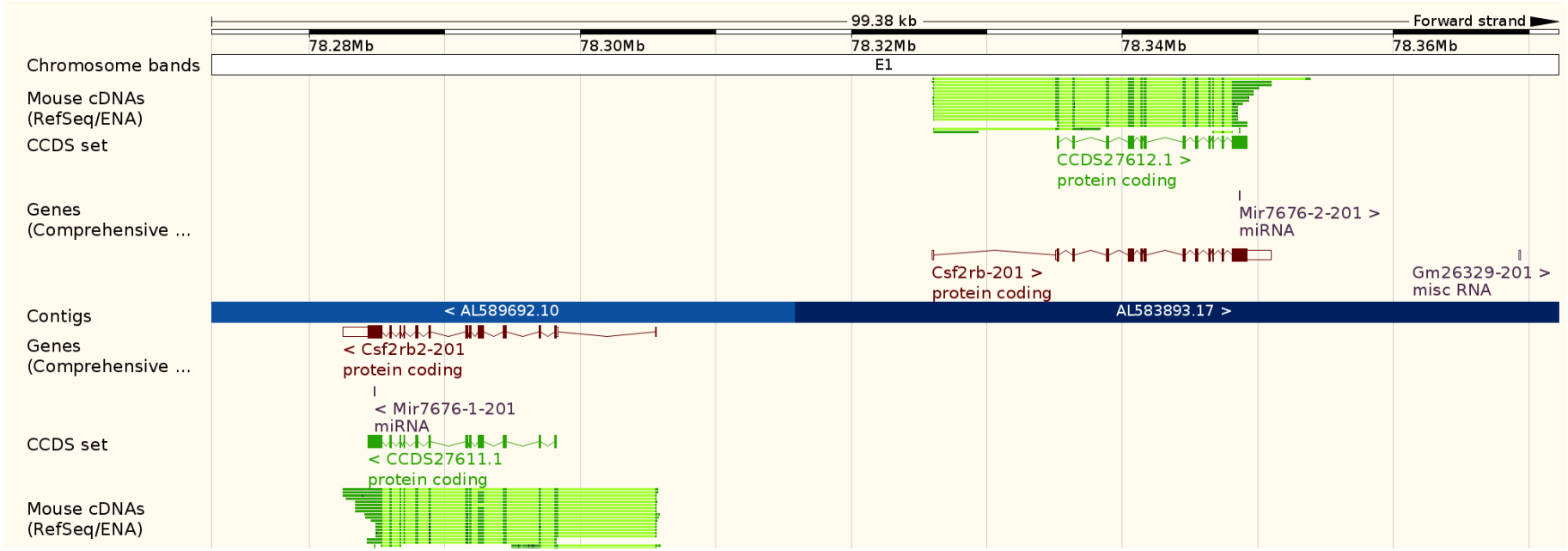
Project WM04/WM07

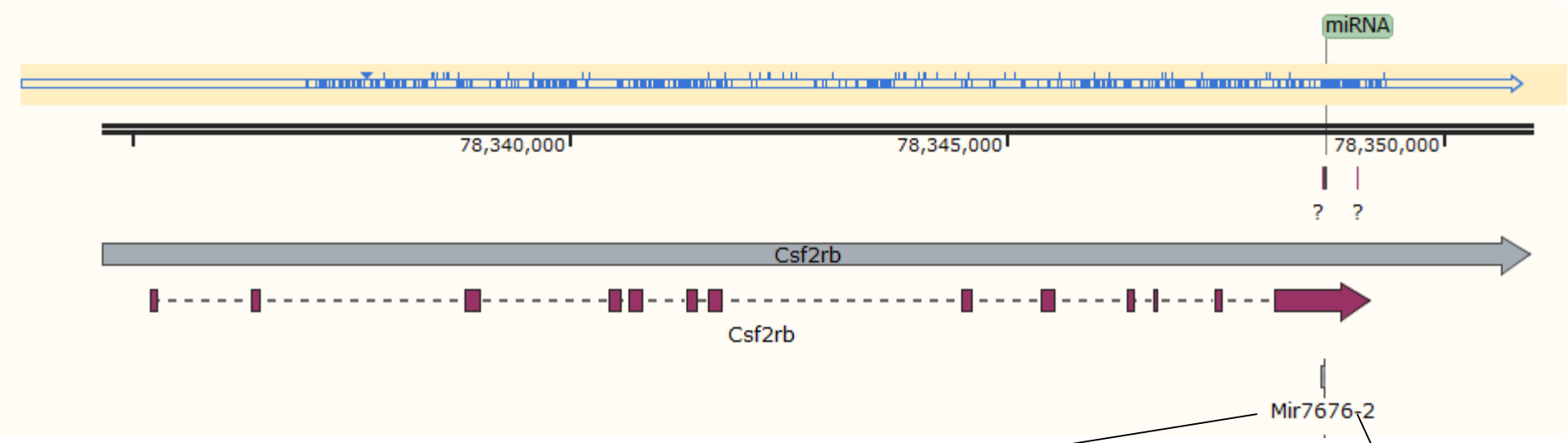
- CSF2RB S708A point mutation
- Werner Muller and Tony Segal (UCL)

Strategy and problems

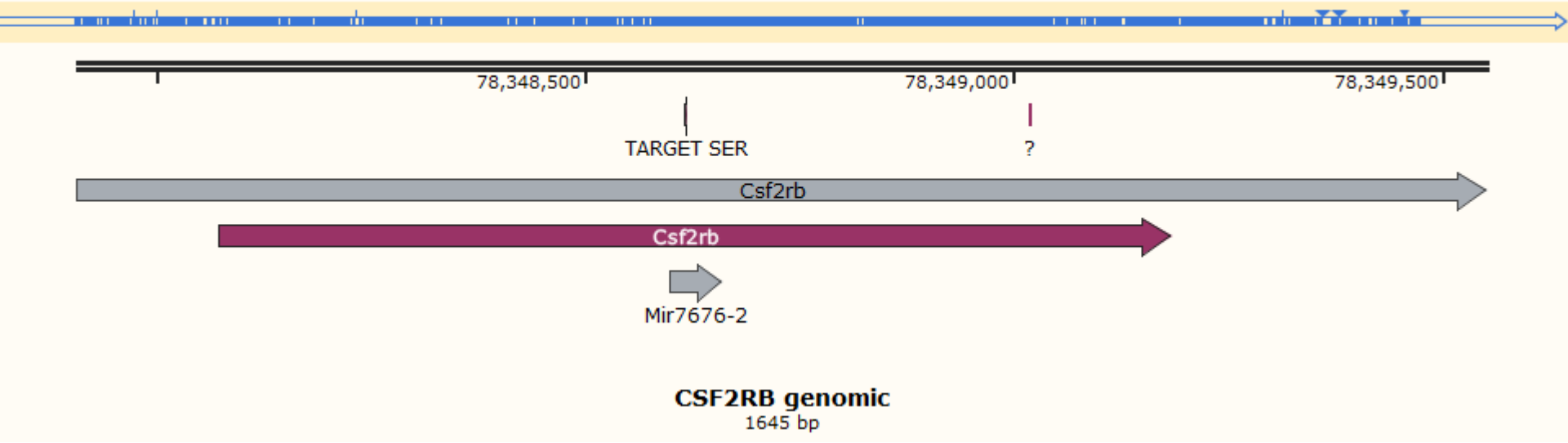
- CSF2RB S708A mutation identified in humans, replicate in a mouse model
- Problem – gene is duplicated in mouse (CSF2RB2)
- Both genes share significant homology, especially in 3’ region where S708 is found



Alignment of CSF2RB2 sequence with CSF2RB sequence, blue blocks indicate perfect alignment

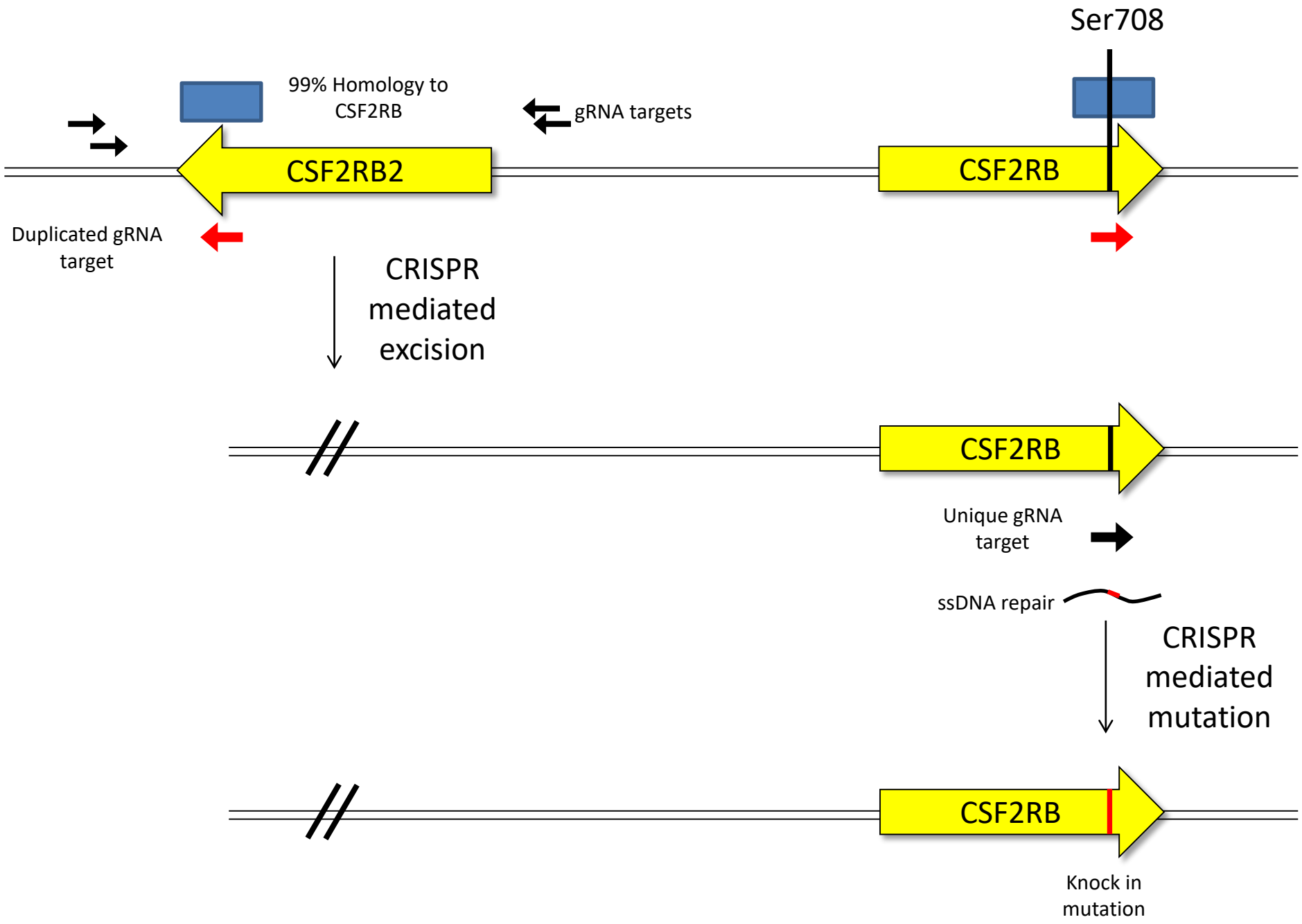


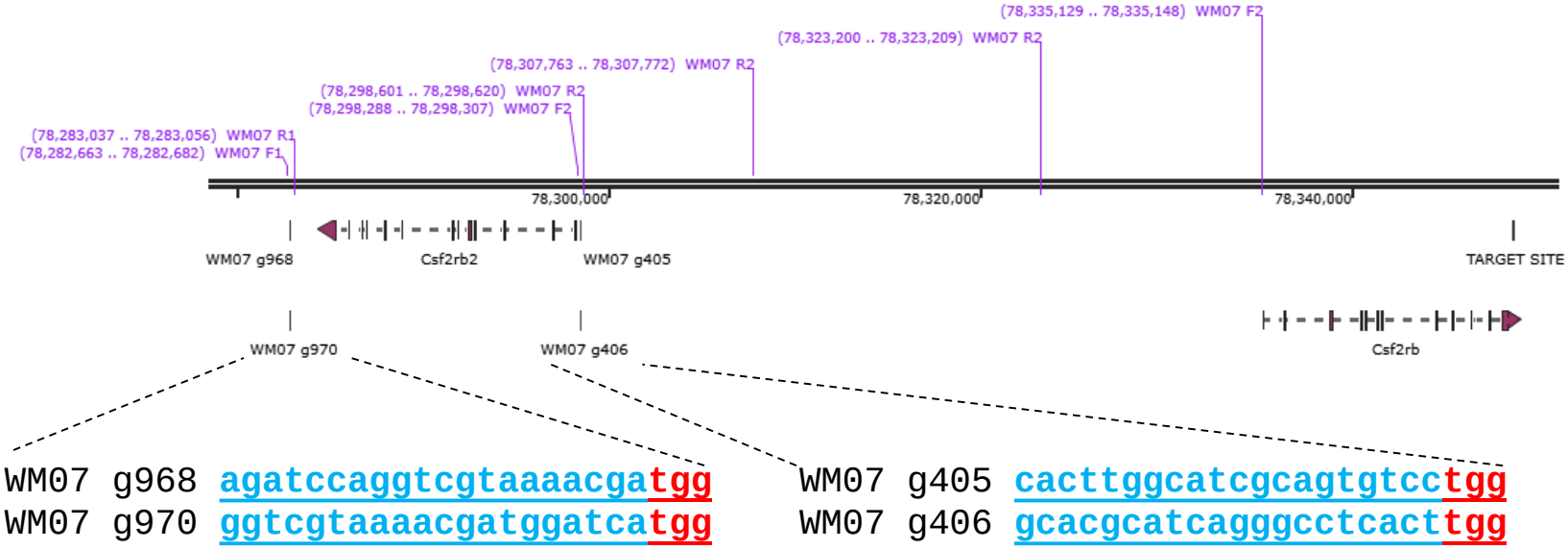
Note the near perfect alignment over Ser708



Strategy

- Use CRISPR-Cas9 to excise the duplicated gene to create CSF2RB2 null mice that have the duplicated target site removed
- Breed this line to homozygosity
- Use CRISPR-Cas9 to precisely generate the S708A point mutation in the CSF2RB gene on this background


Ser708
99% Homology to CSF2RB
gRNA targets
CSF2RB2
CSF2RB
Duplicated gRNA target
CRISPR mediated excision
CSF2RB
Unique gRNA target
ssDNA repair
CRISPR mediated mutation
CSF2RB
Knock in mutation

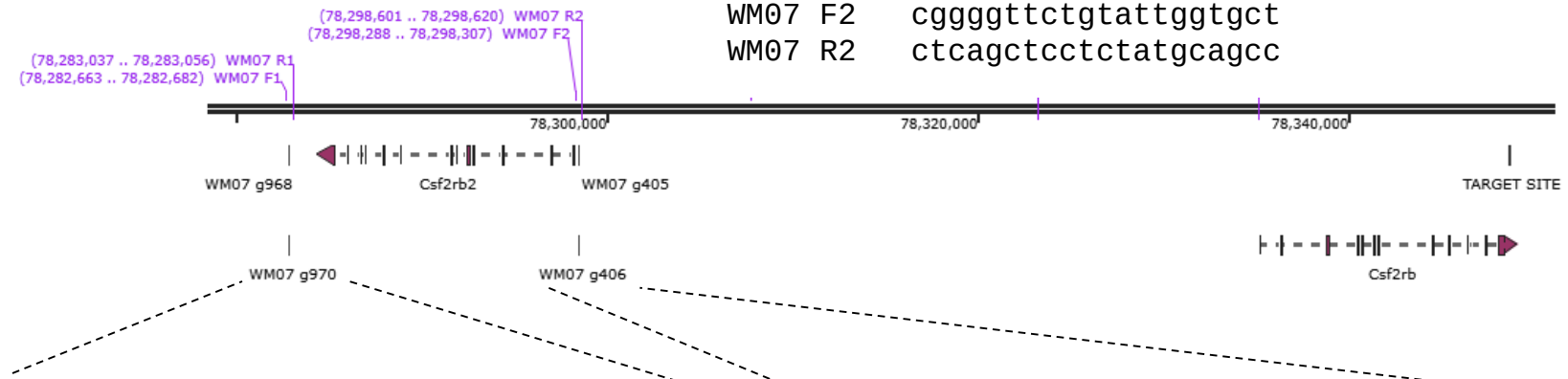


Guides were designed using Sanger website using stringent criteria for off target predictions (guides with mismatch (MM) of 0, 1 or 2 for elsewhere in the genome were discounted. MM3 were tolerated if predicted off targets were NOT exonic)

Alt-R crRNA (IDT) oligos for gRNAs ordered and resuspended in sterile, Rnase free Injection buffer (TrisHCl 1mM, pH 7.5, EDTA 0.1mM) and annealed with tracrRNA (IDT) by combining 2.5ug crRNA with 5ug tracrRNA and heating to 95oC. The mix was allowed to slowly cool to room temperature. After annealing the complex an equimolar amount was mixed with 1000ng Cas9 recombinant protein (NEB; final conc 20ng/ul) and incubated at RT for 15', before adding Cas9 mRNA (final conc; 20ng/ul) and the DNA repair template (final conc 10ng/ul) in a total injection buffer volume of 50ul. The injection mix was centrifuged for 10' at RT and the top 40ul removed to another tube for injection.
2 Days of microinjections using AltR crRNA:tracrRNA:Cas9 complex (20ng/ul; 20ng/ul; 20ng/ul respectively), Cas9 mRNA (20ng/ul), and DNA (ds or ss) HDR template (10ng/ul)

This mix was pronuclear microinjected into one-day single cell mouse embryos. Zygotes were cultured overnight and the resulting 2 cell embryos surgically implanted into the oviduct of day 0.5 post-coitum pseudopregnant mice.
After birth and weaning genomic DNA extracted using Sigma redextract-n-amp tissue pcr kit and used to genotype pups

```
WM07 F1   aacattgccactgcaacacc
WM07 R1   gggccatatttgcagcagac
WM07 F2   cggggttctgtattggtgct
WM07 R2   ctcagctcctctatgcagcc
```



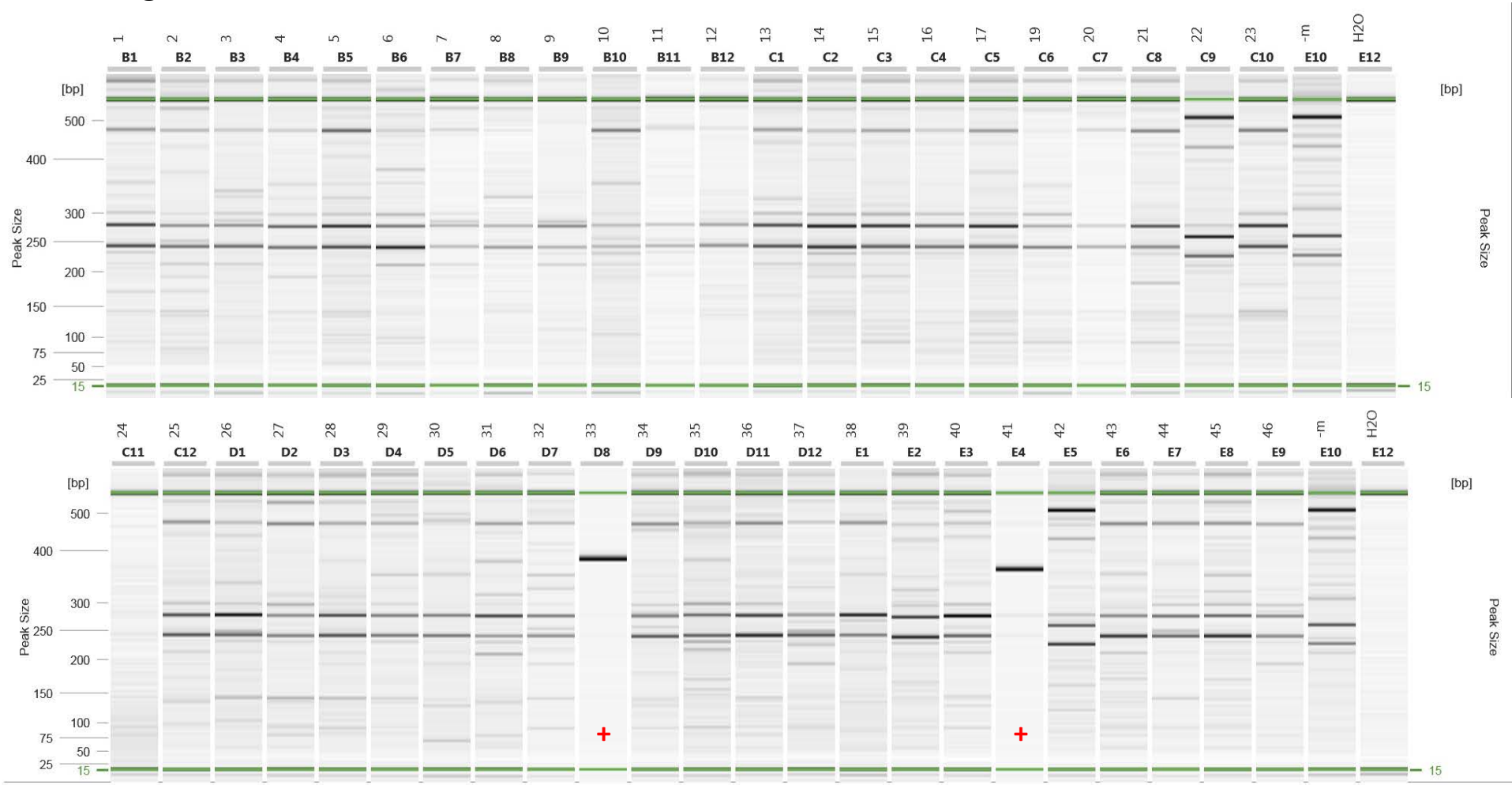

```
WM07 g968 agatccaggtcgtaaaacgatgg     WM07 g405 cacttggcatcgcagtgtcctgg
WM07 g970 ggtcgtaaaacgatggatcatgg     WM07 g406 gcacgcatcagggcctcacttgg
```

Genotyping strategy –

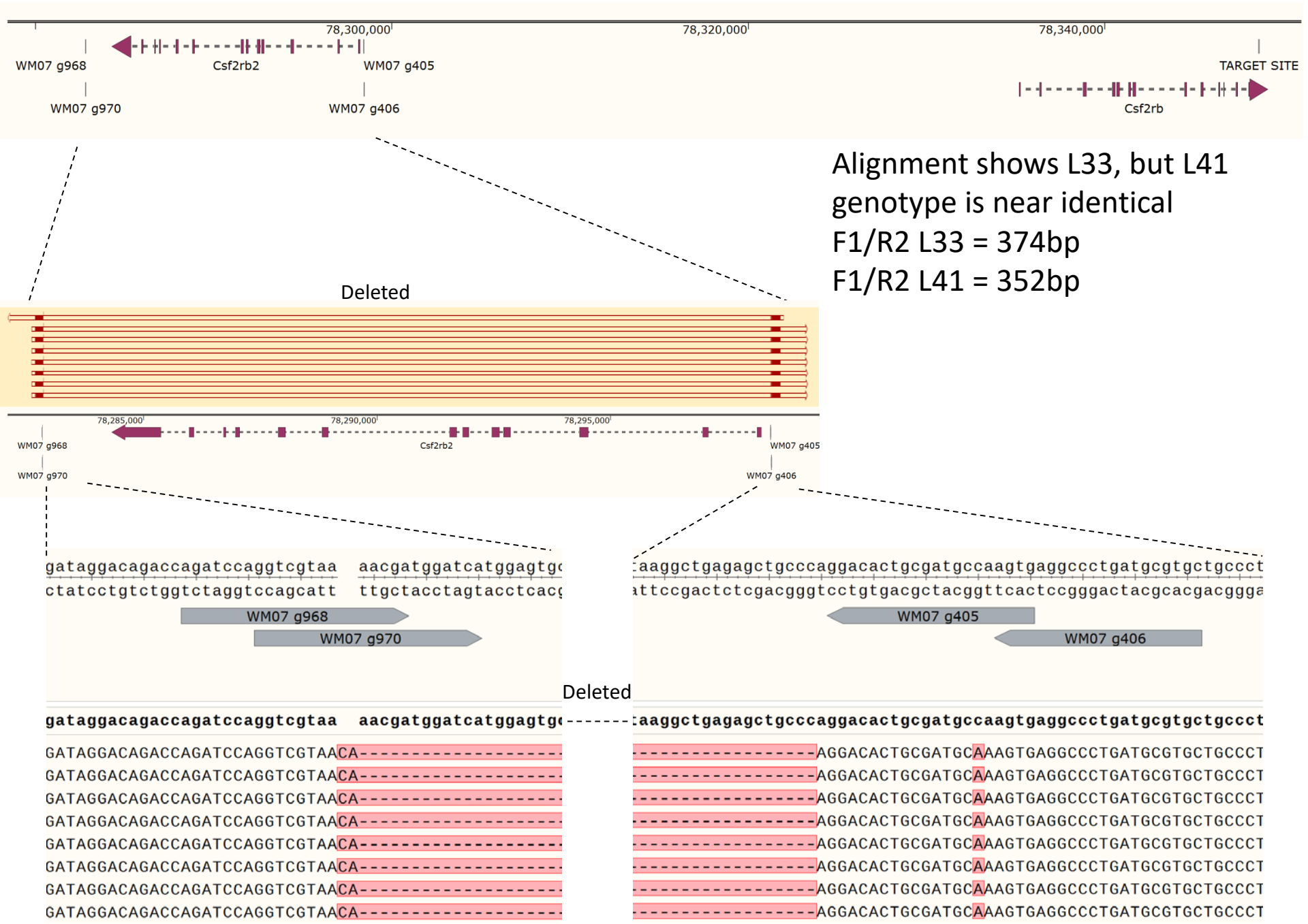
PCRs F1/R1 and F2/R2 can indicate if gRNA are cutting the respective 5’ and 3’ sites
And excision event brings primers F1 and R2 into proximity, a positive result from this PCR indicates likely excision

WM07 F1/R2 1-46 – 23.01.17 (Pup 18 found dead – no e.p.)

NOTE- this primer combination generated a lot of non-specificity, although positive alleles gave clean single band



Products from L33 and 41 were amplified with HF polymerases, and subcloned into pCRBlunt vector for sequencing. 8 colonies per mouse were sent for Sanger sequenced and aligned with the genomic region to confirm specific targeting



Alignment shows L33, but L41 genotype is near identical

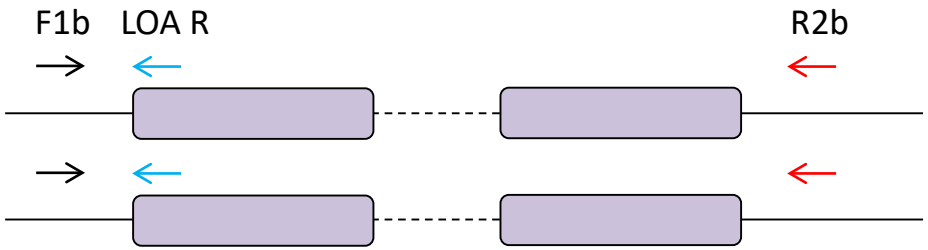
F1/R2 L33 = 374bp

F1/R2 L41 = 352bp

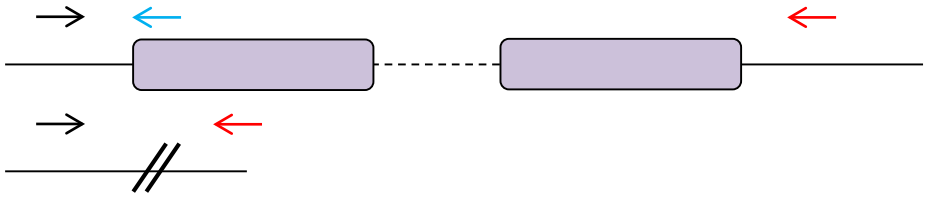
Step 2

- Both L33 and L41 backcrossed with WT BL6 mouse to confirm Germline transmission (GLT)
- Pups from this line 41 (highest number of positives in F1 litter) were then interbred to generate homozygosity
- Triple PCR [slightly re-designed primers for use in single reaction and to clean up specificity issues (F1b, R2b, LOA R)] used to test for WT, Het and Hom alleles, example gel shown on next slide
- Established 10 Female Hom + 1 Male Hom for IVF and CSF2RB S708A targeting on this background

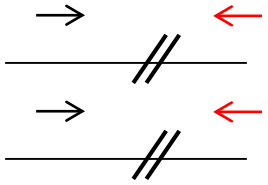
Confirm germline transmission of CSF2RB2 deletion



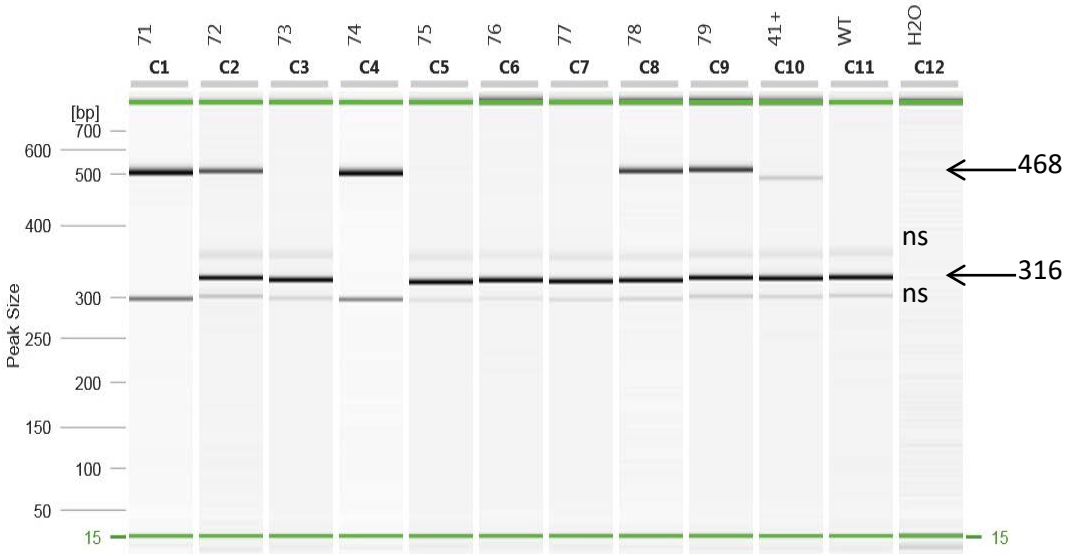
WT mouse



L41 Het



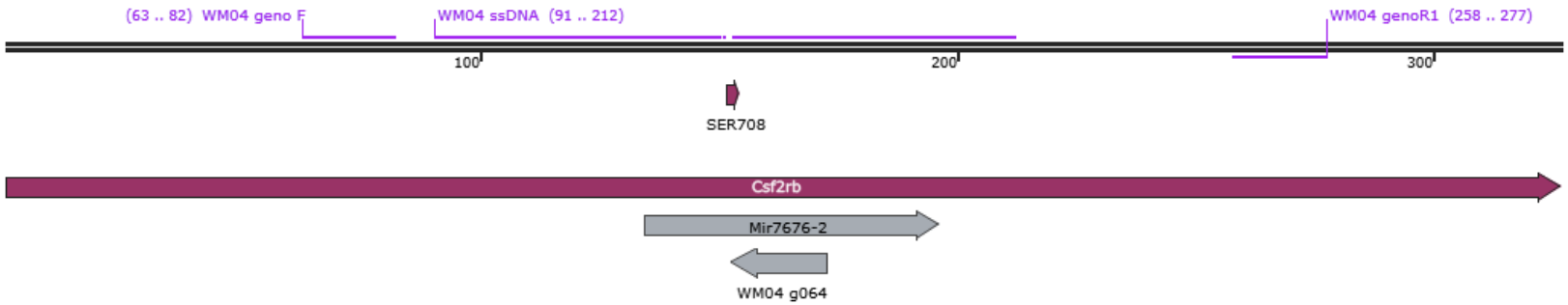
L41 Hom




```
WM07 Geno F1b        ggccgttcctgtttgtttct
WM07 Geno LOA R      atgttctgtggacctggagt
WM07 Geno R2b        caatcacacctaggcttgcc
```

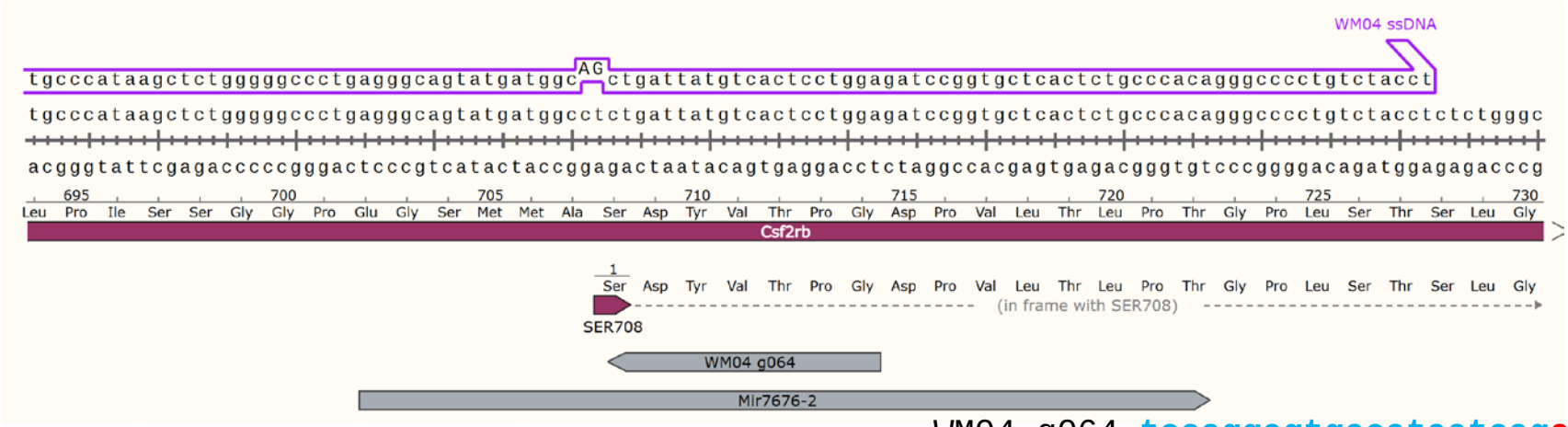
Step 3

- CSF2RB S708A targeting design
- Note the presence of a potential miRNA gene (Mir7676-2) for which we do not know the function (keep in mind in case of unexpected phenotype as mutagenesis of the S708 codon will impact this gene too)

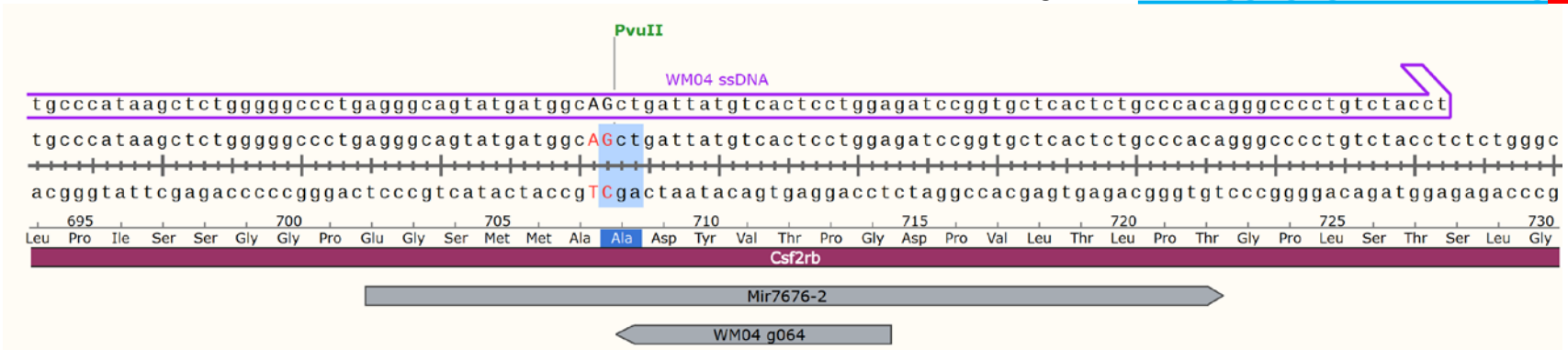


Repair design

- A ssDNA oligo with 60bp flanking homology and harbouring 2 bp substitutions designed that will result in ct > AG mutation
- Double mutation designed to (i) convert Ser708 codon to Ala708 as desired (ii) result in mutation of PAM motif to prevent CRISPR Cas9 re-cutting of repaired region (silent ala707ala) and (iii) create a PvuII site for easy genotyping



WM04 g064 tccaggagtgacataatcagagg



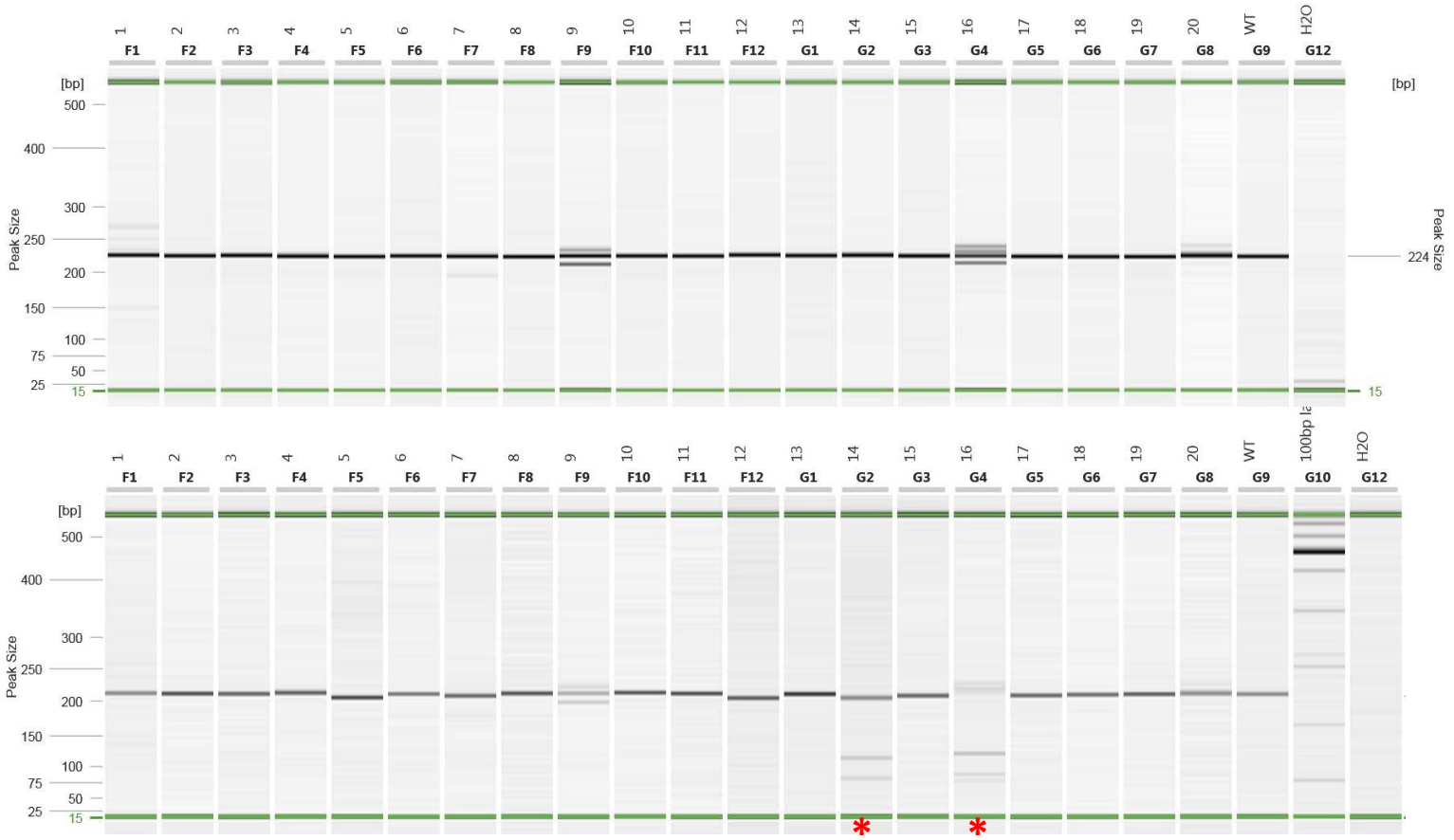
Alt-R crRNA (IDT) oligo for gRNA ordered and resuspended in sterile, optimem and annealed with tracrRNA (IDT) by combining 2.5ug crRNA with 5ug tracrRNA and heating to 95oC. The mix was allowed to slowly cool to room temperature. After annealing the complex an equimolar amount was mixed with 1500ng Cas9 recombinant protein (NEB) and incubated at RT for 15',) and the ssDNA repair template (final conc 10ng/ul) in a total optimem volume of 15ul.

2 Days of embryo electroporations using AltR crRNA:tracrRNA:Cas9 complex (200ng/ul; 200ng/ul; 200ng/ul respectively), ssDNA HDR template (500ng/ul) using a NEPA21 XXXXXX, conditions XXXX

Zygotes were cultured overnight and the resulting 2 cell embryos surgically implanted into the oviduct of day 0.5 post-coitum pseudopregnant mice.
After birth and weaning genomic DNA extracted using Sigma redextract-n-amp tissue pcr kit and used to genotype pups

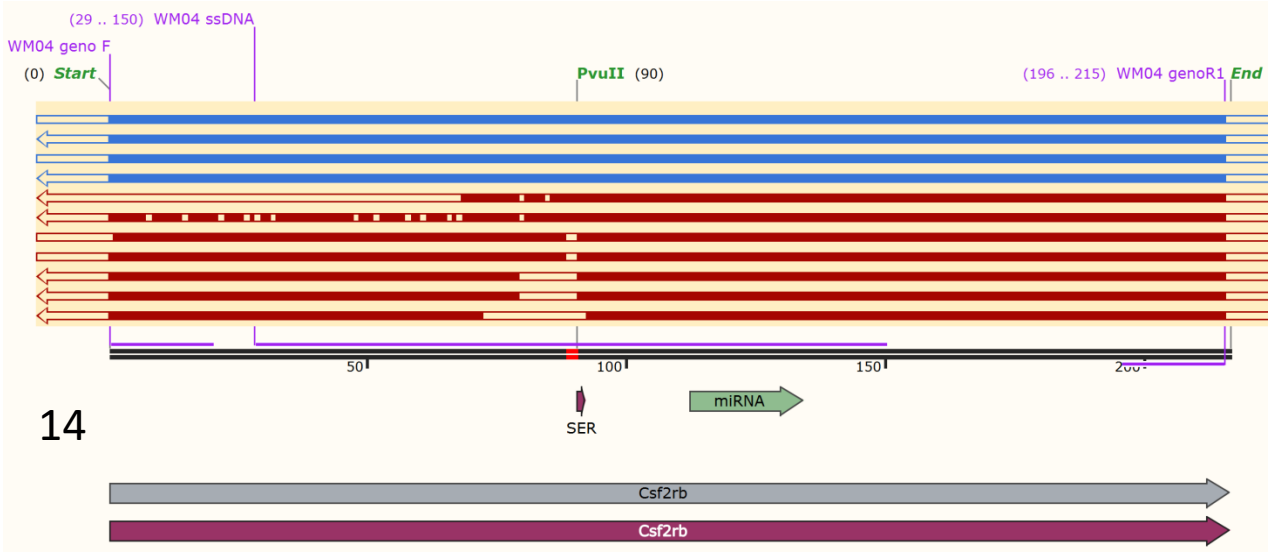
WM04 Geno F1 ttgagctgagcatggaggaa
WM04 Geno R1 cctgggcagcttaagacaga

Genotyping

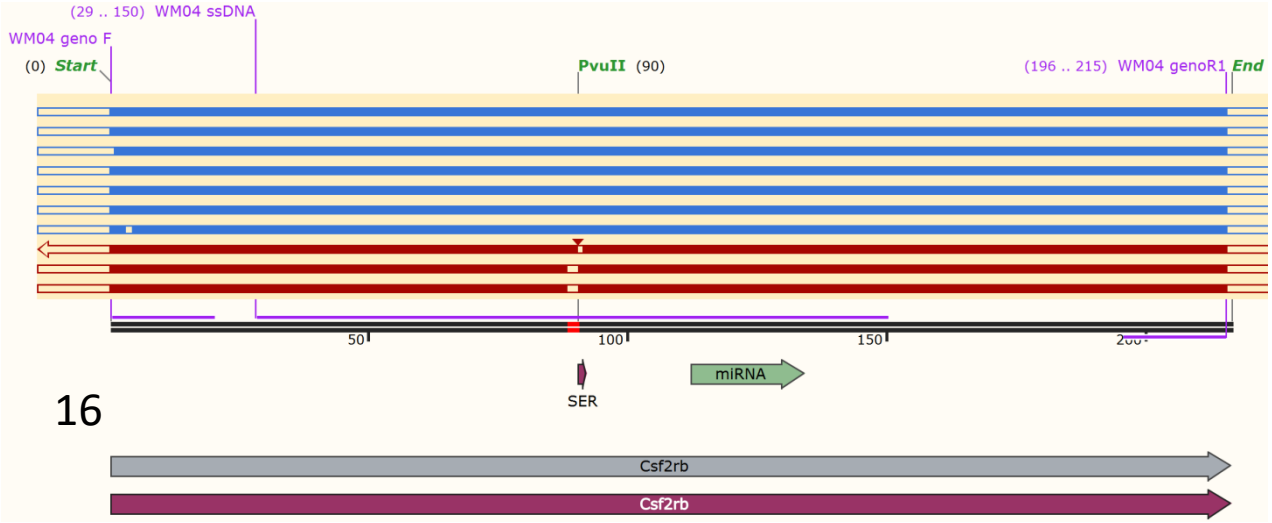


+Pvull

Products from L14 and L16 were amplified with HF polymerases, and subcloned into pCRBlunt vector for sequencing. 8 colonies per mouse were sent for Sanger sequenced and aligned with the genomic region to conform specific targeting



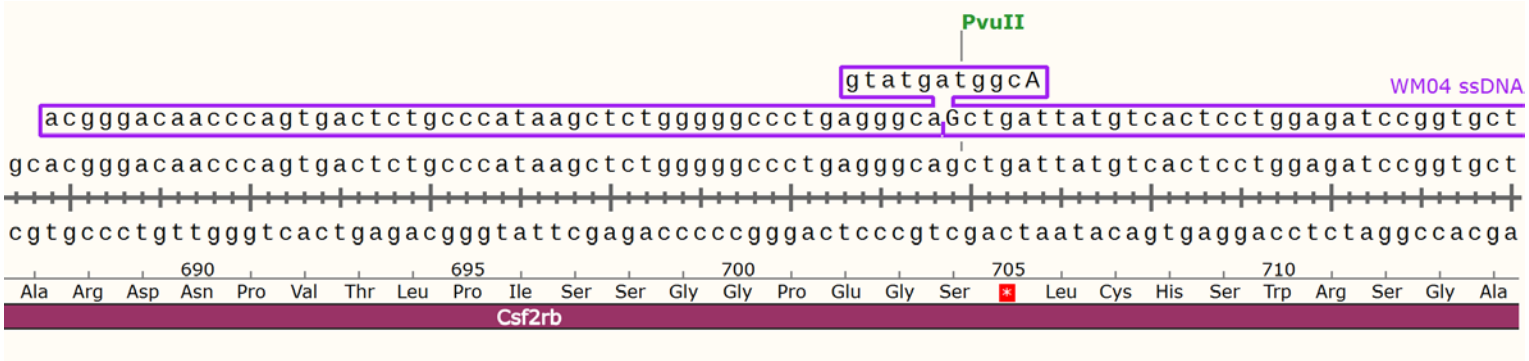
HDR = 6
WT = 2
Other = 2 x -11bp
1 x -20bp



HDR = 7
WT = 2
Other = 1 x +2/-1bp

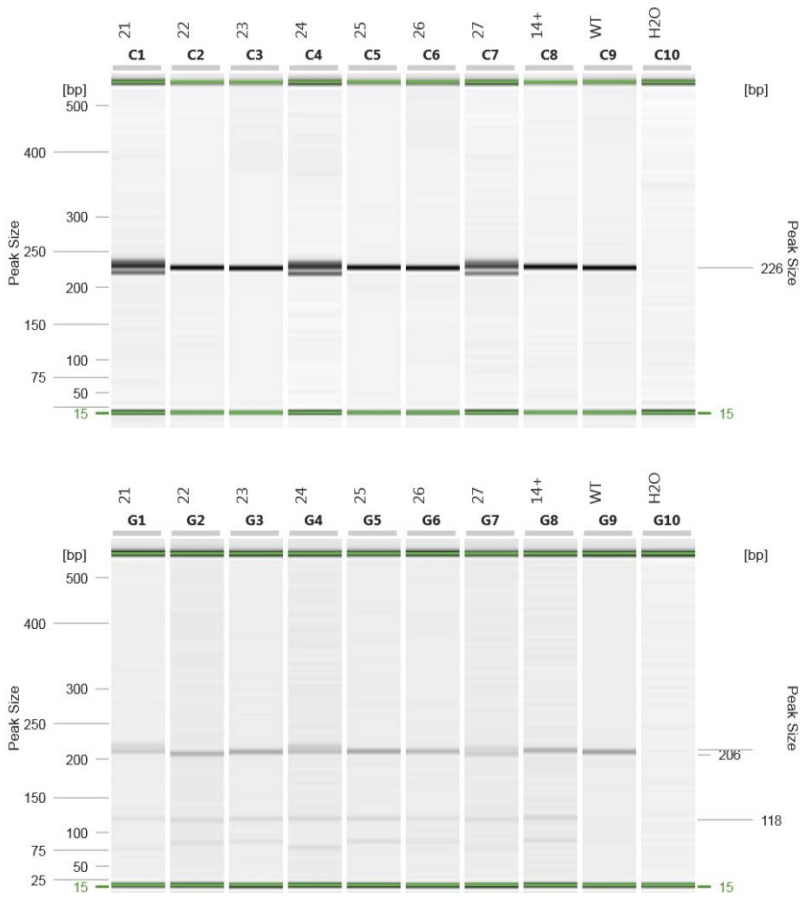
Sequencing summary

	Allele 1	Allele 2	Others?	Notes	
14	Perfect HDR	WT	Possible -20bp and -11bp alleles	Mosaic, the -11bp allele would actually generate a PvuII site!	-11bp allele would be predicted to be C terminal truncation
16	Perfect HDR	WT	+2/-1bp	Mosaic	+2/-1bp allele would be predicted to be C terminal truncation



Animal 14, -11bp allele, not premature STOP codon, allele is likely a C terminal truncation

Germline transmission



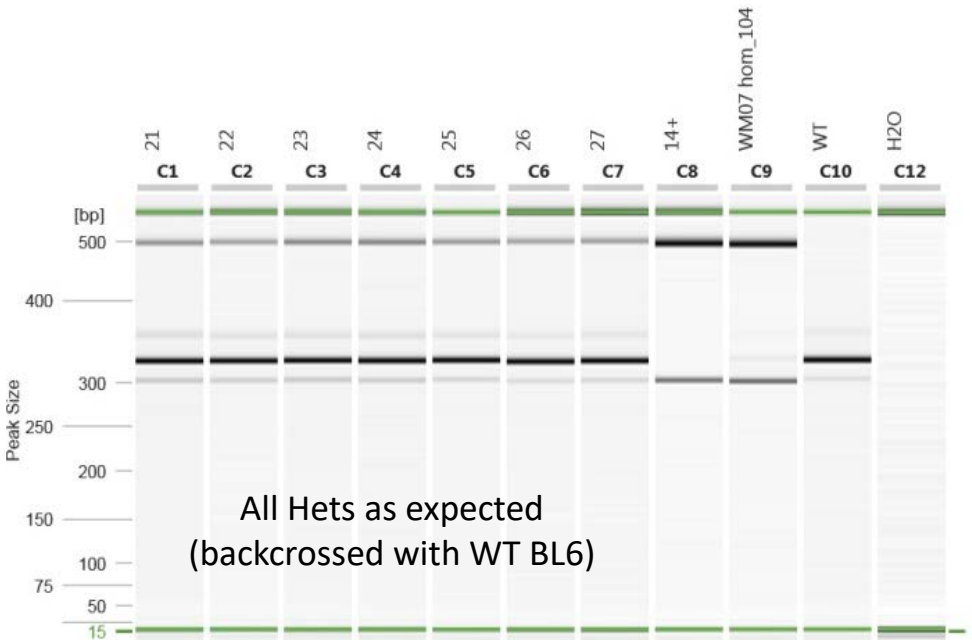
+PvuII

Pups 22, 23, 25, 26 are all HDR positive.

The PvuII digests ALL amplicons, but clearly the -11bp NHEJ InDel allele has been transmitted to 21, 24 and 27

Is this allele useful?

Confirm CSF2RB2 null background



Further Breeding

- These mice were created on a C57BL/6 background. Official nomenclature for the line would be:
- **C57BL/6J.CSF2RB2^{Em1Uman} (Tg41)**
- **C57BL/6J.CSF2RB2^{Em1Uman} .CSF2RB^{Em1Uman} (Tg14) or (Tg16)**
- The genotyping PCRs we describe can be used to genotype your breeding colony
- For experiments with the S708A model we recommend you use the null CSF2RB2 background as a control

- Any issues or questions about breeding and genotyping please contact us

- **Please remember to get in touch at the point of publication, we can provide written methodologies and details, and the relevant co-author details for myself and Neil.**