A

gRNA sequence:

NNNNNNNNNNNNNNNNNNNNGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCU
AGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU

targeting sequence

B

target gene	Ensembl ID	gRNA ID	target sequence *(PAM)*
tardbp	ENSDARG00000040031	bpt1	GGCAGCCGAAGCAACATGGG*(TGG)*
tardbpl	ENSDARG00000004452	bplt4	GGCAGCACTTCAGAGTAGTT*(GGG)*
C13H9orf72	ENSDARG00000011837	C9t2	GGGTTGCTGCTGCTCAGTGA*(CGG)*
C13H9orf72	ENSDARG00000011837	C9t3	GGACAGGCTGAAGACATGAT*(AGG)*

C

D

C9t3/Cas9 injected P0 mutations identified

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.....TATCTATAAGCCCC-----TTCAGCCTGTCCTCCACAATCTCCAGCCGTGGCC....
.....TATAAGCCCCT----------------------ATCTCCAGCCGTGGCC....
.....TATAAGCCCC----------------------CAATCTCCAGCCGTGGCC....
.....TATAAGCCCCTAT-A---CTTCAGCCTGTCCTCCACAATCTCCAGCCGTGGCC....
.....TATAAGCCCCTAT-------TCAGCCTGTCCTCCACAATCTCCAGCCGTGGCC....
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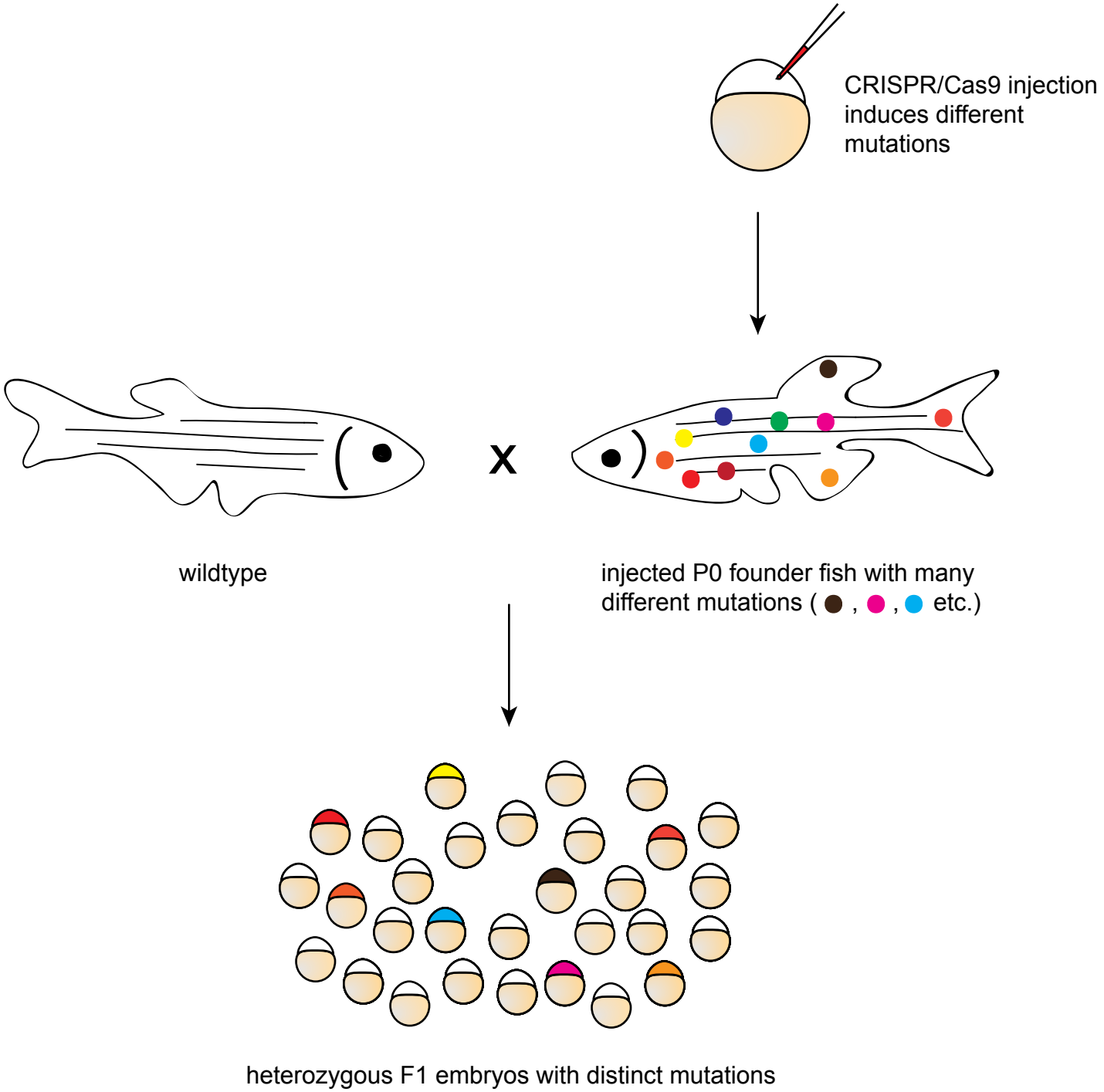
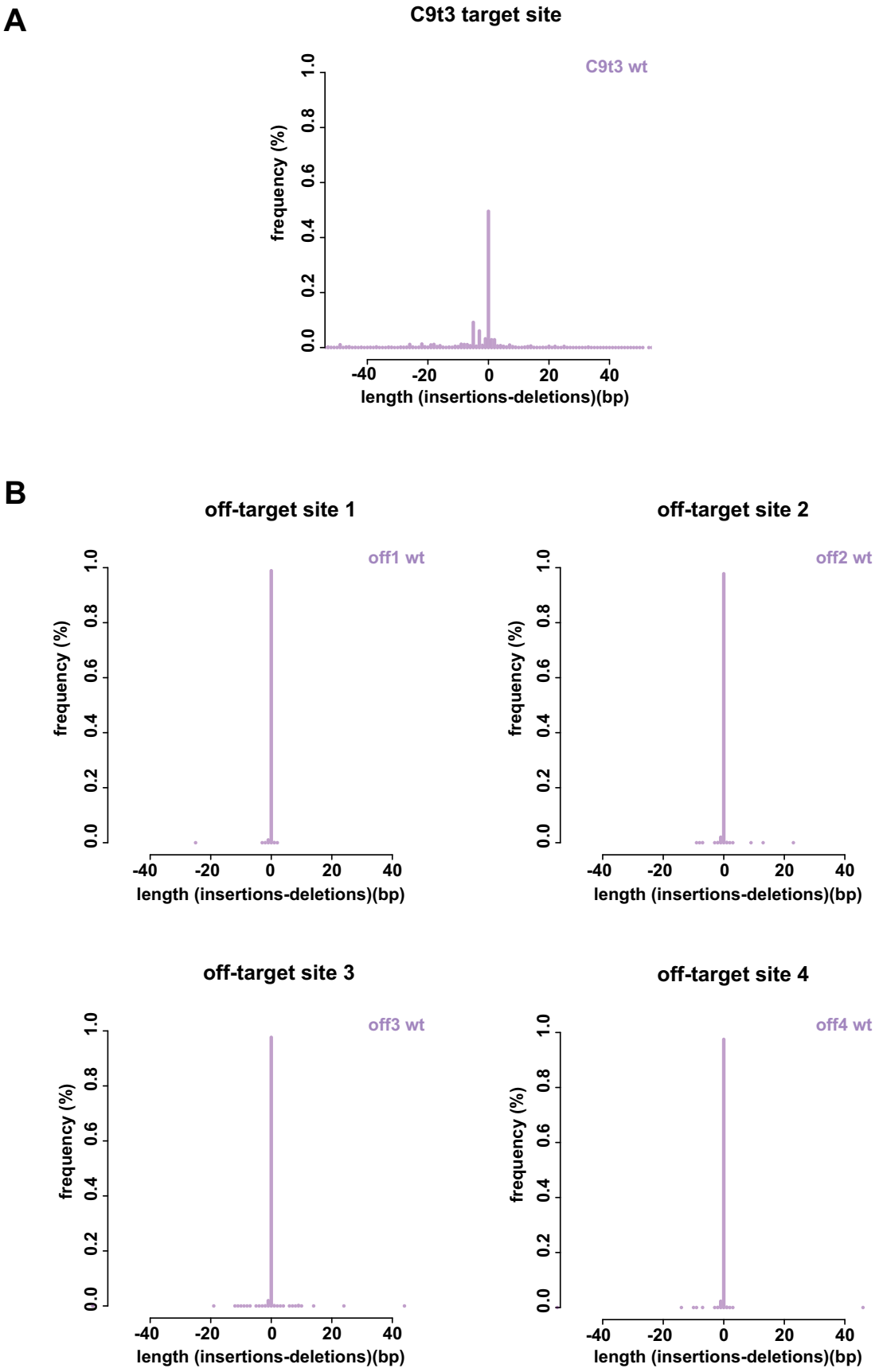
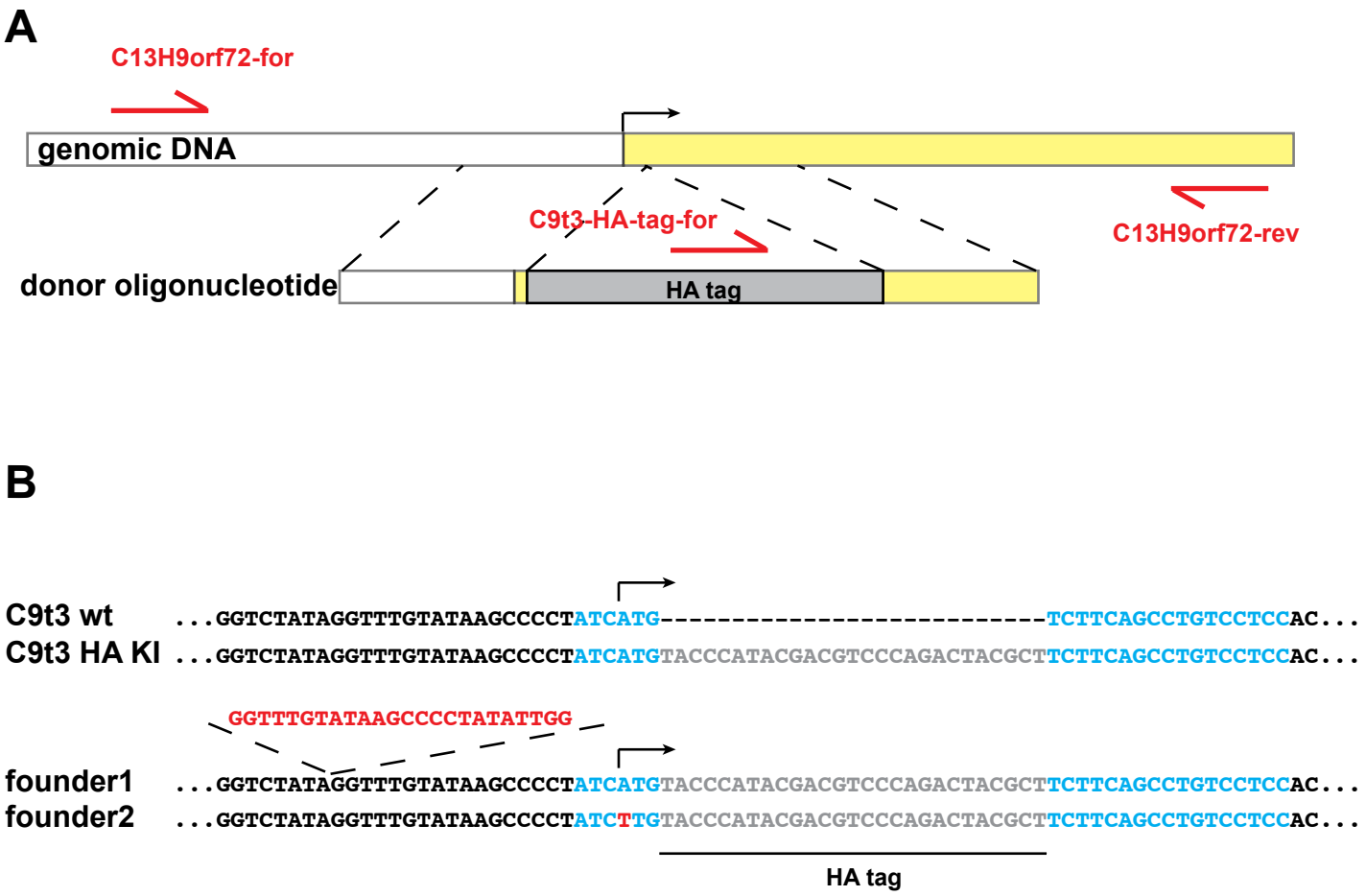
Supplementary Fig. 1. Overview of tools, sequences, and C9t3 induced P0 mutations. (A) gRNA sequence backbone with green N20 representing variable target sequences. (B) Table summarizing the genes targeted, their Ensembl ID, the gRNA ID we assigned, and the specific target sequence with the adjacent PAM sequence in parenthesis and in italics. (C) Schematic representation of the Cas9 protein. Orange box represents nuclear localization signal (NLS), dark blue box the Cas9 coding sequence, and cyan box the HA tag. (D) Wildtype C13H9orf72 sequence (top) and mutant sequences derived from one individual C9t3 gRNA and Cas9 mRNA injected P0 founder embryo. Yellow box indicates coding sequence.



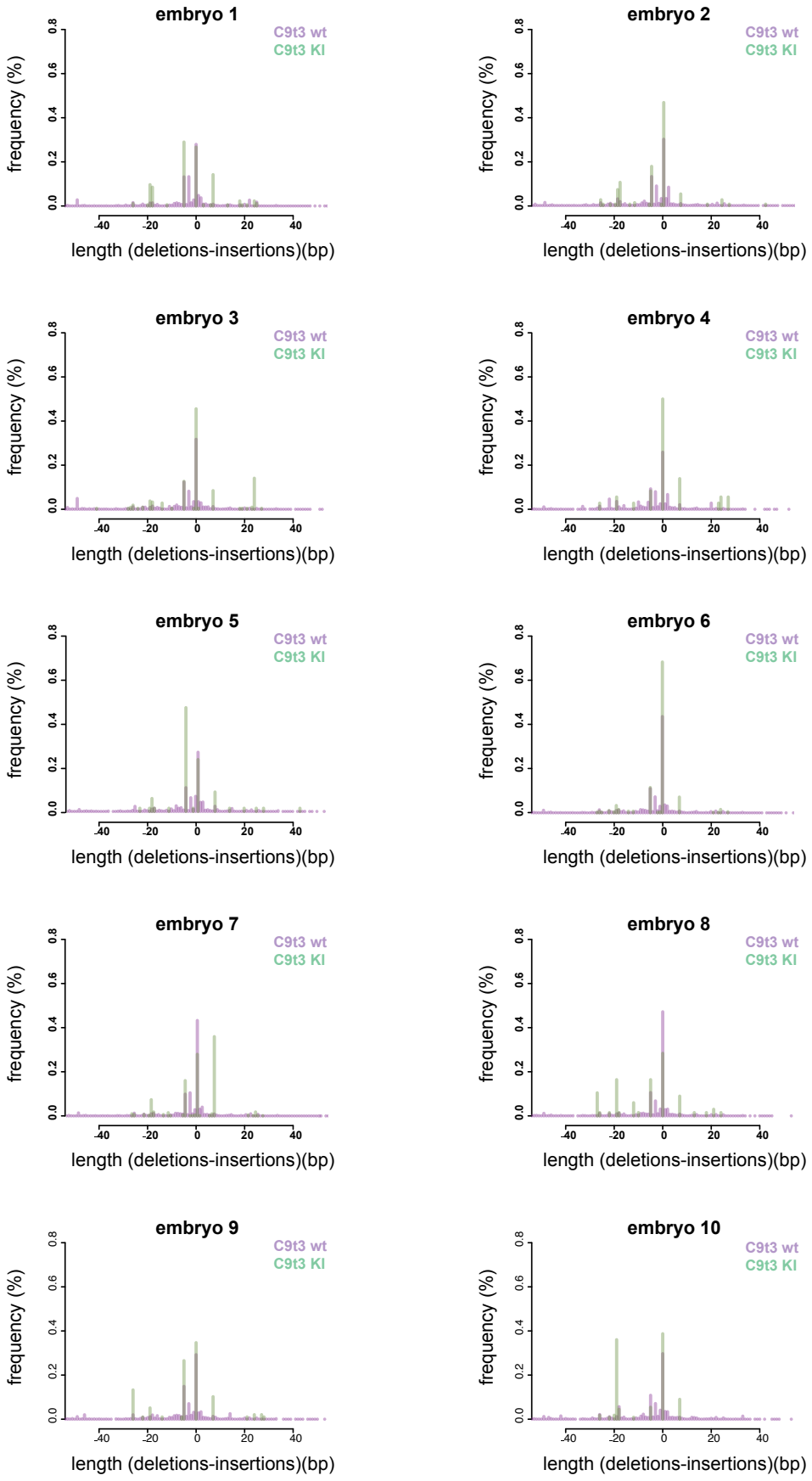
Supplementary Fig. 2. Schematic representation of germline transmission of mutations from a mosaic founder fish. Injected founder fish harbor several different mutations (indicated by colored circles), which can be in the soma or the germline. Mutations in the germline will be propagated to the F1 offspring. The F1 embryos will be heterozygous for the respective mutation(s) (as indicated by the color).



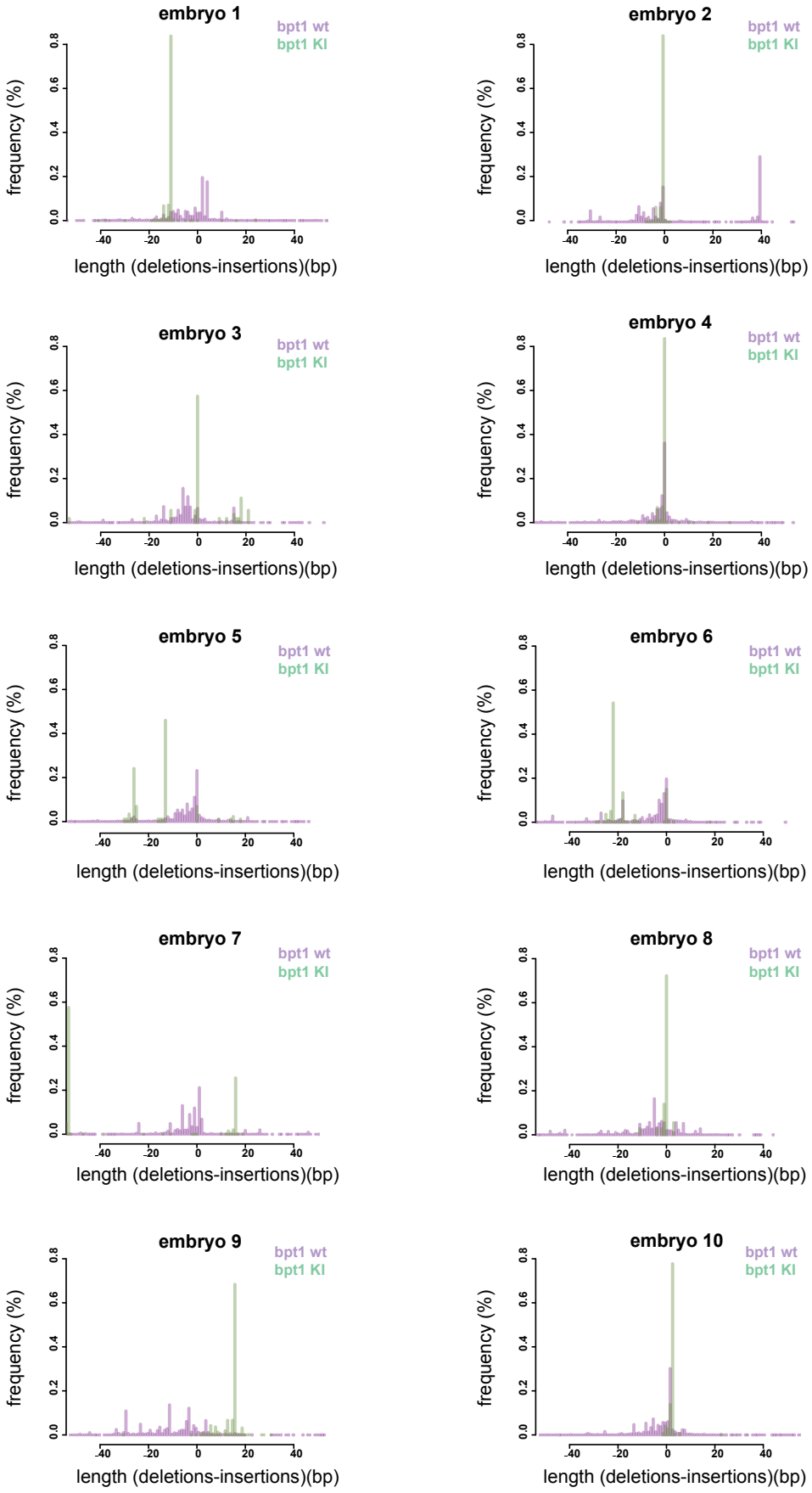
Supplementary Fig. 3. Frequencies of mutations on the C9t3 target site and potential off target sites 1-4. (A) Frequencies of NGS reads with wildtype sequence at the C9t3 target site (value 0) and respective insertions and deletions. (B) Frequencies of NGS reads with wildtype off target site sequence (off target site 1-4) and indels. Sequences without mutations have the value 0.



Supplementary Fig. 4. HA tag KI scheme and C9t3 KI alleles recovered. (A) Schematic representation of HA containing donor oligonucleotide for the C9t3 locus. Primers used for KI screening are indicated in red. HA tag (gray). (B) C9t3 target sequence and C9t3 HA KI sequence as reference sequence on top panel. Lower panel: alleles recovered from founder 1 and founder 2. Target sequence (cyan), HA tag (gray), transcription start site (arrow), mutations (red).



Supplementary Fig. 5. KI and mutagenesis analysis of 10 individual C9t3 KI injected embryos. NGS reads of each embryo were either mapped to the wildtype C9t3 target site (C9t3 wt in purple) or the C9t3 reference sequence with correct HA KI (C9t3 KI in green). Sequences that perfectly map to respective reference sequence have the value 0.



Supplementary Fig. 6. KI and mutagenesis analysis of 10 individual bpt1 KI injected embryos. NGS reads of each embryo were either mapped to the wildtype bpt1 target site (bpt1 wt in purple) or the bpt1 reference sequence with correct HA KI (bpt1 KI in green). Sequences that perfectly map to respective reference sequence have the value 0.