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Supplemental information

A Cas9^{D10A} nickase mouse line for precise genome editing

Janine Trombke, Daniel Bauersachs, Carlota Farre Diaz, Klaus Rajewsky, Van Trung Chu, and Ralf Kühn

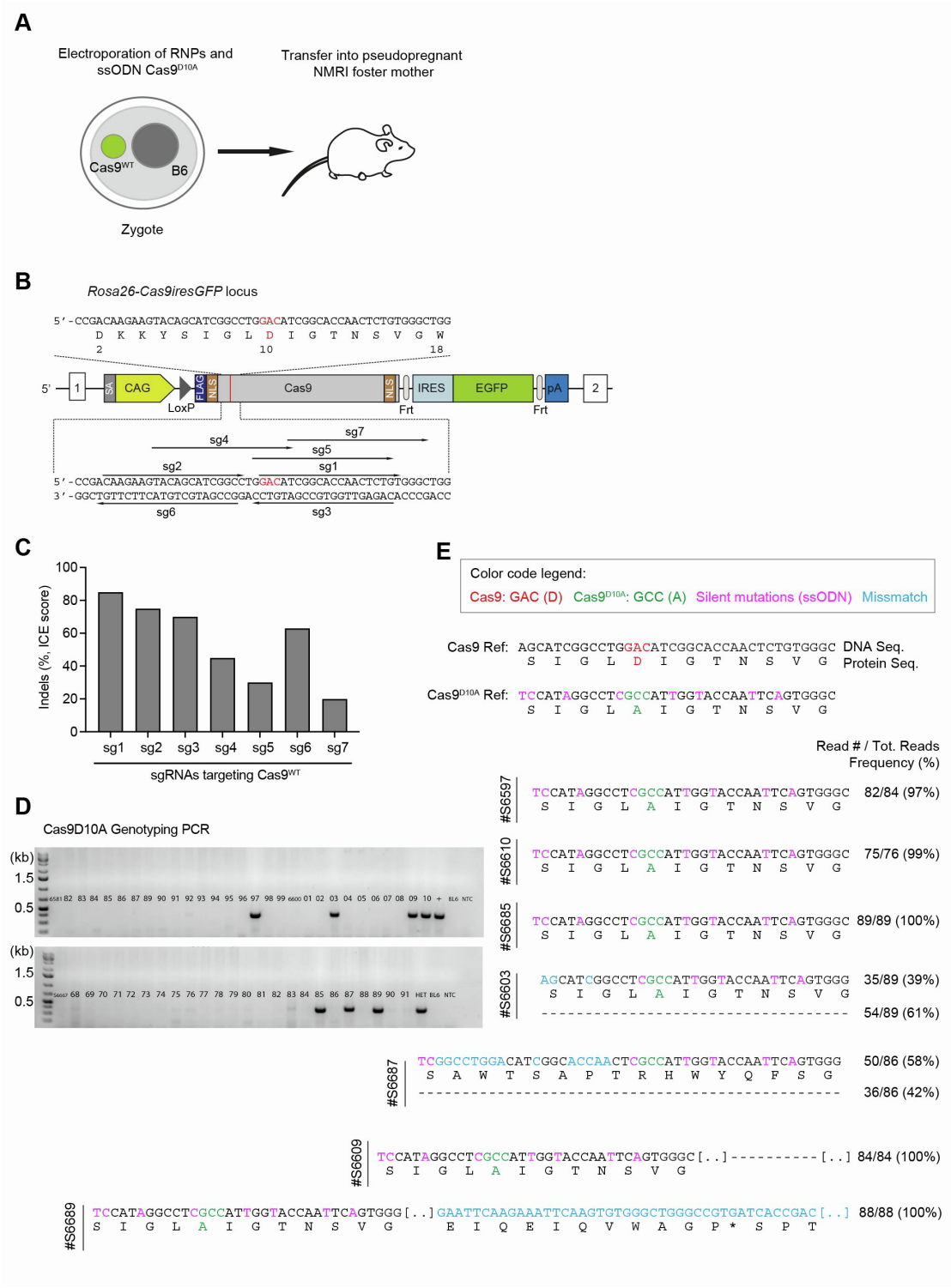
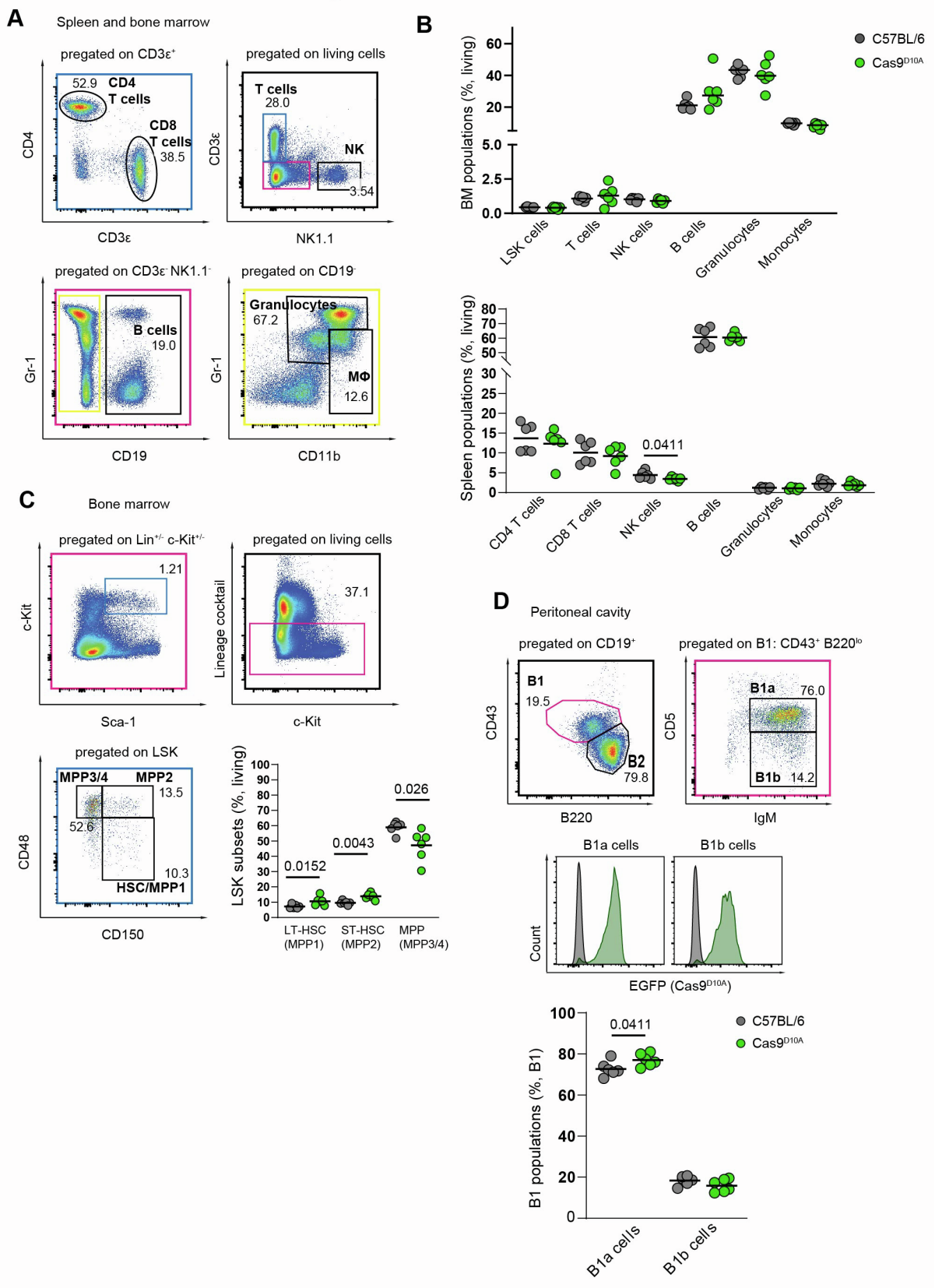


Figure S1. Gene editing strategy for generating the Cas9^{D10A} transgenic mouse line, Related to Figure 1.

(**A**) Schematic depiction of a mouse zygote consisting of male R26-Cas9iEGFP and female C57BL/6 pronuclei. Editing of the *Rosa26-Cas9iresEGFP* allele was performed using RNPs and ssODN. Edited zygotes were transferred into NMRI foster mothers. (**B**) sgRNA targeting sites in the *Rosa26-Cas9iresEGFP* allele. (**C**) Efficiencies of sgRNAs targeting the *Rosa26-Cas9iresEGFP* allele. Data are from a single experiment. (**D**) Genotyping PCR on DNA isolated from pups assessing the D10A codon replacement. (**E**) Sanger Sequencing of *Rosa26-Cas9^{D10A}iresEGFP* alleles from pups.



(*Figure S2 continued on next page*)

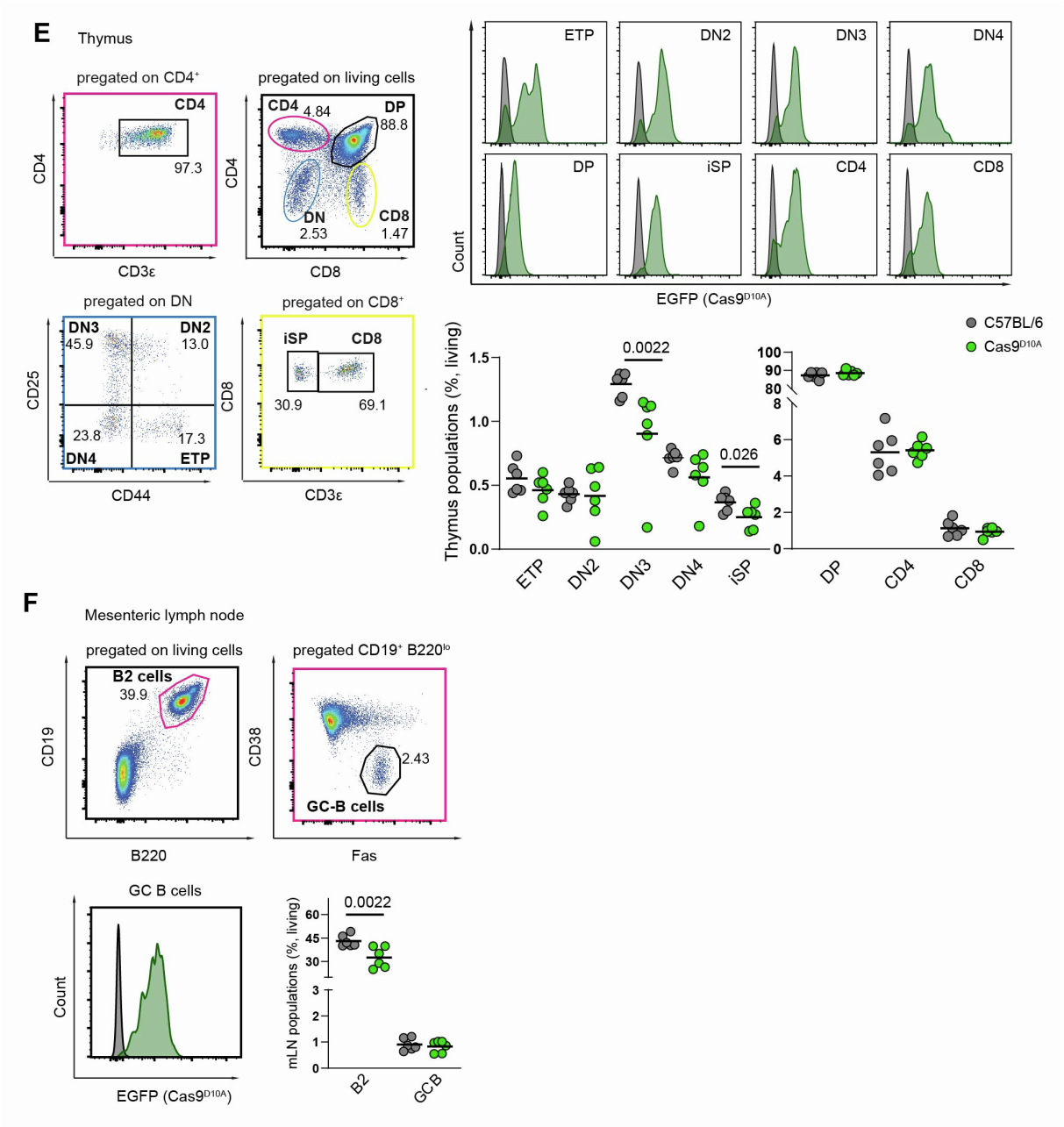


Figure S2. Characterization of the hematopoietic system of Cas9^{D10A} nickase transgenic mice, Related to Figure 1.

(**A**) Flow cytometric analysis showing the gating strategy in spleen and bone marrow of mice (n=6 per group) to determine CD4 T, CD8 T, NK, B, granulocyte, and macrophage (MΦ) cell populations. (**B**) Quantification of the immune cell lineages in the bone marrow (top) and spleen (bottom) of Cas9^{D10A} (green) and C57BL/6 (black) mice. (**C**) Gating strategy for LSK (Lineage$^-$Sca-1$^+$c-Kit$^+$) cells and LSK subsets in the bone marrow: MPP3/4 (CD48$^+$CD150$^-$), ST-HSCs (MPP2; CD48$^-$CD150$^-$), and LT-HSCs (MPP1; CD48$^-$CD150$^+$), with quantification of their frequencies (bottom right). (**D**) Gating strategy (top) for B2 cells (CD43$^-$B220$^+$) and B1 cells (CD43$^+$B220^{lo}) in the peritoneal cavity, including the B1a (CD5$^+$IgM$^+$) and B1b (CD5$^-$IgM$^+$) subsets. Histograms (middle) show EGFP (Cas9^{D10A}) expression in B1a and B1b cells, with quantification of their frequencies (bottom). (**E**) Gating strategy (left) for ETP (CD4$^-$CD8$^-$CD44$^+$CD25$^+$), DN2 (CD4$^-$CD8$^-$CD44$^+$CD25$^+$), DN3 (CD4$^-$CD8$^-$CD44$^-$CD25$^+$), DN4 (CD4$^-$CD8$^-$CD44$^-$CD25$^-$), DP (CD4$^+$CD8$^+$), iSP (CD8$^+$CD3$^-$), CD4 (CD4$^+$CD3$^+$), and CD8 (CD8$^+$CD3$^+$) T-cell

subsets in the thymus. Flow cytometry histograms show EGFP ($Cas9^{D10A}$) expression in thymic cell subsets (top right), with quantification of T cell subset frequencies (bottom right). (**F**) Gating strategy (top) for germinal center (GC) B2 cells ($CD38^{-}Fas^{+}$) in mesenteric lymph nodes. EGFP ($Cas9^{D10A}$) expression (bottom left) and quantification (bottom right) are shown. (**A-F**) Each symbol in the quantification graphs represents one mouse; horizontal lines indicate the mean. Frequencies of each population were compared between $Cas9^{D10A}$ and C57BL/6 mice using a two-tailed Mann–Whitney *U* test (n = 6 mice per genotype). *P* values are shown for comparisons reaching $p < 0.05$; all remaining comparisons were nonsignificant (see also Table S1).

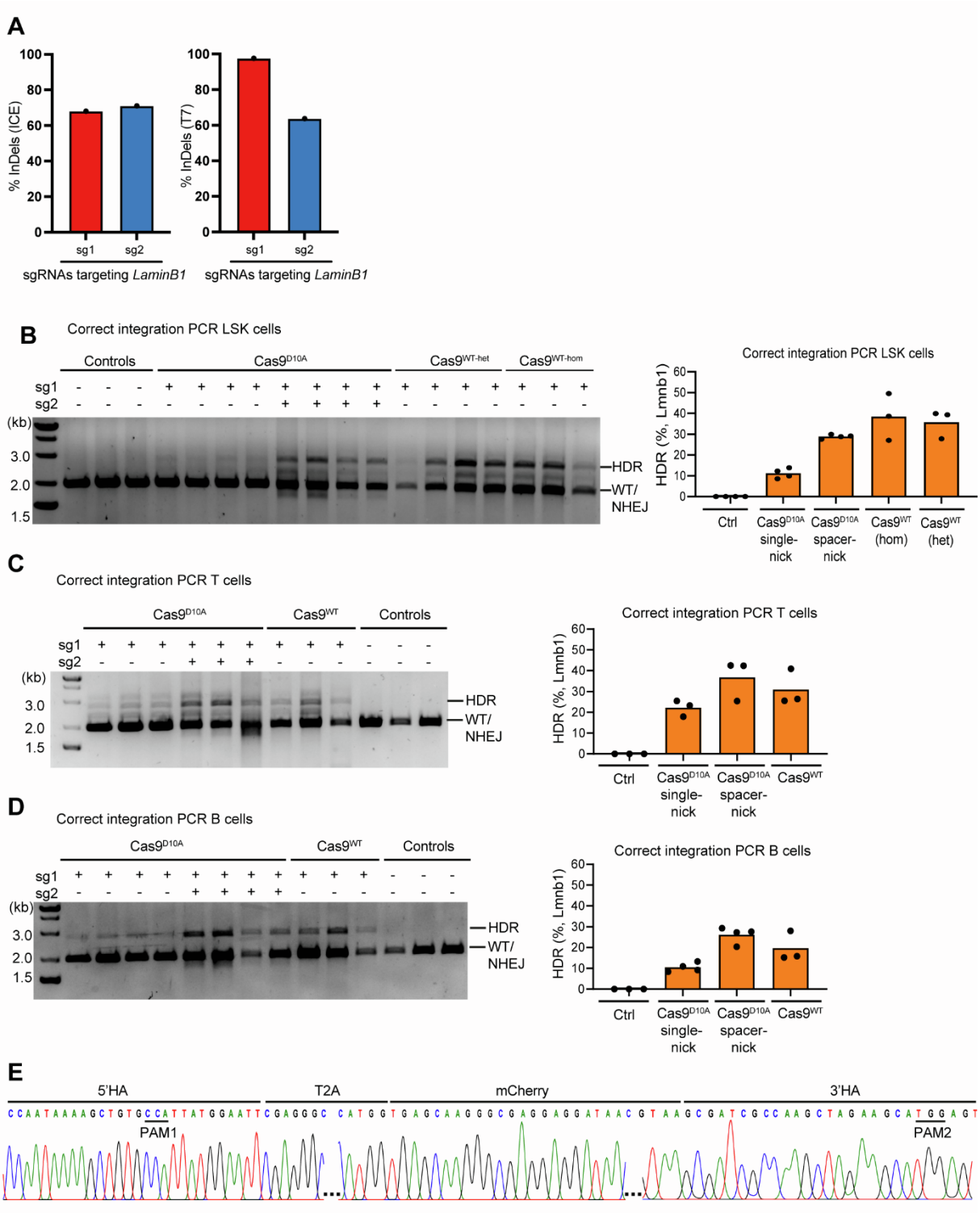


Figure S3. sgRNA screening and validation of T2A-mCherry insertion in the *Lmnb1* gene of various hematopoietic lineages of Cas9^{D10A} transgenic mice, Related to Figure 2.

(**A**) The Indel frequency of sgRNAs targeting the mouse *Lmnb1* locus in a PAM-out fashion was determined by ICE analysis (left) and T7EI assay (right). Data are from a single experiment. (**B-D**) Correct integration PCR of LSK, T, and B cells upon *Lmnb1* gene targeting and HDR quantification through densitometric analysis, respectively. (**E**) Sanger sequencing of mCherry^{+} sorted HSPCs (LSK cells) showing the correct integration of T2A-mCherry in the *Lmnb1* locus. Data are pooled from 2-3 independent experiments and represented as mean ± SD. Each symbol represents one mouse.

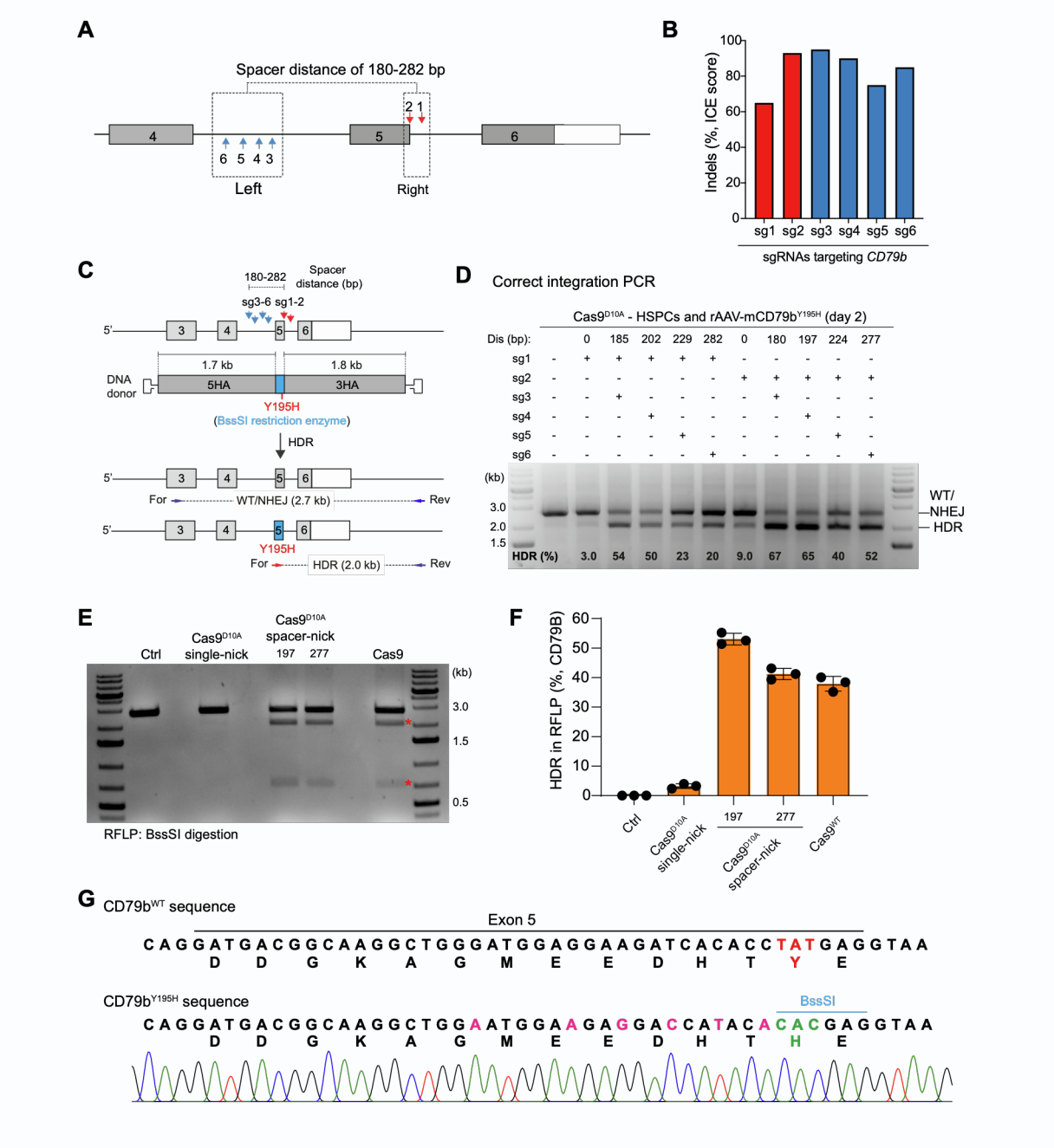


Figure S4. Establishment of Y195H mutation knock-in into the *Cd79b* locus in Cas9^{D10A} nickase transgenic mice, Related to Figure 3.

(**A**) sgRNAs targeting the mouse *Cd79b* in a PAM-out fashion with varying spacer distances. (**B**) Indel frequency of individual sgRNAs targeting *Cd79b*. Data are from a single experiment. (**C**) Targeting scheme of the Y195H mutation knock-in at the *Cd79b* locus, highlighting the PCR strategy to assess knock-in efficiencies and detect the Y195H mutation. (**D**) Correct integration PCR quantifying HDR events in Cas9^{D10A} HSPCs treated with spacer-nick sgRNAs and rAAV-mCD79b^{Y195H} particles carrying a donor template. (**E**) RFLP with *BssSI* of one representative experiment with the sgRNAs used for further experiments. (**F**) Quantification of the HDR efficiency in *BssSI*-mediated RFLP assays shown in (**E**). Data are pooled from 3 independent experiments and represented as mean ± SD. Each symbol represents one mouse. (**G**) Sanger Sequencing of *Cd79b* gene-edited HSPCs showing the Y195H mutation (highlighted in green).

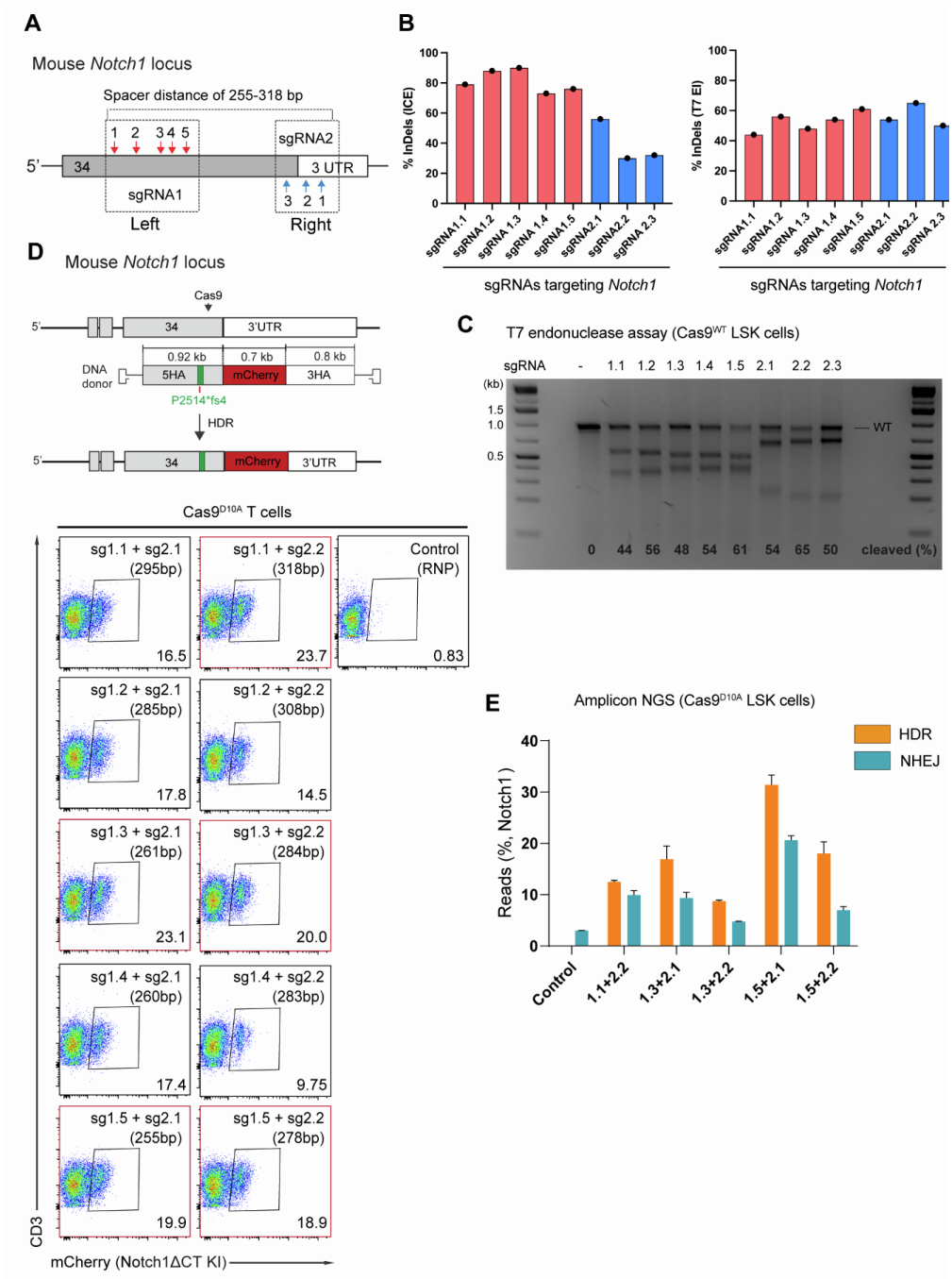


Figure S5. Establishment of the P2514*fs4 mutation knock-in into the *Notch1* locus in Cas9^{D10A} nickase transgenic mice, Related to Figure 3.

(**A**) sgRNAs targeting the mouse *Notch1* in a PAM-out configuration with varying spacer distances. (**B**) Indel frequency of individual sgRNAs targeting the *Notch1* locus, assessed using ICE score prediction (left) or T7EI (right). (**C**) T7EI assay with cleavage proportions determined by densitometry. Data are from a single experiment. (**D**) Strategy to screen pairs of spacer-nick sgRNAs for the *Notch1* locus of Cas9^{D10A} nickase T cells by targeting the last exon (exon 34) with sgRNAs and rAAV-6 particles carrying the P2515*fs4 mutation and an mCherry reporter. (**E**) Best-performing sgRNA pairs (highlighted in red in **D**) were used in Cas9^{D10A} nickase HSPCs. The same targeting strategy as depicted in Figure 3D was used (AAV-6 without mCherry reporter). Amplicon NGS was used to determine HDR and NHEJ events in the *Notch1* locus. Data are pooled from 2 independent experiments and represented as mean ± SD.

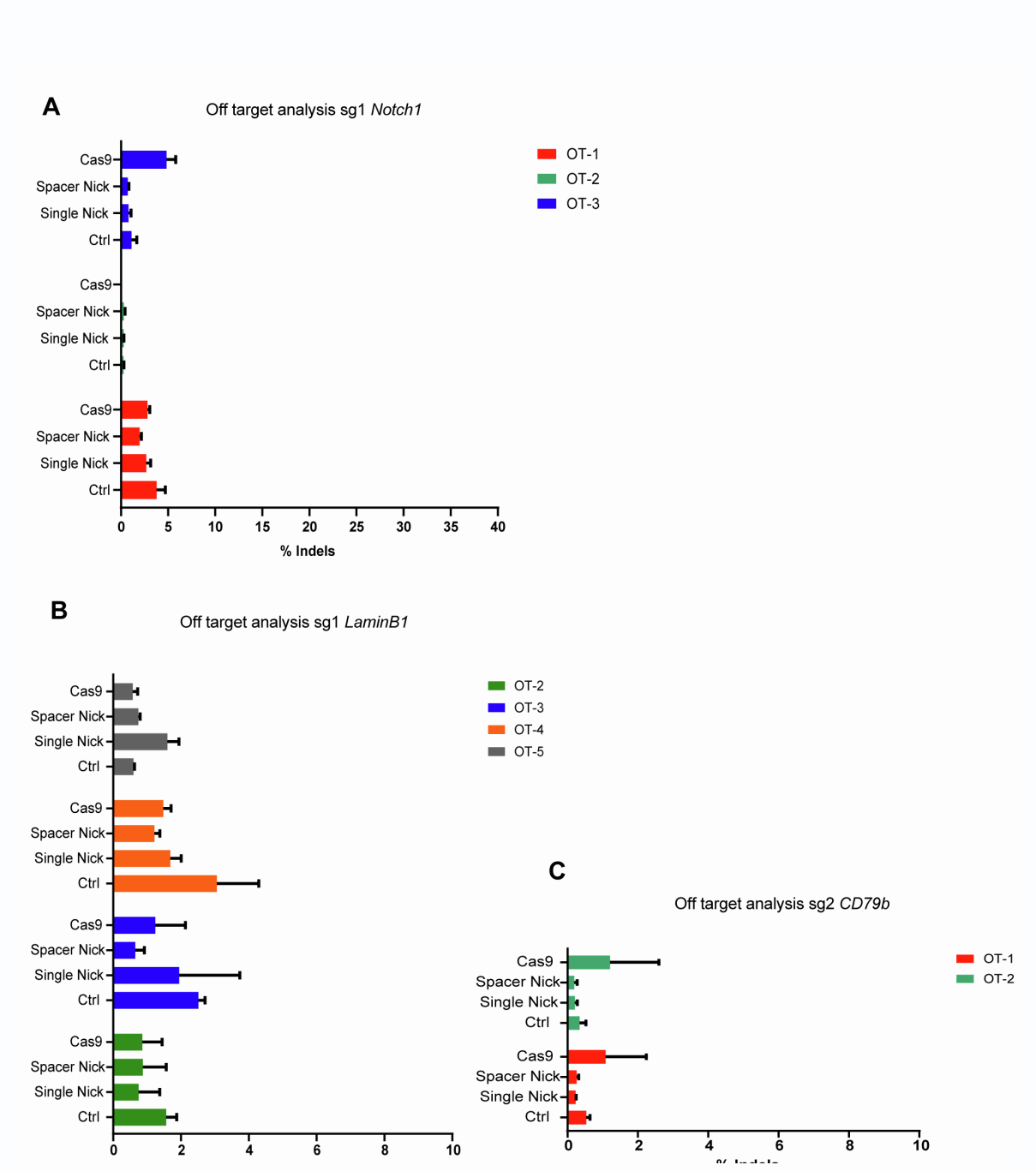


Figure S6. Off-target editing profiles of Cas9^{D10A} nickase and Cas9^{WT} across different gene loci, Related to Table S2.

(**A**) Off-target analysis of the *in silico*-predicted off-target sites of sgRNA 1 at the *Notch1* locus. (**B**) Off-target analysis of the *in silico*-predicted off-target sites of sgRNA 1 at the *Lmnb1* locus. (**C**) Off-target analysis of the *in silico*-predicted off-target sites of sgRNA 2 at the *Cd79b* locus. Data are pooled from two sample sets per off-target and represented as mean + SD.

Table S3: Oligonucleotides used in this study.

Single-stranded ODN containing D10A mutation	**Sequence (5' to 3')**
ssODN-D10A	GCCCCAAAGAAGAAGCGGAAGGTCGGTATCCACG GAGTCCCAGCAGCCGACAAGAAGTACTCCATAGGC CTCGCCATTGGTACCAATTCAGTGGGCTGGGCCGT GATCACCGACGAGTACAAGGTGCCCAGCAAGAAATT CAAGGTGCTGGGC
Primers for Cas9D10A genotyping	**Sequence (5' to 3')**
Forward	GGTTCGGCTTCTGGCGTGTGACCGGCG
Reverse	GCCCACTGAATTGGTACCAATGGCG
Primers for locus PCR	**Sequence (5' to 3')**
LaminB1 (extern PCR)-Forward	ACTCTTCCAGTGTGGGTCAGCCAAGCTTCT
LaminB1 (extern PCR)-Reverse	CTATAAAATGGTGCTGTGCGGGAGGGAGGG
LaminB1 (nested PCR)-Forward	TAGGGAGTGCAGCGCTTTAG
LaminB1 (nested PCR)-Reverse	GGGGACAAGGAGGGTAAGAAA
CD79B (extern PCR)-Forward	ACAGCGCCAACCATAATGTCACCGACAGCT
CD79B (extern PCR)-Reverse	AAGTCACTGCCCAGCGAGGGCGTCTGCTGA
CD79b-HDR-Y195H (correct integration PCR, combined with CD79B extern)-Forward	AAGGCTGGAATGGAAGAGGACCATACACAC
CD79B (nested PCR)-Forward	TCATTGTGCCCATCTTCCTGCTACTT GACA
CD79B (nested PCR)-Reverse	ATAGGTGGCTGTCTGGTCAATGTTC AAGCC
Notch1 (extern PCR)-Forward	ATACTCTCTGAGCCTGGTGGTCTAGGATGC
Notch1 (extern PCR)-Reverse	TGACCCAGACCTTGTGACCTGTAGCTGCCG
Notch1 (nested PCR)-Forward	CACTCTTTCCCTACACGACGCTCTTCCGATCTGTCG GATCGGAGCCCAGTCAGGCAGATGTACAACC
Notch1 (nested PCR)-Reverse	GTCCTTTCCAGTGCTTCAGGATGCTGCAGCGTA GAGATCGGAAGAGCACACGTCTGAACTCCAGTC
Primers for off-target (OT)	**Sequence (5' to 3')**
Notch1-sgRNA1-OT-1-Forward	TTGTGAGCTACTGTGTGGGT
Notch1-sgRNA1-OT-1-Reverse	TGTTCCACTCCAGTCCTTCC
Notch1-sgRNA1-OT-2-Forward	CAAACAAACAAACGCAGGGC
Notch1-sgRNA1-OT-2-Reverse	TCTGAGATCCTGCCAGTGTG
Notch1-sgRNA1-OT-3-Forward	TCAGTCTTTGGAGGAGCTGG
Notch1-sgRNA1-OT-3-Reverse	GGAGAGGTTGCTGTTGTGTG
LaminB1-sgRNA1-OT-1-Forward	GCTACATTGGGCATCTGGTG
LaminB1-sgRNA1-OT-1-Reverse	TTGCTTACTGGCTTGCTTCC
LaminB1-sgRNA1-OT-2-Forward	GCCTGTGAGTGCCTCTTAGA
LaminB1-sgRNA1-OT-2-Reverse	GCCAGACTCCCAAGAAGACT
LaminB1-sgRNA1-OT-3-Forward	GCTTGATATGCACACACAGACA
LaminB1-sgRNA1-OT-3-Reverse	TTGGTGTTTCGAGACAGGGT
LaminB1-sgRNA1-OT-4-Forward	ACACTCATGACACAGCCAGA
LaminB1-sgRNA1-OT-4-Reverse	TGTGTGCCCATATGCTATGC
LaminB1-sgRNA1-OT-5-Forward	TCTGCCACAACACTCCTGAT
LaminB1-sgRNA1-OT-5-Reverse	CTGCCACTTGTTCCACTGTC
LaminB1-sgRNA1-OT-6-Forward	AGAGAAACACAGAGAGCTAGCT
LaminB1-sgRNA1-OT-6-Reverse	CATCTGAGTGCTGTTGGGTAC
CD79B-sgRNA2-OT-1-Forward	GGAAGGTCTTGCTTGGCTAA
CD79B-sgRNA2-OT-1-Reverse	AACTGGTTAGATTGAGTGGCA
CD79B-sgRNA2-OT-2-Forward	TGAGCTATCATCCCGGTCTC
CD79B-sgRNA2-OT-2-Reverse	AGTCCTAGCACCTTCAAGCA
CD79B-sgRNA2-OT-3-Forward	CAAGGTGAGAGCAGTCAGGT
CD79B-sgRNA2-OT-3-Reverse	TGTATGGAGGATATGTGATGGCT

CD79B-sgRNA2-OT-4-Forward	ACTCACAGGTAGTCAAATGTCG
CD79B-sgRNA2-OT-4-Reverse	ATTCGGGCATGGTGGAAGAA
CD79B-sgRNA2-OT-5-Forward	CTCACAGGTAGTCAAATGGCG
CD79B-sgRNA2-OT-5-Reverse	ATTAAGTCGGGCATGGTGGA
CD79B-sgRNA2-OT-6-Forward	CACTCACAGGTAGTCAAATGGTC
CD79B-sgRNA2-OT-6-Reverse	TGGAGGAATAGAGAAGGCCA
CD79B-sgRNA2-OT-7-Forward	ACACGATGGGCCTGAATTCT
CD79B-sgRNA2-OT-7-Reverse	GCAGAGATTTGTTTGTGCTGT
CD79B-sgRNA2-OT-8-Forward	CTCACAGGTAGTCAAATGGCG
CD79B-sgRNA2-OT-8-Reverse	ATTAAGTCGGGCATGGTGGA
CD79B-sgRNA2-OT-9-Forward	AGTCAAATGTCGAACTCCACA
CD79B-sgRNA2-OT-9-Reverse	ATTCGGGCATGGTGGAAGAA
CD79B-sgRNA2-OT-10-Forward	CTCACAGGTAGTCAAATGGCG
CD79B-sgRNA2-OT-10-Reverse	AGTCGGTCATGGTGGAAGAG
sgRNA names	**Sequence (5' to 3')**
sgCas9WT.1	GACATCGGCACCAACTCTGT
sgCas9WT.2	CAAGAAGTACAGCATCGGCC
sgCas9WT.3	CAGAGTTGGTGCCGATGTCC
sgCas9WT.4	TACAGCATCGGCCTGGACAT
sgCas9WT.5	GGACATCGGCACCAACTCTG
sgCas9WT.6	GCCGATGCTGTACTTCTTGT
sgCas9WT.7	TCGGCACCAACTCTGTGGGC
sgLmnB1.1	GTCTTGACAAGTTCACATAA
sgLmnB1.2	ACTGAGCTAACTGTACTGTT
sgCD79B.1	AGATCACACCTATGAGGTAA
sgCD79B.2	GGAGGAAGATCACACCTATG
sgCD79B.3	GTGAATGAACAGGGAGCCAA
sgCD79B.4	CAATGGACAGGACTTGGGGC
sgCD79B.5	GTAGCCAGAGTCTCAGAATA
sgCD79B.6	CCAACCTCCCAGCCTTCCCA
sgNotch1-1.1	TGGGGCAGAATGGTGTGCAC
sgNotch1-1.2	CTGGCTTTCCTGGGGCAGAA
sgNotch1-1.3	GGATGGCAGTGATGTGGGCA
sgNotch1-1.4	AGGATGGCAGTGATGTGGGC
sgNotch1-1.5	CATGGAGGATGGCAGTGATG
sgNotch1-2.1	CCAGATCACCCACATTCCAG
sgNotch1-2.2	CATTTAAATAAACAGAGATG
sgNotch1-2.3	TAAACAGAGATGTGGGATGC

All primers were purchased from Eurofins. The D10A-containing ssODN and sgRNAs were purchased from IDT.