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MODEL GENERATION TECHNICAL REPORT

Generation of mouse model : Cdc27 S93F point mutation-conditional KO

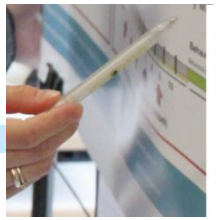
Project code: G4623 / IR4623

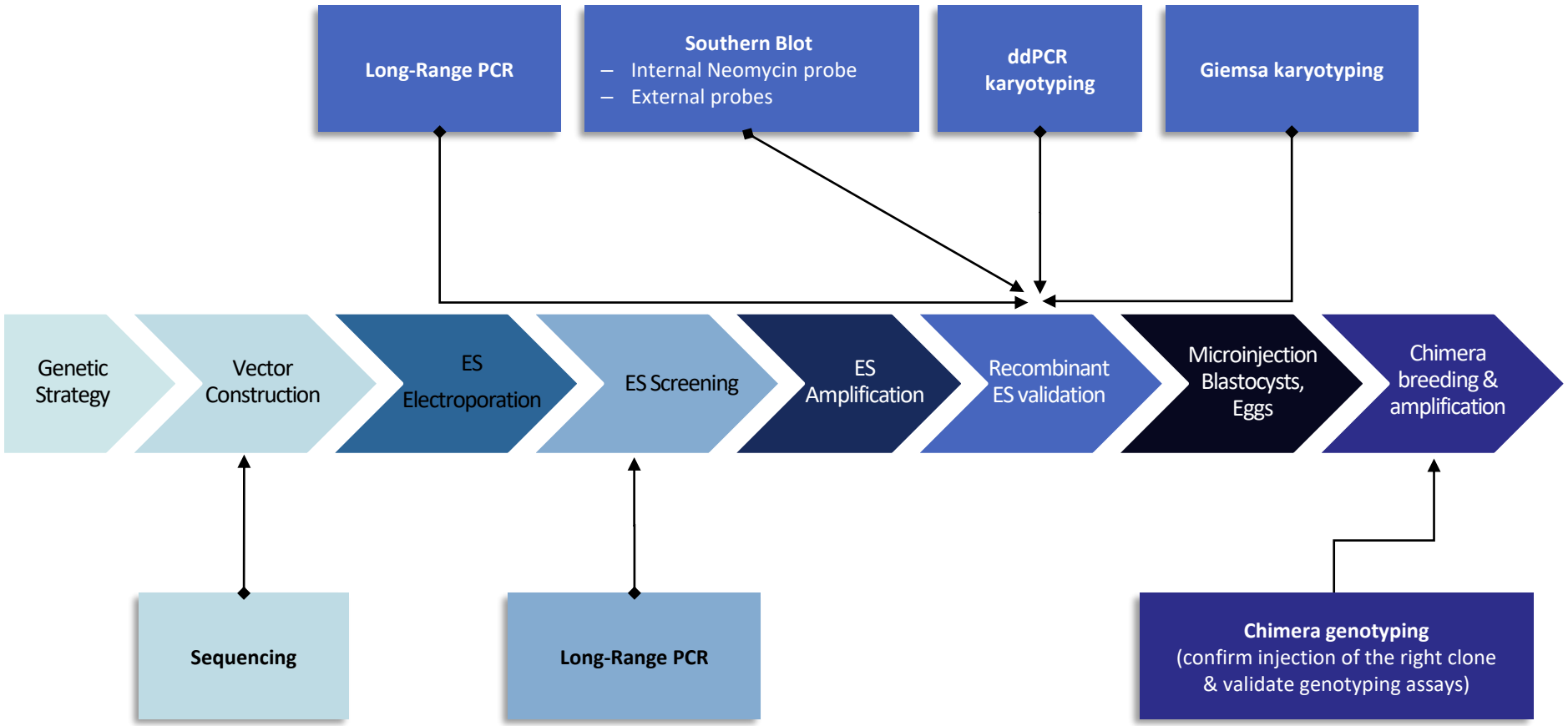
Report finalized: 2023/10/12

cnrs
Université de Strasbourg
Instituts thématiques Inserm institut national de la santé et de la recherche médicale
CERBM centre européen de recherche en biologie et en médecine gie
Aix*Marseille université

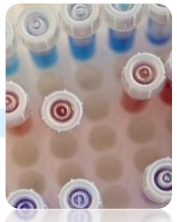
MODEL GENERATION TECHNICAL REPORT

PROJECT PROCESS & QUALITY CONTROLS



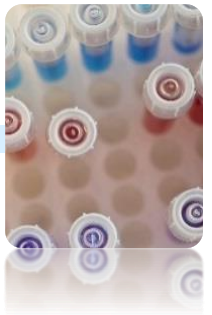


2 GENETIC STRATEGY

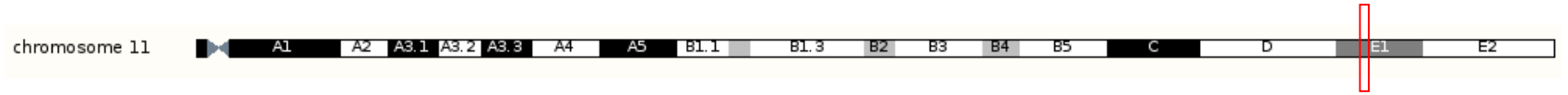


- Target locus structure
- mRNA(s) and protein(s)
- Genetic strategy
- PRO & CONS evaluation of the strategy

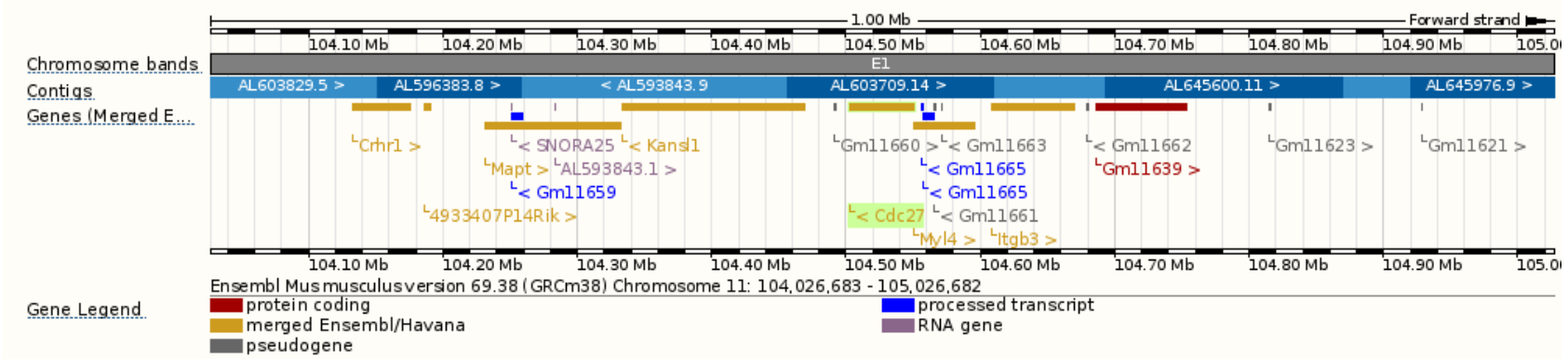
Cdc27 mouse genomic locus – structure

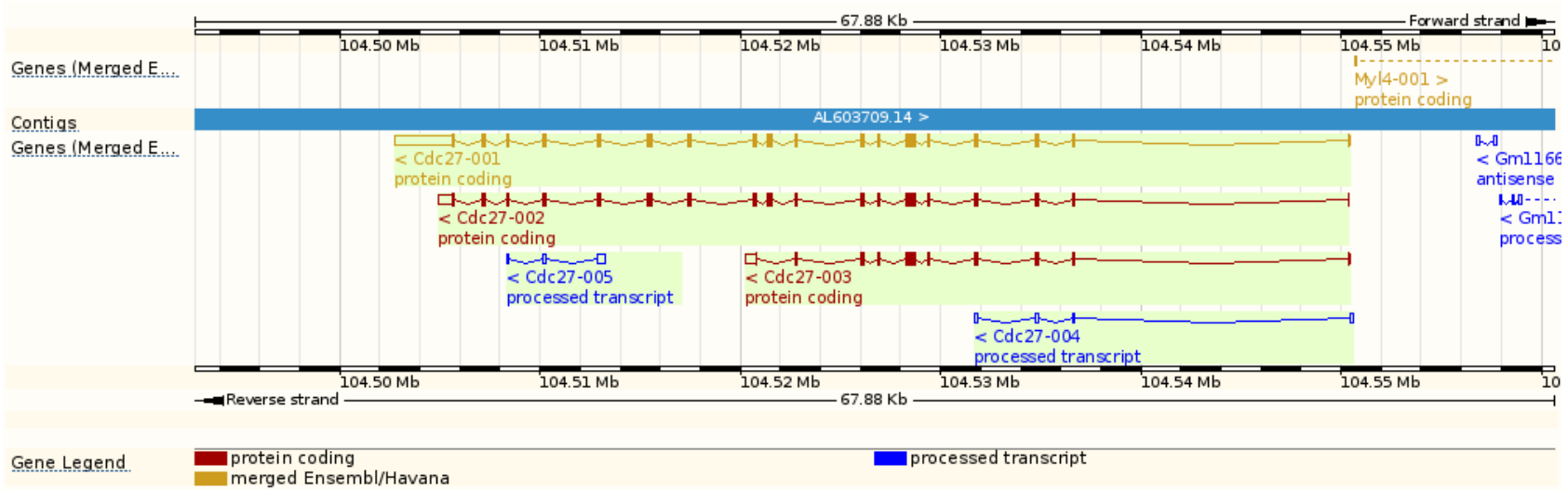


Location:

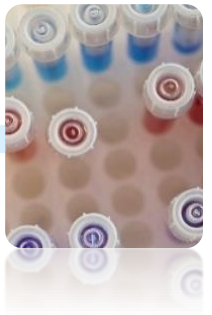


Ensembl Gene ID: **Cdc27 ENSMUSG00000020687**



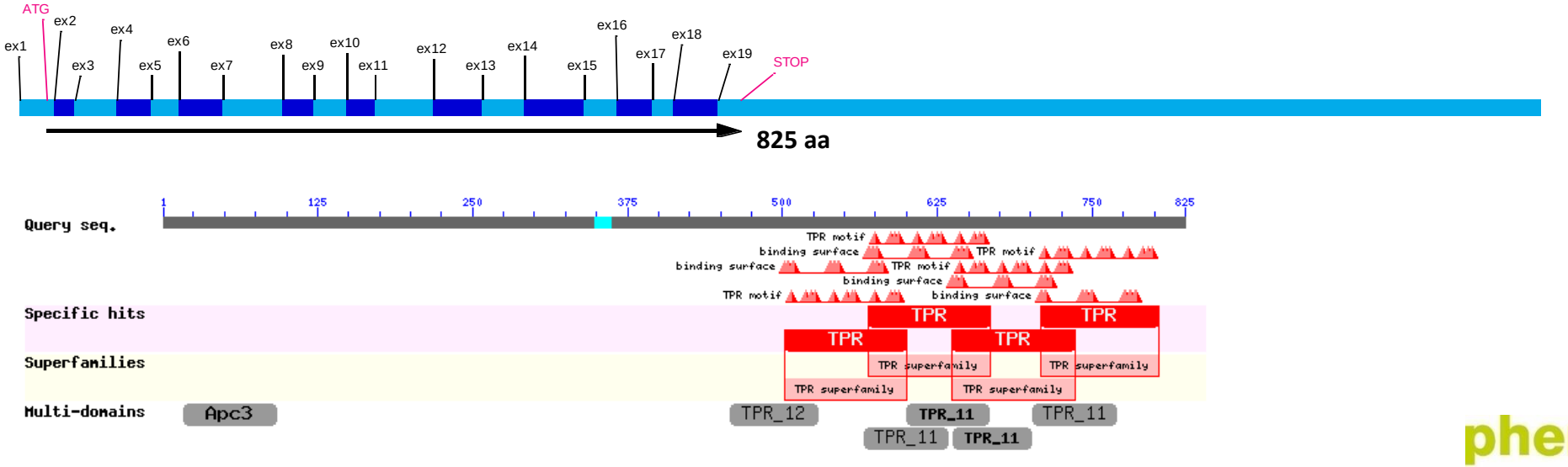


Cdc27 mRNA(s) and protein(s)

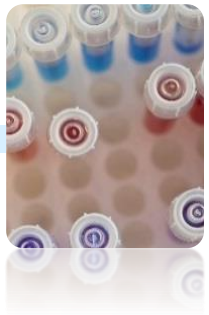


Name	Transcript ID	Length (bp)	Protein ID	Length (aa)	Biotype	CCDS
Cdc27-001	ENSMUST00000093923	5425	ENSMUSP00000091452	825	Protein coding	CCDS36354
Cdc27-002	ENSMUST00000106962	3160	ENSMUSP00000102575	831	Protein coding	-
Cdc27-003	ENSMUST00000106961	1863	ENSMUSP00000102574	399	Protein coding	-
Cdc27-004	ENSMUST00000135303	486	No protein product	-	Processed transcript	-
Cdc27-005	ENSMUST00000127506	580	No protein product	-	Processed transcript	-

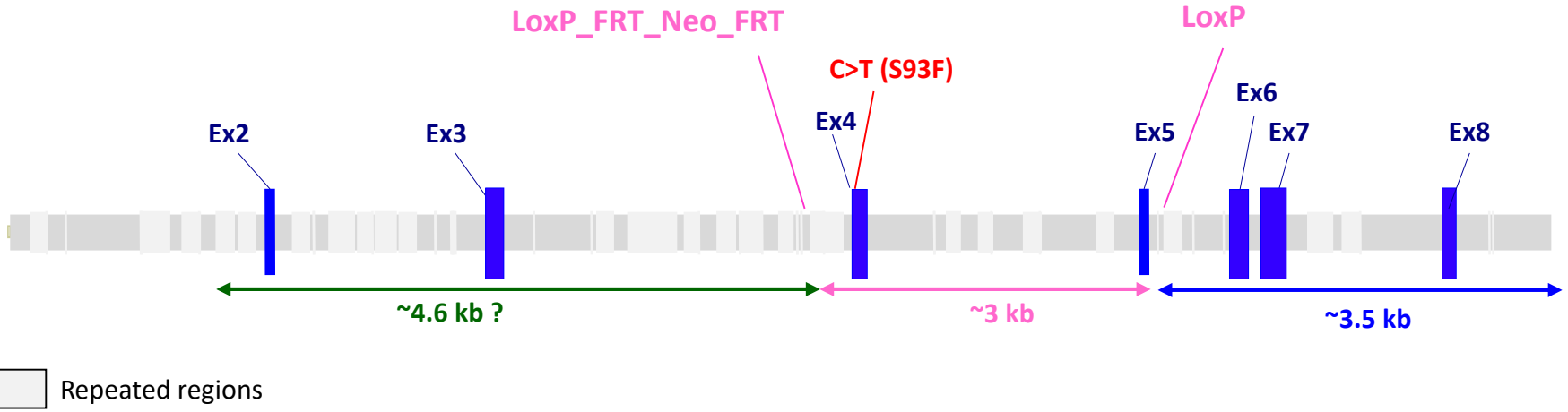
Cdc27-001 ENSMUST00000093923



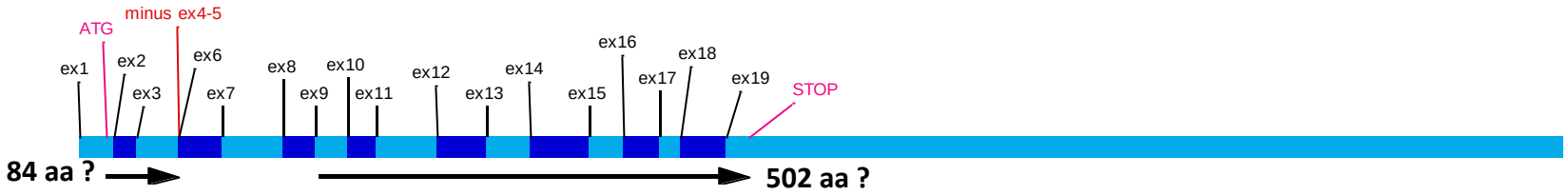
Approach selected: S93F PM with conditional KO potential (24/01/2013)

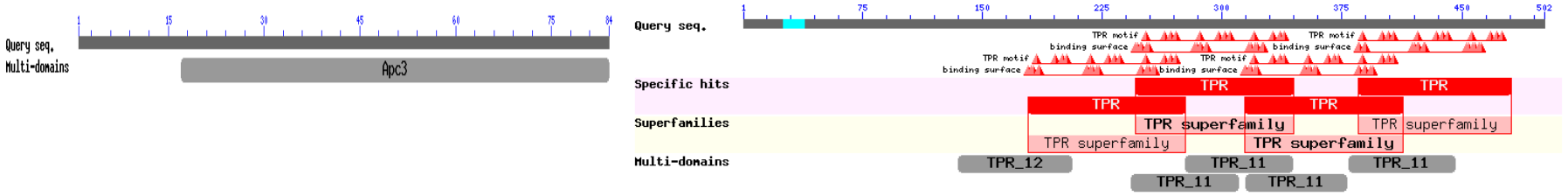


Targeted allele



mRNA and protein obtained after Cre mediated excision (-001)





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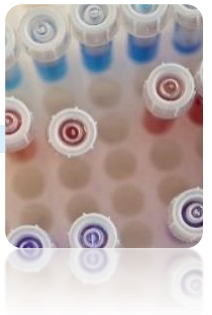
Strategy



Introduiced point mutation

ex1 ATG

TCGCTGGTGAGTTTAAATGAGCCGGGGCTGGCCGGGCTGGAGCCGCTACGGGGGGGGCCTGAGGCACTGCAGAAAGTGGGTCTGAGCCTCAAGG ATG ACG GTG CTG

M T V L

ex2

CAG GAA CCT GTC CAG GCT GCT ATA TGG CAA GCG CTA AAC CAC TAT GCT TAC CGA GAT GCA GTT TTC CTC GCA GAA CGA CTA TAT

Q E P V Q A A I W Q A L N H Y A Y R D A V F L A E R L Y

ex3

GCA GAA GTA CAT TCA GAA GAA GCC TTG TTT TTA CTG GCA ACC TGT TAC TAC CGC TCA GGA AAG GCT TAT AAA GCA TAT AGA CTC

A E V H S E E A L F L L A T C Y Y R S G K A Y K A Y R L

ex4

TTG AAA GGA CAC AGT TGT ACC ACC CCA CAG TGT AAA TAC CTG CTT GCA AAA TGT TGT GTT GAC CTC AGC AAG CTT GCA GAA GGG

L K G H S C T T P Q C K Y L L A K C C V D L S K L A E G

C>T (S93F)

GAA CAG ATC TTA TTT GGT GGA GTG TTT AAT AAG CAG AAA AGC CAT GAC GAC CTT GTC ACT GAG TTT GGA GAT TCA GCT TGC TTC

E Q I L F G G V F N K Q K S H D D L V T E F G D S A C F

ex5

ACT CTT TCC TTG TTG GGA CAT GTG TAT TGC AAG ACA GAT CGG CTT GCC AAA GGG TCA GAA TGT TAC CAA AAG AGC CTT AGT TTA

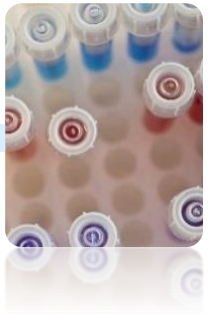
T L S L L G H V Y C K T D R L A K G S E C Y Q K S L S L

ex6

AAT CCT TTC CTC TGG TCT CCC TTT GAA TCG TTA TGT GAA ATA GGT GAG AAG CCA GAT CCT GAC CAA ACA TTT

N P F L W S P F E S L C E I G E K P D P D Q T F

PROs& CONs evaluation of the strategy



Pros

- Only strategy possible

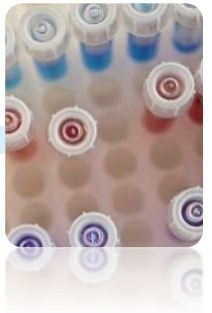
Cons

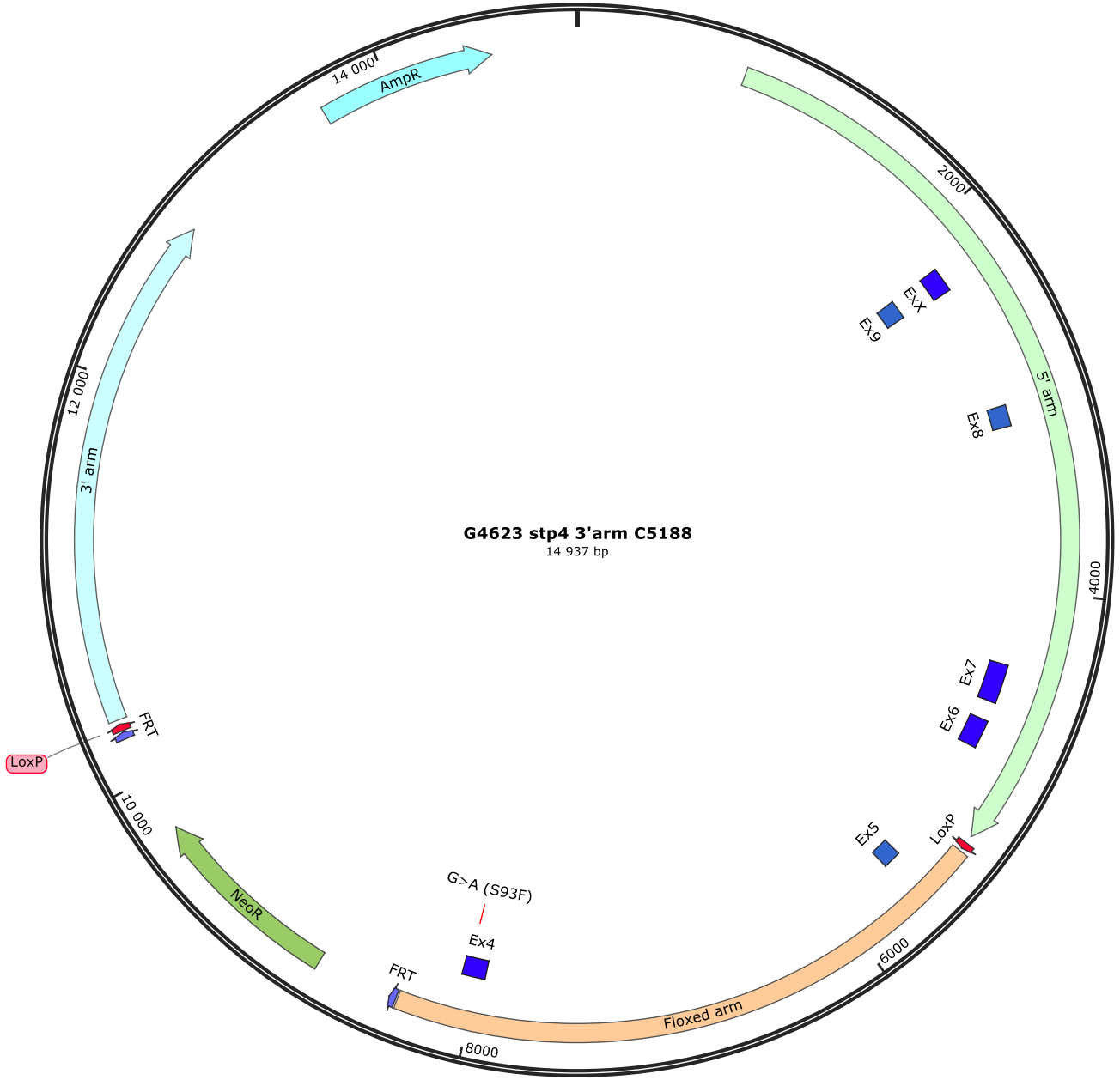
- Large size of the inter LoxP (the 3' LoxP site might be lost after homologous recombination
- A protein of 84 aa might be expressed after Cre mediated excision if RNA decay does not occur
- A protein of 502 aa might be expressed after Cre mediated excision if reinitiation occurs at one of the in frame ATG present in exon 9 or further exon and if RNA decay does not occur
- Presence of repeated regions (in light grey) in both homology arms (green and blue arrows) might render PCR amplification and/or homologous recombination at the locus difficult

The selection cassette (FRT-Neo-FRT) will be removed by breeding male chimera with a flp deleter line which shows maternal contribution (*Birling et al.*, 2012)

Highly-efficient, fluorescent, locus directed cre and FlpO deleter mice on a pure C57BL/6N genetic background.
Birling MC, Dierich A, Jacquot S, Hérault Y, Pavlovic G. Genesis. 2012 Jun;50(6):482-9. doi: 10.1002/dvg.20826.

3 HOMOLOGOUS RECOMBINATION - VECTOR CONSTRUCTION





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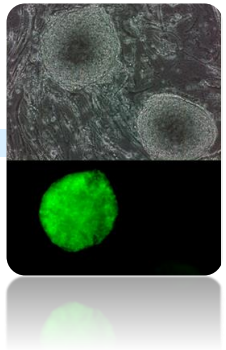
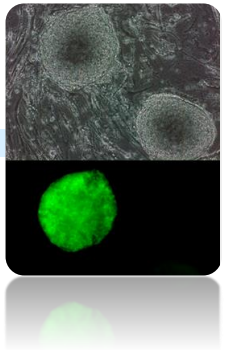
4 ES cell electroporation & Screening of recombinant clones



- Electroporation and screening process
- Long range PCR screening – strategy
- Long-Range 5’ PCR screening – results
- Recombinant ES validation by Long Range PCR
- Recombinant ES clones validation by Southern Blot – internal probe
- Recombinant ES clones validation by Southern Blot – External probe
- Aneuploidy screening in ES recombinant clones

Electroporation and screening process



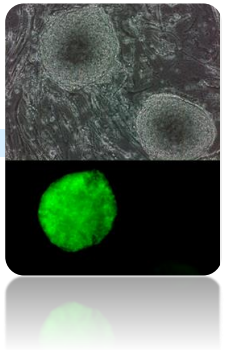
The targeting vector was electroporated in the proprietary C57BL/6N TB1 ES cell line.

Transfected ES clones were submitted to neomycin selection (G418) and 93 resistant ES clones were isolated. The clones were then submitted to the screening process allowing secured identification of those harbouring the expected recombination events at both ends of targeting vector.

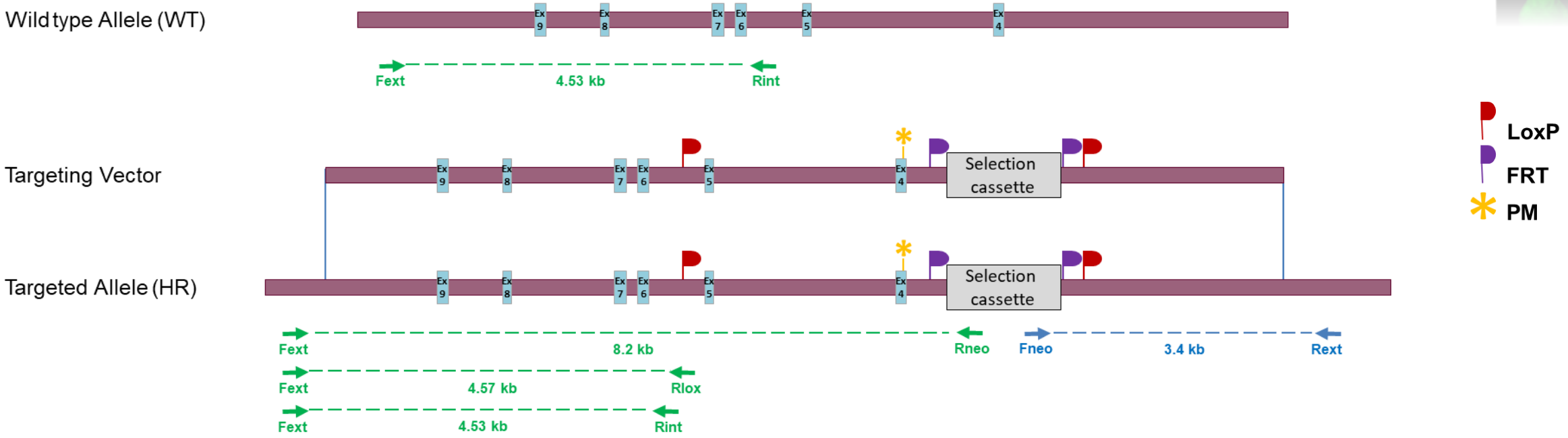
Screening process steps:

1. Identification of candidate recombinant clones by initial 5’ Long-Range PCR
2. Six of 5’ PCR positive clones are confirmed for 3’ recombination event by Long-Range PCR
3. Positive clones in step2 are further validated by Southern blot analysis using internal and external probes
4. The karyotype of at least 2 validated clones is verified using ddPCR aneuploidy screening and Giemsa staining

Long range PCR screening – strategy

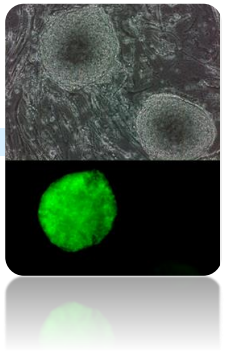


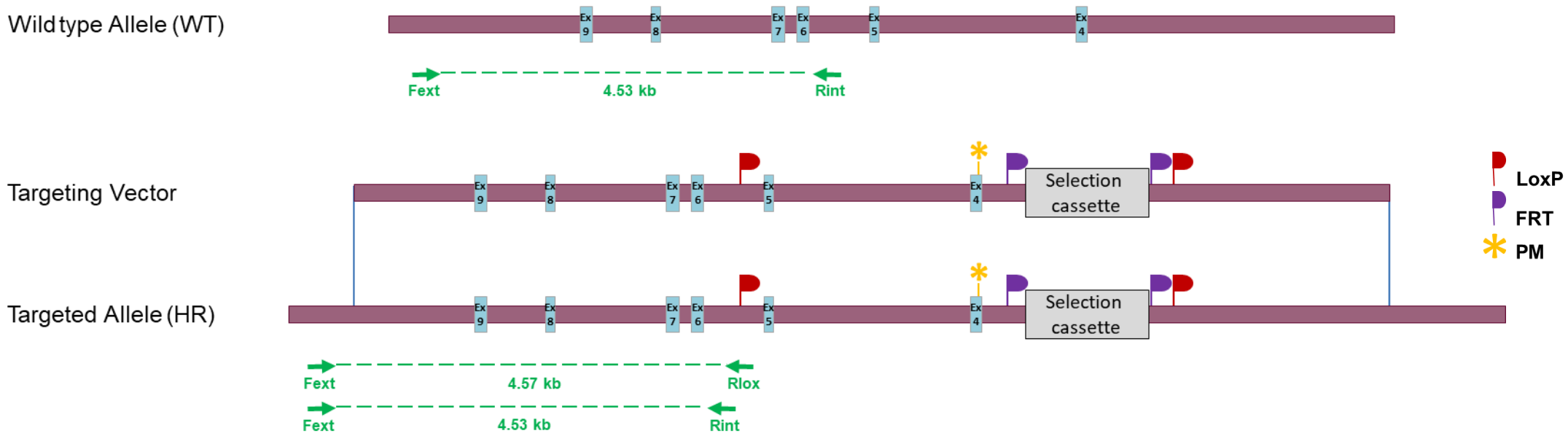
Schematic 5’ and 3’ PCR screening strategy



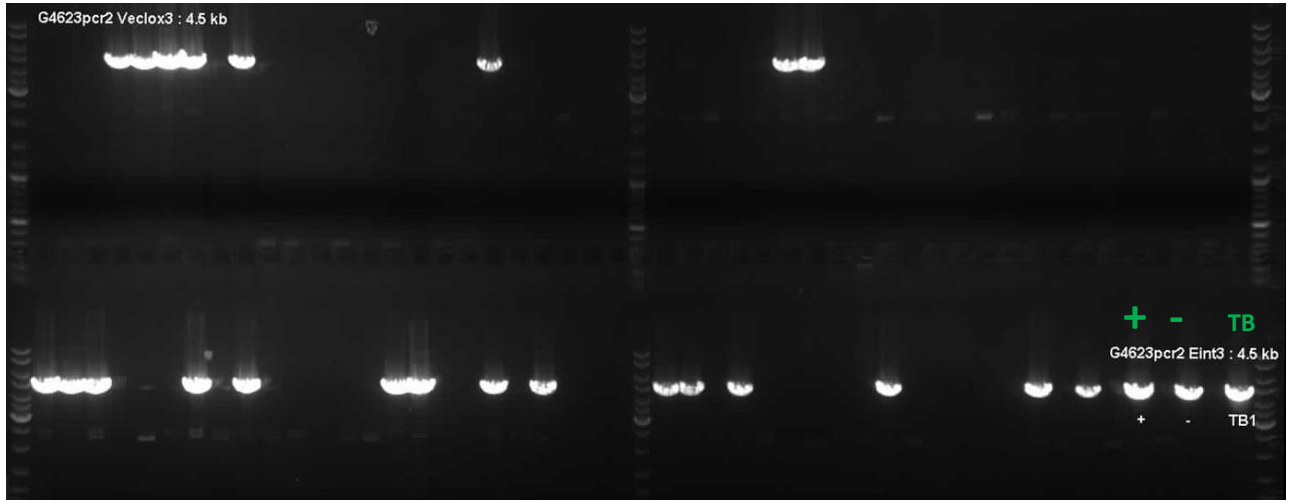
PCR	Primer Name	Primer sequences	PCR product size
5' PCR	Fext	GTTTGCAGCCATCCTAGGATACATG	8.2 kb
	Rneo	GCGGCCGGAGAACCTGCGTGCAATC	
5’ PCR	Fext	GTTTGCAGCCATCCTAGGATACATG	4.57 kb
	Rlox	GTTATCTGCAGGTCGACCTTAAGCT	
5’ PCR	Fext	GTTTGCAGCCATCCTAGGATACATG	4.53 kb
	Rint	GCCTCCTGGCAAGAGGATCTTAAG	
3’ PCR	Fneo	AGGGGCTCGCGCCAGCCGAACTGTT	3.4 kb
	Rext	CTTAACTTGTTTGCTGGCAGACTGA	

Long-Range 5’ PCR screening – results

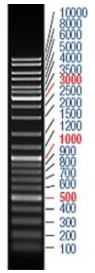




PCR Fext – Rlox : 4.57 kb



+ / - / TB : Controls DNAs



Ladder pattern

PCR Fext – Rint : 4.53 kb

Six candidate clones out of the 23 positive clones were selected for 3’ Long-Range PCR and Southern blot validation.

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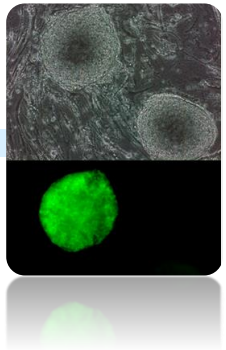
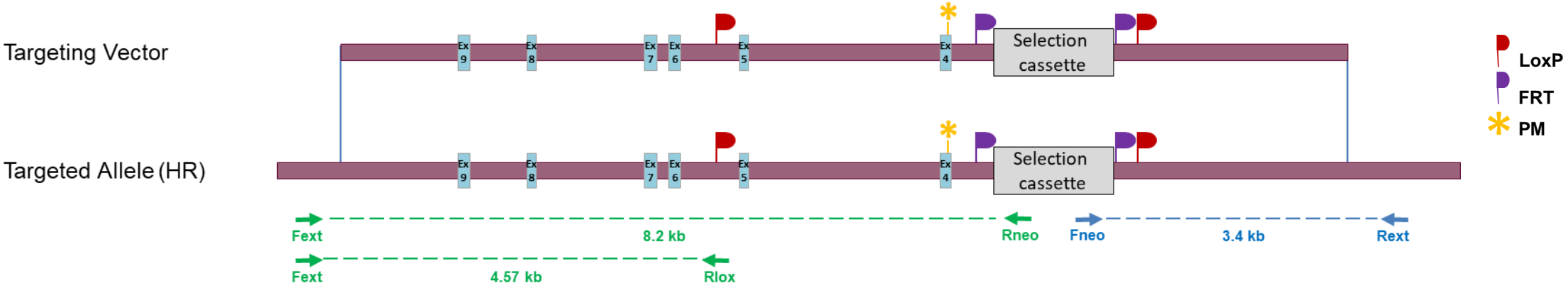
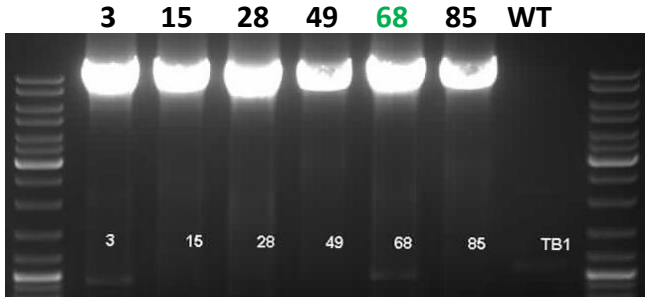
Recombinant ES validation by Long Range PCR

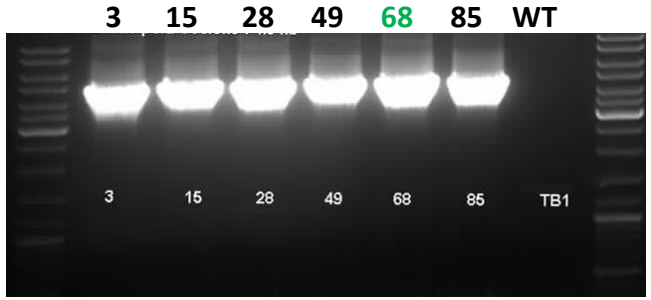


Confirmation and Validation of candidate recombinant ES clones by 5’ and 3’ PCRs

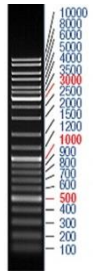




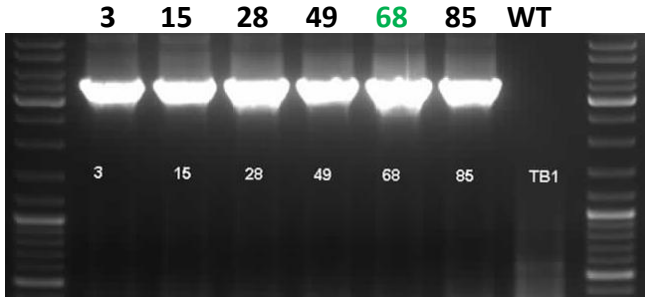
PCR Fext – Rneo : 8.2 kb



PCR Fext – Rlox : 4.57 kb



Ladder pattern



PCR Fneo – Rext : 3.4 kb

Six candidate clones identified by 5’ PCR screening were further analysed by 3’ PCR screening.
Six clones (clones #3, #15, #28, #49, #68 and #85) were confirmed.

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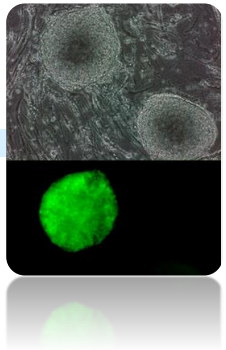
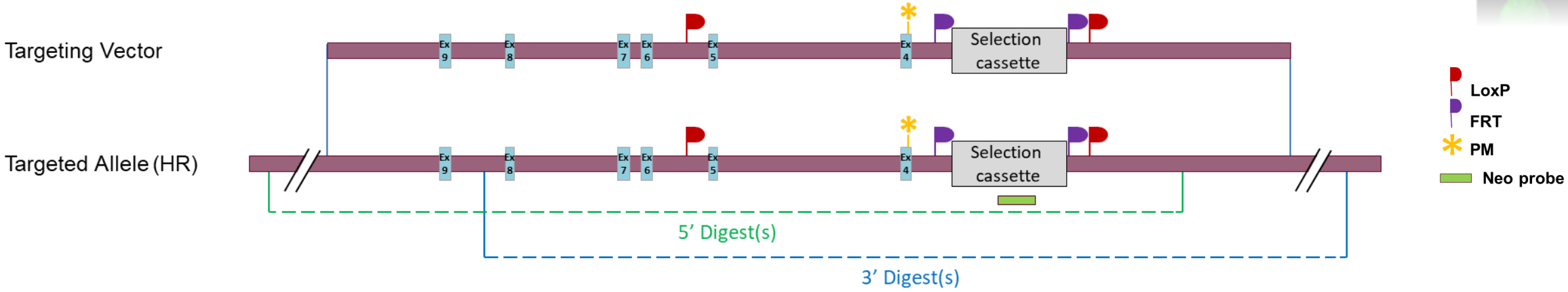
Recombinant ES clones validation by Southern Blot – Internal probe



Schematic Southern Blot validation strategy

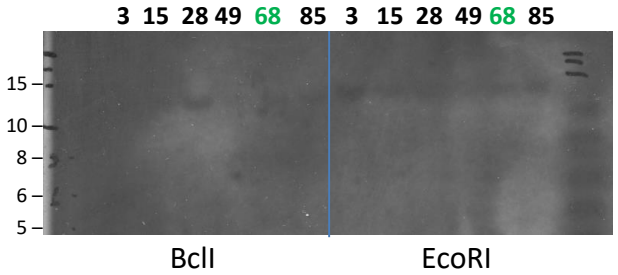
Digests on the scheme illustrate the position of the chosen restriction sites relative to the probe. They don't show the exact position of the restriction sites.



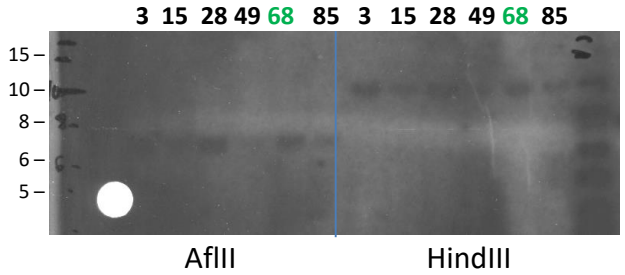
Digestions used to validate the 5' and 3' insertion

Probe		Genomic DNA digest	Targeted Allele (kb)
Neo	5' digests	BclI	12.7
		EcoRI	13.7
	3' digests	AflI	7
		HindIII	10.6

Southern blot - Neo 5'



Southern blot - Neo 3'



Neo probe sequence

CTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTC
ACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCT
GCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGAC
CACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGAC
GAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTC
GTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGT
GGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGC
GAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTT
CTTGACGAGTTCTTCTGAGGGGATCCGCTGTAAGTCT

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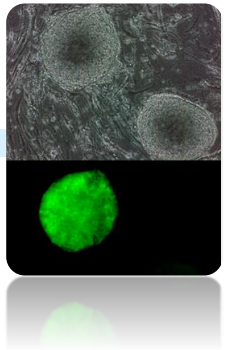
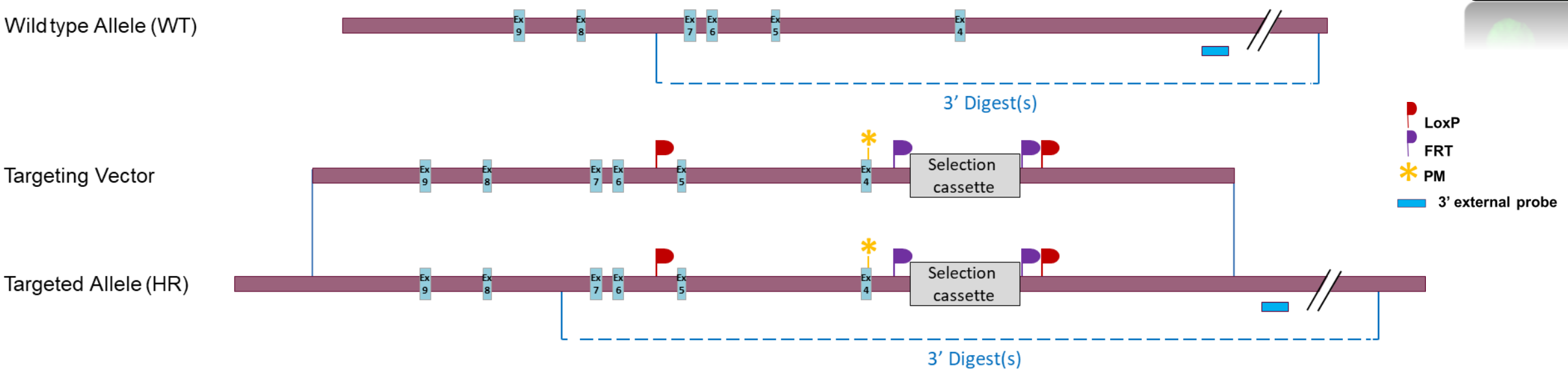
Recombinant ES clones validation by Southern Blot – External probe

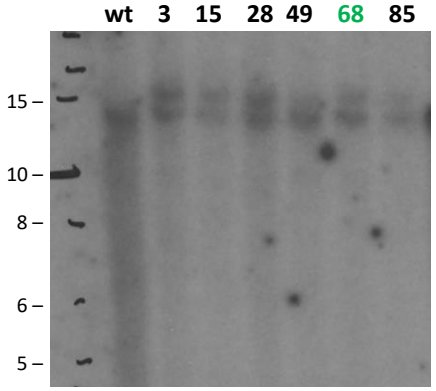


Schematic Southern Blot validation strategy

Digests on the scheme illustrate the position of the chosen restriction sites relative to the probe. They don't show the exact position of the restriction sites.



Southern blot – 3' probe



NdeI 13.5 / 15.5

3' probe sequence

CCTTGCTAGTCACAAAGTGATGGAATTCCATCTATTTTATTCATTTCTGGTT
CTATACAAATGAATAGATCACAAACCTTCTGCATATAGTCGTTCTGCGAGGA
AAACTGCATCTCGGTAAGCATAGTGGTTTAGCGCTTGCCATATAGCAGCCTG
TAAGTAGAGAAGCACATAAATATACACACAGTGACTCTGGCACCGTCTTGAA
CATAATATTCAAGTTAGTACTCGGAATTTGTTTGTATTATTTCTTCATTTAT
TTATTTACTTTGGTATGTGCATGTGTGTGTAGCAGGTGGGCATGTGCGCGCG
GGAAGGAGGTCAAAGATAACTTCTCTCCTACCATGTGGG

Digestions used to validate the 5' and 3' insertion

Probe	Name	Genomic DNA digest	WT allele (kb)	Targeted Allele (kb)
3' external probe	3' first digest	NdeI	13.5	15.5

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Confirmation of the presence of the mutation (CTT>TTT; S93F) on clone #68

Sequencing of PCR product Fext-Rneo

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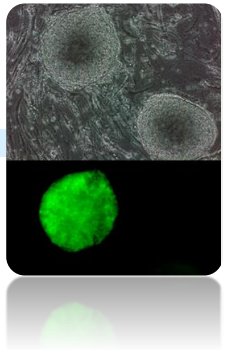
Aneuploidy screening in ES recombinant clones



Selected recombinant ES cells clones were karyotyped by Giemsa metaphase staining.

Results of aneuploidy analysis are presented in the table below.

Clone ID	Giemsa
#3	Failed
#28	**Pass**
#49	Not done
#68	**Pass**
#85	Not done

5 MICROINJECTION & BREEDING

- Microinjection
- Breeding to F1 generation

Microinjection

- The ES cells used in the injection experiment were originally derived from a C57BL/6N mouse strain (which have black coat colour). These cells were injected into blastocysts derived from an BALB/cN strain, which have a white coat colour. The resulting offspring are thus chimeras of two different cell types (ES cell-derived cells and host blastocyst-derived cells) and the degree of chimerism was monitored by the percentage of light and dark patches on these animals.
- Recipient blastocysts were isolated from mated BALB/cN females (Health status SPF Specific Pathogens Free).
- Recombinant ES clones #28 and #68 validated in previous project phase were injected into blastocysts to generate chimeric males. The results are presented in the table below.

Clone ID	Num1ber of chimeric males identified according to chimerism rate (Number of chimeric males bred to F1 generation)			
	5 - 40%	45% - 55%	60-100%	Total
#28	4	2	5	11
#68	**0**	**1**	**13**	**14**

Breeding to F1 generation

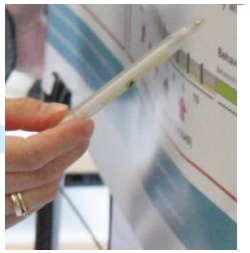
- Eight highly chimeric males generated in the previous phase by blastocyst injection of the ES clones were mated with wild-type C57BL/6NCrl females (health status SPF – Specific Pathogen Free) to investigate whether the recombined ES cells have contributed to the germ layer.

- Germ line transmission was obtained the : 22/10/2014

- The line issued from clone #68 was cryopreserved

- Allele nomenclature (following MGI guidelines) : **Cdc27$^{tm1.1Ics}$**

6 SEQUENCE OF THE DELIVERED ALLELE



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LoxP FRT Exon 4 & 5 (reversed) Intoduced mutation :G>A (S93F)

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REPORT REDACTION & VALIDATION
Protocol finalized on 2023/10/12
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