361	AGAUUCUCCGCACCUGCCCA	-	-	-	-
gRNA N2	362	CCUGCCCAAGGCUUCCCACC	-	-	-	-
gRNA M2	363	CUGCCCAAGGCUUCCCACCU	-	-	-	-
gRNA P3	50	CGAGUGUCUUCACUACAAAA	84	0.96	-	-
gRNA Q3	364	UCACUACAAAACGGAUGCUC	42	0.96	-	-
gRNA J	365	CACUACAAAACGGAUGCUC	51	0.95	-	-
gRNA R3	366	GAUGCUCAGGGAACACGUUAU	77	0.93	-	-
gRNA S3	51	AUGCUCAGGGAACACGUUAUC	86	0.95	-	-
gRNA T3	367	GACAUCUCUCGACUCUCCAG	77	0.94	-	-

gRNA V3	368	UUCUCAAGUUCCACAUC	85	0.95	-	-
gRNA W3	369	AAAGUUCCACAUCUGGUG	66	0.96	-	-
gRNA X3	370	AAGUUCCACAUCUGGUGC	63	0.96	-	-
gRNA I	29	AGUUCCACAUCUGGUGCG	88	0.95	-	-
gRNA Y3	371	CCCACAUCUGGUGCGGGGC	66	0.94	-	-
gRNA H4	372	UUUGUCGUCUUUUCACAGAU	3	0.99	-	-
gRNA I4	373	UCACAGAUUGGUGAGUAGCC	0	1	-	-
gRNA J4	374	CACAGAUUGGUGAGUAGCCC	26	0.96	-	-
gRNA G4	375	GACGACAAACUUAUCUGUGC	0	1	-	-
gRNA F4	376	ACGACAAACUUAUCUGUGCA	0	1	-	-
gRNA E4	377	CGACAAACUUAUCUGUGCAG	25	0.97	-	-
gRNA D4	378	AUCUGUGCAGGGGAUACCGA	81	0.97	-	-
gRNA B4	379	CUGCGCUCCUGCCCCGCACC	88	0.95	-	-
gRNA A4	380	UCCUGCCCCGCACCAGGAUG	14	0.98	-	-
gRNA Z3	381	CCUGCCCCGCACCAGGAUGU	89	0.95	-	-
gRNA U3	382	GUGGGAACUUUGAGAACCGC	3	0.99	-	-
gRNA O3	383	CGUACUGUUGACGCCUGCUG	90	0.96	-	-
gRNA N3	49	UUGACGCCUGCUGCGGUAAG	89	0.96	-	-
gRNA N4	384	AGACACAUUCCUUUAUGCAC	55	0.97	-	-
gRNA P4	385	CUAUGAGCUUCAGAUACAAA	10	0.99	-	-
gRNA O4	386	UCUCAUUUCCAGUGCAUAA	35	0.98	-	-
gRNA M4	387	AUUACACUUUGCAGUCAUGU	54	0.96	-	-
gRNA L4	388	UUACACUUUGCAGUCAUGUU	98	0.98	-	-
gRNA K4	389	CACUUUGCAGUCAUGUUGGG	42	0.97	-	-
gRNA Q4	390	GCAGCCUGUAAUCACAGAAC	80	0.97	84	0.96
gRNA R4	391	CUCACCUGUUCUGUGAUUAC	76	0.94	79	0.93
gRNA U4	392	UCCUUCAGCUACUCAAUCC	-	-	-	-
gRNA Z4	393	ACACAGUACAAAUAAGAGCC	-	-	-	-
gRNA A5	394	CACAGUACAAAUAAGAGCCC	-	-	-	-
gRNA D5	395	UGUAUGAAUUCUUGAGCGCC	-	-	-	-
gRNA E5	396	UGGAGCACCCCCCAGCGCUU	-	-	-	-
gRNA G5	397	CCCCCAGCGCUUCGGUGAG	-	-	-	-

gRNA H5	398	CCCCCAGCGCUUCGGUGAGU	-	-	-	-
gRNA N5	399	GCUUCGGUGAGUGGGCUGUG	-	-	-	-
gRNA L5	400	AGCCCACUCACCGAAGCGCU	-	-	-	-
gRNA K5	401	GCCCACUCACCGAAGCGCUG	-	-	-	-
gRNA I5	402	CCACUCACCGAAGCGCUGGG	-	-	-	-
gRNA F5	403	GAAGCGCUGGGGGGUGCUCC	-	-	-	-
gRNA C5	404	AGAAUUCAUACACUCUUUCC	-	-	-	-
gRNA B5	405	GAAUUCAUACACUCUUUCCC	-	-	-	-
gRNA Y4	406	AUUUGUACUGUGUACGUUCC	-	-	-	-
gRNA X4	407	ACGUUCCAGGAUUGAGUAGC	-	-	-	-
gRNA W4	408	UCCAGGAUUGAGUAGCUGGA	-	-	-	-
gRNA V4	409	AGGAUUGAGUAGCUGGAAGG	-	-	-	-
gRNA T4	410	GAGGUUCUGUCUCUGACCUG	-	-	-	-
gRNA S5	411	UUUACCCCUAGAGUGCGACC	60	0.95	71	0.95
gRNA V5	412	ACCCCUAGAGUGCGACCAGG	92	0.95	95	0.95
gRNA W5	413	CCUAGAGUGCGACCAGGAGG	88	0.96	92	0.96
gRNA X5	414	CUAGAGUGCGACCAGGAGGA	75	0.94	91	0.95
gRNA C6	415	AGGGCGCAAACACACGUGCC	37	0.96	45	0.96
gRNA D6	416	GCGCAAACACACGUGCCUGG	50	0.96	54	0.96
gRNA F6	417	GACGUCGUCGUGAUCGCGC	23	0.98	25	0.98
gRNA G6	418	ACGUCGUCGUGAUCGCGCU	0	1	0	1
gRNA H6	419	CGUCGUCGUGAUCGCGCUG	57	0.94	-	-
gRNA I6	420	GAUCGCGCUGGGGACGCUGC	36	0.97	35	0.97
gRNA J6	421	GCUGGGGACGCUGCUGGCC	79	0.95	84	0.97
gRNA M6	422	GUGUCUUCGUGAUCUGCAGA	0	1	0	1
gRNA N6	423	CUGCAGAAGGUGAGCCCUCG	50	0.96	53	0.98
gRNA O6	424	UGCAGAAGGUGAGCCCUCGA	55	0.96	59	0.92
gRNA L6	425	AGAUCACGAAGACACAGACC	63	0.95	63	0.96
gRNA K6	426	GAUCACGAAGACACAGACCA	95	0.96	97	0.97
gRNA E6	427	GAUCAGCAGCGACGUCCGCC	30	0.97	32	0.97
gRNA B6	428	GUGUGUUUGCGCCCUCCUCC	75	0.94	-	-
gRNA A6	429	CCUCCUCCUGGUCGCACUCU	58	0.96	66	0.95

gRNA Z5	430	CUCCUCCUGGUCGCACUCUA	59	0.95	55	0.95
gRNA Y5	431	UCCUCCUGGUCGCACUCUAG	89	0.95	89	0.94
gRNA U5	432	UGGUCGCACUCUAGGGGUAA	72	0.95	69	0.94
gRNA T5	433	GGUCGCACUCUAGGGGUAAA	92	0.94	95	0.95
gRNA R6	434	UCUCUUUCCUCCGAGGUAUC	90	0.92	91	0.93
gRNA X6	435	CCUCACAUGAAAAGACCCCAU	94	0.97	95	0.97
gRNA D7	436	CAGCUUCCAAAACGACAAGC	68	0.96	72	0.96
gRNA E7	437	AACAUACCAGCUUGUCGUUU	16	0.97	37	0.96
gRNA C7	438	GUUUUGGAAGCUGUCACCGA	48	0.96	46	0.95
gRNA B7	439	UUUUGGAAGCUGUCACCGAU	98	0.99	98	0.99
gRNA A7	440	UUUGGAAGCUGUCACCGAUG	92	0.98	95	0.97
gRNA Z6	441	CCGAUGGGGUCUUUCAUGUG	87	0.95	83	0.89
gRNA Y6	442	CGAUGGGGUCUUUCAUGUGA	97	0.98	95	0.96
gRNA W6	443	GGUCUUUCAUGUGAGGGAUG	91	0.96	95	0.96
gRNA V6	444	GUCUUUCAUGUGAGGGAUGC	84	0.97	88	0.97
gRNA U6	445	UCUUUCAUGUGAGGGAUGCG	88	0.96	88	0.95
gRNA T6	446	CUCUGCAUACCAGAUACCU	93	0.95	91	0.96
gRNA S6	447	UGCAUACCAGAUACCUCGG	91	0.93	92	0.92
gRNA I7	448	UCUCGUCUCUGCAGGUGGUC	53	0.95	57	0.95
gRNA J7	449	CUCGUCUCUGCAGGUGGUCU	81	0.91	86	0.92
gRNA L7	450	GUCUCUGCAGGUGGUCUGGG	92	0.95	93	0.95
gRNA M7	451	UCUCGAGGUGGUCUGGGAGG	42	0.94	-	-
gRNA O7	452	GUCUGGGAGGCGGGCAAAGC	42	0.95	40	0.95
gRNA P7	247	GGAGGCGGGCAAAGCCGGCC	78	0.97	80	0.95
gRNA Q7	453	GGCGGGCAAAGCCGGCCUGG	48	0.95	52	0.94
gRNA R7	454	AGCCGGCCUGGAGGAGUGUC	91	0.96	86	0.96
gRNA U7	455	GUGUCUGGUGACUGAAGUAC	95	0.95	-	-
gRNA V7	456	UCGUGCAGAAAACUUGAGAC	13	0.99	12	0.99
gRNA W7	457	CGUGCAGAAAACUUGAGACU	90	0.96	94	0.97
gRNA Y7	458	AAAACUUGAGACUGGGGUUC	72	0.96	-	-
gRNA T7	459	CAGUCACCAGACACUCCUCC	84	0.94	-	-
gRNA S7	460	CACCAGACACUCCUCCAGGC	76	0.93	-	-

gRNA K7	461	AGACCACCUGCAGAGACGAG	95	0.96	93	0.97
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Screening of gRNAs in primary CD34+ human stem and progenitor cells (HSPCs)

Primary human CD34+ HSPCs were cultured and electroporated with ribonucleoprotein RNP complexes composed of Cas9 protein and one of the 44 gRNAs listed in Table 8. These 44 gRNAs screened include those that were selected from screening performed in the THP-1 cells and/or those gRNAs that had a favorable off-target profile.

Table 8. Sequences of target domains of CD123 gRNAs screened in human CD34+ cells. The corresponding gRNAs comprised a targeting domain consisting of the equivalent RNA sequence.

gRNA	SEQ ID NO:	Sequence	PAM	Exon
gRNA N	69	GTGAGCCAAAGGAGGACCAT	CGG	Exon 2
gRNA A	1	GCCCTGTCTCCTGCAAACGA	AGG	Exon 2
gRNA R2	122	GACCTGCTGGATTCATGACG	TGG	Exon 5
gRNA C3	133	GTCGTA CTGGACGTCCGCGG	GGG	Exon 5
gRNA H	8	GGTCGTA CTGGACGTCCGCG	GGG	Exon 5
gRNA B	2	TGAGCCAAAGGAGGACCATC	GGG	Exon 2
gRNA C	3	TCAGGAGCAGCGTGAGCCAA	AGG	Exon 2
gRNA P	71	GGAGACAGGGCAGGGCGATC	AGG	Exon 2
gRNA D	4	TCCTTCGTTTG CAGGAGACA	GGG	Exon 2
gRNA T	73	TTCCTTCGTTTG CAGGAGAC	AGG	Exon 2
gRNA M	68	CGTTCCCGATGGTCCTCCTT	TGG	Exon 2
gRNA O	70	GGAGCAGCGTGAGCCAAAGG	AGG	Exon 2
gRNA E	5	ATCCACGTCATGAATCCAGC	AGG	Exon 5
gRNA D3	134	AGGTCGTA CTGGACGTCCGC	GGG	Exon 5
gRNA F	6	CAGGTCGTA CTGGACGTCCG	CGG	Exon 5
gRNA E3	135	CGTTCAAGTACAGGTCGTAC	TGG	Exon 5
gRNA G	7	TTTCTTGAGCTGCAGCTGGG	CGG	Exon 5
gRNA B8	122	GACCTGCTGGATTCATGACG	TGG	Exon 5
gRNA C8	133	GTCGTA CTGGACGTCCGCGG	GGG	Exon 5
gRNA D8	8	GGTCGTA CTGGACGTCCGCG	GGG	Exon 5
gRNA E4	158	CGACAAACTTATCTGTGCAG	GGG	Exon 6

gRNA I	9	AGTTCCACATCCTGGTGCG	GGG	Exon 6
gRNA D4	157	ATCTGTGCAGGGGATACCGA	AGG	Exon 6
gRNA J	10	CACTACAAAACGGATGCTCA	GGG	Exon 6
gRNA K	66	TTCCGGAGCTGCGTTCCCGA	TGG	Exon 2
gRNA K3	141	ACCTTACCGCTTACCGCAGC	AGG	Exon 6
gRNA L3	142	GCTGCGGTAAGCGGTAAGGT	TGG	Exon 6
gRNA M3	143	GCCTGCTGCGGTAAGCGGTA	AGG	Exon 6
gRNA N3	42	TTGACGCCTGCTGCGGTAAG	CGG	Exon 6
gRNA W	76	GATCTAAAACGGTGACAGGT	TGG	Exon 3
gRNA X	77	TTTGATCTAAAACGGTGAC	AGG	Exon 3
gRNA Z	79	AGGTTTCGTGATTGGTGGGTT	TGG	Exon 3
gRNA A1	80	ACCCACCAATCACGAACCTA	AGG	Exon 3
gRNA B1	81	TCCTTAGGTTTCGTGATTGGT	GGG	Exon 3
gRNA C1	82	ATCCTTAGGTTTCGTGATTGG	TGG	Exon 3
gRNA D1	40	TTCATCCTTAGGTTTCGTGAT	TGG	Exon 3
gRNA G1	85	CAAAGGCTCAGCAGTTGACC	TGG	Exon 3
gRNA I1	87	CACATTTCTGTAAAGGTCCC	AGG	Exon 3
gRNA J1	88	TATCGGTCACATTTCTGTTA	AGG	Exon 3
gRNA L	67	GACCATCGGGAACGCAGCTC	CGG	Exon 2
gRNA O3	144	CGTACTGTTGACGCCTGCTG	CGG	Exon 6
gRNA P3	44	CGAGTGTCTTCACTACAAAA	CGG	Exon 6
gRNA S3	46	ATGCTCAGGGAACACGTATC	GGG	Exon 6
gRNA Z3	153	CCTGCCCCGCACCAGGATGT	GGG	Exon 6

The editing frequency of these gRNAs in primary human CD34+ HSPCs was calculated and is depicted in Figure 9 and Figure 10. Of the 44 gRNAs tested, 7 demonstrated an editing efficiency above 80% (Figure 9 and Figure 10). These gRNAs included gRNA A, gRNA G, gRNA I, gRNA N3, gRNA P3, and gRNA S3 and their calculated mean editing efficiencies are shown in Table 9.

Table 9. Mean editing efficiencies of gRNAs screened in primary human CD34+ HSPCs

Guide	Mean Editing Frequency
gRNA A	80.4

gRNA G	95.3
gRNA I	83.3
gRNA N3	86.0
gRNA P3	89.3
gRNA S3	79.0

The INDEL (insertion/deletion) distributions for gRNA A, gRNA G, gRNA I, gRNA N3, gRNA P3, and gRNA S3 as evaluated in the primary human CD34+ cells was quantified and are shown in Figure 11. Each gRNA led to INDELs ranging from -14 to +2. The INDEL that occurred at the greatest percentage for all the gRNAs tested was +1. gRNAs N, G, I, and P3 led to INDELs of smaller sizes compared to gRNA P3 and S3, which led to INDELs of up to -14. The INDEL distribution of gRNA D1 as evaluated in the primary human CD34+ cells is also shown in Figure 12. gRNA D1 led to INDELs of -15, -11, -7, -6, -2, 0, +1, and +2, with an INDEL of +1 occurring at the greatest frequency.

The off-target effects of gRNA A, gRNA G, gRNA I, gRNA N3, gRNA P3, and gRNA S3 were also predicted, as shown in Table 10. gRNAs were prioritized based on minimizing off-target effects. These off-target predictions were based on sequence complementarity with up to 1 nucleotide mismatch or gap allowed between the PAM and the target or up to 3 nucleotide mismatch or gap between the guide and the target.

Table 10. Off-target predictions for gRNAs targeting human CD123

Guide	No mismatch/gap in PAM		1 mismatch/gap in PAM		
	# of mismatch/gap in guide		# of mismatch/gap in guide		
	2	3	1	2	3
gRNA A	3	151	0	12	488
gRNA G	13	373	1	23	560
gRNA N3	1	19	0	0	57
gRNA P3	5	202	0	9	408
gRNA S3	2	100	0	2	161
gRNA I	3	127	0	4	125

Among other gRNAs targeting human CD123 investigated in this Example, three gRNAs (gRNA A, gRNA I, and gRNA P3) were selected that demonstrated particularly

efficient on-target editing in primary human CD34+ HSPCs, few or no predicted off-target effects, and a desirable INDEL distribution.

Example 4: Evaluation of CD123KO CD34+ cells *in vivo*

5

Editing in CD34+ human HSPCs

gRNAs (Synthego) were designed as described in Example 1 and Example 3. The human CD34+ HSPCs were then edited via CRISPR/Cas9 as described in Example 1 using the CD123-targeting guide RNAs: gRNA I, gRNA D1. Non-edited, electroporated control (EP Ctrl) HSPCs were also generated.

After *ex vivo* editing, the genomic DNA was harvested from cells, PCR amplified with primers flanking the target region, purified, and analyzed by TIDE (gRNA I) or amplicon sequencing (gRNA D1), in order to determine their editing efficiency in the CD34+ HSPCs. As shown in Table 11, gRNA I and gRNA D1 had high editing efficiencies, specifically 77.2% and 76.5%, respectively.

15

Table 11. Gene editing efficiency of CD123 gRNAs

CD34 ⁺ HSPCs	Editing efficiency
EP only control	N/A
CD123 gRNA I-edited	77.2% (TIDE)
CD123 gRNA D1-edited	76.5% (Amp seq)

Investigating engraftment efficiency and persistence of CD123KO CD34+ HSPCs in vivo

Female nonirradiated NOD.B6.SCID Il2r γ ^{-/-} Kit(W41/W41) (NBSGW) mice (n=15) were engrafted with the CD123KO HSPCs edited with gRNA I or gRNA D1, or non-edited (EP Ctrl) (Figure 13). At weeks 8 and 12 following engraftment, peripheral blood was collected from each mouse for analysis by FACS for measuring engraftment. At week 16, following engraftment, mice were sacrificed and blood, spleens, and bone marrow were collected for analysis by FACS for multilineage differentiation(Figure 13).

25

Results from cell samples obtained from the bone marrow of engrafted animals

At week 16 following engraftment, rates of human leukocyte chimerism in mice were calculated as percentage of human CD45+ (hCD45+) cells in the total CD45+ cell population (the sum of human and mouse CD45+ cells) were quantified in the three groups of mice
 5 (n=15 mice/group) that received the non-edited control cells (EP Ctrl) or the CD123KO cells (edited by gRNA: I or D1, as depicted on the X-axis) (Figure 14A). As shown in Figure 14A, the bone marrow chimerism and percentage of hCD45+ cells was equivalent across control or the CD123 KO groups, indicating no loss of nucleated bone marrow frequency.

Additionally, at week 16 post-engraftment, the percentage of hCD45+ cells that were
 10 also positive for human CD34 (hCD34+) in the bone marrow was quantified (Figure 14B). As shown in Figure 14B, the percentage of hCD45+ cells also expressing hCD34+ was equivalent across control and the CD123 KO groups.

At week 16 post engraftment, the percentage of hCD45+ cells that were B-cells, T cells, monocytes, neutrophils, conventional dendritic cells (cDCs), plasmacytoid dendritic
 15 cells (pDCs), eosinophils, basophils, and mast cells were quantified in the bone marrow (Figure 14C). The percentages of these various immune cell subtypes were equivalent between the control and CD123 KO groups. These data indicate multilineage human hematopoietic reconstitution from the edited CD123KO cells in the mice.

The percentages of CD123KO cells that were hCD45+ were quantified in the bone
 20 marrow of control and CD123KO cell engrafted mice at week 16 post-engraftment (Figure 15). The percentage of hCD123+ hCD45+ cells was significantly lower in the CD123KO groups (cells edited with gRNA I or gRNA 25) compared to the control group, indicating loss of CD123 from nucleated blood cells in these groups. These data also demonstrate the long term persistence of CD123KO HSCs in the bone marrow of NBSGW mice.

25

Example 5: Evaluation of CD123KO CD34+ cells *in vitro**Editing in CD34+ human HSPCs*

gRNAs (Synthego) were designed as described in Example 1 and Example 3. The
 30 human CD34+ HSPCs were then edited via CRISPR/Cas9 as described in Example 1 using the CD123-targeting guide RNAs: gRNA I, gRNA D1, as well as a non-edited, electroporated control (EP Ctrl).

After *ex vivo* editing, the genomic DNA was harvested from cells, PCR amplified with primers flanking the target region, purified, and analyzed by TIDE (gRNA I) or

amplicon sequencing (gRNA D1), in order to determine their editing frequency in the CD34+ HSPCs. As shown in Figure 16A, gRNA I and gRNA D1 showed editing frequencies of 75.8% and 71.1%, respectively. Cell surface expression of CD123 was also quantified by FACs in the CD123KO cells (edited by gRNA I or gRNA D1), the non-edited control (EP ctrl), or the FMO (fluorescent minus one) control. CD34+ HSPCs edited by gRNA I or gRNA D1 exhibited lower expression of CD123 compared to the non-edited control (EP Ctrl) (Figure 16A).

Non-edited control cells (EP Ctrl) or CD123KO cells edited by gRNA I or gRNA D1 were cultured with myeloid differentiation media, inducing either granulocytic (Figure 16B) or monocytic (Figure 16C) lineages, and the cell numbers were quantified over time. The CD123KO cells demonstrated comparable cell growth to the non-edited control cells in both granulocytic (Figure 16B) and monocytic (Figure 16C) differentiation culture.

Additionally, the percentage of cells that were CD123+ in granulocytic differentiation (Figure 17, top) or cells that were CD123+ in monocytic differentiation (Figure 17, bottom), were quantified at day 0, 7, and 14 following editing and culture of non-edited control cells or CD123KO cells edited by gRNA I or gRNA D1. The granulocytes and monocytes generated from CD123KO cells exhibited sustained, loss of CD123 expression over time, as compared to the non-edited control cells (Figure 17). The ability of the CD123KO cells to differentiate into myeloid cells *in vitro* was also evaluated. The percentage of CD15+ (Figure 18, top left) or CD11b+ positive granulocytes (Figure 18, top right) was quantified at day 0, 7, and 14 following editing and culture of non-edited control cells or CD123KO cells edited by gRNA I or gRNA D1. Expression of these granulocyte markers were not affected by loss of CD123. The percentage of CD14+ (Figure 18, bottom left) or CD11b+ positive monocytes (Figure 18, bottom right) was also quantified at day 0, 7, and 14 following editing and culture of non-edited control cells or CD123KO cells edited by gRNA I or gRNA D1. Similar to the granulocyte markers, expression of these monocyte markers were not affected by loss of CD123. Expression of CD33 (marker for myeloid cells) and HLA-DR (antigen presentation) were also unaltered by CD123 disruption. These data indicate the loss of CD123 did not affect *in vitro* myeloid differentiation.

The function of CD123KO cells was also evaluated *in vitro*. The percentage of phagocytosis performed by granulocytes (Figure 19A, top) and monocytes (Figure 19A, bottom) was quantified in the control cell population and the CD123KO cell populations. Phagocytosis activity was equivalent between the control and CD123KO cells for both granulocytes and monocytes, demonstrating the CD123KO cells retained phagocytosis

activity (Figure 19). The ability of CD123KO cells to produce inflammatory cytokines upon stimulation was also evaluated. Granulocytes (Figure 19A) and monocytes (Figure 19B) produced from non-edited control cells or CD123KO cells edited by gRNA I or gRNA D1 were unstimulated or stimulated with LPS or R848. The levels of IL-6 (Figure 19A or 19B, left) and TNF- α (Figure 19A or 19B, right) were subsequently quantified. CD123KO granulocytes and monocytes exhibited intact inflammatory cytokine production upon TLR agonist stimulation and cytokine production was equivalent to non-edited control cells. Production of other cytokines, including IL-1 β and MIP-1 α was also not altered by CD123 disruption. Taken together, these data demonstrate that loss of CD123 did not affect *in vitro* myeloid cell function.

The differentiation potential of the gene-edited CD34+ CD123KO cells (edited by gRNA I or gRNA D1) was also measured by a colony formulation assay. Following electroporation, CD34+ edited cells were plated and cultured for two weeks. Colonies were then counted and scored using StemVision (Stem Cell Technologies). Cells edited for CD123 by gRNA I (editing frequency of 77.9%) or gRNA D1 (editing frequency of 72.5%) produced fewer BFU-E, CFU-G/M/GM, and CFU-GEMM colonies compared to non-edited control cells (Figure 20A). However, cells edited for CD123 produced similar distributions and percentages of BFU-E colonies (Burst forming unit-erythroid), CFU-G/M/GM colonies, and CFU-GEMM colonies, as non-edited control cells, showing that the CD123 edited cells retain significant differentiation potential in this assay (Figure 20B). Colony forming unit (CFU)-G/M/GM colonies refer to CFU-G (granulocyte), CFU-M (macrophage), and CFU-GM (granulocyte/macrophage) colonies. CFU-GEMM (granulocyte/erythroid/macrophage/megakaryocyte) colonies arise from a less differentiated cell that is a precursor to the cells that give rise to CFU-GM colonies. Taken together, the differentiation assays indicate that human CD34+ cells edited at the CD123 locus retain the capacity to differentiate into variety of cell types.

Example 6: Evaluation of resistance of CD123 edited cells to CART effector cells

This Example describes evaluation of resistance of CD123 edited cell to CART effector cells targeting CD123. CD123KO cells that lack CD123 expression are resistant to CD123 CAR killing, compared to wild-type CD123+ cells, as measured by the assays described herein.

Editing in CD34+ human HSPCs

gRNAs (Synthego) are designed as described in Example 3. The human CD34+ HSPCs are then edited via CRISPR/Cas9 as described in Example 1 using the CD123 targeting gRNAs, e.g., a CD123 targeting gRNA of Table 2, 6, or 8.

5 *CAR constructs and lentiviral production*

Second-generation CARs are constructed to target CD123. The CAR consists of an extracellular scFv antigen-binding domain, using a CD8 α signal peptide, a CD8 α hinge and transmembrane region, a 4-1BB or CD28 costimulatory domain, and a CD3 ξ signaling domain. The anti-CD123 scFv sequence is obtained from clone 32716 in a heavy-to-light chain orientation of the scFv. The heavy and light chains are connected by (GGGS)₃ linker (SEQ ID NO: 63). The CD123 CAR cDNA sequence is sub-cloned into the multiple cloning site of the pCDH-EF1 α -MCS-T2A-GFP expression vector, and lentivirus is generated following the manufacturer's protocol (System Biosciences). Lentivirus can be generated by transient transfection of 293TN cells (System Biosciences) using Lipofectamine 3000 (ThermoFisher).

CAR transduction and expansion

Human primary T cells are isolated from Leuko Pak (Stem Cell Technologies) by magnetic bead separation using anti-CD4 and anti-CD8 microbeads according to the manufacturer's protocol (Stem Cell Technologies). Purified CD4+ and CD8+ T cells are mixed 1:1, and activated using anti-CD3/CD28 coupled Dynabeads (Thermo Fisher) at a 1:1 bead to cell ratio. The T cell culture media is CTS Optimizer T cell expansion media supplemented with immune cell serum replacement, L-Glutamine and GlutaMAX (all purchased from Thermo Fisher) and 100 IU/mL of IL-2 (Peprotech). T cell transduction is performed 24 hours post activation by spinoculation in the presence of polybrene (Sigma). CAR-T cells are cultured for 9 days prior to cryopreservation. Prior to all experiments, T cells are thawed and rested at 37°C for 4-6 hours.

Flow Cytometry based CAR-T cytotoxicity assay

The cytotoxicity of target cells is measured by comparing survival of target cells relative to the survival of negative control cells. For CD123 assays, wildtype and CRISPR/Cas9 edited human CD34+ HSPCs cells are used as target cells. Wildtype Raji cell lines (ATCC) are used as a negative control. Target cells and negative control cells are

stained with CellTrace Violet (CTV) and CFSE (Thermo Fisher), respectively, according to the manufacturer's instructions. After staining, target cells and negative control cells are mixed at 1:1.

Anti-CD123 CAR-T cells are used as effector T cells. Non-transduced T cells (mock
5 CAR-T) are used as control. The effector T cells are co-cultured with the target cell/negative control cell mixture at a 1:1 effector to target ratio in duplicate. A group of target cell/negative control cell mixture alone without effector T cells is included as control. Cells are incubated at 37°C for 24 hours before flow cytometric analysis. Propidium iodide (ThermoFisher) is used as a viability dye. For the calculation of specific cell lysis, the
10 fraction of live target cell to live negative control cell (termed target fraction) is used. Specific cell lysis is calculated as ((target fraction without effector cells – target fraction with effector cells)/(target fraction without effectors)) × 100%.

The analysis described above shows that CD123 KO HPSCs (and their progeny) are
15 resistant to anti-CD123 CAR-T-mediated killing, while non-edited control HPSCs (and their progeny) are susceptible to anti-CD123 CAR-T-mediated killing.

Example 7: Treatment of Hematologic Disease

An exemplary treatment regimen using the methods, cells, and agents described
20 herein for acute myeloid leukemia or MDS is provided. Briefly, a subject having AML or MDS that is a candidate for receiving a hematopoietic stem cell transplant (HSCT) is identified. A suitable HSC donor, e.g., an HLA-matched donor, is identified and HSCs are obtained from the donor, or, if suitable, autologous HSCs from the subject are obtained.

The HSCs so obtained are edited according to the protocols and using the strategies
25 and compositions provided herein, e.g., a suitable guide RNA targeting a CD123 target domain described in any of Tables 2, 6, or 8. In an exemplary embodiment, the editing is effected using a gRNA comprising a targeting domain described herein for gRNA A, gRNA I, and gRNA P3. Briefly, a targeted modification (deletion, truncation, substitution) of CD123 is introduced via CRISPR gene editing using a suitable guide RNA and a suitable
30 RNA-guided nuclease, e.g., a Cas9 nuclease, resulting in a loss of CD123 expression in at least 80% of the edited HSC population.

The subject having AML or MDS may be preconditioned according to a clinical standard of care, which may include, for example, infusion of chemotherapy agents (*e.g.*,

etoposide, cyclophosphamide) and/or irradiation. Depending on the health status of the subject and the status of disease progression in the subject, such pre-conditioning may be omitted, however.

5 A CD123-targeted immunotherapy, e.g., a CAR-T cell therapy targeting CD123 is administered to the subject. The edited HSCs from the donor or the edited HSCs from the subject are administered to the subject, and engraftment, survival, and/or differentiation of the HSCs into mature cells of the hematopoietic lineages in the subject are monitored. The CD123-targeted immunotherapy selectively targets and kills CD123 expressing malignant or pre-malignant cells, and may also target some healthy cells expressing CD123 in the subject,
10 but does not target the edited HSCs or their progeny in the subject, as these cells are resistant to targeting and killing by a CD123-targeted immunotherapy.

The health status and disease progression in the subject is monitored regularly after administration of the immunotherapy and edited HSCs to confirm a reduction in the burden of CD123-expressing malignant or pre-malignant cells, and to confirm successful
15 engraftment of the edited HSCs and their progeny.

EQUIVALENTS AND SCOPE

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the exemplary embodiments described herein.
20 The scope of the present disclosure is not intended to be limited to the above description.

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 two or more members of a group are considered satisfied if one, more than one, or all of the group members are present, unless indicated to the contrary or otherwise evident
25 from the context. The disclosure of a group that includes “or” between two or more group members provides embodiments in which exactly one member of the group is present, embodiments in which more than one members of the group are present, and embodiments in which all of the group members are present. For purposes of brevity those embodiments have not been individually spelled out herein, but it will be understood that each of these
30 embodiments is provided herein and may be specifically claimed or disclaimed.

It is to be understood that the invention encompasses all variations, combinations, and permutations in which one or more limitation, element, clause, or descriptive term, from one or more of the claims or from one or more relevant portion of the description, is introduced into another claim. For example, a claim that is dependent on another claim can be modified

to include one or more of the limitations found in any other claim that is dependent on the same base claim. Furthermore, where the claims recite a composition, it is to be understood that methods of making or using the composition according to any of the methods of making or using disclosed herein or according to methods known in the art, if any, are included,
5 unless otherwise indicated or unless it would be evident to one of ordinary skill in the art that a contradiction or inconsistency would arise.

Where elements are presented as lists, it is to be understood that every possible individual element or subgroup of the elements is also disclosed, and that any element or subgroup of elements can be removed from the group. It is also noted that the term
10 “comprising” is intended to be open and permits the inclusion of additional elements, features, or steps. It should be understood that, in general, where an embodiment, is referred to as comprising particular elements, features, or steps, embodiments, that consist, or consist essentially of, such elements, features, or steps, are provided as well. For purposes of brevity those embodiments have not been individually spelled out herein, but it will be understood
15 that each of these embodiments is provided herein and may be specifically claimed or disclaimed.

Where ranges are given, endpoints are included. Furthermore, it is to be understood that unless otherwise indicated or otherwise evident from the context and/or the understanding of one of ordinary skill in the art, values that are expressed as ranges can
20 assume any specific value within the stated ranges in some embodiments, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise. For purposes of brevity, the values in each range have not been individually spelled out herein, but it will be understood that each of these values is provided herein and may be specifically claimed or disclaimed. It is also to be understood that unless otherwise indicated or otherwise evident
25 from the context and/or the understanding of one of ordinary skill in the art, values expressed as ranges can assume any subrange within the given range, wherein the endpoints of the subrange are expressed to the same degree of accuracy as the tenth of the unit of the lower limit of the range.

All publications, patent applications, patents, and other references (e.g., sequence
30 database reference numbers) mentioned herein are incorporated by reference in their entirety. For example, all GenBank, Unigene, and Entrez sequences referred to herein, e.g., in any Table herein, are incorporated by reference. Unless otherwise specified, the sequence accession numbers specified herein, including in any Table herein, refer to the database

entries current as of August 28, 2019. When one gene or protein references a plurality of sequence accession numbers, all of the sequence variants are encompassed.

In addition, it is to be understood that any particular embodiment of the present invention may be explicitly excluded from any one or more of the claims. Where ranges are
5 given, any value within the range may explicitly be excluded from any one or more of the claims. For purposes of brevity, all of the embodiments in which one or more elements, features, purposes, or aspects is excluded are not set forth explicitly herein.