

Fig. S1

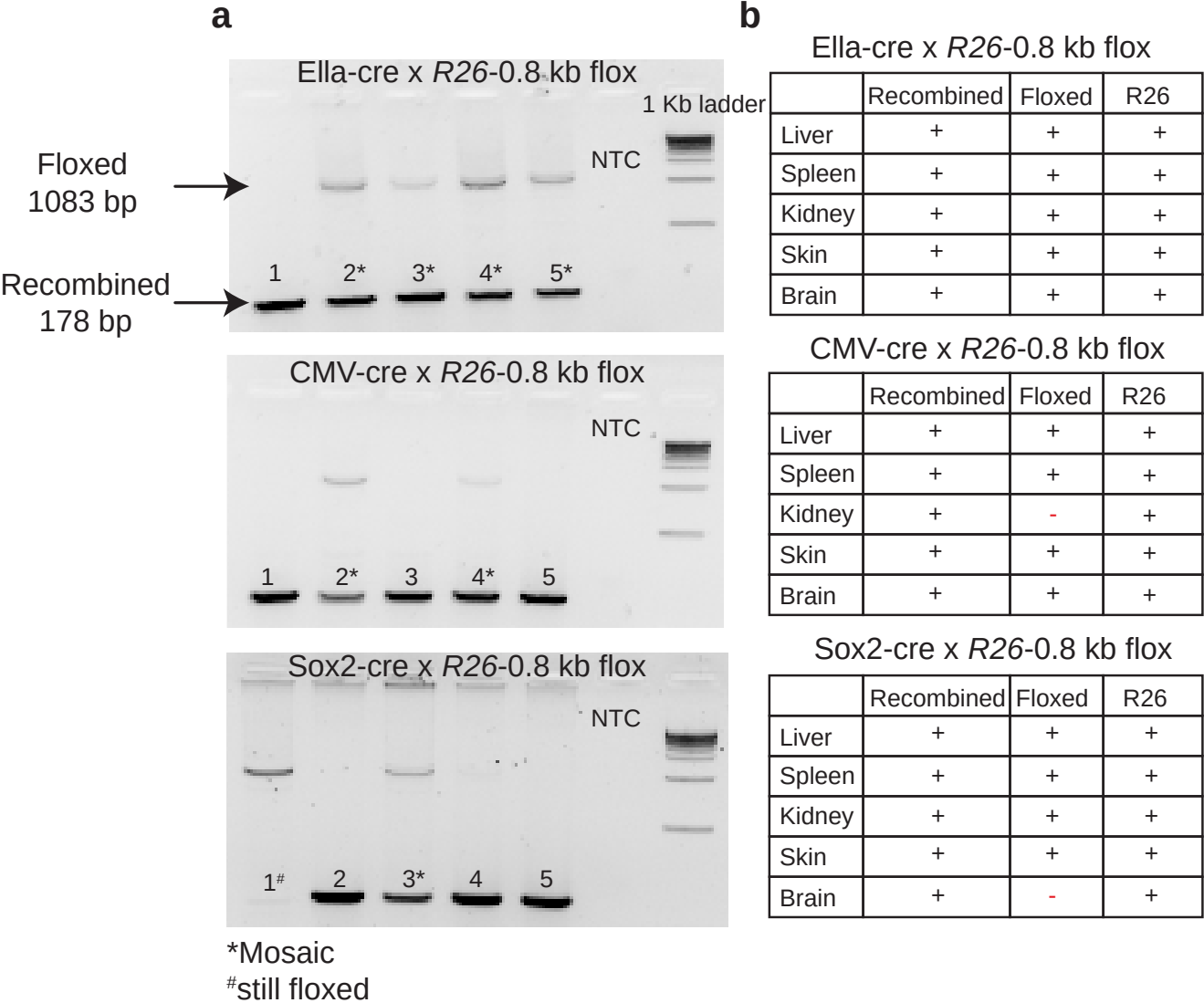
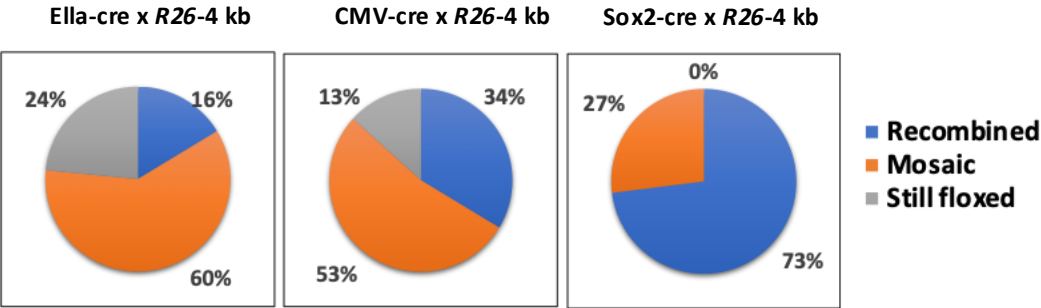
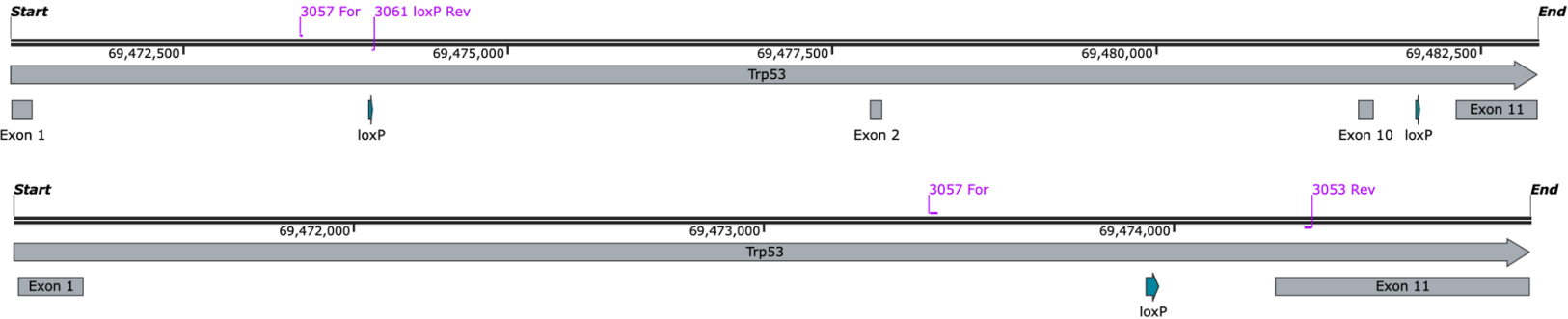
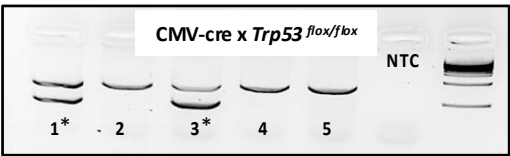
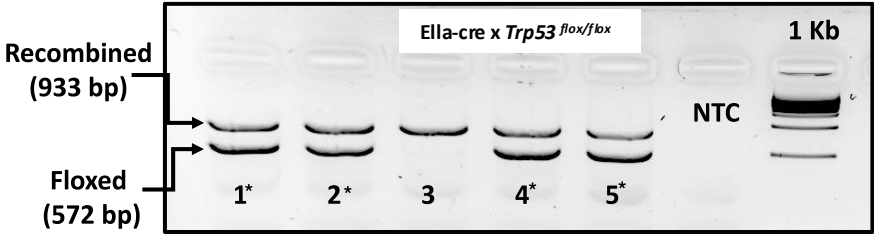
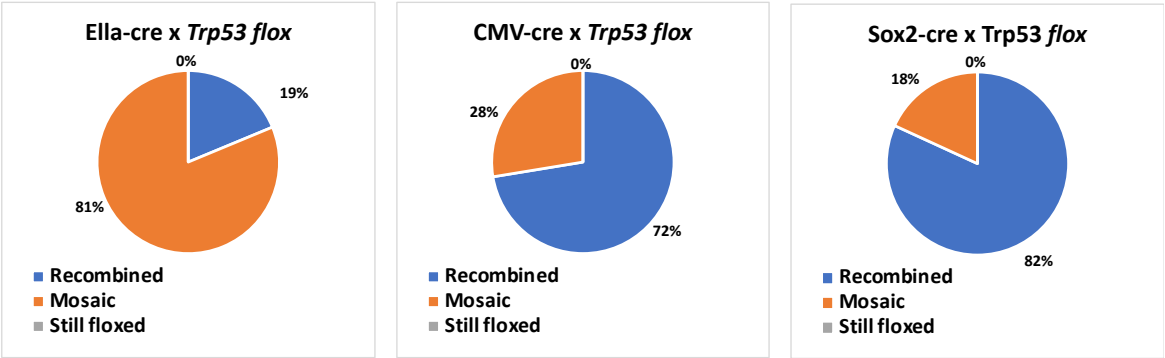


Fig. S2

a



b

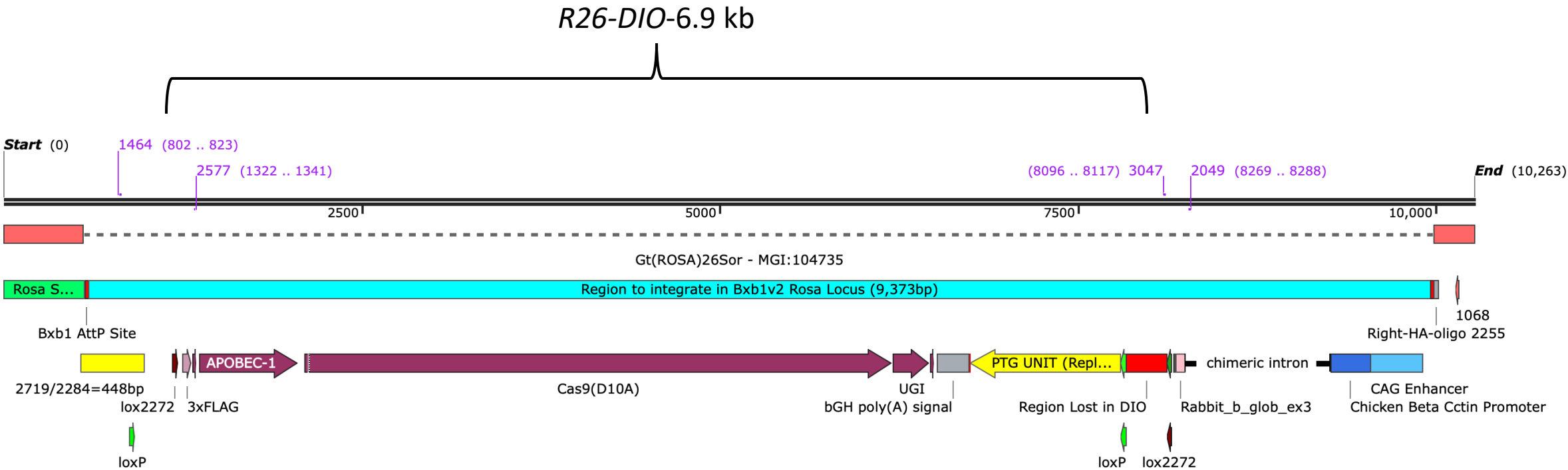


Trp53 floxed allele

Trp53 recombined allele

Fig. S3

a



b

Ella-cre X <i>R26-DIO</i> F1 offspring	
Strain name	n
Rosa-DIO-1.1 kb	25
Rosa-DIO-2.2 kb	42
Rosa-DIO-5.3 kb	26
Rosa-DIO-6.9 kb	24
Rosa-DIO-8.1 kb	32

CMV-cre X <i>R26-DIO</i> F1 offspring	
Strain name	n
Rosa-DIO-1.1 kb	26
Rosa-DIO-2.2 kb	29
Rosa-DIO-5.3 kb	35
Rosa-DIO-6.9 kb	23
Rosa-DIO-8.1 kb	28

Sox2-cre X <i>R26-DIO</i> F1 offspring	
Strain name	n
Rosa-DIO-1.1 kb	43
Rosa-DIO-2.2 kb	14
Rosa-DIO-5.3 kb	26
Rosa-DIO-6.9 kb	24
Rosa-DIO-8.1 kb	55

Fig. S4

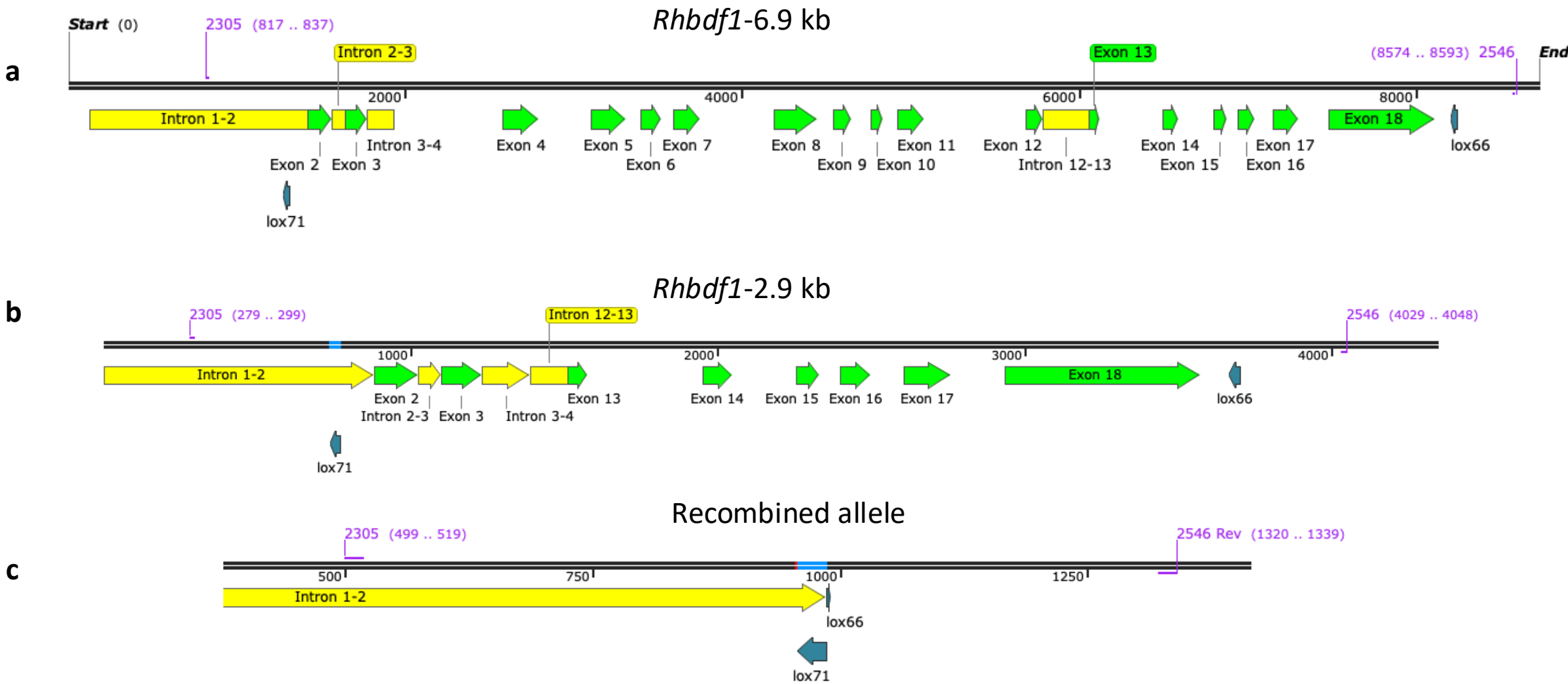
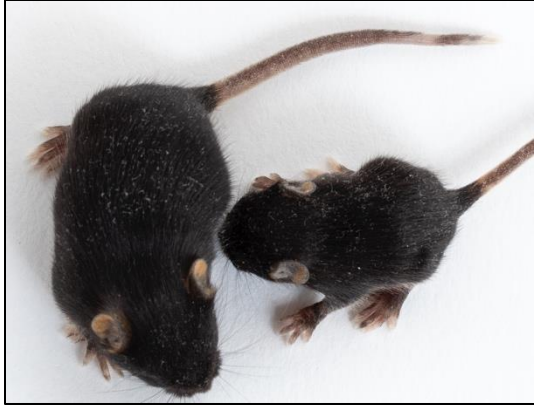


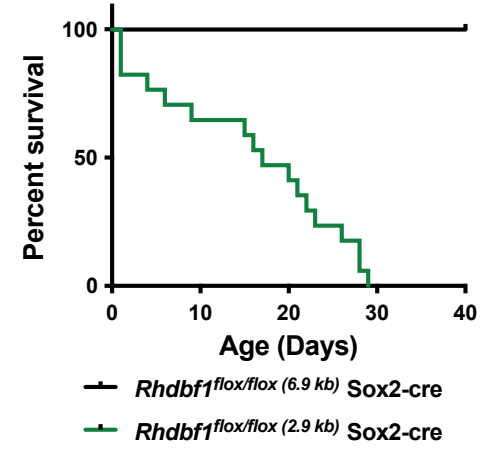
Fig. S5

a

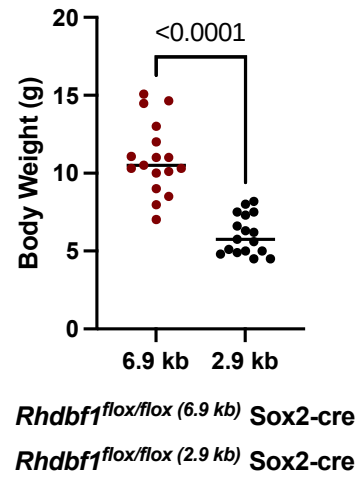


Rhdbf1^{flox/flox} (6.9 kb) Sox2-cre *Rhdbf1*^{flox/flox} (2.9 kb) Sox2-cre

b



c



d

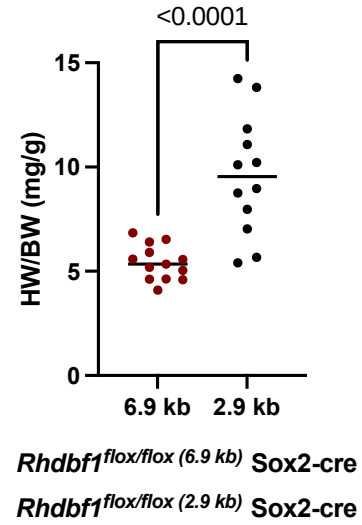


Fig. S6

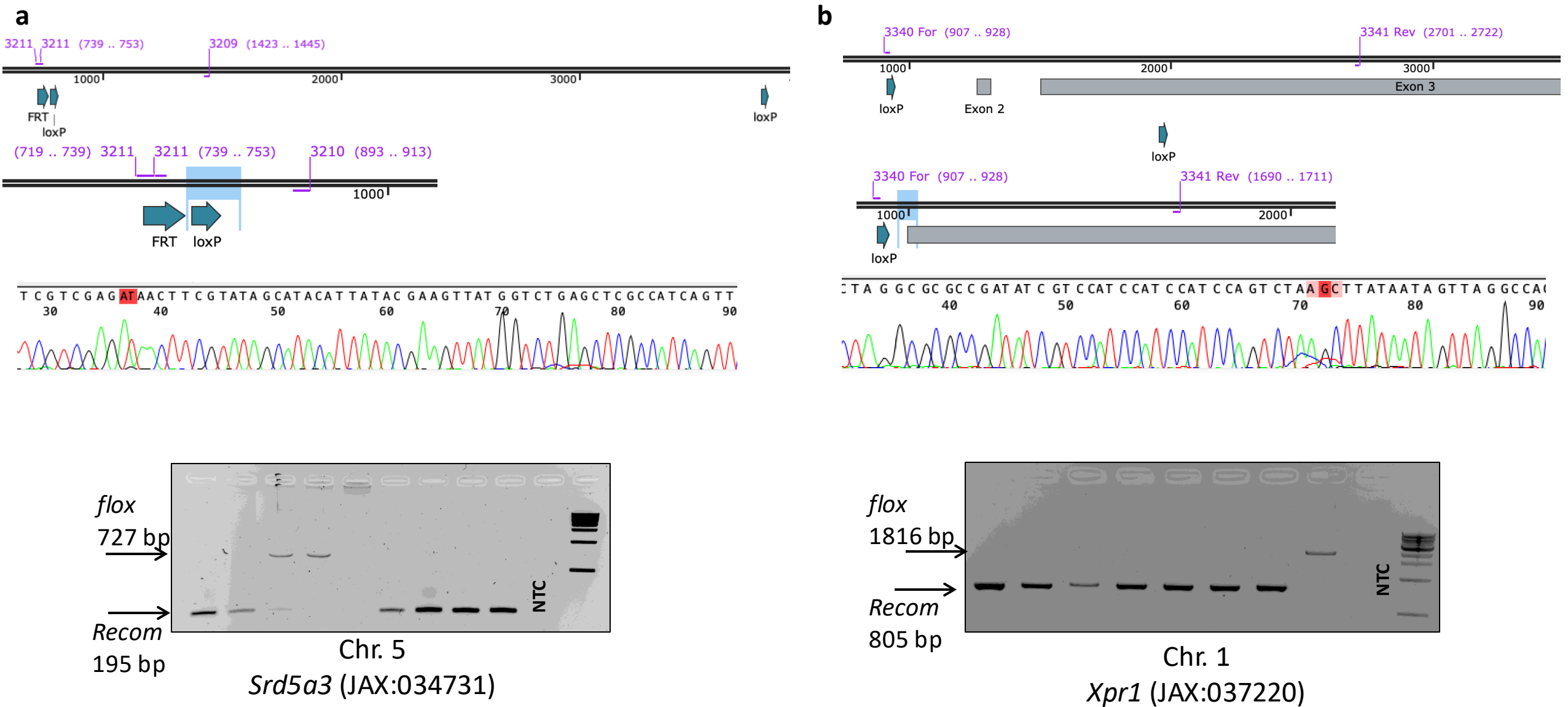
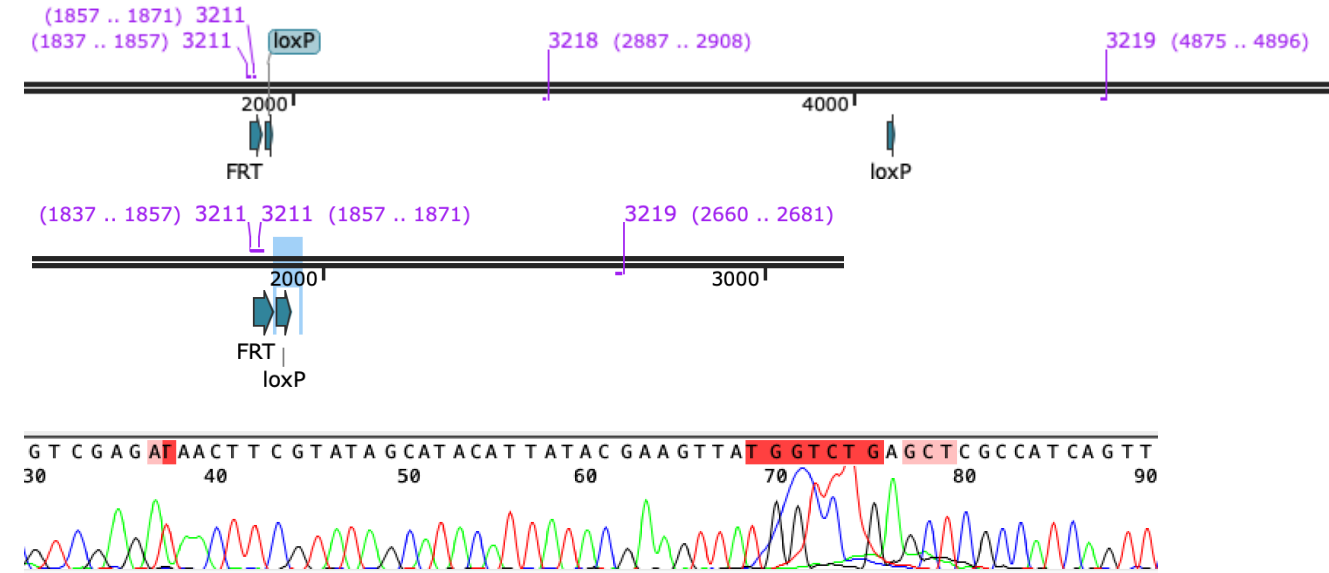


Fig. S7

a



b

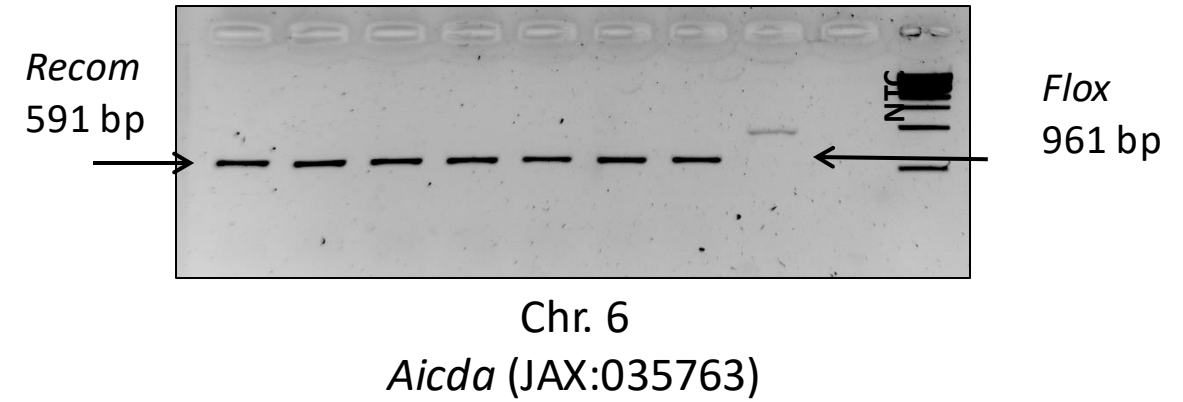
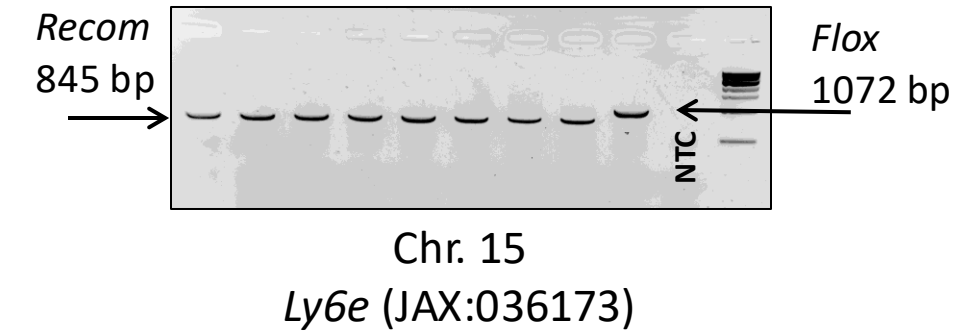
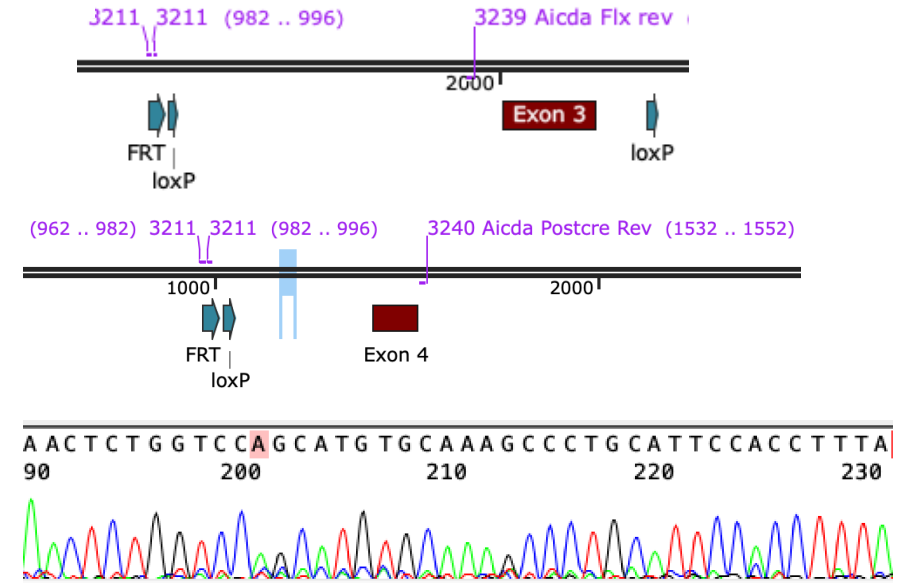


Fig. S8

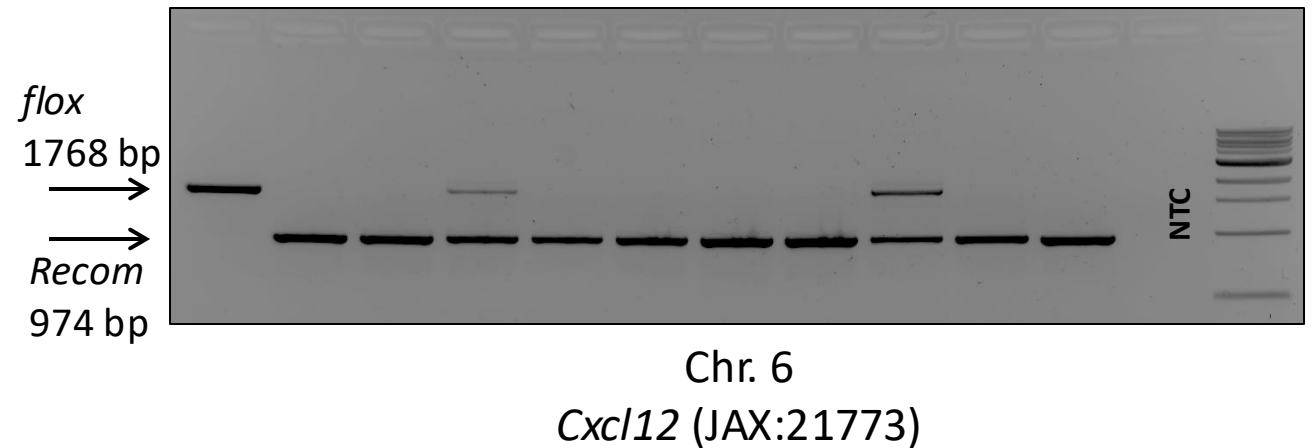
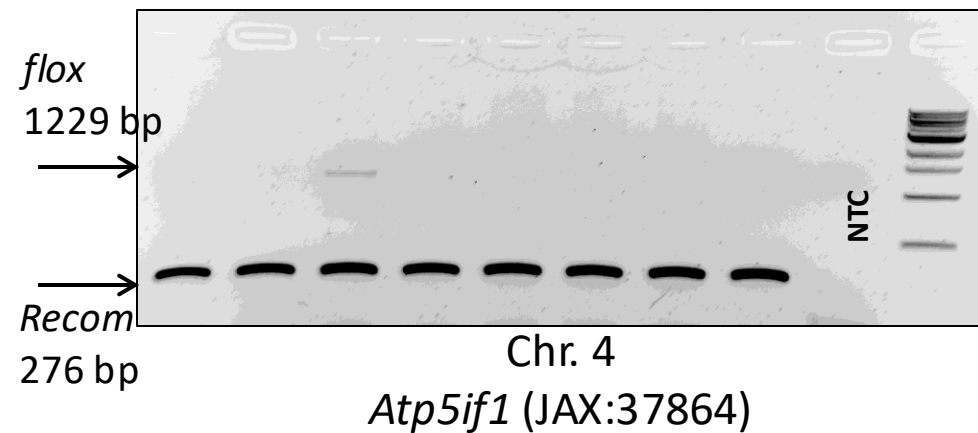
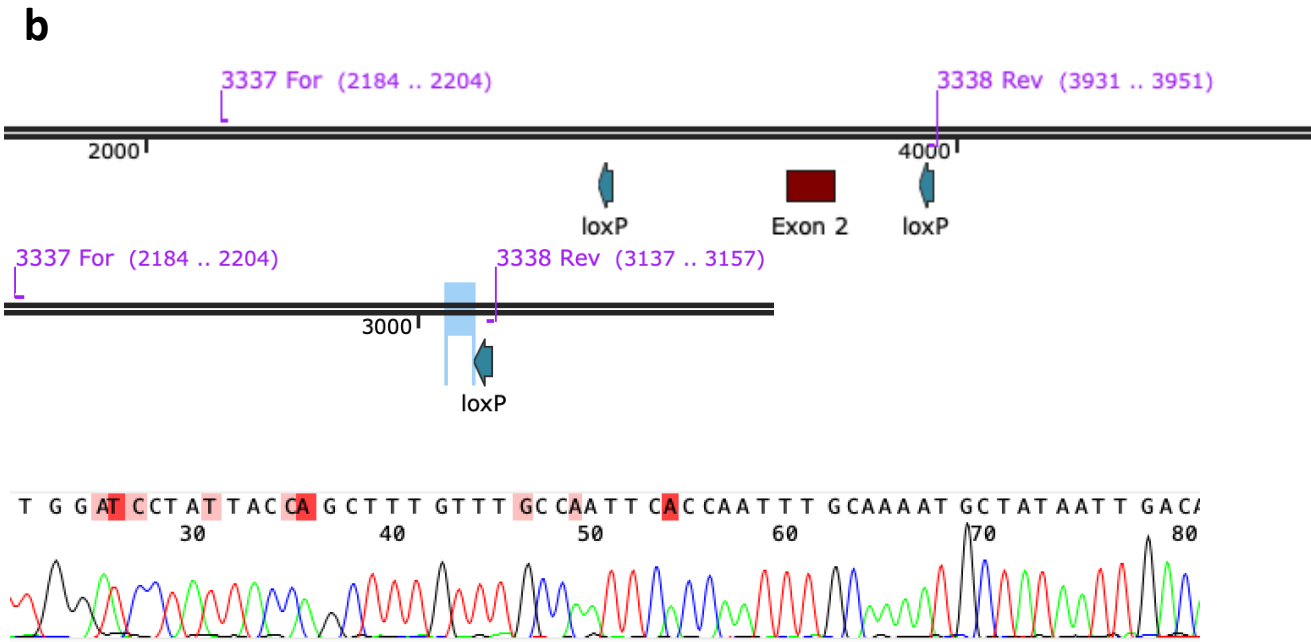
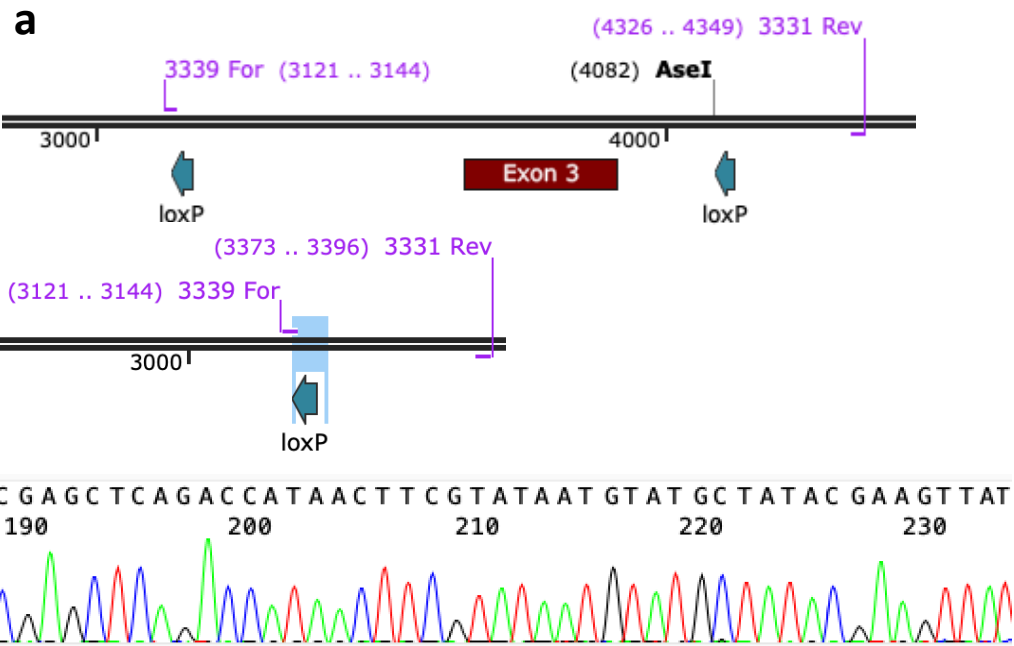
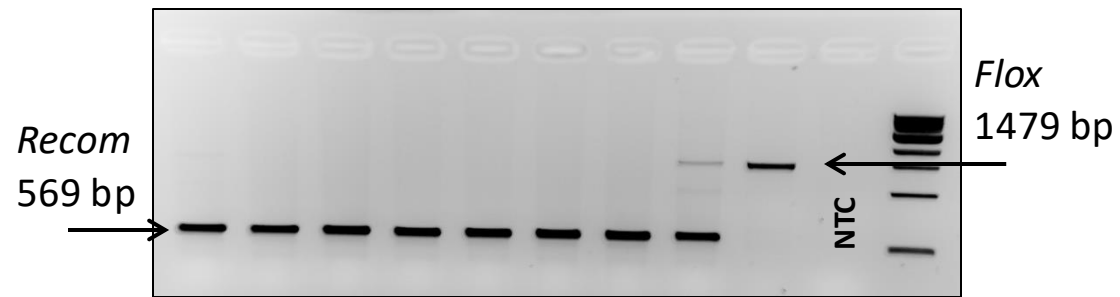
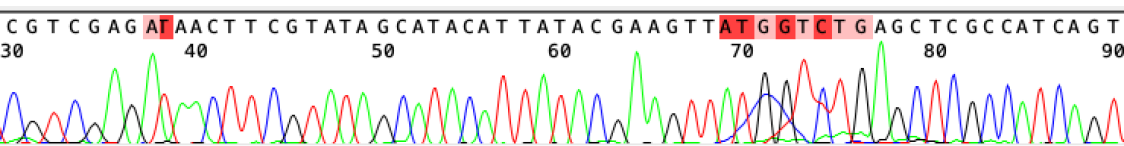
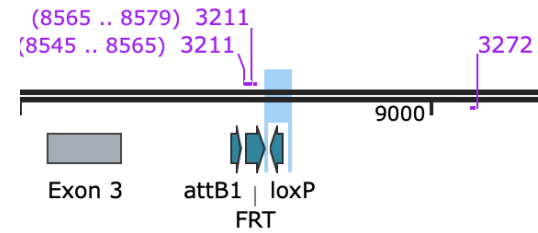
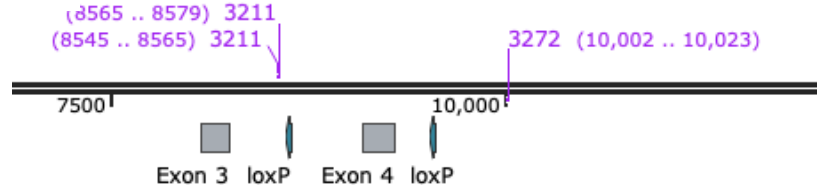


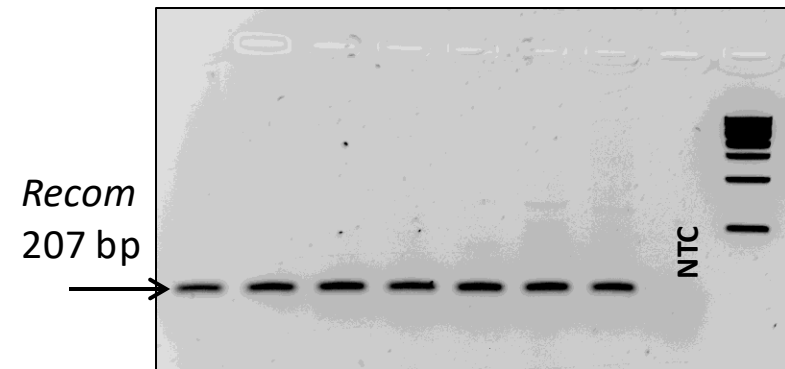
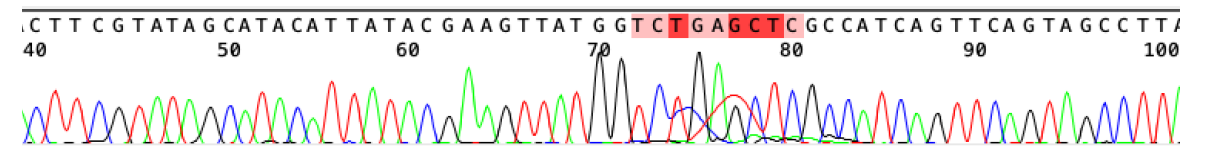
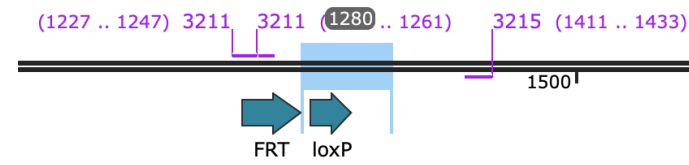
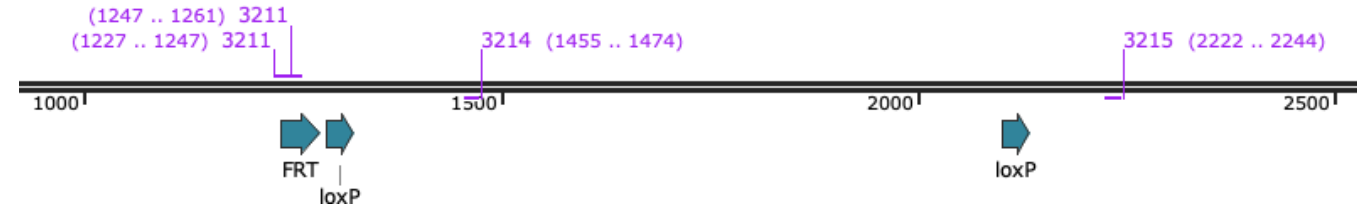
Fig. S9

a



Chr. 1
Mogat1 (JAX:37509)

b



Chr. 19
Cpt1a (JAX:32778)

Fig. S10

a

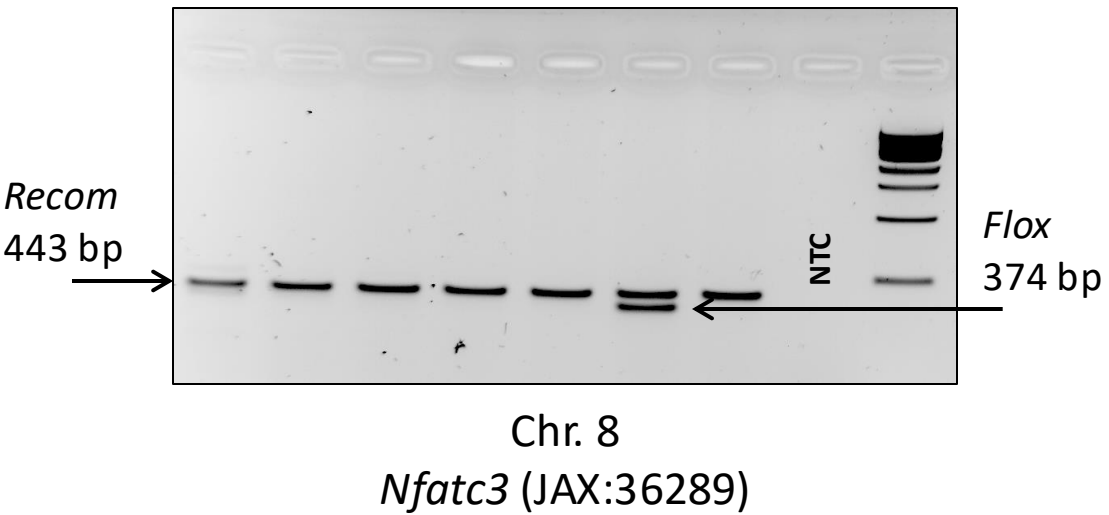
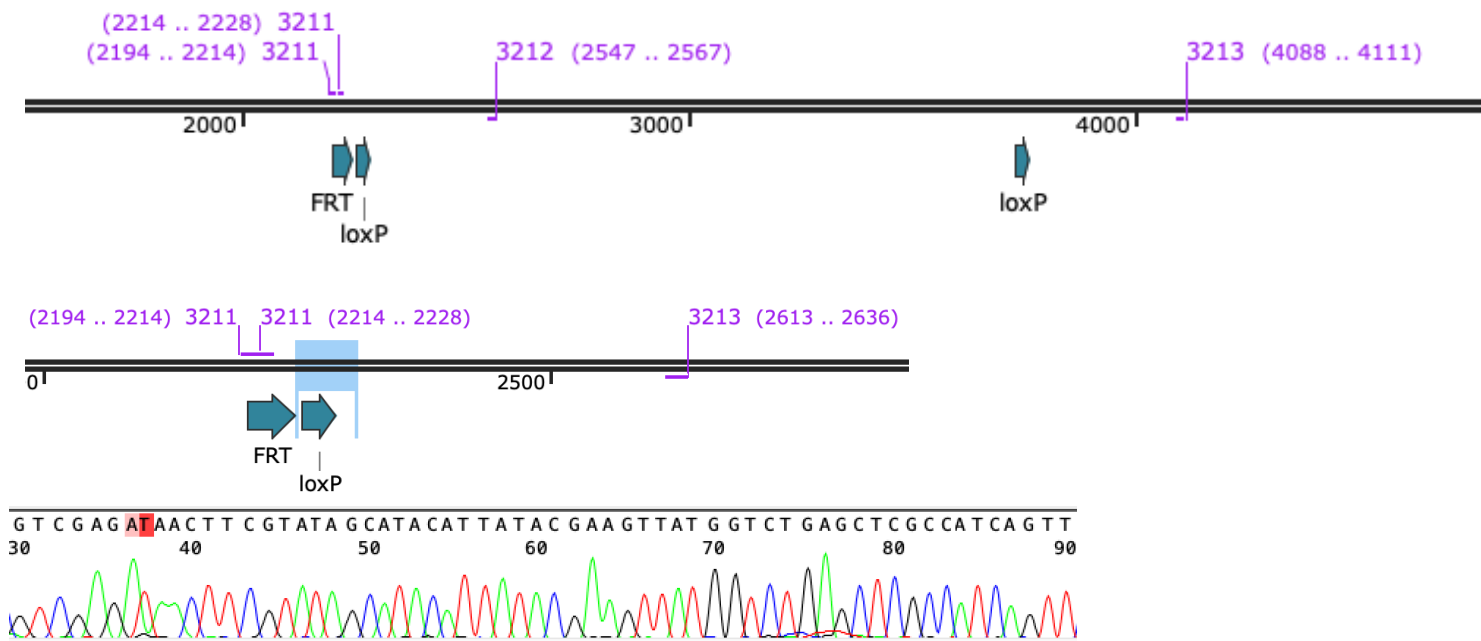
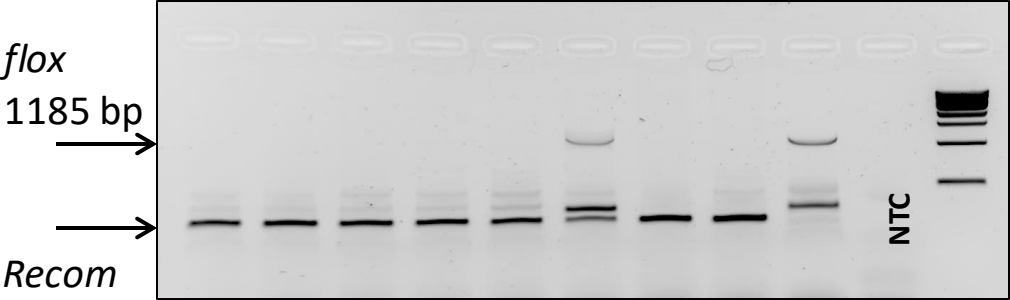
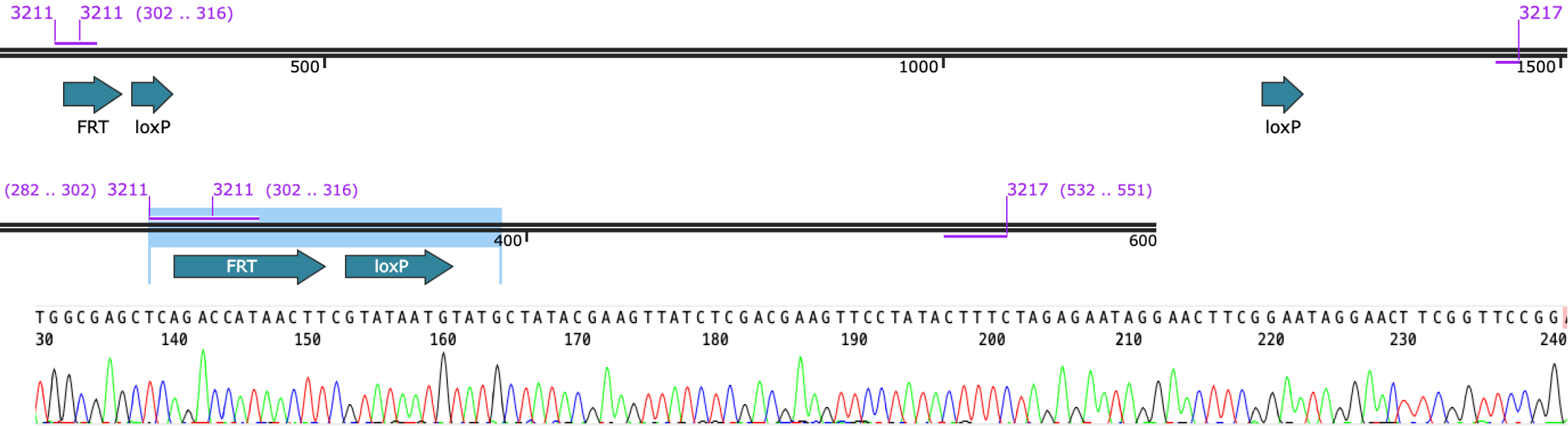


Fig. S11

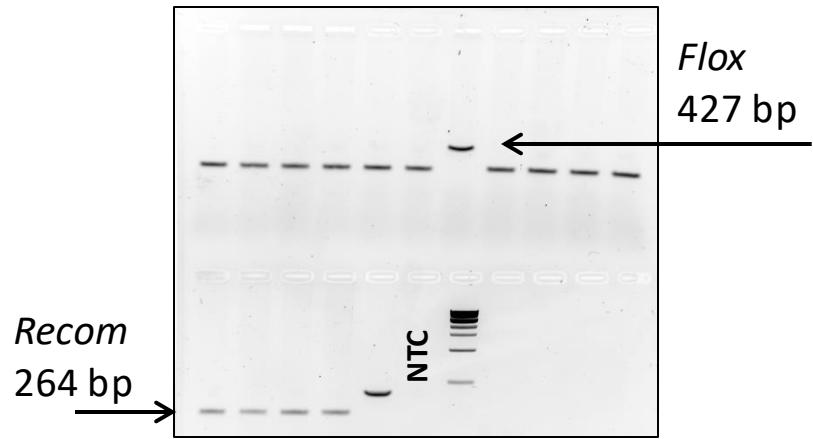
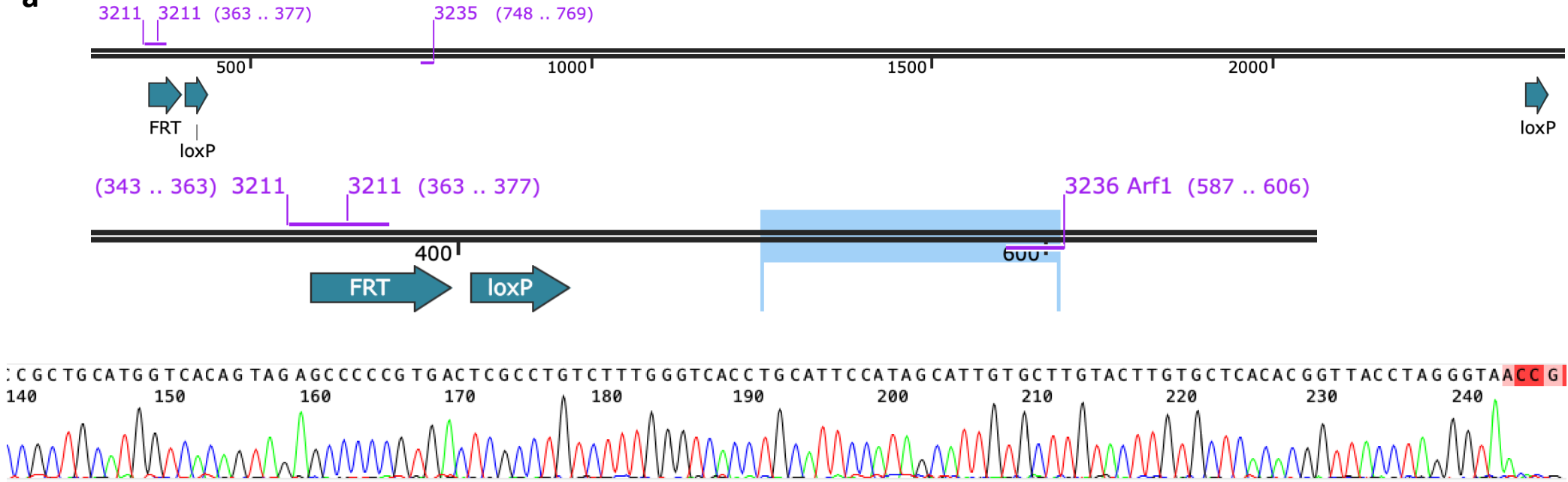
a



Chr.7
Ldha (JAX:30112)

Fig. S12

a



Chr. 11
Arf1 (JAX:37055)

Fig. S13

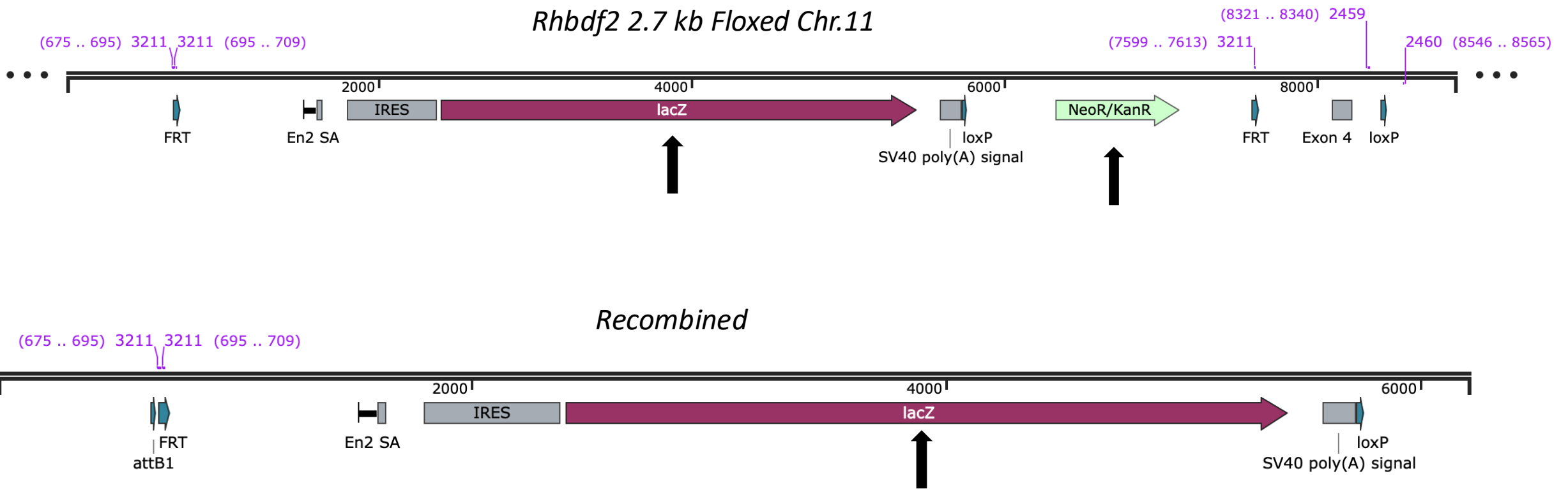
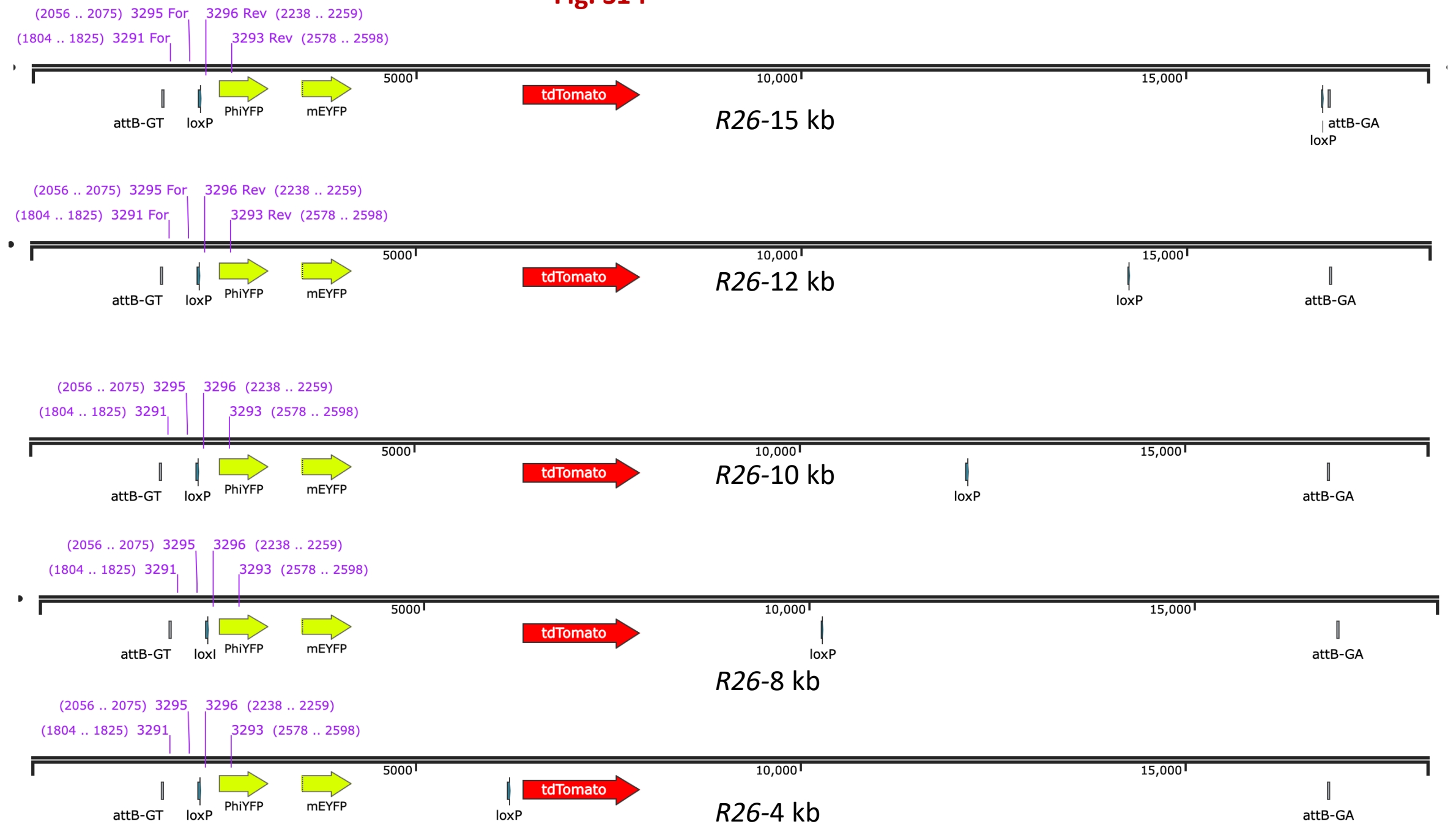


Fig. S14



Supplemental figure legends

Fig. S1. a) PCR of tail genomic DNA using forward and reverse primers reveals distinct banding patterns for recombined and mosaic (recombined + still floxed) genotypes. Recombined mice exhibit a single band (178 bp PCR product), mosaic mice display both a recombined band (178 bp) and a floxed band (1083 bp), while still-floxed mice show only the floxed band (1083 bp). A no template control (NTC) was included. The New England Biolabs 1 kb DNA Ladder served as a molecular marker. b) The DNA was isolated from the indicated tissues of mosaic F1 pups, and the genotype was determined using real-time PCR with specific probes targeting the recombined and floxed alleles (*Transnetyx*). An *R26* probe served as a positive control.

Fig. S2. a) Male homozygous *R26*-4 kb floxed mice were bred with female *Ella*-cre, *CMV*-cre, or *Sox2*-cre mice. The F1 offspring were then genotyped using PCR from tail DNA to identify floxed and recombined alleles. Offspring with complete recombination were categorized as recombined, those with no recombination as still floxed, and those with both recombined and floxed alleles as mosaic. b) Male homozygous *Trp53* floxed mice were bred with female *Ella*-cre, *CMV*-cre, or *Sox2*-cre mice. The F1 offspring were then genotyped using PCR from tail DNA to identify floxed and recombined alleles. Offspring with complete recombination were categorized as recombined, those with no recombination as still floxed, and those with both recombined and floxed alleles as mosaic. PCR of tail genomic DNA using forward and reverse primers reveals distinct banding patterns for recombined and mosaic genotypes. Recombined mice exhibit a single band (933 bp PCR product), mosaic mice display both a recombined band (933 bp) and a floxed band (572 bp). A no template control (NTC) was included. The New England Biolabs 1 kb DNA Ladder served as a molecular marker. Mosaic mice are indicated by an asterisk (*).

Fig. S3. a) A representative double-*floxed* inverse open reading frame (DIO) construct with wildtype loxP and mutant lox2272 sites. b) The number of offspring genotyped to assess the percentage of progeny that are recombined, mosaic, and not recombined is summarized in Supplementary Fig. 3b

Fig. S4. A detailed schematic representation of the *Rhdbf1* alleles, including the a) 6.9 kb, b) 2.9 kb, and c) the post-Cre recombined alleles.

Fig. S5. a) A representative image of two-week-old *Rhdbf1*^{flox/flox} (6.9 kb) Sox2-cre (left) and *Rhdbf1*^{flox/flox} (2.9 kb) Sox2-cre (right) female pups. b) Kaplan–Meier survival analyses show that the lifespan of *Rhdbf1*^{flox/flox} (2.9 kb) Sox2-cre mice is shorter than that of *Rhdbf1*^{flox/flox} (6.9 kb) Sox2-cre mice. c) Comparison of body weights between *Rhdbf1*^{flox/flox} (6.9 kb) Sox2-cre and *Rhdbf1*^{flox/flox} (2.9 kb) Sox2-cre mice of both sexes. d) The comparison of the heart-to-body-weight ratio between *Rhdbf1*^{flox/flox} (6.9 kb) Sox2-cre and *Rhdbf1*^{flox/flox} (2.9 kb) Sox2-cre mice of both sexes shows a significantly higher ratio *Rhdbf1*^{flox/flox} (2.9 kb) Sox2-cre , indicating cardiac hypertrophy in *Rhdbf1*^{flox/flox} (2.9 kb) Sox2-cre mice.

Figs. S6-12. PCR of tail genomic DNA using forward and reverse primers reveals distinct banding patterns for recombined and mosaic (recombined + still floxed) genotypes. Recombined mice exhibit a single band, mosaic mice display both a recombined band and a floxed band, while still-floxed mice show only the floxed band. A no template control (NTC) was included. The New England Biolabs 1 kb DNA Ladder served as a molecular marker. All recombined PCR products were sequenced to verify complete recombination.

Fig. S13. Probe-based assays targeting the LacZ and NeoR/kanR regions were used to genotype floxed and recombined alleles. Notably, a similar probe-based approach can be applied to genotyping floxed and recombined KOMP strains.

Fig. S14. We synthesized five different constructs based on a common 18.5 kb prototype, all exhibiting similar sequence patterns. Each of these constructs contains attB-GT and attB-GA sites that are designed to pair with the corresponding attP-GT and attP-GA sites located in the *R26* locus of the *Bxb1.v2* mouse strain for Bxb1-mediated RMCE (Fig. 4a). In addition, every construct is equipped with a *loxP* site positioned at the 5' end, accompanied by another *loxP* site at the 3' side, and notably lacks a promoter. The distinguishing feature of these constructs is the varying lengths of the internal segment, which spans 4, 8, 10, 12, or 15 kb, measured between the two *loxP* sites.