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MODEL GENERATION TECHNICAL REPORT

Generation of mouse model : Med23 R614Q PM (corresponding to R617Q in human)

Project code: G3 / IR00003698

Final report

CNRS · Université de Strasbourg · Instituts thématiques Inserm – Institut national de la santé et de la recherche médicale · CERBM gie – centre européen de recherche en biologie et en médecine · Aix*Marseille université

MODEL GENERATION TECHNICAL REPORT

1 PROJECT PROCESS & QUALITY CONTROL

2 GENETIC STRATEGY

3 HOMOLOGOUS RECOMBINATION VECTOR CONSTRUCTION

4 ES ELECTROPORATION & SCREENING OF RECOMBINANT CLONES

5 MICROINJECTION & BREEDING

6 CONFIRMATION OF THE INTRODUCED MUTATION

7 SEQUENCE OF THE DELIVERED ALLELE

PROJECT PROCESS & QUALITY CONTROL

Long-Range PCR

Southern Blot
- Internal Neomycin probe
- External probes

ddPCR karyotyping

Giemsa karyotyping

Genetic Strategy → Vector Construction → ES Electroporation → ES Screening → ES Amplification → Recombinant ES validation → Microinjection Blastocysts, Eggs → Chimera breeding & amplification

Sequencing

Long-Range PCR

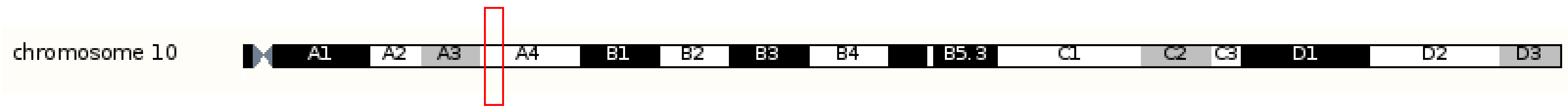
Chimera genotyping
(confirm injection of the right clone & validate genotyping assays)

2 GENETIC STRATEGY

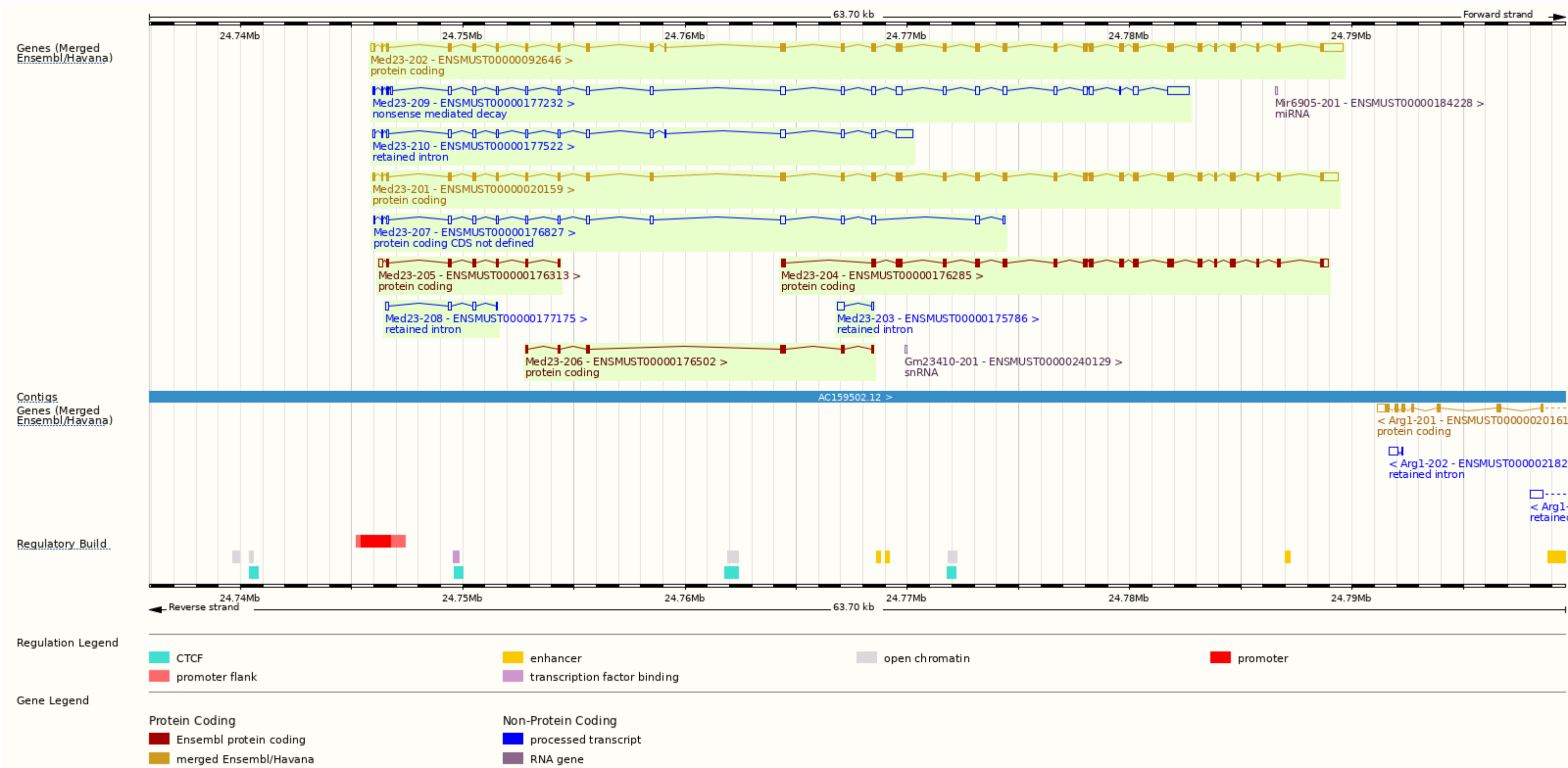
- Target locus structure
- mRNA(s) and protein(s)
- Genetic strategy
- PRO & CONS evaluation of the strategy

Med23 mouse genomic locus – structure

Location: Chromosome 10: 24,745,884-24,789,579



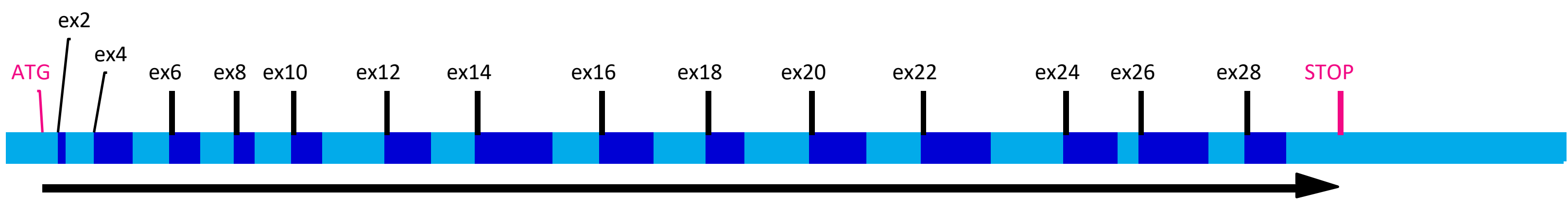
Gene: Med23 ENSMUSG00000019984



Med23 mRNA(s) and protein(s)

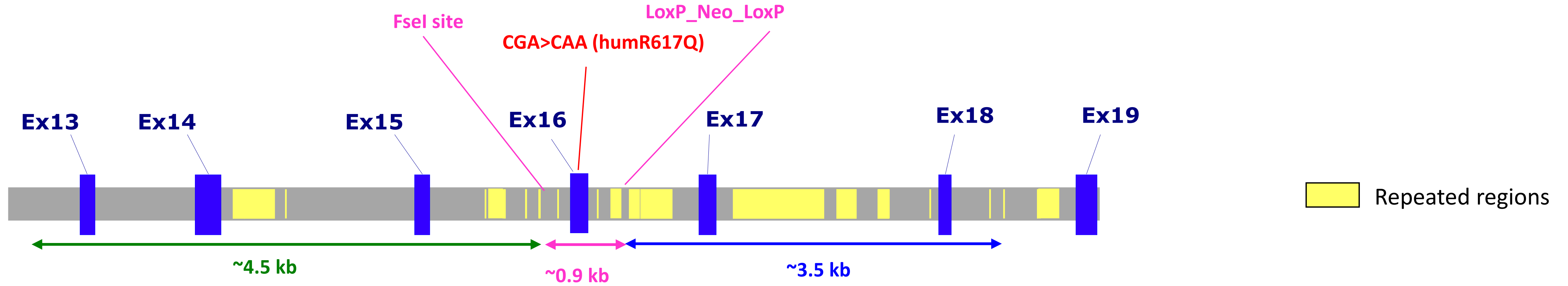
Name	Transcript ID	Length (bp)	Protein ID	Length (aa)
Med23-201	ENSMUST00000020159	4928	ENSMUSP00000020159	1370
Med23-202	ENSMUST00000092646	4946	ENSMUSP00000090316	1376
Med23-204	ENSMUST00000171625	3953	ENSMUSP00000128671	960
Med23-203	ENSMUST00000168046	4928	ENSMUSP00000129267	1367

Med23-201



Strategy proposed: point mutation R611Q

Targeted locus



Sequence detail (partial)

ex16

GTG GAA ACT TAC AGC CGT TTA CTG GTC TAC ATG GAA ATT GAG TCT TTG GGC ATC AAA GGA TTT ATC AGT CAG CTC CTG CCC
V E T Y S R L L V Y M E I E S L G I K G F I S Q L L P

CGA>CAA (humR617Q)

ACT GTC TTC AAG TCC CAC GCC TGG GGC ATC CTG CAC ACA CTG CTG GAG ATG TTC AGC CAC cAA ATG CAC CAC ATT CAG CCC
T V F K S H A W G I L H T L L E M F S H Q M H H I Q P

CAC TAC CGA GTT CAG CTC CTG AGC CAT CTC CAC ACA CTG GCT GCA GTC GCA CAG ACC AAC CAG AAC CAG CTC CAT CTG TGT
H Y R V Q L L S H L H T L A A V A Q T N Q N Q L H L C

GTG GAG AGC ACT GCA CTG AGG CTC ATC ACA GCC CTG GGA AGC TCA GAG GTC CAG CCG CAG TTC ACG CGT TTC CTC AAT GAT
V E S T A L R L I T A L G S S E V Q P Q F T R F L N D

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PROs& CONs evaluation of the strategy

I. Pros

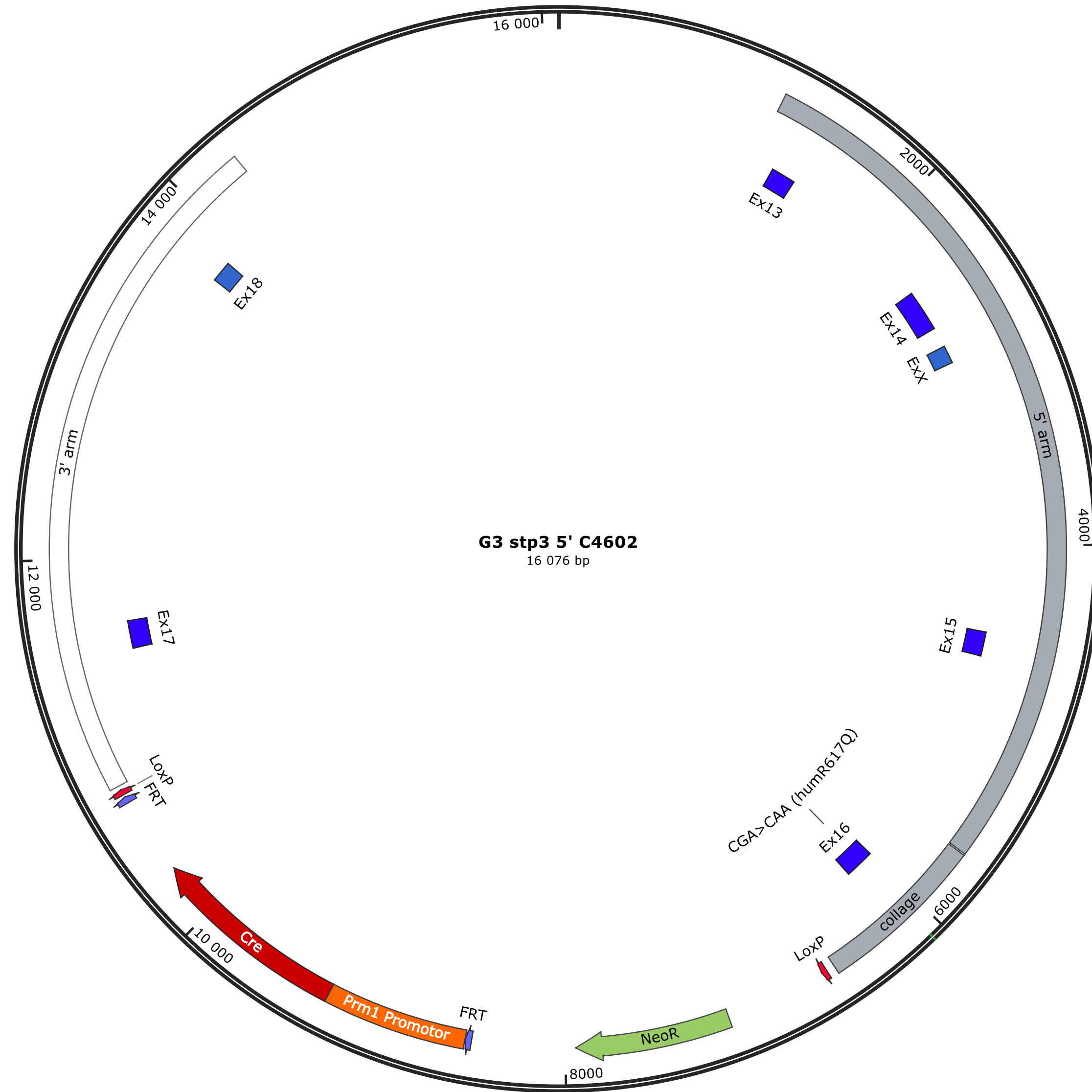
- Human R617Q mutation introduced, in the mouse it correspond, for isoform Med23-201, to the amino acid 614

I. Cons

- An FseI restriction site will be inserted in intron 15 for cloning purpose

- Presence of repeated regions (in yellow) in most of the 3’ homologous arm might render PCR amplification or PCR screening difficult

3 HOMOLOGOUS RECOMBINATION - VECTOR CONSTRUCTION



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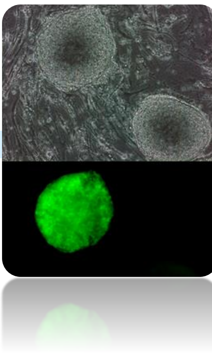
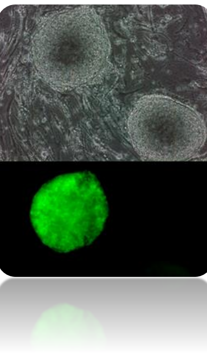
4 ES cell electroporation & Screening of recombinant clones



- Electroporation and screening process
- Long range PCR screening – strategy
- Long-Range 3’ 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



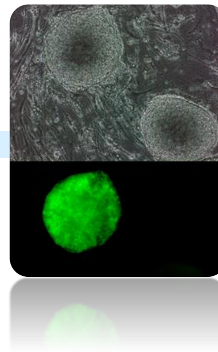
The targeting vector was electroporated in the proprietary C57BL/6NCrl S3 cell line.

Transfected ES clones were submitted to neomycin selection (G418) and 186 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 3’ Long-Range PCR
2. Eight of 3’ PCR positive clones are confirmed for 5’ 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

Wild type Allele (WT)

Ex 13 Ex 14 Ex X Ex 15 Ex 16 Ex 17 Ex 18

Fint 3.6 kb Rext

Targeting Vector

Ex 13 Ex 14 Ex X Ex 15 Ex 16 Selection cassette Auto-excision cassette PA Ex 17 Ex 18

Targeted Allele (HR)

Ex 13 Ex 14 Ex X Ex 15 Ex 16 Selection cassette Auto-excision cassette PA Ex 17 Ex 18

Fext 6 kb Rneo

Fneo 6.7 kb Rext

Fcre 5.1 kb Rext

Fint 3.6 kb Rext

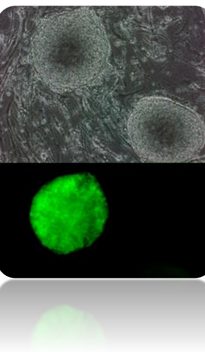
LoxP

FRT

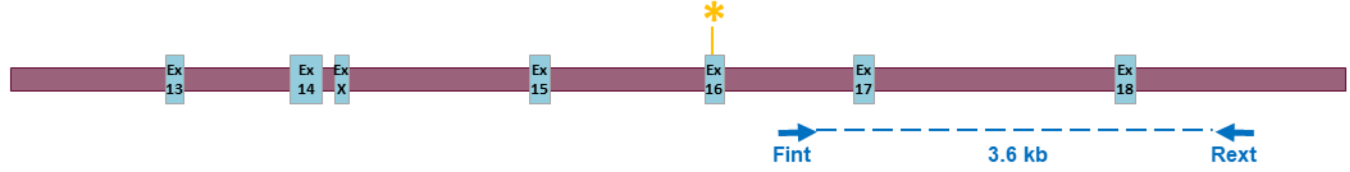
PM

PCR	Primer Name	Primer sequences	PCR product size
5' PCR	Fext	GTAGAAACTTGCCAACAAGAACCTC	6 kb
	Rneo	GCGGCCGGAGAACCTGCGTGCAATC	
3’ PCR	Fneo	AGGGGCTCGCGCCAGCCGAACTGTT	6.7 kb
	Rext	CGACACTTTAGCTTCACTCAAGTCC	
3’ PCR	Fcre	GGCCAAGCCAGCACCATGTCCA	5.1 kb
	Rext	CGACACTTTAGCTTCACTCAAGTCC	
3’ PCR	Fint	TAACTTAATGGAGGAGGGGACACAG	3.6 kb
	Rext	CGACACTTTAGCTTCACTCAAGTCC	

Long-Range 3’ PCR screening – results

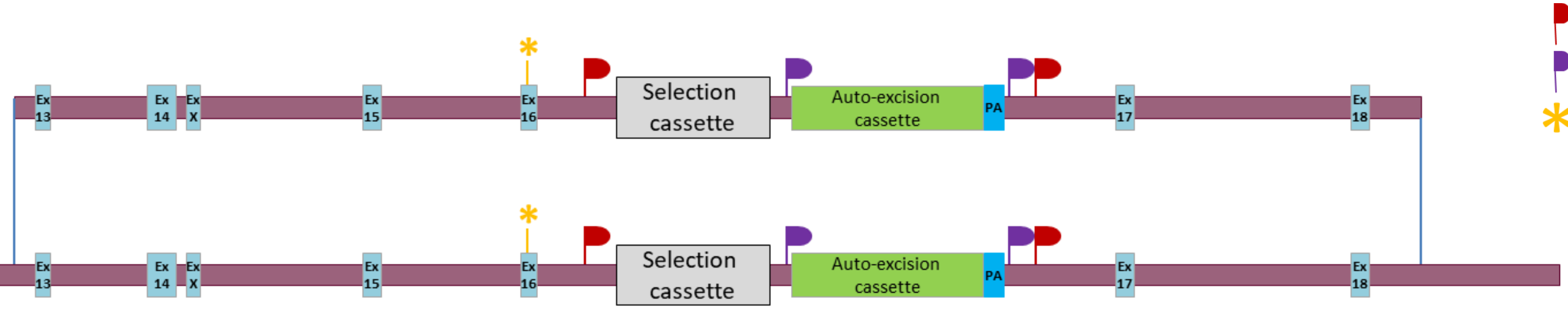


Wild type Allele (WT)



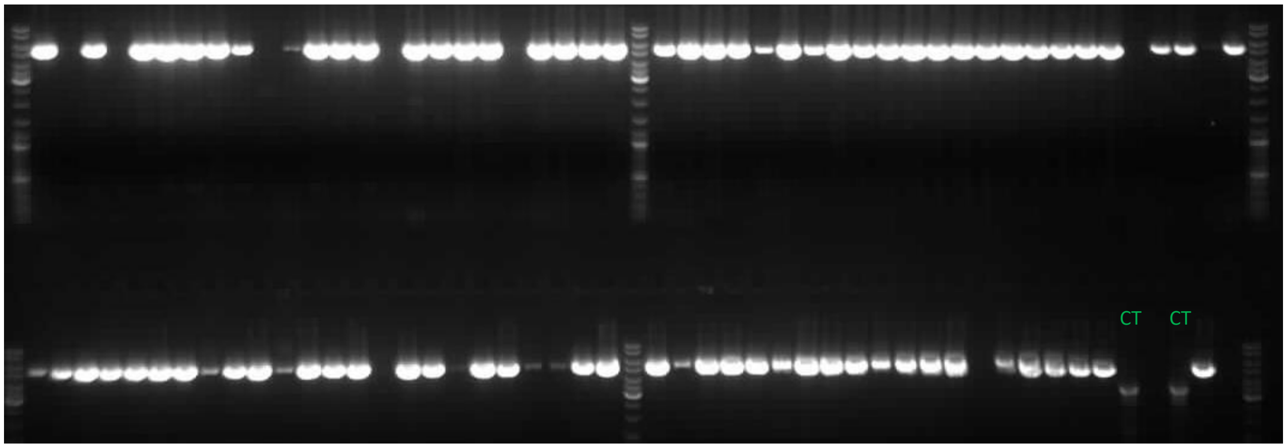
Targeting Vector

Targeted Allele (HR)



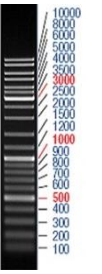
LoxP
FRT
PM

Pcr Fcre – Rext : 5.1 kb



CT = control DNAs

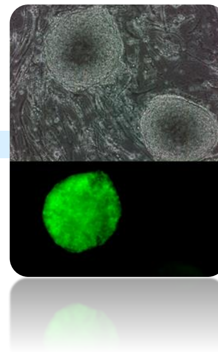
Pcr Fint – Rext : 3.6 kb



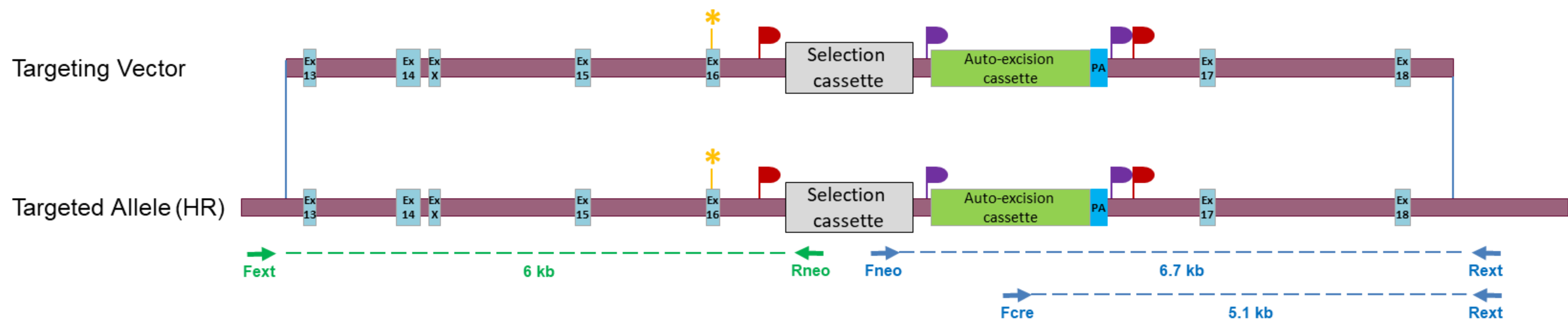
Ladder pattern

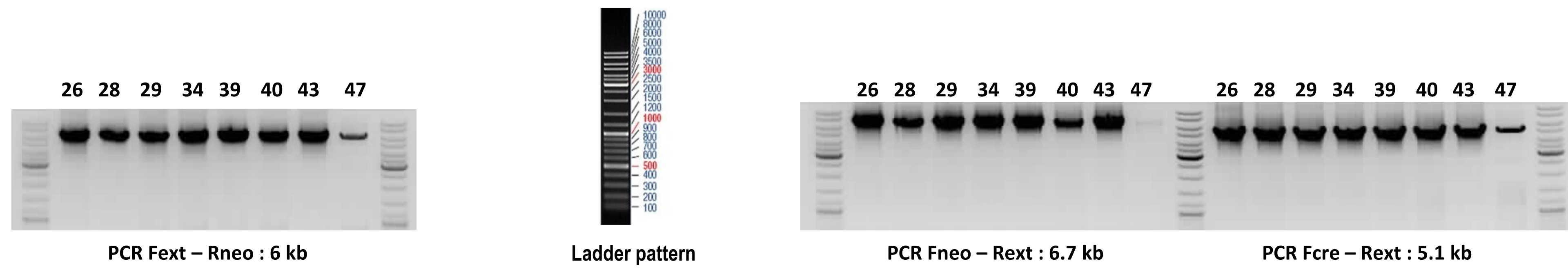
Eight candidate clones out of the positive clones were selected for 5’ Long-Range PCR and Southern blot validation.

Recombinant ES validation by Long Range PCR



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





PCR Fext – Rneo : 6 kb

Ladder pattern

PCR Fneo – Rext : 6.7 kb

PCR Fcre – Rext : 5.1 kb

Eight candidate clones identified by 5' PCR screening were further analysed by 3' PCR screening.
Seven clones (clones #26, #28, #29, #34, #39, #40 and #43) were confirmed.

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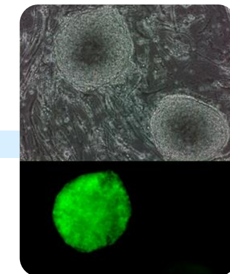
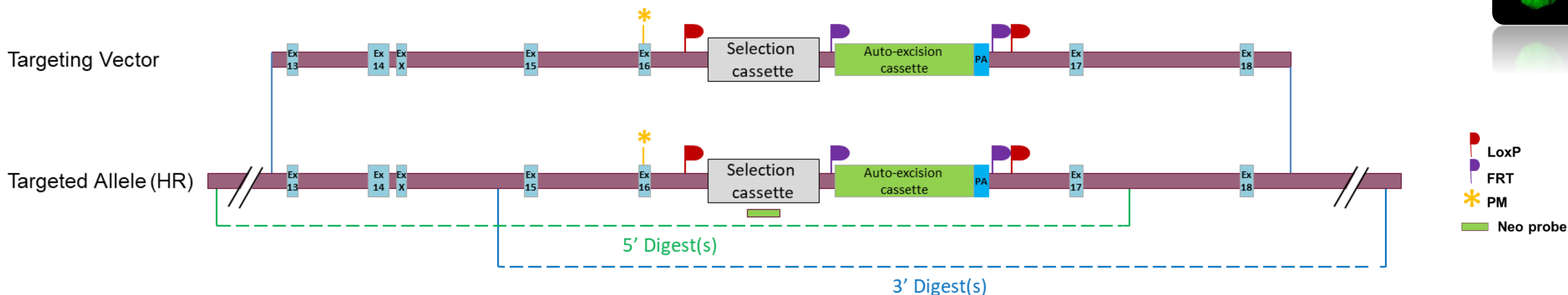
Recombinant ES clones validation by Southern Blot – Internal probe



Schematic Southern Blot validation strategy

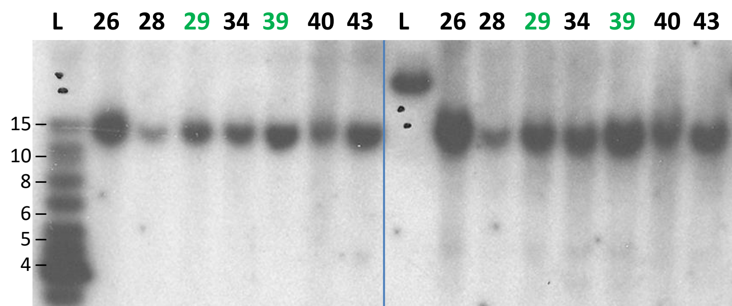
Digests on the scheme illustrate the position of the chosen restriction sites relative to the probe. They don't show the exact position of the restriction sites.



Digestions used to validate the 5' and 3' insertion

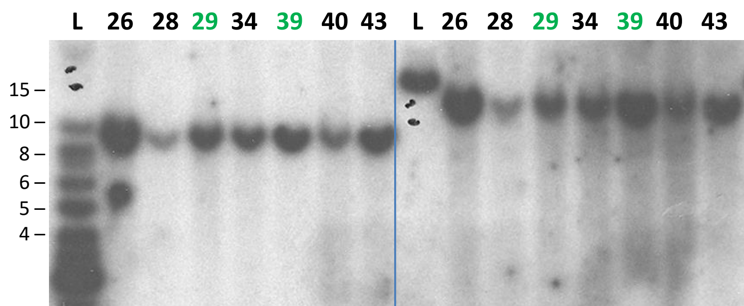
Probe		Genomic DNA digest	Targeted Allele (kb)
Neo	5' digest	SexAI	9.4
		DraIII	9.6
	3' digest	XcmI	9.7
		HincII	12.8

Southern blot - Neo 5'



SexAI DraIII

Southern blot - Neo 3'



XcmI HincII

Neo probe sequence

AGAAGAACTCGTCAAGAAGGCGATAGAAGGCGATGCGCTGCGAATCGGGAG
CGGCGATACCGTAAAGCACGAGGAAGCGGTCAGCCCATTCGCCGCCAAGCT
CTTCAGCAATATCACGGGTAGCCAACGCTATGTCCTGATAGCGGTCCGCCA
CACCCAGCCGGCCACAGTCGATGAATCCAGAAAAGCGGCCATTTTCCACCA
TGATATTCGGCAAGCAGGCATCGCCATGGGTCACGACGAGATCCTCGCCGT
CGGGCATGCGCGCCTTGAGCCTGGCGAACAGTTCGGCTGGCGCGAGCCCCT
GATGCTCTTCGTCCAGATCATCCTGATCGACAAGACCGGCTTCCATCCGAG
TACGTGCTCGCTCGATGCGATGTTTCGCTTGGTGGTCGAATGGGCAGGTAG
CCGGATCAAGCGTATGCAGCCGCCGCATTGCATCAGCCATGATGGATACTT
TCTCGGCAGGAGCAAGGTGAGATGACAGGAGATCCTGCCCCGGCACTTCGC
CCAATAGCAGCCAGTCCCTTCCCGCTTCAGTGACAACGTCGAGCACAGCTG
CGCAAGGAACGCCCGTCGTGGCCAGCCACGATAGCCGCGCTGCCTCGTCCT
GCAG

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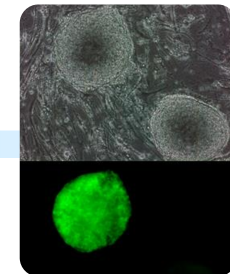
Recombinant ES clones validation by Southern Blot – External probe



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.

Wild type Allele (WT)

Ex 13, Ex 14, Ex X, Ex 15, Ex 16, Ex 17, Ex 18

3' Digest(s)

Targeting Vector

Ex 13, Ex 14, Ex X, Ex 15, Ex 16, Selection cassette, Auto-excision cassette, PA, Ex 17, Ex 18

Targeted Allele (HR)

Ex 13, Ex 14, Ex X, Ex 15, Ex 16, Selection cassette, Auto-excision cassette, PA, Ex 17, Ex 18

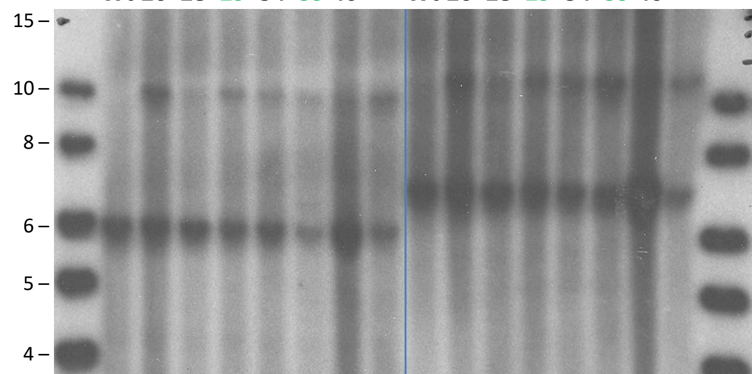
3' Digest(s)

LoxP

FRT

PM

Southern blot – 3' probe



EcoNI 6 / 10.3 AflII 7 / 11.3

3' probe sequence

CCACAGCACGGTGGACTTGAGTGAAGCTAAAGTGTCGCTTGTC
ACAGATCACAGATTCTGGATCCCAGCCTCAGAGCTGCTGGCTG
AGCAGATCTGGGCAGGGCCTGAAGACTGGCTGAGCGTGCGCCT
CAGGGACCAGAATCCGAGCCACTGGCCTGCCATAGCCGATTCT
CCTTGTGGAGACACTGCATATATAACCTATGTAACCTCACACT
TCTTTTCCTTCCTCTCTTCCTTCTCTCAAAGATTAGCATAAAA
TGATTGAAGAGACTTTGAATTTAAACTTGCTTAGAATTTCTGT
TTAGGACTTAGAAAAAAGGAACTTAGAGTTGCTAGGCCAGCCT
GTAACGTTCAGGC

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	EcoNI	6	10.3
	3' second digest	AflII	7	11.3

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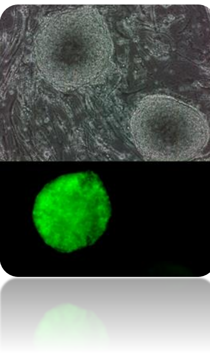
Aneuploidy screening in ES recombinant clones



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

Results of aneuploidy analysis are presented in the table below.

Clone ID	Giemsa
#26	Pass
#29	**Pass**
#34	Limit
#39	**Pass**
#40	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/6 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 #29 and #39 validated in previous project phase were injected into blastocysts to generate chimeric males. The results are presented in the table below.

Clone ID	Number of chimeric males identified according to chimerism rate (Number of chimeric males bred to F1 generation)			
	5 - 40%	45% - 55%	60-100%	Total
#29	0	3	4	7
#39	3	1	2	6

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Breeding to F1 generation

- Two 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 : 28/03/2012

- Allele nomenclature (following MGI guidelines) : **$\mathbf{Med23^{tm2.1(R614Q)Ics}}$**

6 CONFIRMATION OF THE INTRODUCED MUTATION

Clone #29

Clone #39

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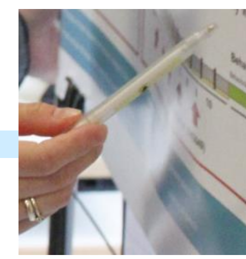
7 SEQUENCE OF THE DELIVERED ALLELE



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GGATTTTTTACCCCTACAATTGTAGTGGAAATATGTAAATACTTGTGTGTGTGTGTGTATGTCTGTGTTTATAGTCATTTTCACATACATCCTGAGCGACATTCTTCTGATATATACAGGGATGTCCTTTTGCTAACTGGTC
CCTGGAATTCTGCCCTGGTAGGTCAGCTCCTGCCCACTGTCTTCAAGTCCCACGCCTGGGGCATCCTGCACACACTGCTGGAGATGTTCAGCCAC**CAA**ATGCACCACATTCAGCCCCACTACCGAGTTCAGCTCCTG
AGCCATCTCCACACACTGGCTGCAGTCGCACAGACCAACCAGAACCAGCTCCATCTGTGGTGAGTGAGCAGCCTGCAGGCTGCCTCCCCTAGGGTTTAGTCCACCCATGCTTGGAAGTGGGGGTCTTAATCCCCCT
GCCCCCAGGTCCTTTTTTTTTCTTATTTGCTGTAAAATTAAAATTCTGCTTATAAGACAAAAGAGTGAAACCCAAGTTTTTAAAAGAATTTGGATTTACATGTTTCAAGTCATGATTAAAGTACCATTTTATGGAAGGC
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GTGTGTGCTGCCTACCTAGTAGTGAGGTGCCATCTCTCTAGGAGAAGGG

LoxP

Exon 16

CGA>CAA (R614Q)

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INVESTIR L'AVENIR

REPORT REDACTION & VALIDATION
Protocol finalized on 2023/08/25
Prepared by Romain LORENTZ, IE
Verified by Marie-Christine BIRLING, PhD

CONTACT US
By email at mutagenesis@igbmc.fr
By phone at +33 (0)3 88 65 56 57

www.phenomin.fr

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