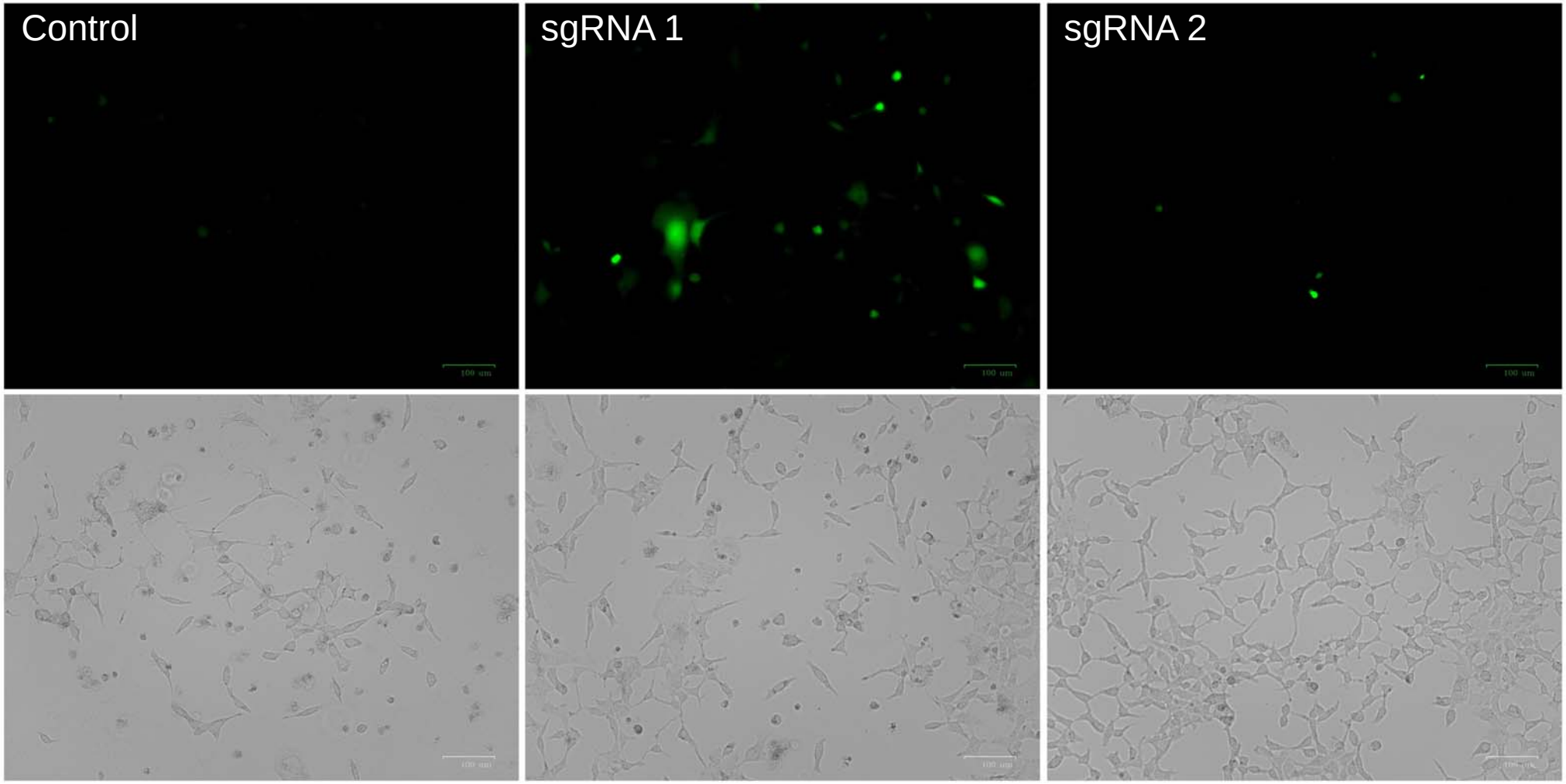
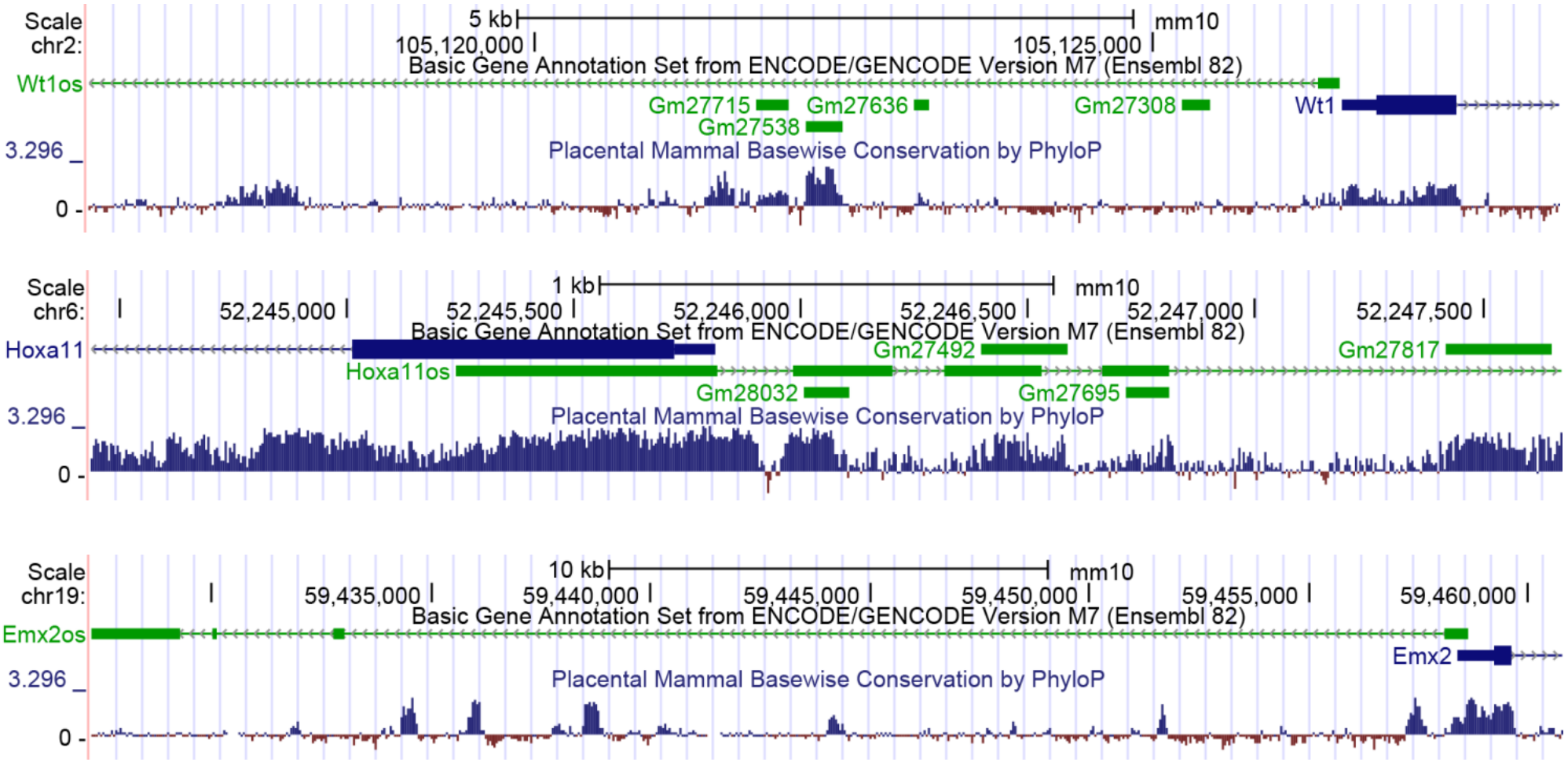
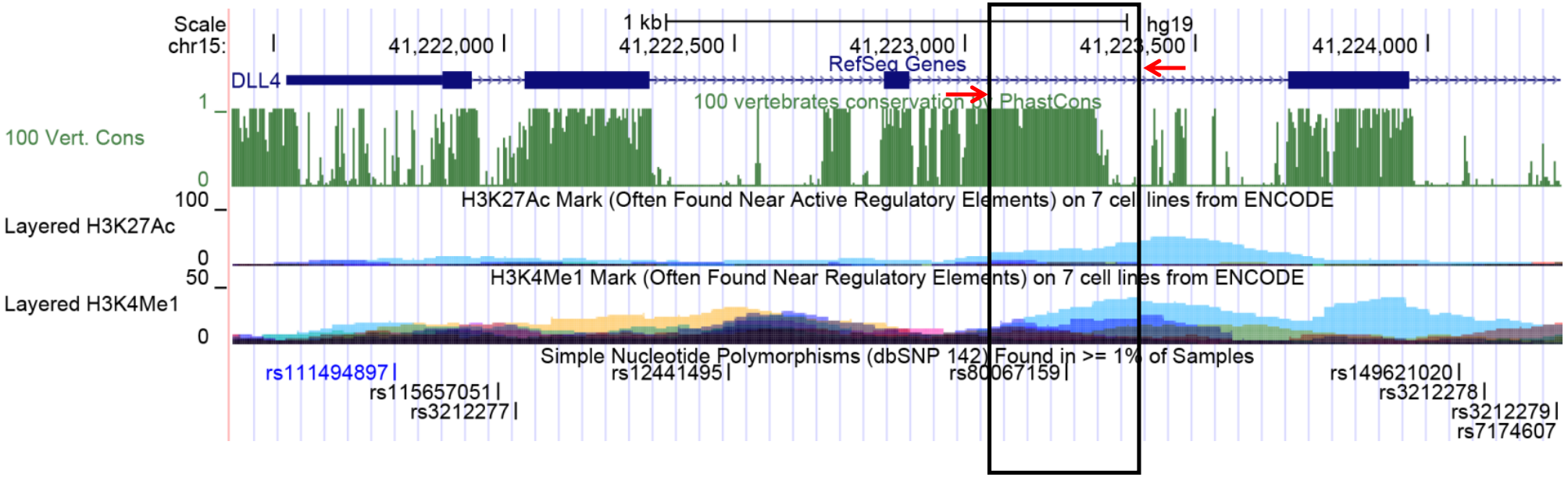


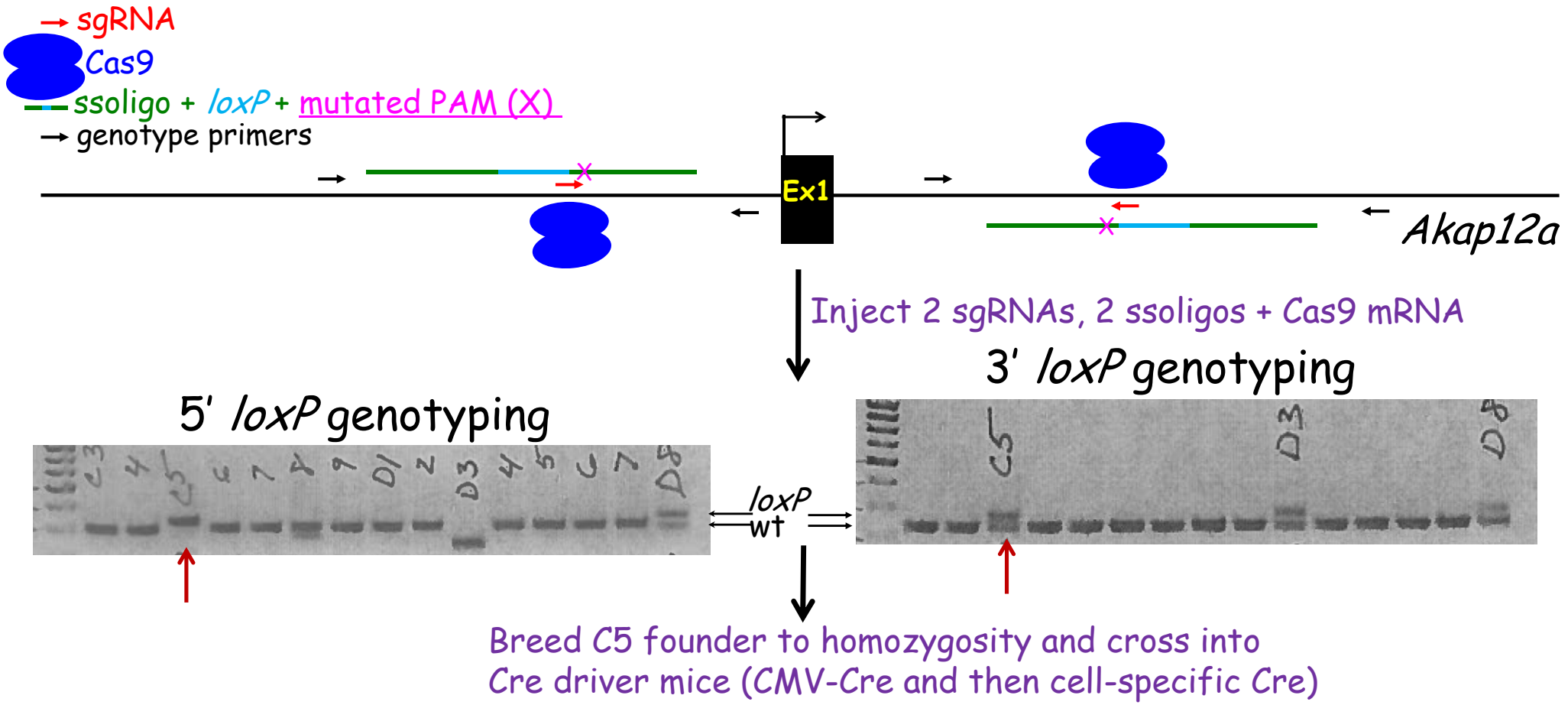
Supplemental Figure I. **Testing sgRNAs in vitro**. Immunofluorescence microscopy for GFP in HEK-293 cells co-transfected with empty EGxxFP split reporter plasmid and sgRNA (Control) or EGxxFP slit reporter carrying one of two target sequences and either sgRNA 1 or sgRNA 2. Variable GFP signal presumably reflects level of sgRNA activity. Please note, the empty control EGxxFP reporter occasionally exhibits some GFP fluorescence. This “background” signal is uniformly reduced across all conditions. Below are phase contrast images of the same fields as above. Note that results from this assay and Surveyor may not reflect actual activity in a mouse zygote. Some labs find it useful to test sgRNAs in early embryos; this however, is not practical for the vast majority of labs.



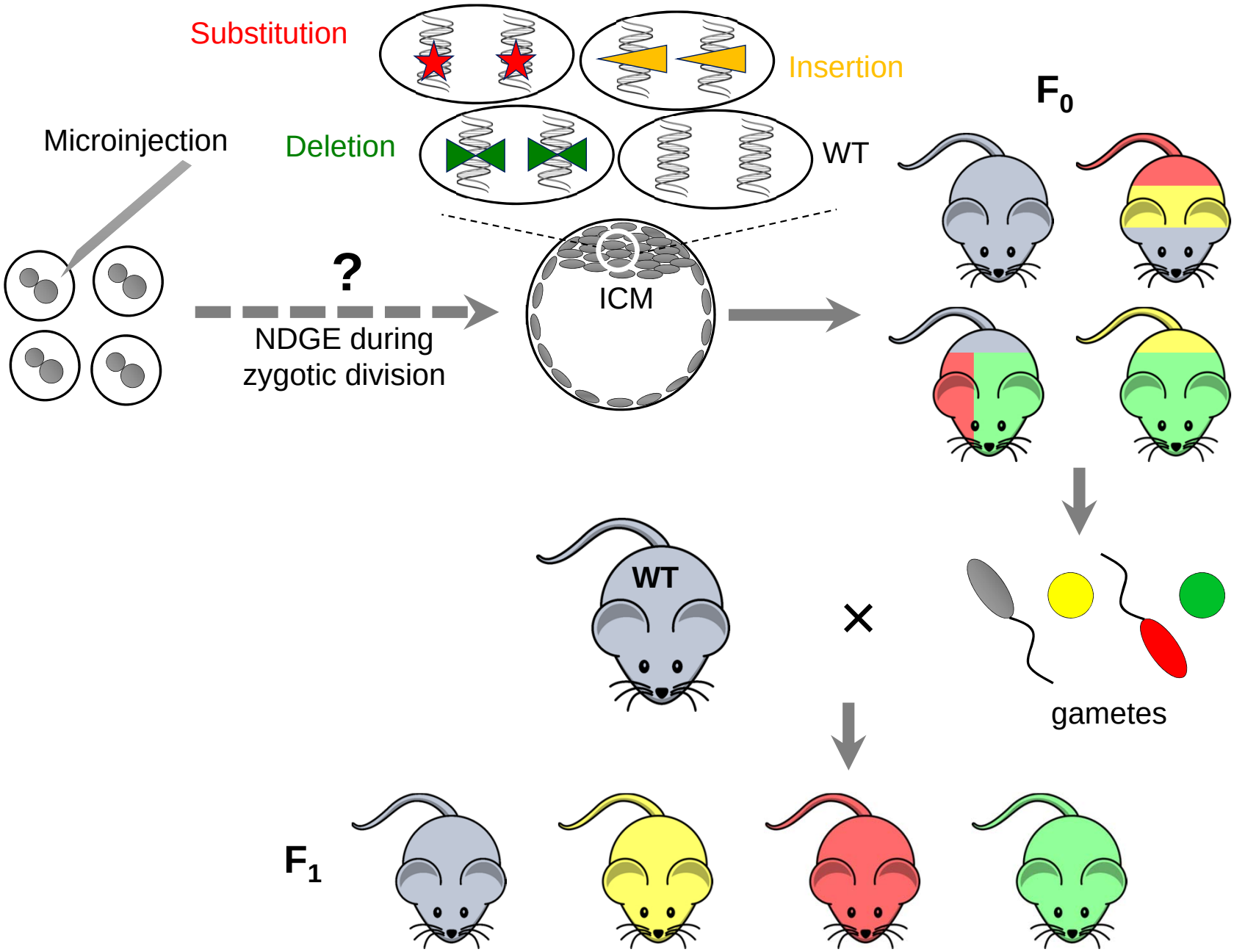
Supplemental Figure II. Sense-antisense gene pairs. UCSC Genome Browser tracks depicting several protein-coding genes (blue labeled Wt1, Hoxa11, and Emx2) that overlap or are in close proximity to adjacent 5' antisense long non-coding RNA genes (labeled in green). These three examples are representative of a large number of similarly arrayed coding/non-coding gene pairs in the mouse and human genomes. Targeting the first exon of either coding or non-coding gene with 2c CRISPR or 3c CRISPR using *loxP* sequences could complicate interpretation of phenotypes. Thus alternative strategies are needed to differentiate effects of inactivating a protein-coding (or non-coding) gene from the neighboring gene. The suffix "os" stands for opposite strand.



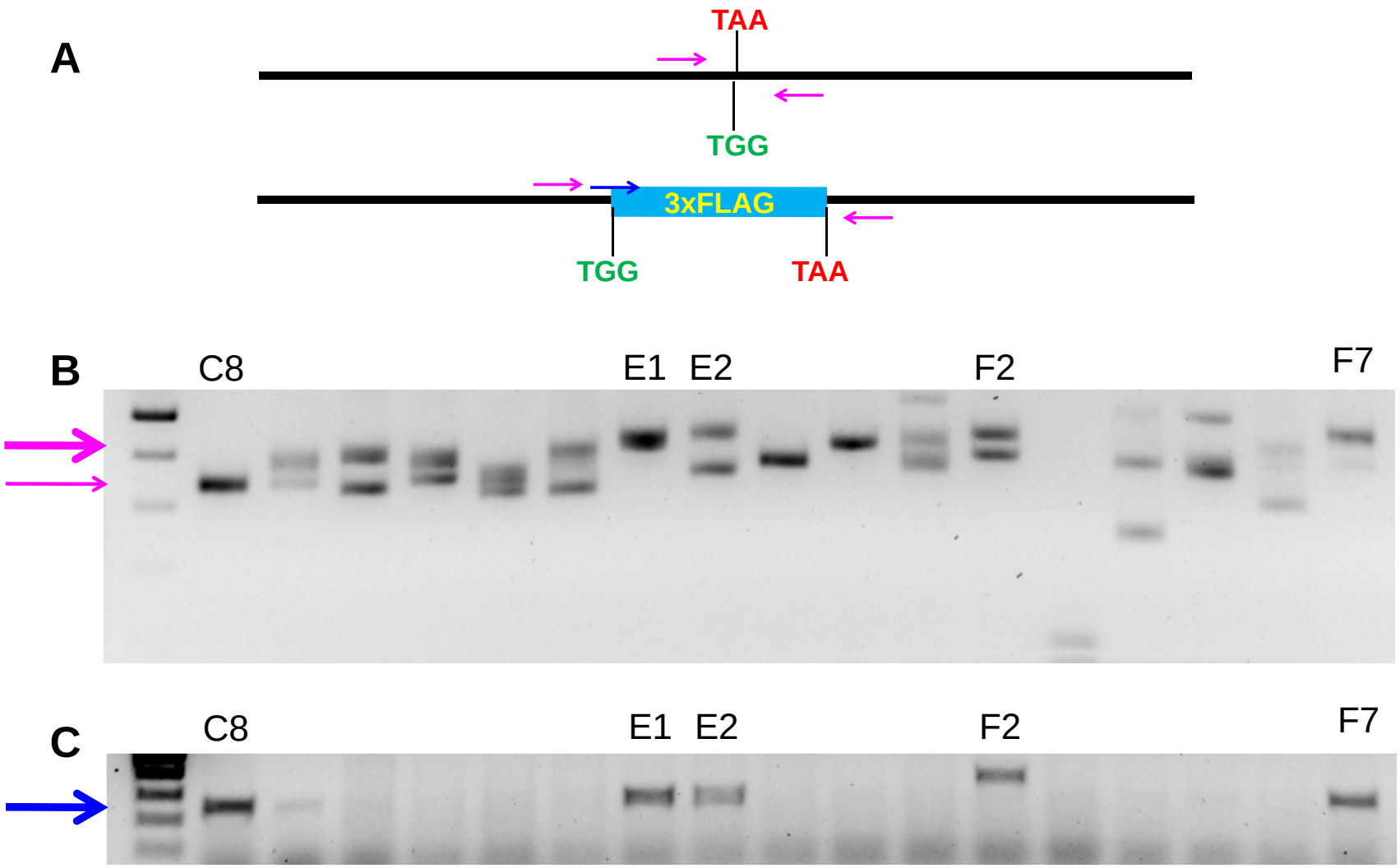
Supplemental Figure III. **Strategy for deleting enhancer using ENCODE data**. UCSC Genome Browser tracks showing the human *DLL4* gene, an endothelial restricted gene, with a highly conserved intron 3 region containing an experimentally-defined enhancer (black rectangle) that would be an excellent target for 2c CRISPR-mediated deletion in the orthologous position of the mouse genome. ENCODE track data below intron 3 depict chromatin marks (H3K27Ac and H3K4Me1) supportive of an enhancer. The teal color in both chromatin mark tracks reflects the binding activity of each chromatin factor in HUVEC, consistent with the EC-restricted expression of *DLL4*. Small red arrows at top represent hypothetical positions for sgRNAs, the remote position of which from exon-intron boundaries would not likely have an effect on splicing of the *DLL4* gene. Note presence of common SNP within enhancer region at bottom. There are thousands of similar genome snapshots that can be generated to assist investigators in the design of 2c CRISPR-mediated deletion of enhancer regions in the mouse genome as a first approximation of important regulatory sites (and variants therein) controlling target gene expression.



Supplemental Figure IV. **3c CRISPR targeting of the *Akap12a* locus for integrating *loxP* sequences**. Schematic of 5' *Akap12a* locus and two sgRNAs (red arrows) with attending Cas9 protein (blue) and HDR template containing centrally-positioned *loxP* site (green-teal lines). These components were injected into mouse zygotes for the generation of several founder pups that were genotyped with primers (black arrows) flanking the 5' and 3' *loxP* sequences. Founder C5 (vertical red arrows) had bi-allelic targeting of the 5' *loxP* site and mono-allelic targeting of the 3' *loxP* site (confirmed by Sanger sequencing). Breeding these mice to homozygosity followed by crossing into the CMV-Cre mouse allowed us to confirm expected excision as determined by Sanger sequencing. This was the only founder (of 40) that yielded both *loxP* sites on same allele, though the 5' *loxP* site had a single base deletion. See text for more details and compare with genotyping strategies in Supplemental Figures VIa and VII. Note, the field is changing rapidly and this successful targeting appears to be a rare event; most attempts using the above approach yield only one *loxP* site integrated and/or show mutations in the *loxP* sequence. Consequently, other approaches are emerging such as sequential targeting of each *loxP* site in two rounds of microinjection (i.e., screening for correctly targeted 5' *loxP* site followed by injection of zygotes carrying the 5' targeting event with ssoligo carrying the 3' *loxP* site). Some labs continue to use traditional ESC-based methods for making a conditional mouse.



Supplemental Figure V. Genetic mosaicism in CRISPR mice. The dotted line indicates the period leading up to day 3.5 (blastocyst stage with inner cell mass [ICM] indicated). The question mark indicates our uncertainty as to how long CRISPR components persist following initial injection. The mosaicism schematized in cells of the inner mass reflects a number of events, many of which are unpredictable, but clearly demonstrated in Supplemental Figures IV, VI, and VII, that reflect the ongoing editing following zygotic divisions, leading to mosaic genotypes in F_0 mice. Allelic segregation during gametogenesis allows one to screen F_1 pups with robust genotyping strategies (see text and figures below).



Supplemental Figure VI. **3c CRISPR targeting of *Myocd* locus with 3xFLAG epitope**. (**A**) Schematic of 3xFLAG knockin and genotyping strategy. The pink primers amplify a wild type band of ~330 bp (lower thin arrow in **B**) whereas the presence of 3xFLAG shifts the PCR product up to ~393 bp (upper thick arrow in **B**). Note diversity in alleles across founders with more than two alleles in some individual founder pups, consistent with mosaicism. (**C**) Higher specificity genotyping using a hybrid forward primer (blue in **A**) of genomic/3xFLAG sequence with reverse pink primer. Only 5 pups show positive results (blue arrow), though C8 would not have been predicted based on the genotyping results in panel **B**. See Supplemental Figure VID for sequencing data derived from careful excision of upper bands in E1, E2, F2, and F7) and TA cloning.

D

CAGCAG**TGG**TAAACACCCG crRNA

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Query  235  CTTAATCTGAATTCCCCTATGGATCTCCACTTACAGCAGTGGGACTATAAGGACCACGAC  294
Sbjct  151  CTTAATCTGAATTCCCCTATGGATCTCCACTTACAGCAGTGGGACTATAAGGACCACGAC  210

Query  295  GGAGACTACAAGGATCATGATATTGATTACAAAGACGATGACGATTAAACACCCGAGGTA  354
Sbjct  211  GGAGACTACAAGGATCATGATATTGATTACAAAGACGATGACGATTAAACACCCGAGGTA  270

Query  355  CAAGAGCTACGAGAGCTCAGTGGGAATTCAATGGAGGAAAGCACGATACCGGAAATGTGT  414
Sbjct  271  CAAGAGCTACGAGAGCTCAGTGGGAATTCAATGGAGGAAAGCACGATACCGGAAATGTGT  330
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E2

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Query  296  GAGACTACAAGGATCATGATATTGATTACAAAGACGATGACGATTAAACACCCGAGGTAC  355
Sbjct  202  GAGACTACAAGGATCATGATATTGATTACAAAGACGATGACGATTAAACACCCGAGGTAC  261

Query  356  AAGAGCTACGAGAGCTCAGTGGGAATTCAATGGAGGAAAGCACGATACCGGAAATGTGTG  415
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F2

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Sbjct  269  AAGAGCTACGAGAGCTCAGTGGGAATTCAATGGAGGAAAGCACGATACCGGAAATGTGTG  328
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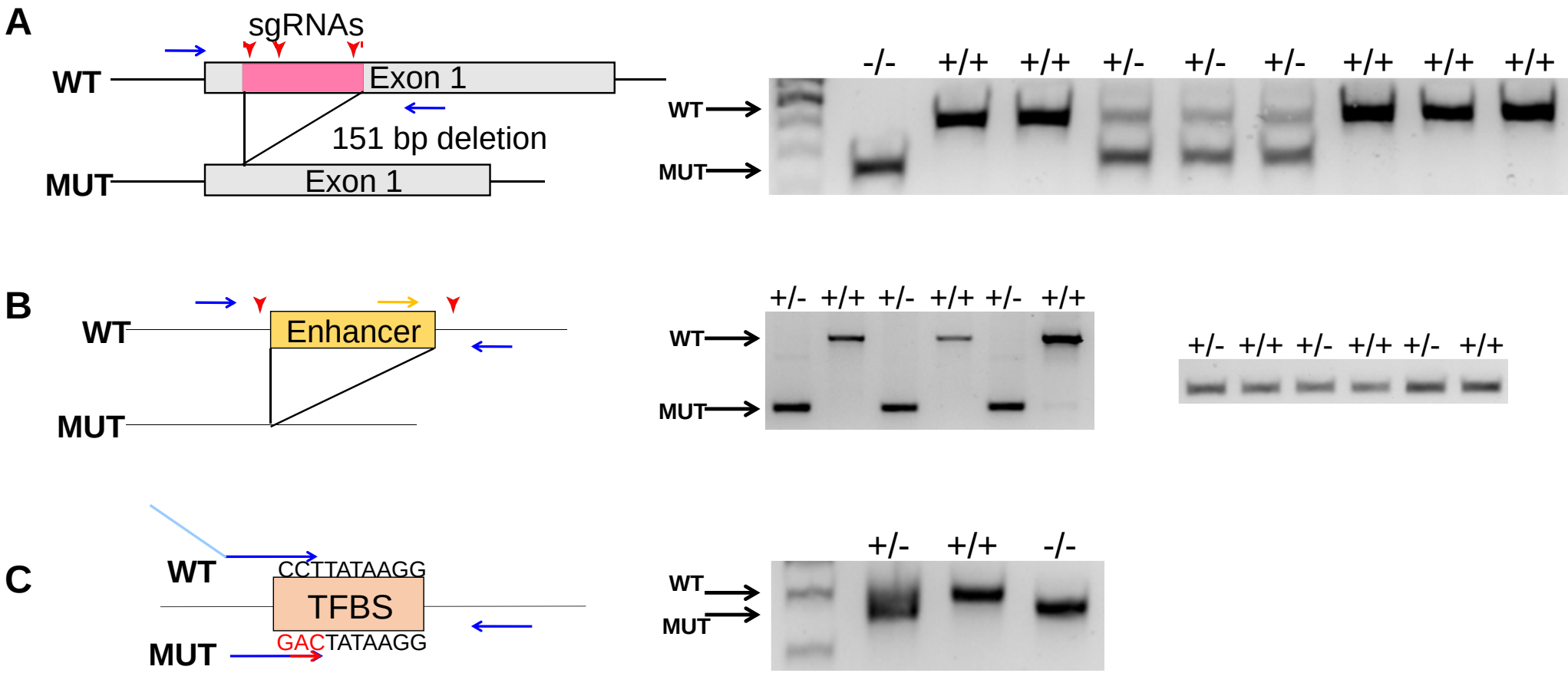
E1, F7

Supplemental Figure VI continued. (**D**) Sanger sequence analysis of four founders (E1, E2, F2, and F7) that genotyped positive with the 5' FLAG-specific forward primer (see Suppl. Fig VIC). The green boxed sequence is the last amino acid; the red boxed sequence is the stop codon and the intervening sequence between the TGG and TAA represents the 63nt 3xFLAG. The blue boxed sequence is the PAM. The crRNA of the sgRNA used for editing is shown at top. Although each of these founders yielded seemingly positive results in both genotyping assays (supportive of 3xFLAG), only E1, E2, and F7 showed correct sequence. Note that mutation in the PAM (AGG>AGC) is not seen in E2 and F2. We surmise this occurs via repair or incomplete recombination. Also note that in F2, while 3xFLAG recombined in, subsequent NHEJ (due to absence of PAM mutation?) resulted in deletions. In addition, there appears to be some discrepancy in the size of the PCR product of F2 and the sequencing result here. We emphasize the need to analyze at least two sequence-confirmed lines of mice to address this and other variables.



Supplemental Figure VII. **Strategies for genotyping CRISPR mice**. (**A**) 2c CRISPR deletion in exon 1 of a gene. Three sgRNAs (red arrowheads) used to create a 151 bp deletion resulting in a frameshift and premature termination codon (not shown). Primers (in blue) flanking the deletion yield the corresponding PCR products in the gel at right. (**B**) 2c CRISPR deletion of enhancer. Here, primers yield only one band in each lane of gel at left, requiring a separate PCR with a primer to the enhancer (orange) to verify the heterozygous pups in gel at right. It may be possible to combine all 3 primers and resolve the bands in a 2% agarose gel. (**C**) 3c CRISPR genotyping strategy depicting two forward primers of different length (30 nt) that specifically anneal to either wild type (upper) or mutant (lower) sequence (edits shown in red). Combining these forward primers with a common reverse primer yields expected bands in 2% gel at right. This assay requires optimization during the generation of CRISPR mice (see Supplemental Method 3 for more details). Note that we have successfully genotyped mice with only 1 base difference at the 3' end of the mutant primer. Schematics are not drawn to scale.