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Supplemental Information

An engineered Cas12i nuclease that is an efficient genome editing tool in animals and plants

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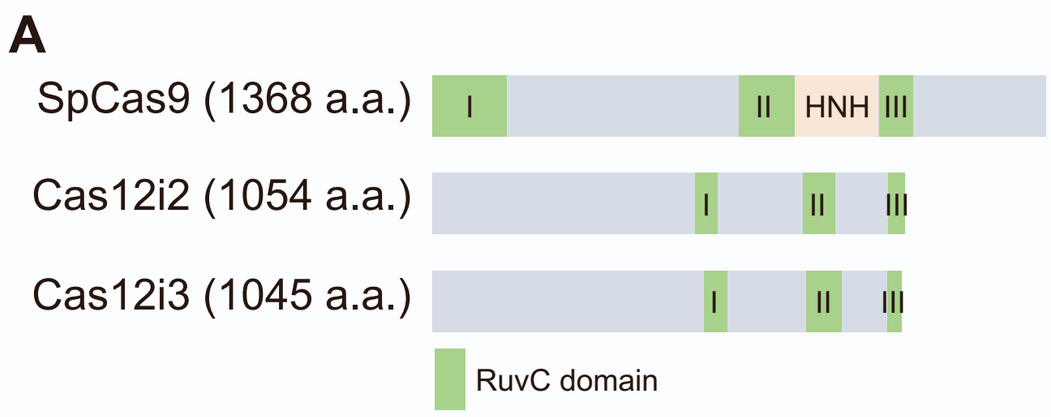
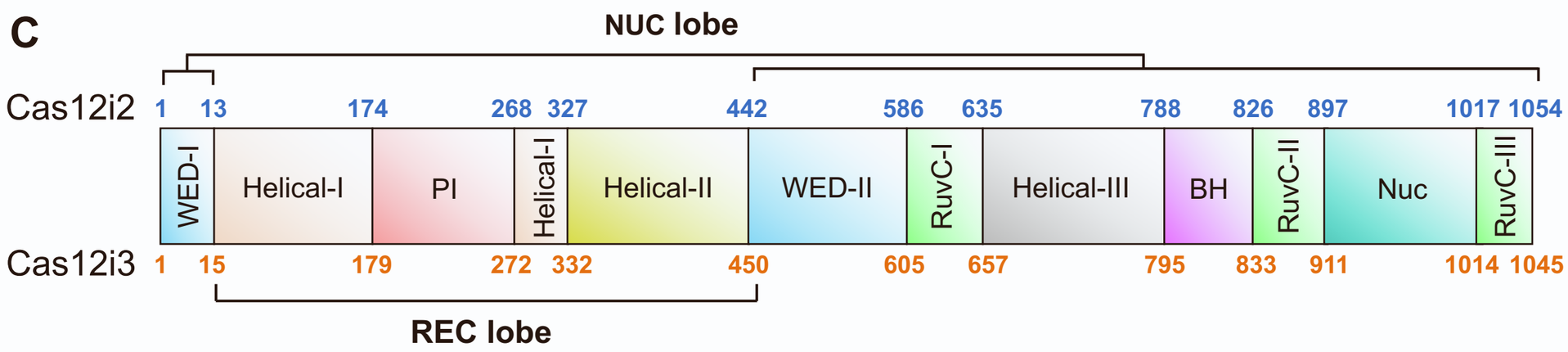
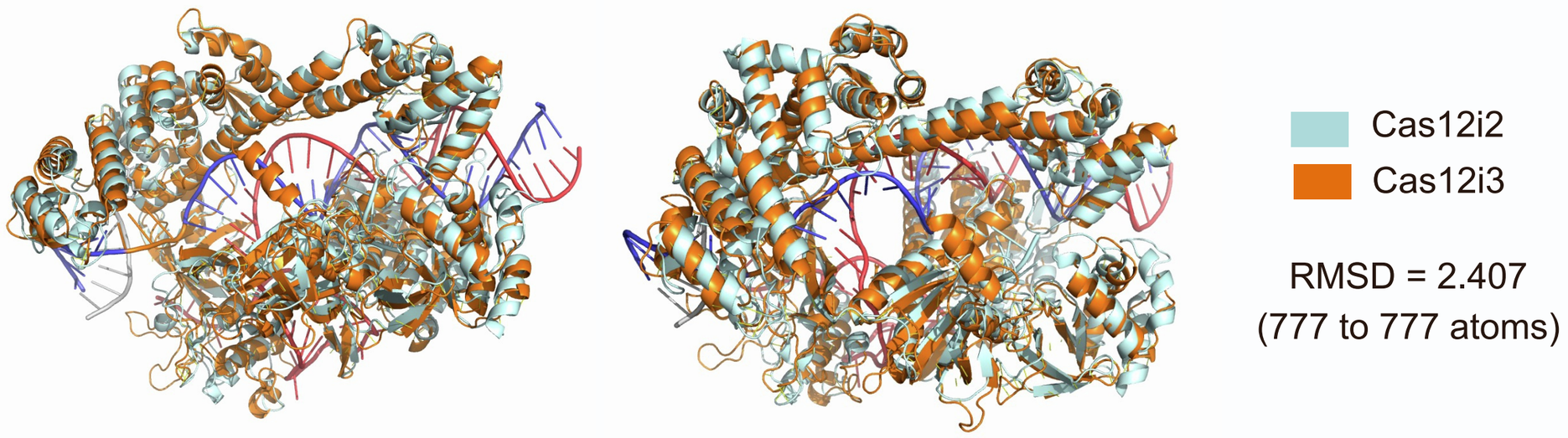
Supplemental Figure 1



B Cas12i3 blasts with Cas12i2

Score	Expect	Method
348 bits (892)	2e-10^{7}	Compositional matrix adjust.
Identities	**Positives**	**Gaps**
312/1077 (29%)	512/1077 (47%)	112/1077 (10%)





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MKKVEV**SRP**Y**QS**LL**LPN**HR**K**FKYLDETWNAYKSVKSLLHRFLVCAYGAVPFNKFVEVVEKVDNDQLVLAFAV
RLFRLVPVESTSFAKVDKANLAKSLANHLPVGTAIPANVQSYFDSNFDPKK**Y**MWIDCAWEADRLAREMGLSA
SQFSEYATTMLWEDWLPLNKDDV**NG**W**G**SV**S**GLF**GEGKKE**DRQQ**K**VKMLNNLLNGIKKNPPKDYTQYLKIL
LNAFDAKSHKE**A**VKNYK**GDSTGRT**AS**Y**LSEKSGEITELMLEQLMS**N**IQ**RD**IG**DK**QKE**I**SLPKKDVVKKYLESE
SGVP**Y**D**Q**NLWSQAY**R**NAA**S**SI**KKT**D**TR**NF**N**STLEKFKNEVELRGLLSEGDDVEILRSKFFSSEFH**KTPD**K**F**V
IK**PEH**IG**F**NNKYNVVAELYKLKAEATDFESAFATVKDEFEEK**G**I**KH**P**IKNI**L**E**YIWNNEVPVEKWGRVARFNQS
EE**K**LL**RIKANP**TVE**CNQ**GM**T**F**G**NSA**M**VGE**V**L**R**S**N**Y**V**SK**KGALV**SGEHGGRLIGQNNMI**W**LEMRLLNKGKW
E**T**H**H**V**P**T**HNMK**FFEEVHAYNPSLADSVNVR**NR**LYRSED**YTQL**PS**SITD**G**LKGNPKAK**LLKRQHCALNNMTA
NVLNPKLSFT**I**NKKNDDYTV**I**I**V**HSVEVSKPRREVLVGDYLVGM**DQNQT**ASNTYAVMQVVKPKSTDAIPFRNM
WVRFVESGSIESR**TLN**S**R**G**E**Y**V**D**Q**LN**H**DG**V**DLF**EI**GDT**EW**VDSARKFFNKLGVK**H**KD**G**TLVDLSTAPRKA**Y**
AFNNFYFKTM**L**NHL**R**SNEVDLTLLRNEILRVANG**RF**SPM**RLGSL**SWTTLKALGSFK**S**LV**LS**YF**D**RLG**AKEMV**
DKEAKDKSLFDLLVAI**N**NKRS**NKR**E**E**RT**SR**IA**S**SLMTVAQKYKVDNAVVHVVV**E**G**NLSSTDR**SA**SK**AH**NR**NT
MD**WCSRA**VV**KK**LE**DM**CN**L**YGFNIKGVP**AFYTSH**QDPLVHRADYDDPKPALRC**R**YSSYSRADFSKWGQNA
LAAVVRWASNKK**S**NTCYKVGAVEFLKQHGLFA**D**KKLTVEQFLSKVKDEEILIPR**RG**G**R**VFLTTHRLLAESTFV
YLNGVKYHSCNADEVAAVNICLNDWVIPCKKKMKEESSASG

Supplemental Figure 1. The predicted protein structure of Cas12i3 is similar to that of Cas12i2.

(**A**) Protein domain organization of SpCas9, Cas12i2 and Cas12i3. Protein lengths are indicated.

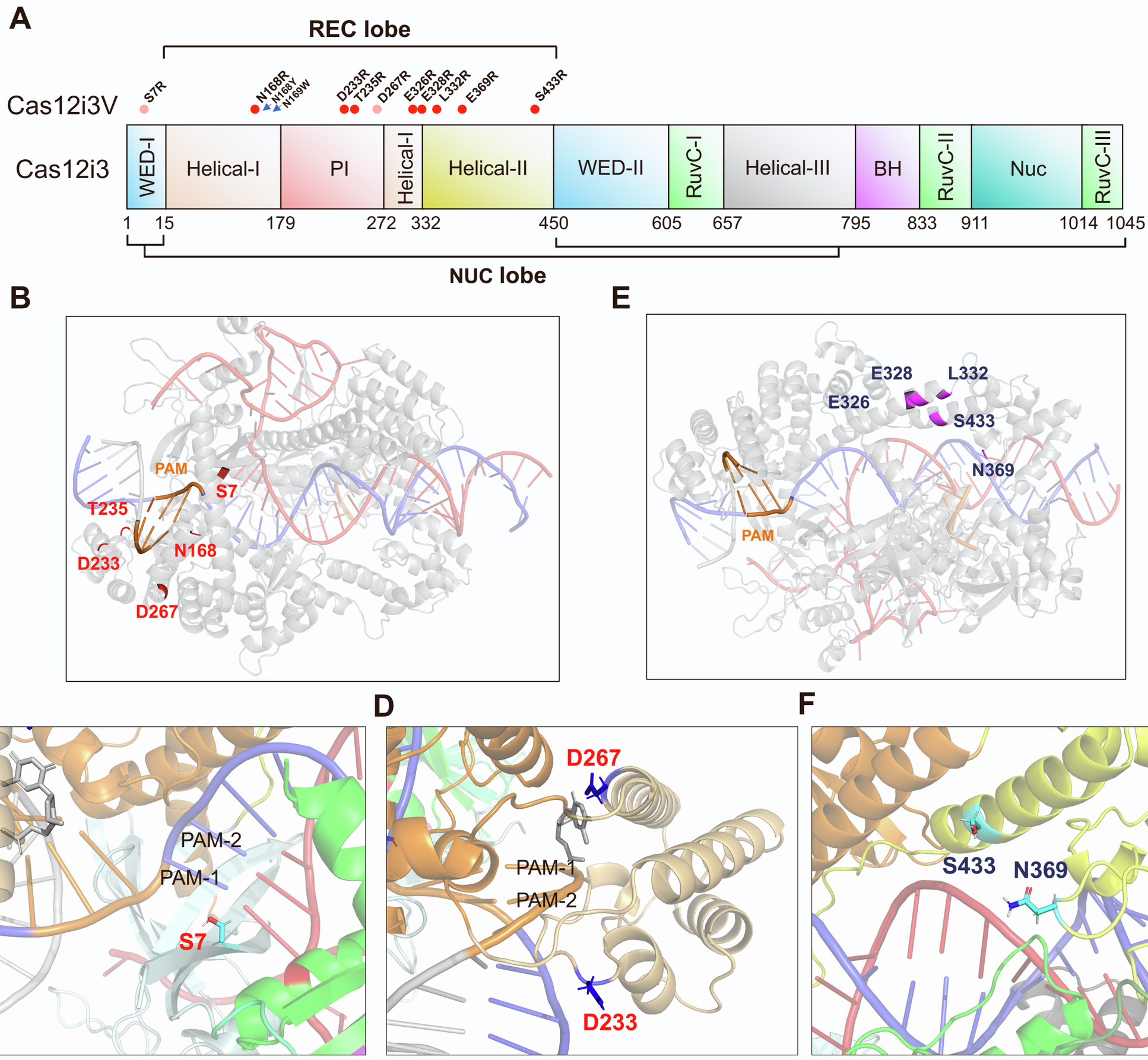
(**B**) Protein blast result for Cas12i3 and Cas12i2.

(**C**) Detailed domain organizations of Cas12i3 and Cas12i2.

(**D**) The structural similarity between Cas12i3 (orange) and Cas12i2 (light blue) as predicted by Alpha-fold.

(**E**) The protein sequence of Cas12i3 and the predicted residues (highlighted by red, bold) interacting with crRNA or DNA.

Supplemental Figure 2



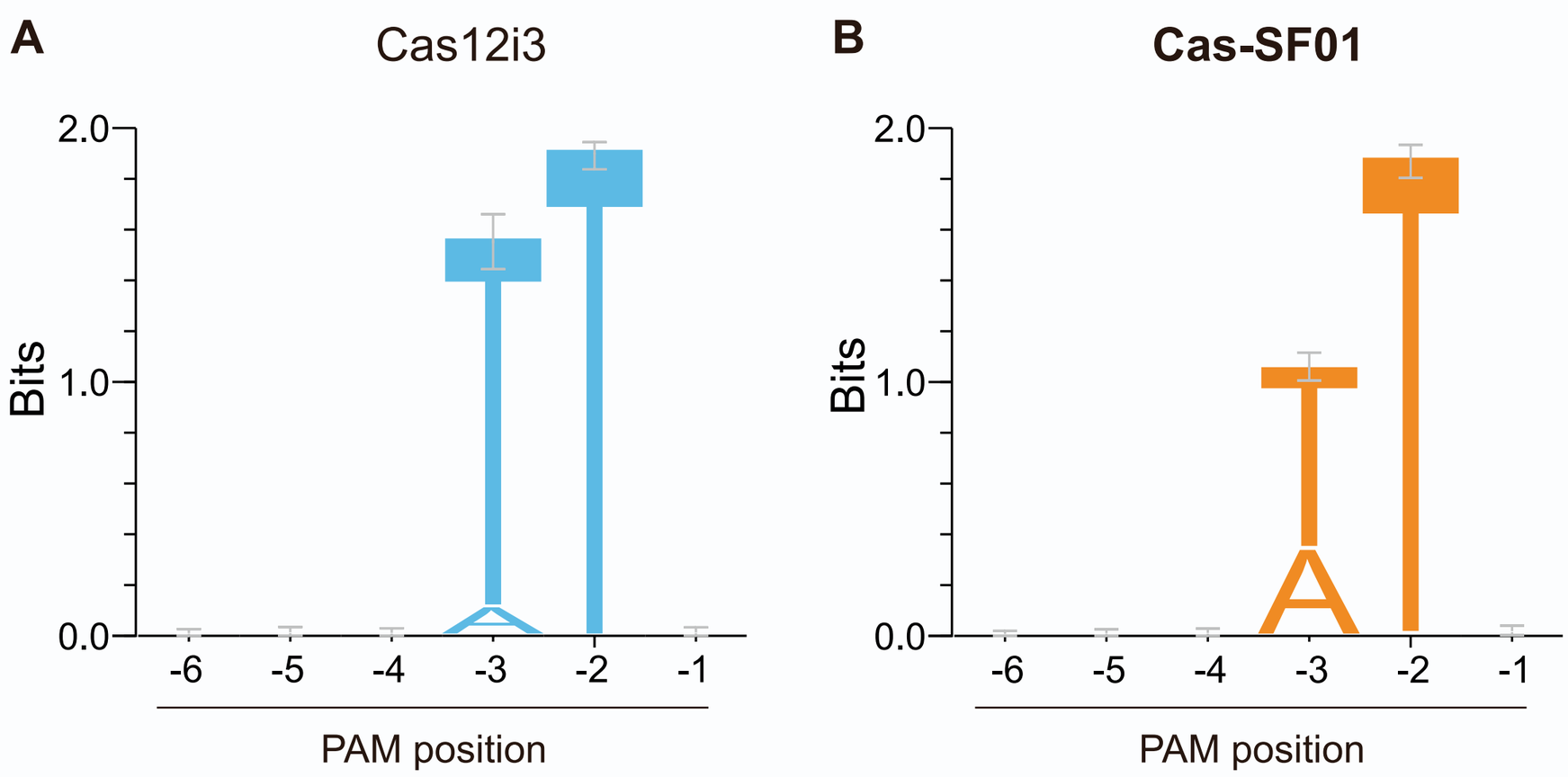
Supplemental Figure 2. Cas12i3 variants selected for combinatorial engineering.

(**A**) The position information of variants with increased editing activity.

(**B - D**) Amino acids chosen for enhancing interaction between Cas-SF01 and PAM, indicated in red.

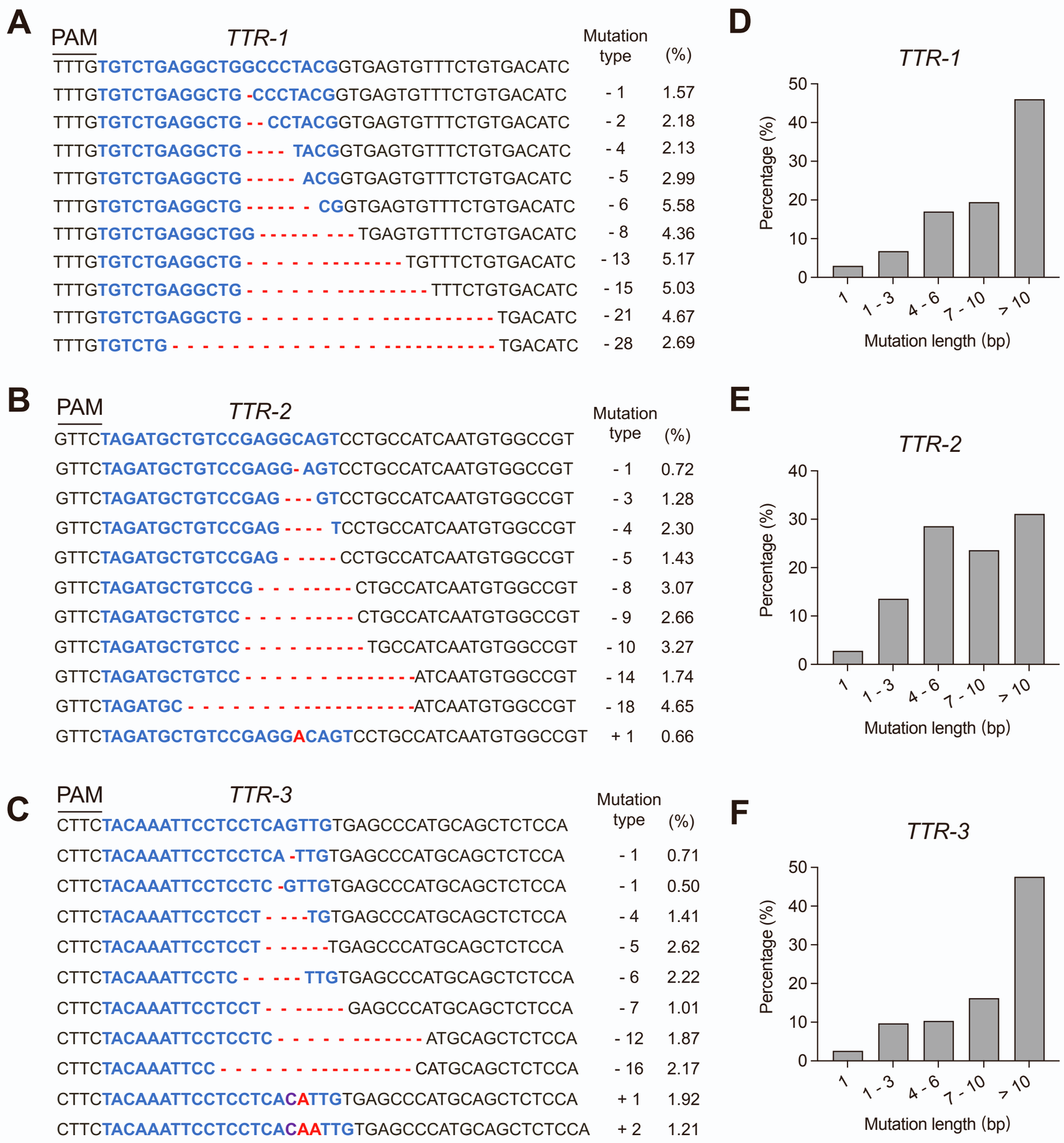
(**E, F**) Amino acids chosen for promoting interaction with DNA, indicated in blue.

Supplemental Figure 3



Supplemental Figure 3. WebLogo diagrams of Cas12i3 and Cas-SF01 were generated based on deep sequencing data.

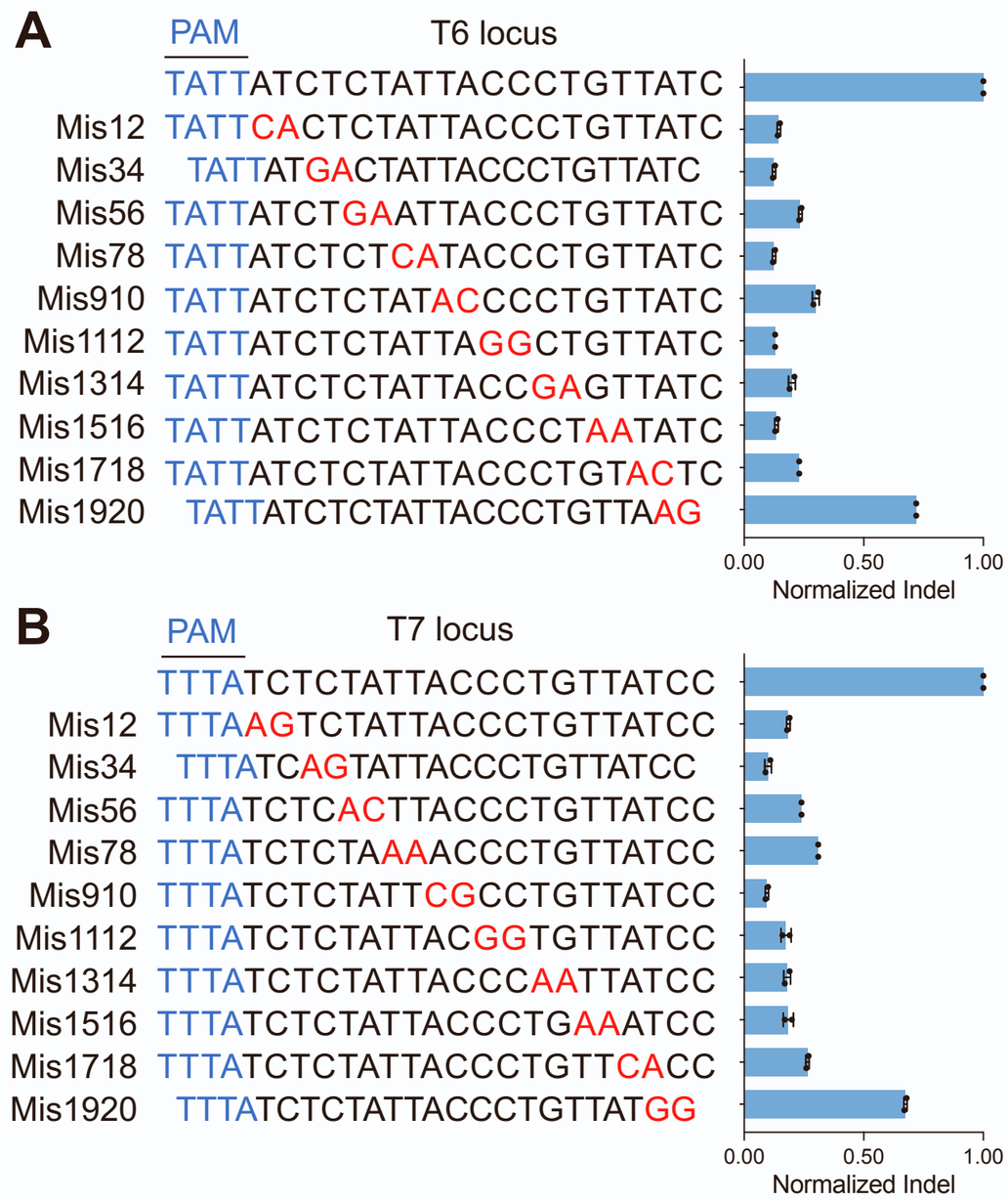
Supplemental Figure 4



Supplemental Figure 4. The distribution of different types of mutations generated by Cas-SF01 at the *TTR* locus.

The mutant alleles at three *TTR* sites, *TTR-1* to *TTR-3* (**A–C**), generated by Cas-SF01. The percentages of various mutation lengths are shown in (**D–F**).

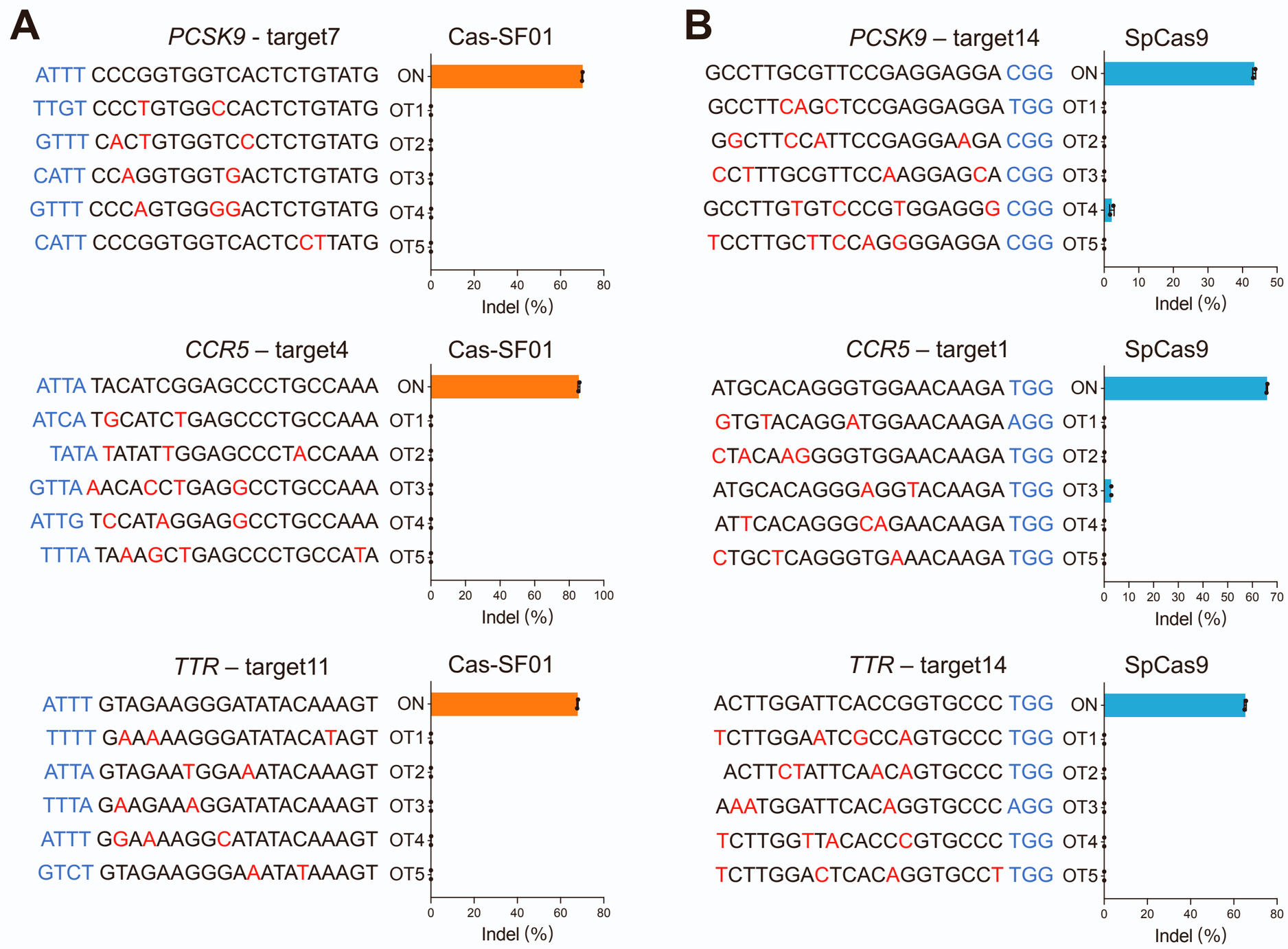
Supplemental Figure 5



Supplemental Figure 5. Mismatch tolerance of Cas-SF01 at T6 and T7 sites.

(**A, B**) The mismatch tolerance of Cas-SF01 was examined by designing dinucleotide mutations at T6 (**A**) and T7 (**B**) target sites, and then the EGFP florescence was determined.

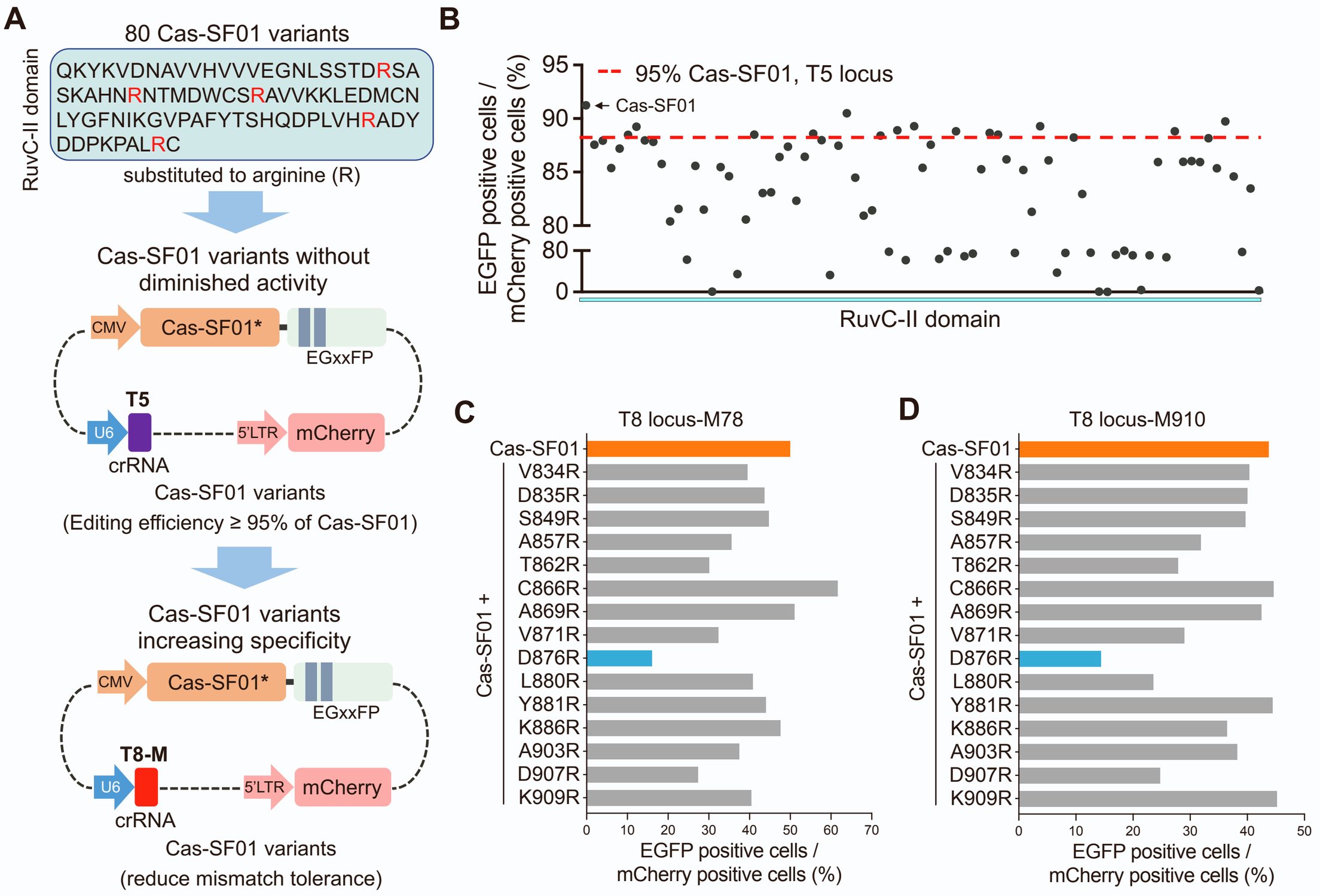
Supplemental Figure 6



Supplemental Figure 6. Targeted deep sequencing showed the high specificity of Cas-SF01 in mammalian cells.

(**A, B**) The editing efficiency of Cas-SF01 (A) and SpCas9 (B) at three different target sites (ON) of *PCSK9*, *CCR5* and *TTR* and five *in silico* predicted off-target sites (OT).

Supplemental Figure 7



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Cas nuclease	Cas12i3	Cas-SF01	Cas-SF01^{HiFi}
Size (aa)	1,045	1,045	1,045
PAM	TTN	NWTN, TTNN	ND
Editing activity	+	++++	+++
Specificity	ND	++	+++

Supplemental Figure 7. Screening of variants of Cas-SF01 for increased specificity.

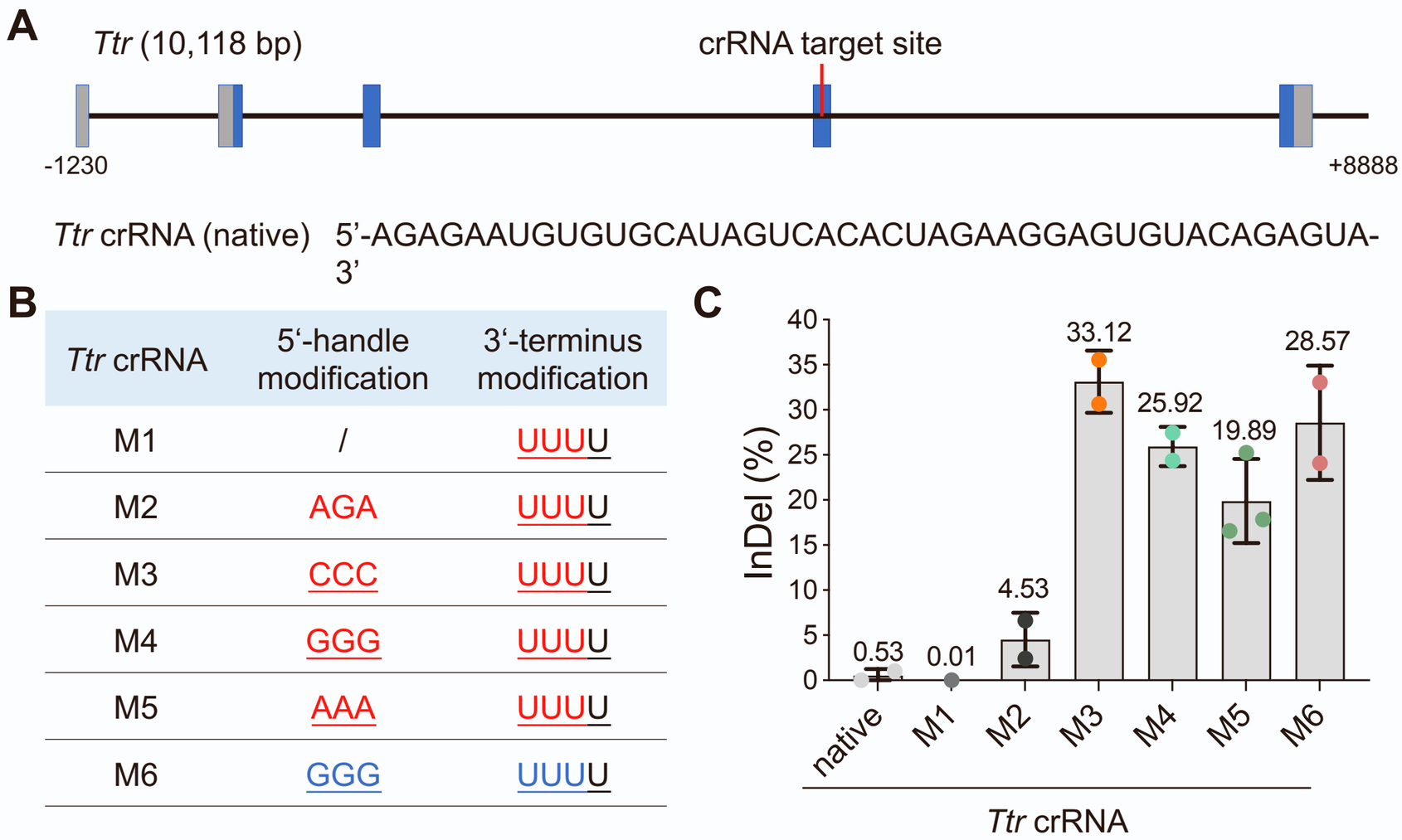
(**A**) Strategy for identifying Cas-SF01 variants with reduced mismatch tolerance. A Cas-SF01 variant pool was generated by individually mutating the amino acid residues in RuvC-II to arginine. Variants with similar editing activity as Cas-SF01 at the T5 locus of the EGFP-reporter system were selected, and finally, their mismatch tolerance was examined using T8-M78 and T8-M910 crRNA.

(**B**) The editing efficiency of a total of 80 Cas-SF01 variants at the T5 locus was determined using a reporter system. The red dotted line represents 95% editing efficiency of Cas-SF01.

(**C, D**) Screening for single mutations reducing the mismatch tolerance of Cas-SF01. A total of sixteen variants with similar editing activity were chosen to analyze their mismatch tolerance using T8 locus-M78 (C) and -M910 (D). Note that the D876R variant reduced the mismatch tolerance the most.

(**E**) The comparison of Cas12i3, Cas-SF01 and Cas-SF01^{HiFi}. ND, not determined.

Supplemental Figure 8



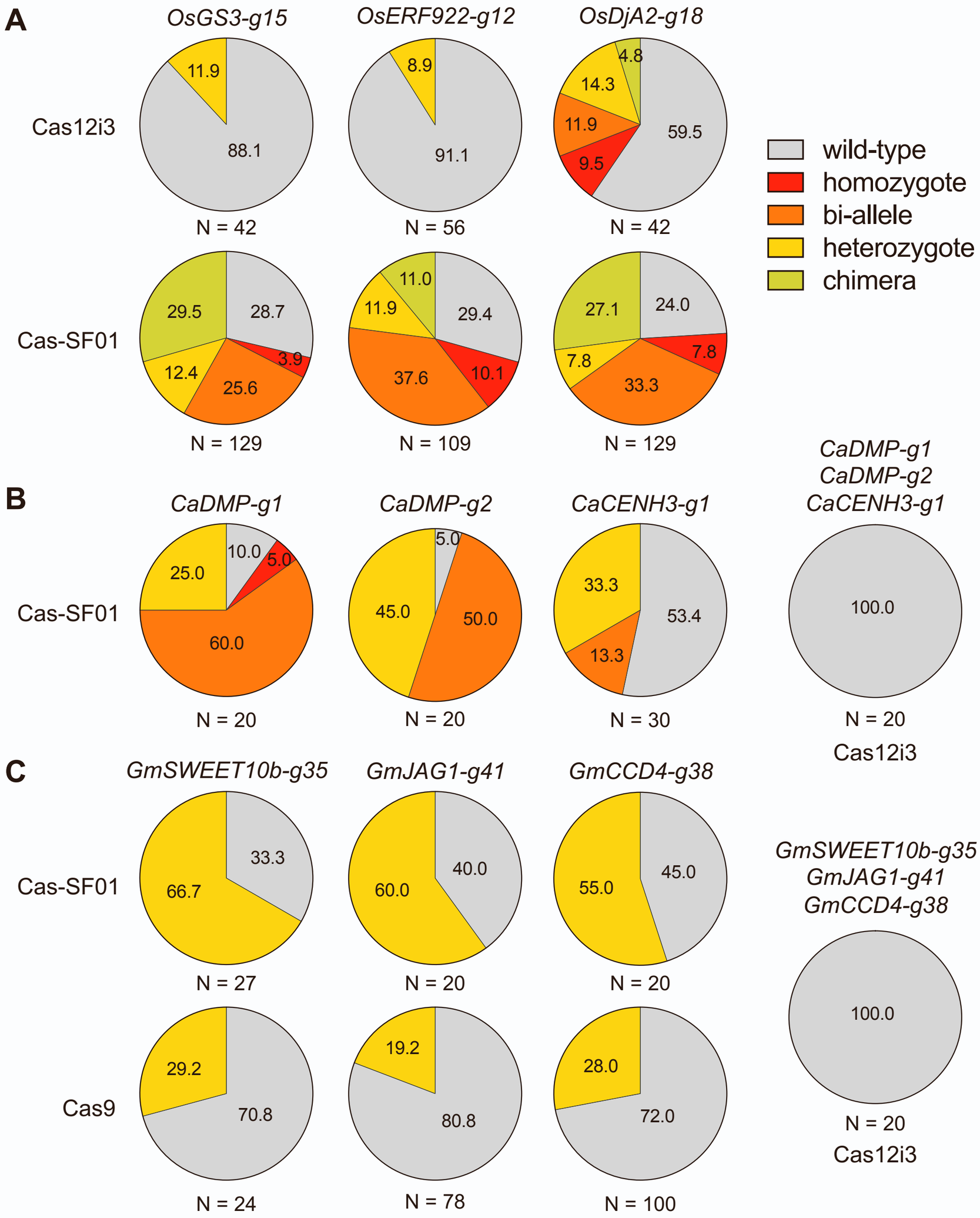
Supplemental Figure 8. Analysis of *in vivo* editing activity of Cas-SF01 at the *Ttr* locus using different chemically modified crRNAs.

(**A**) Schematics of *Ttr* gene and sequence of native *Ttr* crRNA.

(**B**) Six chemically synthesized *Ttr* crRNAs with 5'-handle and/or 3'-terminus modifications. Sequence extension was underlined. Phosphorothioate modification was shown in red, and phosphorothioate combined with 2′-*O*-methyl modifications were shown in blue.

(**C**) Determination of indel activity of Cas-SF01 with native and six modified crRNAs via LNP delivery in C57 mice.

Supplemental Figure 9



Supplemental Figure 9. Analysis of genotypes of E0 generation plantlets of rice, and hairy roots of pepper and soybean.

(**A–C**) Distribution of the genotypes from E0 plantlets edited at the *OsGS3*, *OsERF922* and *OsDjA2* sites in rice (A), hairy roots edited at *CaDMP* and *CaCENH3* sites in pepper (B), and hairy roots edited at *GmSWEET10b*, *GmJAG1* and *GmCCD4* in soybean (C).