#### Supplementary figures and legends



# Supplementary Fig. 1. pegRNA containing additional base substitutions induced higher editing efficiencies of single-base substitution at more target sites.

(a) Sequences of the PBS and RTT of pegRNAs and WT on-target genomic sites.
Intended single-base edits are in cyan, additional base substitutions are in blue and PAMs are in brown. (b-d) The intended single-base editing frequencies (b), unintended indel frequencies (c) and fractions of editing product (d) were induced by the pegRNAs in (a) at the indicated target sites under the PE3 setting. Means ± s.d. are from three independent experiments. Source data are provided as a Source Data file.



# Supplementary Fig. 2. pegRNA containing SSMs induced higher editing efficiencies to generate or repair pathogenic point mutations at more target sites. (a) Sequences of the PBS and RTT of pegRNAs and spegRNAs for generating pathogenic point mutations and WT on-target genomic sites. Pathogenic point mutations are in red and SSMs are in blue. (b-d) The intended single-base editing frequencies (b), unintended indel frequencies (c) and fractions of editing product (d) were induced by the indicated pegRNAs and spegRNAs in (a). (e) Sequences of the PBS and RTT of pegRNAs and spegRNAs for repairing pathogenic mutations and ontarget genomic sites with pre-installed mutations. Corrected bases are in green, preinstalled point mutations are in red and SSMs are in blue. (f-h) The intended single-

base editing frequencies (**f**), unintended indel frequencies (**g**) and fractions of editing product (**h**) were induced by the indicated pegRNAs and spegRNAs in (**e**). (**b**, **c**, **d**, **f**, **g**, **h**) Means  $\pm$  s.d. are from three independent experiments. Source data are provided as a Source Data file. а

pegCXCR4+5G-to	Γ-1		pegPRNP+6G-to-T	-2	
WT	AGAACCAGCGGTTACCATGGAGGGGATCAGTGTAA		WT .	GTGGGGGGCCTTGGCGGCTACATGCTGGGAAGTGC	
Complete product	AGAACCAGCGGTTACCATGGCTGGGATCAGTGTAA	(40.57%)	Complete product	GTGGGGGGCCTTAGCATCTACATGCTGGGAAGTGC	(24.83%)
Intended products	AGAACCAGCGGTTACCATGGATGGGATCAGTGTAA	(0.03%)		GTGGGGGGCCTTGGCGTCTACATGCTGGGAAGTGC	(0.03%)
Incomplete products	AGAACCAGCGGTTACCATGGCGGGGATCAGTGTAA	(0.05%)	Intended products	GTGGGGGGCCTTAGCGTCTACATGCTGGGAAGTGC	(0.02%)
				GTGGGGGGCCTTGGCATCTACATGCTGGGAAGTGC	(0.10%)
pegFMX1+4G-toC-	2			GTGGGGGGCCTTAGCGGCTACATGCTGGGAAGTGC	(0.17%)
WT			Incomplete products	GTGGGGGGCCTTGGCAGCTACATGCTGGGAAGTGC	(0.03%)
Complete product	CCGAGCAGAAGAAGGACAGCACCCATCACATCAACCGGTGGCG	(27.50%)		GTGGGGGGCCTTAGCAGCTACATGCTGGGAAGTGC	(0.02%)
		(0.01%)			
	CCGAGCAGAAGAAGGACGGCTCCCATCACATCAACCGGTGGCG	(0.01%)	pegRUNX1+6G-to-	C-2	
Intended products		(0.06%)	WT	CAGGAGGAAGCGATGGCTTCAGACAGCATATTTG	
		(0.15%)	Complete product	CAGGAGGAAGTGATGCCTTCAGACAGCATATTTG	(24.33%)
		(0.06%)	Intended products	CAGGAGGAAGCGATGCCTTCAGACAGCATATTTG	(3.91%)
Incomplete producte		(0.01%)	Incomplete products	CAGGAGGAAGTGATGGCTTCAGACAGCATATTTG	(0.52%)
incomplete products		(0.13%)			
1	CCOAGCAGAAGAAGAAGGGCACCCATCACATCAACCGGTGGCG	(0.12/6/			
peaSITE3+5G-toT-	1		peaVEGFA+5G-to-	T-1	
WT			WT	TCTGCAGGCCAGATGAGGGCTCCAGATGGCACAT	
Complete product	CAGACTGAGCACGAGATTGCAGAGGAAAGGAAGCCCTGCTTCC	(11.69%)	Complete product	TCTGCAGGCCAGACGAGTGCTCCAGATGGCACAT	(57.40%)
Intended products		(0.08%)	Intended products	TCTGCAGGCCAGATGAGTGCTCCAGATGGCACAT	(1.31%)
Incomplete products	CAGACTGAGCACGAGATGGCAGAGGAAAGGAAGCCCTGCTTCC	(0.12%)	Incomplete products	TCTGCAGGCCAGACGAGGGCTCCAGATGGCACAT	(0.47%)
b					
DegACTG1_TROL 4			pegCFTR-L23M-3		
улотат-тозі-ч мт			WT	GATACAGACAGCGCCTGGAATTGTCAGACATATACCAAATCCC	
Complete product		(45.06%)	Complete product	GATACAGACAGCGTATGGAGTTGTCAGACATATACCAAATCCC	(16.61%)
Complete product		(0.75%)		<u>ΑΤΑΓΑGACAGCGCATGGAATTGTCAGACATATACCAAATCCC</u>	(0.02%)
		(6.73%)	Intended products		(0.56%)
Intended products		(0.24%)	interface producto	GATACAGACAGCGCATGGCAGTTGTCAGACATATACCGAATCCC	(0.04%)
		(0.36%)			(0.04%)
		(0.13%)	Incomplete products		(4.01%)
		(0.03%)	1		(4.0170)
		(0.57%)			
		(0.02%)	nogUPP K19Torm	9	
Incomplete products	CGITGTAGAAGGTGTGGTGCCAGATTTTCTCCATGTCGTCCCA	(0.03%)	реупов-ктотенн-		
	CGIIGIAGAAGGIGIGAIGCCAIAICIICICCAIGICGICCCA	(0.03%)	Complete avertuat		(0.010)
	CGTTGTAGAAGGTGTGATGCCAGATTTTCTCCATGTCGTCCCA	(0.01%)	Complete product		(9.01%)
	CGTTGTAGAAGGTGTGGTGCCATATTTTCTCCATGTCGTCCCA	(0.76%)	Interlated products		(0.03%)
I	CGTTGTAGAAGGTGTGATGCCATATTTTCTCCATGTCGTCCCA	(2.61%)	incomplete products	GCCCTGTGGGGCTAGGTGAACGTGGATGAAGTTGGTGGTG	(0.00%)
С					
pegACE2-A19S-3			pegHBB-V7E-2		
WT	CTGTTCCTCAATGGTGGCCTGAGCAGCAGTTACAGCAACAAGG		WT		
Complete product	CTGTTCCTCAATTGTAGACTGAGCAGCAGTTACAGCAACAAGG	(70.91%)	Complete product		(39.22%)
	CTGTTCCTCAATGGTGGACTGAGCAGCAGTTACAGCAACAAGG	(0.21%)	Somplete product		(0 11%)
Intended products	CTGTTCCTCAATTGTGGACTGAGCAGCAGCAGTTACAGCAACAAGG	(0.05%)	Intended products		(0.019/)
	CTGTTCCTCAATGGTAGACTGAGCAGCAGTTACAGCAACAAGG	(0.37%)	intended products		(0.01%)
	CTGTTCCTCAATTGTGGCCTGAGCAGCAGTTACAGCAACAAGG	(0.06%)			(0.03%)
Incomplete products	CTGTTCCTCAATTGTAGCCTGAGCAGCAGTTACAGCAACAAGG	(0.02%)	Incomplete products		(0.01/0)
1				CACCTGACTCCTGTAGAGAAGTCTGCCGTTACTGCCCTGTGGG	(0.04%)
			I		
pegPGM3-S111L-1	l				
WT .	TTGGGTGAAATGTCGGCACCATCCTGGGAGGAACATGCCACCT				
Complete product	TTGGGTGAAATGTTAGCACCATCCTGGGAGGAACATGCCACCT	(46.39%)			
Intended products	TTGGGTGAAATGTTGGCACCATCCTGGGAGGAACATGCCACCT	(6.80%)			
Incomplete products	TTGGGTGAAATGTCAGCACCATCCTGGGAGGAACATGCCACCT	(0.09%)			

# Supplementary Fig. 3. Incomplete products induced by the pegRNAs containing additional base substitutions at on-target sites.

(a) The frequencies of complete products (with intended single-base editing and all additional base substitutions), intended products (with intended single-base editing but not all additional base substitutions) and incomplete products (with additional base substitutions but no intended single-base editing) induced by the optimized pegRNAs with additional base substitutions in Fig. **1a** and Supplementary Fig. **1a**. (**b**) The frequencies of complete products (with intended single-base editing and all SSMs), intended products (with intended single-base editing but not all SSMs) and incomplete products (with SSMs but no intended single-base editing) induced by the optimized spegRNAs in Fig. **1a** and Supplementary Fig. **2a**. (**c**) The frequencies of complete products (with intended single-base editing and all SSMs), intended products (with intended single-base editing and all SSMs), intended products (with intended single-base editing and all SSMs), intended products (with intended single-base editing but not all SSMs) and incomplete products (with intended single-base editing but not all SSMs) and incomplete products (with SSMs but no intended single-base editing) induced by the optimized spegRNAs in Fig. **1a** and Supplementary Fig. **2e**. The frequencies are means from three biologically independent experiments.

_			RT-template Sca	ffold insertion
а	PegCXCR4+5G-to-T WT A6AACCA6CGGTTACCATGGAGGGGATCAGTG Complete product AGAACCA6CGGTTACCATGGATGGGATCAGTG PegCXCR4+5G-to-T_1	TAA TAA (0.0329%)	pegPRNP+6G-to-T WT CTCCCCCCCCTTGCCCCTACATGCTGCGAAGTGCCATGAC Complete product CTGCGCGCCCTTGCCCTACATGCCGAAGTGCCATGACG Byproducts CGGGGGGCCTTGCCCTCGCCTCACATGCTGCGAAGTGCC	A A (11.73%) A (0.0026%)
	WT AGAACCACCGGTTACCATGGACGGATCAGTG Complete product AGAACCACGGTTACCATGGTGGATCAGTG Byproducts AGAACCACGGTTACCATGGTGGATGGATG AGAACCACGCGGTTACCATGGTGGATGGATG	TAA (40.57%) TAA (0.0213%) TAA (0.0137%) TAA (0.0017%) TAA (0.0001%)	pegPRNP+6G-to-T_2 WT CFCGCCCCCTTGC_CCTACATCCTGCGAACTCCCTCACA Complete product GFCGCGCCCTTACATCTACATCCTGCGAACTCCCATCACA Byproducts GFCGGCGCCTTACATCTACATCTGCGAAGTCCCATCACA DeprElitik 1_GC_GC	A A (24.83%) A (0.0038%)
	pegEMX1+4G-to-C WT CCGAGCAGAAGAAGAAGAACGGCTCCCATCACATC Complete product CCGAGCAGAAGAAGAACGGCTCCCATCACATC	AACCGGTGGCG AACCGGTGGCG (2.56%)	WT CAGAGGAAGGACGA GCTTCAGACAGGATATTTGAGTCATT Complete product: CAGGAGGAGGAGGATG: CTTCAGACAGCATATTTGAGTCATTT Byproducts   CAGGAGGAGGAGCGATG: CTTCAGACAGCATATTTGGGTCATTT pegRUNX1+6G-to-C_2	(16.65%) (0.1107%)
	Byproducts 1 CLCARCAUAAGAAGAACGACCCCCTCCATCACATC pegEMX1+4G-to-C_2 WT CCGACGAGAAGAAGGACACGCCCCCATCACATC/ Complete product CCGACGAGAAGAAGGACACCATCACATC/ 1 CCGACGAGAAGAAGGACACGCCCCATCACATC/	MACCGGTGGCG (0.0812%)	WT CACGACGAAGCGATISCTTCAGACGACGATATTCGATCATT Complete product CACGACGACGATGATTCGATCATT CACGACGACGATGATGATCCTTCAGACACGCATATTCGATCATT CACGACGAAGCGATCCTTCAGACACGATATTCGATCATT Byproducts CACGACGATCGATCCTCAGACACGATATTCGATCATT CACGACGAGAGCGATCCTTCAGACACGATATTCGATCATT	(14.91%) (0.2096%) (0.0472%) (0.0027%)
	CCCAGCAGAAGAAGAAGAAGGCCCCCCATCACATC Byproducts CCCGAGCAGAAGAAGAAGAACAACCACCCCATCACATC CCCGAGCAGAAAGAAGAACAACGCCCCCATCACATC CCCGAGCAGAAGAAGAAGAAGAAGAAGCACCCCATCACATCA	MCGGGTGGCG (0.0284%) MCGGGTGGCG (0.0028%) MCGGGTGGCG (0.0003%) MCGGGTGGCG (0.0002%)	CAGGAGGAGGTGATC.CTTCAGACAGCATATTTGGCACATT pegVEGFA+SG-to-T WT TCTCCAGGCCAGATGACGGCCTCCAGATGGCACATTGTCAGA Byrootus TCTCCAGGCCAGATGACGCCCCGATGTCCAGATGGCACATTGTCAGA Byrootus	(0.0024%) (58.13%) (0.0219%)
	PegSITE3+5G-to-T WT CACACTGACCACGTGATGCCAGAGGAAAGGA Complete product CAGACTGAGCACGTGATTGCAGAGGAAAGGA Byproducts CAGACTGAGCACGTGATTGCAGAGGAAAGGA	AGCOCTGCTTCC AGCCCTGCTTCC (3.20%) AGCC <mark>G</mark> TGCTTCC (0.0001 <i>7%</i> )	pegVEGFA+5G-to-T TCTGCAGGCCAGATGATGCTCCAGATGGCACTTGTCAGA WT TCTGCAGGCCAGATGCTCCAGATGGCACTTGTCAGA Complete product TCTGCAGGCCAGATGCTCCAGATGGCACTTGTCAGA TCTGCAGGCCCAGCAGCCGCTCCCAGTGCCAGTGGCCAGTGTCCAG	(0.0043%) (57.40%) (0.0208%)
	pegSITE3+5G-to-1_1 WT CAGACTGAGCACGTGATGCCAAGGAAAGGAA Complete product CAGACTGCAGCACGTGATGCCAAGGAAAGGAA Byproducts CAGACTGAGCACGAGATTGCAGAGGAAAGGAA	GCCCTGCTTCC GCCCTGCTTCC (11.69%) GCC <mark>G</mark> TGCTTCC (0.001%)	TCTGCAGGCCAAGAC GAT GCTCCAGATGGACATGGACA Byproducts TCTGCAGGCCAAGAGG TCCTCCAGATGGACATGGACAA TCTGCAGGCCAAGAGGAC TCTCCAGATGGACATGGACAA TCTGCAGGCCAAGAGAC TCTCCAGATGGACATGGTCAGA	(0.0004%) (0.0003%) (0.0001%) (0.0001%)
b				
	pegACTG1-T89I WT CGTIGTAGAAGTGTGGGGCAGATCTTCTCC Complete product CGTTGTAGAAGATGTGGTGCCAGATCTTCTCC CGTTGTAGAAGATGTGGTGCCAGATCTTCTCC CGTTGTAGAAGATGTGGTGCCAGATCTTCTCC	ATGTCGTCCCA ATGTCGTCCCA (8.43%) ATGGCGTCCCA (0.0546%) ATGGGCTCCCA (0.0034%)	PegCFTR-L23M WT GATACAGAGGCCCCCAATTGTCAGACATAACCAATTC Complete product GATACAGACAGCCCCATCGATTGTCAGACATATACCAATTC Byproducts   GATACAGACAGCGCATCGAATTGTCAGACATATACCAATTC SpeeCFTR-L23M 3	C (2.24%) C (0.104%)
	SpegACTG1-T891_4 WT CGTTGTAGAAGGTGTGGTGCCAGATCTTCTCC Complete product CGTTGTAGAAGGTGTGATGCCATATTTTCTCC	ATGTCGTCCCA (0.0005%) ATGTCGTCCCA (45.06%) ATGCCGTCCCA (0.0455%) ATCCCCTCCA (0.0455%)	WT GATACAGCAGCCCCCCCCCCCCCCCCCCCCCCCCCCCCC	C (16.61%) C (0.3796%) C (0.1479%) C (0.0128%) C (0.003%)
	CGT 10 IAGANASI 10 CGI DCLA IA 11 TI CI CLA TANGGI CLA GETTO TAGANASI CONTRECTO AT CONTRECTO TAGO CANA CGT TO TAGANASI CONTRECTO AT CONTRECTO AT CONTRECTO CGT TO TAGANASI CONTRECTO AT CONTRECTO AT CONTRECTO Byproducts CGT TO TAGANAST CONTRECTO AT CONTRECTO CGT TO TAGANAST CONTRECTO AT CONTRECTO AT CONTRECTO CGT TO TAGANAST CONTRECTO AT CONTRECTO CONTRECTO AT CONTRECTO CONTRECTO AT CONTRECTO CONTRECTO AT CONTRECTO CONTRECTO AT CONTRECTO CONTRECTO AT CONTRECTO CONTRECTO AT CONTRECTO CONT	ATGGGTCCCA (0.0022%) ATGGGTCCCA (0.0059%) ATGGGTCCCA (0.0043%) ATGGGTCCA (0.0043%) ATGGGCTCCA (0.009%) ATGGGCTCCA (0.0019%) ATGGGCTCCA (0.0019%) ATGGGCTCCA (0.0019%)	pegHBB-K18Term WT CCCTGTGGGGCAGGTGGAACGTGGATGAAGTTGGTGGTG Complete product CCCTGTGGGGCTAGGTGAACGTGGATGAAGTTGGTGGTG Byproducts CCCTGTGGGGCTACGTGAACGTGGATGAAGTGGTG CCCTGTGGGGCTACGTGAACGTGGATGAAGTGCATGGTG	(5.01%) (0.0196%) (0.0069%)
	CGTTGTAGAAGATGTGATGCCATATCTTCTCC CGTTGTAGAAGGTGTGGTGCCATATCTTCTCC CGTTGTAGAAGATGTGATGCCATATCTTCTCC CGTTGTAGAAGATGTGATGCCATATCTTCTCC	ATG <mark>G</mark> CGTCCCA (0.0006%) ATG <mark>GG</mark> TCCCA (0.0002%) ATG <mark>GGA</mark> TCCCA (0.0002%)	WT CCCCTGTGGGGCANGCTGAACGTGGATGAAGTTGGTGGT Complete produst CCCTGTGGGGCAAGTGAAGTGGATGAAGTTGGTGGT GCCCTGTGGGGCAAAGTGAAGCTGGATGAAGTGGGTGGT Byproducts CCCTGTGGGGCAAAGTGAAAGTGGATGAAGTGGGTGAGT	(9.01%) (0.0499%) (0.0005%)
С	224052 4105			
	WT CTGTTCCTCAATGGTG:CCTGAGCAGCAGTAAC Complete product CTGTTCCTCAATGGTG:GACTGACCAGCAGTAAC Byproducts CTGTTCCTCAATGGTGGACTGACCAGCAGTAAC CTGTTCCTCAATGGTGGACTGAGCAGCAGTAAC	AGCAACAAGG (AGCAACAAGG (25.28%) (AGGGACAAGG (0.0802%) (AGGAACAAGG (0.0093%)	WT CACCTGACTCCTGTGCAGAAGTCTGCCGTTACTGCCTGTGC Complete product CACCTGACTGCAGGAAGTCTGCCCGTGTGC Byproducts CACCTGACTGCCGTGAGGAGAGTCTGCCGTTGCGGTGTGC CACCTGACTCCTGACGAGAAGTCTGCCGTTGCGGTGTGCG	G (12.20%) G (0.1258%) G (0.0014%)
	WT CGTTCCTCATGGTGGCCTGAGCAGCAGTAA Complete product CGTTCCTCATGTAACTGAGCAGCAGTAA Byproducts CTGTTCCTCATTGTAACTGAGCAGCAGTTAA CTGTTCCTCAATTGTAGACTGAGCAGCAGTTAA	AGCAACAAGG AGCAACAAGG (70.91%) AGGGACAAGG (0.0833%) AGGAACAAGG (0.0099%) AGGGACAAGG (0.0021%)	SpegriDo-V/E_2 WT CACCTGACTCCG100AGAAGCTGCCGCTACTGCCCTGTGG Complete product CACCTGACTCCGGAAGAAGACGTGCCCCGTACTGCCCTTACTGCCCTGTG CACCTGACTCCCGAAGAAGACTGTGCCGTTACTGCGCTGTGG CACCTGACTCCGGAAGAAGCTGCCGCTACTGCGCTGTGG Byproduct CACCTGACTCGGAGAAGCTGCGCGTTACTGCGCTGTGG	G (39.22%) G (0.086%) G (0.0006%) G (0.0003%) G (0.0002%)
	PegPGM3-S111L WT TTGGTGAAATGTTGCACCATCCTGGAAGAA Complete product TTGGGTGAAATGTTGCACCATCCTGGGAGGA Byproducts TTGGGTGAAATGTTGCACCATCCTGGGAGGAA TTGGGTGAAATGTTGCACCATCCTGGGAGGAA	CATGCCACCT (CATGCCACCT (27.57%) (CAGGCACCT (1.726%) (CAGGACACCT (0.044%)	CACCTGACTCCGGTGGAGAAGTCTGCCGTTACTGC <mark>G</mark> CTGTG	G (0.0002%)
	spegPGM3-S111L_1 WT TTGGGTGAAATGTCGCACCATCCTGGGAGGA Complete product TTGGGTGAAATGTCACCATCCTGGGAGGA TTGGGTGAAATGTTACCACCATCCTGGGAGGA TTGGGTGAAATGTTACCACCATCCTGGGAGGA	CATGCCACCT (CATGCCACCT (46.39%) (CAGGCCACCT (1.495%) (CAGGACACCT (0.1751%)		
	TTGGGTGAAATGTTGCACCATCCTGGGAGGAA TTGGGTGAAATGTCGCACCATCCTGGGAGGAA TTGGGTGAAATGTCGCACCATCCTGGGAGGAA TTGGGTGAAATGTCAGCACCATCCTGGGAGGAA	NCA <mark>G</mark> GCCACCT (0.1724%) NCA <mark>G</mark> GCCACCT (0.0045%) NCA <mark>GGA</mark> CACCT (0.0014%) NCA <mark>GGA</mark> CACCT (0.0011%)		

# Supplementary Fig. 4. Byproducts induced by the pegRNAs containing additional base substitutions at on-target sites.

(a) The frequencies of complete products and byproducts (with pegRNA scaffold incorporation) induced by regular pegRNAs and the optimized pegRNAs containing

additional base substitutions in Fig. **1a** and Supplementary Fig. **1a**. (**b**) The frequencies of complete products and byproducts induced by regular pegRNAs and the optimized spegRNAs in Fig. **1a** and Supplementary Fig. **2a**. (**c**) The frequencies of complete products and byproducts induced by regular pegRNAs and the optimized spegRNAs in Fig. **1a** and Supplementary Fig. **2e**. The frequencies are means from three biologically independent experiments.



Supplementary Fig. 5. Off-target editing induced by the pegRNAs containing additional base substitutions.

(a) The sequences of on- and off-target sites for the pegRNAs in Fig. 1a andSupplementary Fig. 1a are shown. The base substitution frequencies were determinedat the indicated off-target sites. (b) The sequences of on- and off-target sites for the

pegRNAs in Fig. **1a** and Supplementary Fig. **2a** are shown. The base substitution frequencies were determined at the indicated off-target sites. (c) The sequences of onand off-target sites for the pegRNAs in Fig. **1a** and Supplementary Fig. **2e** are shown. The base substitution frequencies were determined at the indicated off-target sites. Means  $\pm$  s.d. are from three independent experiments. Source data are provided as a Source Data file.



Supplementary Fig. 6. Splicing patterns of the exons edited by the pegRNAs containing additional base substitutions.

(a) Schematic diagrams illustrating the position of PCR primers to detect the splicing pattern of the edited exon 3 of *EMX1*. (b) RT-PCR results showed that no aberrant splicing of *EMX1* exon 3 was triggered by the indicated pegRNAs. (c) Schematic diagrams illustrating the position of PCR primers to detect the splicing pattern of the edited exon 3 of *ACGT1*. (d) RT-PCR results showed that no aberrant splicing of

*ACGT1* exon 3 was triggered by the indicated pegRNAs. Agarose gel data are representative of three independent experiments. Uncropped gels for (**b**) and (**d**) are provided.

### **U2OS**



#### HeLa



#### HeLa mutation correction



# Supplementary Fig. 7. The pegRNA containing additional base substitutions or SSMs induced higher single-base editing efficiencies in different cells.

(a) Sequences of the PBS and RTT of pegRNAs and spegRNAs and WT on-target genomic site in U2OS cells. Intended single-base edits are in cyan, pathogenic point mutations are in red and additional base substitutions and SSMs are in blue. (b, c) The

intended single-base editing frequencies (b) and unintended indel frequencies (c) were induced by the indicated pegRNAs and spegRNAs in (a) in U2OS cells. (d) Sequences of the PBS and RTT of pegRNAs and spegRNAs and WT on-target genomic site in HeLa cells. Intended single-base edits are in cyan, pathogenic point mutations are in red and additional base substitutions and SSMs are in blue. (e, f) The intended single-base editing frequencies (e) and unintended indel frequencies (f) were induced by the indicated pegRNAs and spegRNAs in (d) in HeLa cells. (g) Sequences of the PBS and RTT of spegRNAs and on-target genomic site in the HeLa cells with a pre-installed point mutation. Corrected bases are in green, pre-installed point mutations are in red and SSMs are in blue. (h, i) The intended single-base editing frequencies (h) and unintended indel frequencies (i) were induced by the indicated pegRNAs in (g) in the HeLa cells with the pre-installed point mutation. (b, c, e, f, h, i) Means  $\pm$  s.d. are from three independent experiments. Source data are provided as a Source Data file.



# Supplementary Fig. 8. Effect of additional base substitution position in RTT on

## editing efficiency.

(a) Heatmaps show the intended single-base editing frequencies induced by the

pegRNAs containing one additional base substitution at the indicated positions in

RTT. (**b**) Heatmaps show the intended single-base editing frequencies induced by the pegRNAs containing two additional base substitution at the indicated positions in RTT. Source data are provided as a Source Data file.



Supplementary Fig. 9. Effects of the mutation type and PBS length of spegRNA on editing efficiency.

(a) Sequences of PBS and RTT of the spegRNAs with different kind of SSMs (*e.g.*, transition and transversion). Pathogenic point mutations are in red and SSMs are in blue. (**b**, **c**) The intended single-base editing frequencies (**b**) and unintended indel frequencies (**c**) were induced by the spegRNAs in (**a**). (**d**, **e**) The intended single-base editing frequencies (**c**) and unintended indel frequencies (**c**) and unintended indel frequencies (**c**) mutation (**d**) and unintended indel frequencies (**e**) induced by the spegRNAs with different length of PBS at the indicated target sites. (**b**, **c**, **d**, **e**) Means

 $\pm$  s.d. are from three independent experiments. Source data are provided as a Source Data file.

а	PAM	Intended single base	Unintended indel	Fraction of editing
WT SITE1 5	'-GGGAAAGACCCAGCATCCGTGGGTCG-3'	editing frequency (%)	frequency (%)	product (%)
pegSITE1+8C-to-A spegSITE1+8C-to-A Non-transfected	Primer-binding siteH1-template 3'-UUCUGGGUCGUAGGCACCCA.CGACUUUUCGACA-5' 3'-UUCUGGGUCGUAGGCACGCA.CGACUUUUCGACA-5'			9 9
WT SITE10	5'-ATTTCCAAAGTCCCACTGGGCGGCAGCATAGTGA-3'			
pegSITE10+12TA-to-GC spegSITE10+12TA-to-GC Non-transfected	3'-GUUUCAGGGUGACCCGGCGUCGUCGCACU-5' 3'-GUUUCAGGGUGACACGGCGUCGUCGCACU-5'	9 9 9	9	
WT SITE11	5'-CAATGTGTCAACTCTTGACAGGGCTCTATTT-3'			
pegSITE11+11A-to-C spegSITE11+11A-to-C Non-transfected	3'-CAGUUGAGAACUGUCCCGAGAGAAAAUAU-5' 3'-CAGUUGAGAACUGGCCGGAGAGAAAAUAU-5'		9 9 9	• <del>8</del> • <del>•</del> 8 • • •
WT SITE12	5'-ACAAGTCATTGGGGTAGAAGCGGTCA-3'			
pegSITE12+4C-to-G spegSITE12+4C-to-G Non-transfected	3'-CAGUAACCCCAUCUUCCCCAGU-5' 3'-CAGUAACCCCAUCUUCCGCAGU-5'	¢Ø	8 ⊪8≕↔ 8	9 9 9
WT EMX1	5'-GAGTCCGAGCAGAAGAAGAAGAAGGGCTC-3'			
pegEMX1+1G-to-A spegEMX1+1G-to-A	3'-GGCUCGUCUUCUUUCUCGAGGGUAG-5' 3'-GGCUCGUCUUCUUUCUCGAGGGUAG-5'	e e	8 8 8	9 9 9 9 9 9 9 9 9 9 9 9 9 9 9 9 9 9 9
WT EMX1 pegEMX1+3A-to-G spegEMX1+3A-to-G	5'-GAGTCCGAGCAGAAGAAGAAGAAGGGCTC-3' 3'-GGCUCGUCUUCUUCU.CCCGAGGGUAG-5' 3'-GGCUCGUCUUCUUCCCCUCGAGGGUAG-5'	e	ð 	<u>%</u> %%
b		¢	9	•
WT SITE3 5' pegSITE3+5G-to-T spegSITE3+5G-to-T_1 Non-transfected	-GGCCCAGACTGAGCACGTGATGGCAG-3' 3'-GUCUGACUCGUGCACUAACGUCUCCUUUCCUUCGG-5' 3'-GUCUGACUCGUGCUCUAACGUCUCCUUUCCUUCGG-5'	₽ ₽ ₽	0 0 10	8
WT PRNP 5'-G pegPRNP+6G-to-T spegPRNP+6G-to-T_2 Non-transfected	CAGTGGTGGGGGGCCTTGGCGGCTA-3? 3'-CACCCCCCGGAACCGCAGAUGUACGACCCUUCACG-5' 3'-CACCCCCCGGAAUCGUAGAUGUACGACCCUUCACG-5'	ds ∲ 0	₩ ₩ ₽ ₽	
WT RUNX1 5'-0 pegRUNX1+6G-to-C spegRUNX1+6G-to-C_2 Non-transfected	CATTTTCAGGAGGAAGCGATGGCTT-3' 3'-GUCCUCCUUCGCUACGGAAGUCUGUCGUAUAAAC-5' 3'-GUCCUCCUUCACUACGGAAGUCUGUCGUAUAAAC-5'	₽ ₽	8	
WT VEGFA pegVEGFA+5G-to-T pegVEGFA+5G-to-T_1 Non-transfected	5'-ATGTCTGCAGGCCAGATGAGGGCTC-3' 3'-AGACGUCCGGUCUACUCACGAGGUCUACCGUGUA-5' 3'-AGACGUCCGGUCUGCUCACGAGGUCUACCGUGUA-5'	, , , , , , , , , , , , , , , , , , ,	9 9	•
WT ACTG1 5'-G pegACTG1-T89I spegACTG1-T89I_2	CAGCTCGTTGTAGAAGGTGTGGTGC-3' 3'-GCAACAUCUUCUACACCACGGUCUAGAAGAGGUAC-5' -GCAACAUCUUCUAUACUACGGUCUAGAAGAGGUAC-5'	} 		9 9 9
WT HBB 5' pegHBB-K18Term spegHBB-K18Term_2 Non-transfected	-CGTTACTGCCCTGTGGGGCAAGGTGA-3' 3'-CGGGACACCCCGAUUCACUUGCACCUACUUCA-5' 3'-CGGGACACCCCGAUUCACUUGCACCUACUUCA-5'		p	
ACE2-S19A 5'-C pegACE2-A19S spegACE2-A19S_3	TTGGCCTGTTCCTCAATGGTGGCCT-3' 3'-GACAAGGAGUUACCACCUGACUCGUCGUCAAUGUC-5' 3'-GACAAGGAGUUAACAUCUGACUCGUCGUCAAUGUC-5'	θα φ	66	•
HBB-E7V 5'-CA pegHBB-V7E spegHBB-V7E_2 Non-transfected	TGGTGCACCTGACTCCTGTGGAGA-3' 3'-GUGGACUGAGGACUCCUCUUCAGACGGCAAUGACG-5' 3'-GUGGACUGAGGCCUUCUCUUCAGACGGCAAUGACG-5'			- 
PGM3-L111S 5'-TG pegPGM3-S111L spegPGM3-S111L_1	ATCCTTTGGGTGAAATGTCGGCAC-3' 3'-AACCCACUUUACAACCGUGGUAGGACCCUCCUUGU-5' 3'-AACCCACUUUACAAUCGUGGUAGGACCCUCCUUGU-5'	<u></u> ⊕∘ ■₽	Intended product	Byproduct

Supplementary Fig. 10. Comparison of the editing efficiencies by pegRNA and spegRNA at more target sites.

(a) The intended single-base editing frequencies, unintended indel frequencies and fractions of editing product were induced by the indicated pegRNAs and spegRNAs

at newly tested target sites. (b) Reanalyzing the intended single-base editing frequencies, unintended indel frequencies and fractions of editing product that were induced by the indicated pegRNAs and spegRNAs at previously tested target sites. Means  $\pm$  s.d. are from three independent experiments. The data are from Fig. 1a and Supplementary Fig. 1 and 2. (a, b). Source data are provided as a Source Data file.



### Supplementary Fig. 11. Effect of pegRNA scaffold structure on editing efficiency.

(a) Schematic diagrams illustrating the predicted secondary structures of a regular pegRNA and the apegRNAs with one A/U-to-G/C base pair change in the small hairpin.(b) Intended indel frequencies were induced by the pegRNAs in (a) under the

PE3 setting. (c) Schematic diagrams illustrating the predicted secondary structures of the apegRNAs with two A/U-to-G/C base pair change in the small hairpin or with a previously published structure<sup>17</sup>. (d) Intended indel frequencies were induced by apegRNA-2 and the pegRNAs in (c) under the PE3 setting. (b, d) Means  $\pm$  s.d. are from three independent experiments. Source data are provided as a Source Data file.



Supplementary Fig. 12. Unintended indels induced by regular pegRNA and apegRNA-2 at on-target sites.

On-target unintended indel frequencies induced by the regular pegRNA and apegRNA-2 at the indicated sites under PE3 setting. Means  $\pm$  s.d. are from three independent experiments. Source data are provided as a Source Data file.  
 GCH1 (+1 GAT ins)

 WT
 GGGCGAGAACCCC---CAGCGGCAAGGGCTGCTCAAGA
 pegRNA apegRNA-2

 Complete product
 GGGCGAGAACCCCGATCAGCGGCAAGGGCTGCTCAAGA
 29.72%
 32.53%

 Byproducts
 GGGCGAGAACCCCGATCAGCGGCAAGGGCTGCTGCAAGA
 0.2600%
 0.2470%

 GGGCGAGAACCCCGATCAGCGGCCAAGGGCTGGGAAAGA
 0
 0.0170%
 RT template RP2 (+1 GAA ins) scaffold insertion HT2 (+1 GAA IIS) WT ACAGCTITGAGCT---CTTCGGTAGTAGGGAACAT PegRNA apegRNA-Complete product ACAGCTITGAGCTGAACTTCGGTAGTAGGAACAT 5.25% 13.74% Byproducts ACAGCTITGAGCTGAACTTCGGTAGGTAGGGACAT 0.2970% 0.8030% RP2 (+1 AGTT ins) 
 NPC (+ 1 AGT 1 IIIs)

 WT
 ACAGCTITIGAGCT----CITICGGTAGTAGGTATAGGAACAT
 pegRNA-2

 Complete product
 ACAGCTITIGAGCTAGTTCTCGGTAGTAGGTATAGGAACAT
 5.75%
 11.54%

 Byproducts
 ACAGCTITIGAGCTAGTCTCCGGTAGTAGGTAGTGGAACAT
 0.3430%
 1.0500%
 GCH1 (+1 CAG del) 
 WT
 GGCCCAGAACCCCCCAGGCCCAAGGGCTGCTCAAGA
 pegRNA apegRNA apegRNA 2

 Complete product GGGCGAGAACCCCC---CGCCAAGGGCTGCTCAAGA
 3.16%
 16.80%

 Byproducts
 GGGCGAGAACCCCC---CGGCAAGGGCTGCTCAAGA
 3.16%
 16.80%
 Byproducts RP2 (+2 GCG ins) 
 The Construction of the GCH1 (+2 GGC ins) WT GGGCGAGAACCCCC---AGCGCAAGGGCTGCTCAAGACGCCCC pegRNA apegRNA-Complete product GGGCGAGAACCCCCGGCAGCGGCAAGGGCTGCTCAAGACGCCCC 17.65% 29.32% Byproducts GGGCGAGAACCCCCGGCAGCGGCAAGGGCTGCTCAAGGCGCCCT 0.2600% 0.0959% 
 RP2 (+2 TTCGG del)

 WT
 ACACTITICGCTETICGCTAGTAGGATATAGGATATAGGACATAGTCC

 Complete product
 ACACTITICAGCTC-----TAGTAGGTATAGGAACATAGTCC
 0.29%
 1.67%

 Byproduct
 ACAGCTITIGAGCTC-----TAGTAGGTATAGGAACATAGTCC
 0.29%
 1.67%
 GCH1 (+1 GACC ins) 
 WT
 GGGCGAGAACCCCC
 ---CAG<sup>+</sup>CAGCGAGGGCTGCTCAGGACCCCCT
 pegRNA:
 apegRNA:

 Complete product GGGCGAGAACCCCCGACCGGCAAGGGCTGCTCAAGACGCCCCT
 10.73%
 27.87%

 Byproducts
 GGGCGAGAACCCCCGACCCGGCCAAGGGCTGCTCAAGGCCCCCT
 0.2988%
 ACTG1 (+2 TAA ins) ACI G1 (2: IAA IIIs)
 VIT
 CTCGTTGTAGAAGG---TGTIGGTGCCAGATCTTCTCCATGTCGTCCC
 PegRNA apegRNA:
 Complete product
 CTCGTTGTAGAAGGTATTGTGGTGCCAGATCTTCTCCATGTCGTCCC
 S2%
 14.18%
 Byproducts
 CTCGTTGTAGAAGGTATTGTGGTGCCAGATCTTCTCCATGGCGTCCC
 0.0170%
 0.0130% GCH1 (+5 GAT ins) 
 WT
 GGCCGAGAACCCCCBAC---GCCAAGGGCTGCTCAAGACGCCCCT
 pegRNA-2

 Complete product
 GGGCGAGAACCCCCAGCAGGGCAAGGGCTGCTCAAGACGCCCCT
 3.11%
 8.12%

 Byproducts
 GGGCGAGAACCCCCCAGCAGGGCAAGGGCTGCTCAAGGGCGCCCCT
 0
 0.9724%
 ACTG1 (+4 AACC ins) WT CTCGTTGTAGAAGGTG----TGGTGCCAGATCTTCTCCATGTCGTCGTCCC pegRNA apegRNA-2 Complete product CTCGTTGTAGAAGGTGAACCTGGTGCCCAGATCTTCTCCATGTCGTCCC 6.84% 12.59% GCH1 (+1 CAGCG del) CTCGTTGTAGAAGGTGAACCTGGTGCCAGATCTTCTCCATGGCGTCCC 0.0030% 0.0100% 
 WT
 GGGCGAGAACCCCCCAGCGGCTGATGGCCCCCT
 PegRNA:

 WG
 GGGCGAGAACCCCC-----GCAAGGGCTGCTCAAGACGCCCCT
 PegRNA:

 WT
 GGGCGAGAACCCC-----GCAAGGGCTGCTCAAGACGCCCCT
 PegRNA:

 Byproducts
 GGGCGAGAACCCC-----GCAAGGGCTGCTCAAGGGCCCCT
 PegRNA:
 Byproducts ACTG1 (+3 ACTC ins) WT CICGTIGTAGAAGGT----GTGCTGCCAGATCTTCTCCATGCTGCCC pegRNA apegRNA-2 Complete product CICGTIGTAGAAGGTAC': GTGCTGCCAGATCTTCTCCATGCGTCC 388% 4.32% CICGTIGTAGAAGGTAC': GTGCTGCCAGATCTTCTCCATGCGCCC 0.0020% 0.0025% CTCGTTGTAGAAGGTAC': GTGGTGCCAGATCTTCTCCATGCGCCC 0.0020% 0.0025% GCH1 (+4 CGG del) WT GGGCGAGAACCCCCAGCGAGAGGGCTGCTCAAGACGCCCT pegRNA apegRNA-2 Complete product GGGCGAGAACCCCCAG---CAAGGGCTGCTCAAGACGCCCT 7.33% 29.77% Byproducts GGGCGAGAACCCCCAG---CAAGGGCTGCTCAAGGCGCCCT 0.0329% 0.0802% GGGCGAGAACCCCCAG---CAAGGGCTGCTCAAGGA GGGCGAGAACCCCCCAG---CAAGGGCTGCTCAAGGA RNF2 (+1 TTA ins) 
 TCTTAGTCATTAC---CTGAGCTGTTCGTTGACTCATATA
 pegR4A
 apegR4A
 GCH1 (+2 AGCG del) 
 WT
 GGGCCAGAACCCCCCAGGGCCCAAGACGCCCT
 pegRNA-2

 Complete product
 GGGCCAAGACCCCCC----GCAAGGGCTGCTCAAGACGCCCT
 9.07%
 28.36%

 Byproducts
 GGGCGAGAACCCCCC----GCAAGGGCTGCTCAAGACGCCCT
 0.071%
 0.2711%
 RNF2 (+1 GTA ins) 
 VF2 (+ GTA IIIS)
 CTAGTCATTAC---CTGAGGTGTTCGTTCGTAACTCA
 pegRNA

 pipelee product
 TCTTAGTCATTACGTACTGAGGTGTTCGTTGGAACTCA
 16.46%

 products
 TCTTAGTCATTACGTACTGAGGTGTTCGTTGGAACCGA
 0.4862%
 24.23% SITE1 (+2 TAG ins) WT AGACCCAGCATCC---GTGGTTCGCTGAAAAGCTGTGGGAAATCGGTC pegRNA apegRNA-2 Complete product AGACCCAGCATCCTAGTGGTCGGTCGAAATCGGGTC 107% 8.23% AGACCCCAGCTCTAGTGGTCGCTCGAAAAGCTGTGGGAAGTGGGTC 0.0037% AGACCCCAGCTCTAGTGGTCGCTCGAAAAGCTGTGGGAAGCGGC 0.0089% 0 0.1965% Byproducts 
 Byproducts
 Increment

 RINF2 (+1 CTG del)
 TCTTAGTCATTACCTGAGGTGTTGGTTGTAACTCATATAA
 pegRNA
 apegRNA 

 VT
 TCTTAGTCATTACCTGAGGTGTTGGTTGTAACTCATATAA
 6.01%
 19.48%

 Complete products
 TCTTAGTCATAC---AGGTGTGTCGTTGTAACTCATATAA
 6.01%
 19.48%

 Byproducts
 TCTTAGTCATATAC---AGGTGTGTCGTTGTAACCGTATATAA
 0.000%
 0.2470%
 SITE1 (+2 GTGG del 
 WT
 AGACCCAGCATCCGTGGTCGCTGGTGGGGAÄATCCGGTC
 PegRNA-2

 Complete product AGACCCAGCATCC----GTCGCTGAAAAGCTGTGGGAAATCCGGTC
 20.1%
 18.49%

 Byproducts
 AAGACCCAGCATCC----GTCGCTGAAAAGCTGTGGGAAATCGGGTC
 0.0140%
 0.0076%
 WT TCTTAGTCATTACC----TGI/G0TGTTCGTTGTAACTCATATA P09RNA apogRNA apogRNA complete product TCTTAGTCATTCGGATGAGCTGTTCGTTGACTCATATA 0.799% Byproducts TCTTAGTCATCCGGATGAGCGTTCGTTGTGTGTGACTCATATA 0.0718% 0.0569% TCTTAGTCATTACCGGATGAGCGTGTCGTTGTGAACGGATATAA 0.0718% 0. SITE1 (+4 GGG del) 
 WT
 AAGACCCAGCATCGGTGGGTGGGTGGGTGGGGAAATCGGGC
 pegRNA:
 apegRNA:

 Complete product AAGACCCAGCATCCGT---TCGCTGAAAAGCTGTGGGAAATCGGGTC
 15.55%
 22.59%

 Byproducts
 AAGACCCAGCATCCGT---TCGCTGAAAAGCTGTGGGAAGTCGGGTC
 0.0022%
 0.0160%
 VHL (+3 TTA ins) 
 VIT
 GAGGGAATGCCCCGG---ACGCCGGAGAACTGGGACGAG
 pegRNA apegRNA-2

 Complete product
 GAGGGAATGCCCCGGTMAAGGGCGGAGAACTGGGACGAG
 21.71%
 30.82%

 Byproducts
 GAGGGAATGCCCCGGTMAAGGGCGGAGAACTGGGACGAG
 0.1088%
 0.1088%
 SITE1 (+7 TCGC del) 
 STEEL (\* TOGE dei))

 WT
 AAGACCCAGCATCEGTG00TCGCTGAAAAGCTGTGGGAAATCGGGTC

 Pompleiz produkt
 AAGACCCAGCATCCGTG00----TGAAAAGCTGTGGGAAATCGGGTC

 Byprodukts
 AAGACCCAGCATCGGG6-----TGAAAAGCTGTGGGAAATCGGGTC
 RUNX1 (+1 CGA del) TTTCAGGAGGAAGCGATGGCTTCAGACAGCATATTT WT pegRNA apegRNA-2 11.60% 16.04% Complete product TTTCAGGAGGAAG---TGGCTTCAGACAGCATATTT Byproducts TTTCAGGAGGAAG---TGGCTTCAGACAGCATATTT SITE3 (+1 TGA del) 0.0368% 0.1635% SITE5 (+1 CTT ins) 
 STIEE (+1 CTT ins)
 CLGACTCGACGCACG---TGCTIGCAGCCCAAGAATGCACAG
 pegRNA - 2
 apegRNA - 2

 Vmr
 CAGACTGACGCCCTTGCTGCAGCCCAAGAATGCACAG
 pegRNA - 2
 3.68%
 23.68%

 Complete product
 CAGACTGAGCACGCTTGCTGCAGCCCAAGAATGCACAG
 0.032%
 0.0957%

 Byproducts
 CAGACTGAGCACGCTTGCTGCAGCCCAAGAAGGCAGAG
 0.032%
 0.0978%

 CAGACTGAGCACGCTTGCTGCGACCCCAAGAAGGCAAGGGCAGAG
 0.00043%
 0.00043%
 0.00043%
 SITE3 (+4 TGGC del) SITE8 (+2 GTGG del) WT CAGACTCAGCAGGTGTGGGCGAGCGTGTGTTTTTG Complete product: CAGACTGAGCAGGT----GCAGCGAGCCTTGTCTTTG Byproducts CAGACTGAGCAGGT-----GCAGCGAGCCTGCTTTG CAGACTGAGCAGGT-----GCAGCGAGCCTGCGACTTTG SITE3 (+7 CAGAGG del) 
 STIE3 (\*/ CAGARG BRI)

 WT
 CAGACTGAGCACGTGATGGCAGAGGAAAGGAACGCCTGCTTCCTCCAGAGGGCGT

 Complete product CAGACTGACCACGTGATGG------AAAGGAACCCTGCTTCCTCCAGAGGGCGT
 8.11%
 22.85%

 Byproducts
 CAGACTGAGCACGTGATGG------AAAGGAAGCCCTGCTTCCTCCAGGGGCGT
 0.0133%
 0.0262%
 11.14% 14.38% 0.26% 0.3854% 0.0131% 0.0141% 
 SITE9 (+1-+5 TGAGG del)

 WT
 CAGACTGAGCAAGTGAGGGGCTGAGCCTCCCATGGAG

 Complete product
 CAGACTGAGCAAG-----GGCTCAGCCTCCCATGGAG

 Byproducts
 CAGACTGAGCAAG-----GGCTCAGCCTCCCATGGAG

 CAGACTGAGCAAG-----GGCTCAGCCTCCCATGGAG
 CAGACTGAGCAAG-----GGCTCAGCCTCGGATGGAG
 SITE3 (+1 6\*HIS ins) 
 STEES (+ 16 HIS HIS)
 TGATO/CAGAGGAAGGAAGGAAGCCCTGCTTCCTCCAGAGG
 PeopRNA apogNNA -2

 WT
 CAGACTGAGCACGACCATCATCACCATCATCATCATCATGATG/CAGAGGAAGGAAGCCCTGCTTCCTCCCAGAGG
 PeopRNA apogNNA -2

 Complete product CAGACTGACCACCATCATCATCATCATCATCATCATCATCATGATG/CAGAGGAAGGAAGCCCTGCTTCCTCCCAGAGG
 25.0%
 25.11%

 Byproducts
 CAGACTGAGCACGACCATCATCATCATCATCATCATCATCATCATGATG/CAGAGGAAGGAAGCCCTGCTTCCTCCCAGAGG
 0.0200%
 0.0001%
 1.27% 4.65% 0.0002% 0.0079% 0.0137% 0 SITE3 (+1 LoxP ins) Byproducts CAGACTGAGCACGATTGTTATAACTTCGTATAGCATACATTATACGAAGTTATTGATGGCAGAGGAAAGGAAAGCACCTGCTTCCTCCAGG 0 0.0100%

### Supplementary Fig. 13. Byproducts induced by regular pegRNA and apegRNA-2

at on-target sites.

The frequencies of complete products and byproducts (with pegRNA scaffold incorporation) induced by the regular pegRNA and apegRNA-2 in Fig. **2b**. The frequencies are means from three biologically independent experiments.



Supplementary Fig. 14. Off-target editing induced by regular pegRNA and apegRNA-2.

(a) The sequences of on- and off-target sites for the pegRNAs in (b). (b) The indel frequencies were determined at the predicted off-target sites. 293FT cells were either treated with the regular pegRNA, apegRNA-2 or left non-transfected (NT). Means ±

s.d. are from three independent experiments. Source data are provided as a Source Data file.



Supplementary Fig. 15. apegRNA-2 induced higher intended indel efficiencies than regular pegRNA in U2OS cells.

(a) On-target intended indel editing frequencies induced by the regular pegRNA and apegRNA-2 at the indicated sites in U2OS cells. (b) Statistical analysis of normalized editing frequencies, setting the frequencies induced by regular pegRNAs as 1. n = 15 editing from three independent experiments shown in (a). (c) On-target unintended indel frequencies induced by the regular pegRNA and apegRNA-2 at the indicated sites in U2OS cells. (d) Statistical analysis of normalized indel frequencies, setting the frequencies induced by regular pegRNAs as 1. n = 15 editing from three independent experiments shown in (c). (a, c) Means  $\pm$  s.d. are from three independent

experiments. (**b**, **d**) *P* value, Wilcoxon one-tailed signed-rank test. The median and interquartile range (IQR) are shown. Source data are provided as a Source Data file.



Supplementary Fig. 16. The effect of sgRNA scaffold structure on Cas9-mediated gene editing efficiency and the effect of apegRNA-2 PBS length on PE3-mediated prime editing efficiency.

(a) Schematic diagrams illustrating the predicted secondary structures of the regular sgRNA and asgRNA-2, which contains the same small hairpin as apegRNA-2. (b) On-target indel frequencies were induced by the co-expression of Cas9 nuclease and the regular sgRNA or asgRNA-2. (c) Statistical analysis of normalized indel frequencies, setting the frequencies induced by the regular sgRNA as 1. n = 9 editing from three independent experiments shown in (b). *P* value, Wilcoxon one-tailed

signed-rank test. The median and interquartile range (IQR) are shown. (d) The intended indel editing frequencies induced by the regular pegRNA and apegRNA-2 with different length of PBS at the indicated target sites. (b, d) Means  $\pm$  s.d. are from three independent experiments. Source data are provided as a Source Data file.



Supplementary Fig. 17. Efficacy of spegRNA and apegRNA in PE2 system.

(**a**, **b**) The intended single-base editing frequencies (**a**) and unintended indel frequencies (**b**) were induced by the regular pegRNA and the pegRNAs containing additional base substitutions under the PE2 setting. (**c**, **d**) Intended indel editing frequencies (**c**) and unintended indel frequencies (**d**) induced by the regular pegRNA and apegRNA at the indicated target sites under the PE2 setting. Means  $\pm$  s.d. are from three independent experiments. Source data are provided as a Source Data file.

## Uncropped gels for Supplementary Fig. 6b



# Uncropped gels for Supplementary Fig. 6d



Uncropped gels Supplementary Fig. 6.