Generation of a KO of MIF in rat by CRISPR/Cas9
Final report

Kur7103 / IM7103

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phenomin

EXCELLENCE IN MOUSE PHENOGENOMICS

INVESTISSEMENTS D'AVENIR

Strategy validated

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Université de Strasbourg

Instituts thématiques Inserm

Institut national de la santé et de la recherche médicale

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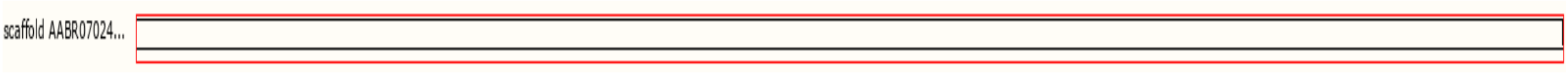
centre européen de recherche en biologie et en médecine

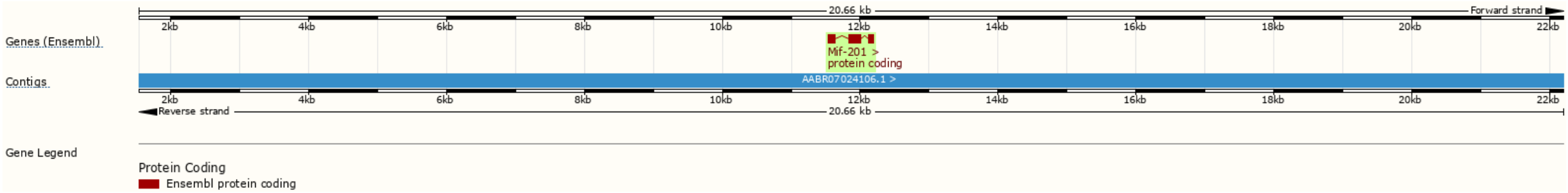
Aix*Marseille université

Mif Gene Rat Genomic locus

Scaffold AABR07024106.1: 11,534-12,195

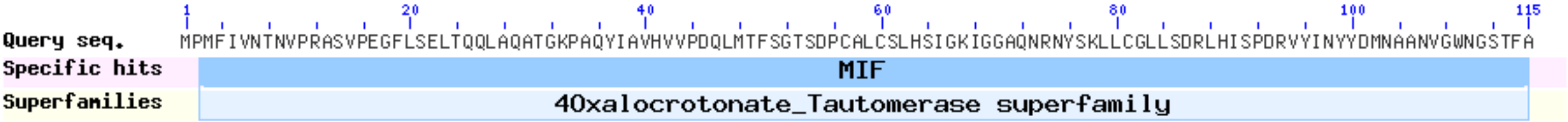


Gene: Mif ENSRNOG00000056076



Mif mRNAs and proteins

Name	Transcript ID	bp	Protein	Biotype	UniProt
Mif-201	ENSRNOT00000077564.1	348	115aa	Protein coding	A0A0F7RQL3 P30904



Both 5’ and 3’ UTR are not defined in rat but must exist.

Strategy 1: Selection of the 5’ sgRNAs

http://crispor.tefor.net/crispor.py?batchId=fsuAE7PvZzA0GS95RJCu

Guide Sequence + *PAM* + Restriction Enzymes Only G- Only GG- Only A-	Specificity Score	Predicted Efficiency Show all scores Doench '16	Mor.-Mateos	Out-of-Frame score Click on score to show micro-homology	Off-targets for 0-1-2-3-4 mismatches + next to PAM	Genome Browser links to matches sorted by CFD off-target score exons only chrom 20 only
GTTTCCTAAGATAGAAACGG *AGG* Enzymes: *BtsCI, TspGWI* **Cloning / PCR primers**	83	71	58	63	0 - 0 - 4 - 12 - 120 0 - 0 - 0 - 0 - 0 136 off-targets	3:intergenic:Rnpc3-Col11a1 4:intergenic:PCDH7-RGD1562755 4:intergenic:RGD1559682-ENSRNOG00000008814 show all...
GCATCCTCCGTTTCTATCTT *AGG* Enzymes: *BstDEI* **Cloning / PCR primers**	79	37	23	78	0 - 0 - 0 - 7 - 103 0 - 0 - 0 - 0 - 0 110 off-targets	4:intron:Plxna4a 4:intergenic:ETV1-ENSRNOG00000004239 4:intergenic:Vgll4-Tamm41 show all...
ACTACCTAGCTTATTAAATG *AGG* Enzymes: *LweI* **Cloning / PCR primers**	76	61	32	63	0 - 0 - 1 - 25 - 119 0 - 0 - 0 - 12 - 3 145 off-targets	4:intron:Skap2 4:intergenic:Nbea-ENSRNOG00000031758 4:intergenic:ENSRNOG00000021496-RGD1564736 show all...

Strategy 1: Selection of the 3' sgRNAs

http://crispor.tefor.net/crispor.py?batchId=VHN9oFt3O5Hg7a7Lg8N4

Guide Sequence + *PAM* + Restriction Enzymes Only G- Only GG- Only A-	Specificity Score	Predicted Efficiency Show all scores Doench '16	Mor.-Mateos	Out-of-Frame score Click on score to show micro-homology	Off-targets for 0-1-2-3-4 mismatches + next to PAM	Genome Browser links to matches sorted by CFD off-target score exons only chrom 20 only
TCCAGGCTGGGAACGTGCGA *TGG* Enzymes: *BtgZI* **Cloning / PCR primers**	94	46	30	63	0 - 0 - 1 - 6 - 80 0 - 0 - 0 - 0 - 0 87 off-targets	4:intergenic:RGD1563169-GRIA1 4:intergenic:Msi2-1700106J16Rik 4:intergenic:Cntn3-ENSRNOG00000032820 show all...
GAACGTGCGATGGGGCGTGA *TGG* **Cloning / PCR primers**	93b	55	61	59	0 - 0 - 0 - 4 - 31 0 - 0 - 0 - 1 - 0 35 off-targets	4:intergenic:Pramel1-Pramef8 3:intergenic:Slc44a3-F3 4:intergenic:Mtmr1-F8 show all...
CCCATCGCACGTTCCCAGCC *TGG* Enzymes: *BseYI, StyD4I, BstNI* **Cloning / PCR primers**	87	53	24	65	0 - 0 - 0 - 6 - 56 0 - 0 - 0 - 1 - 0 62 off-targets	4:intron:Katnal2 4:intergenic:ENSRNOG00000011225-ENSRNOG00000050774 4:intergenic:Litaf-Rmi2 show all...

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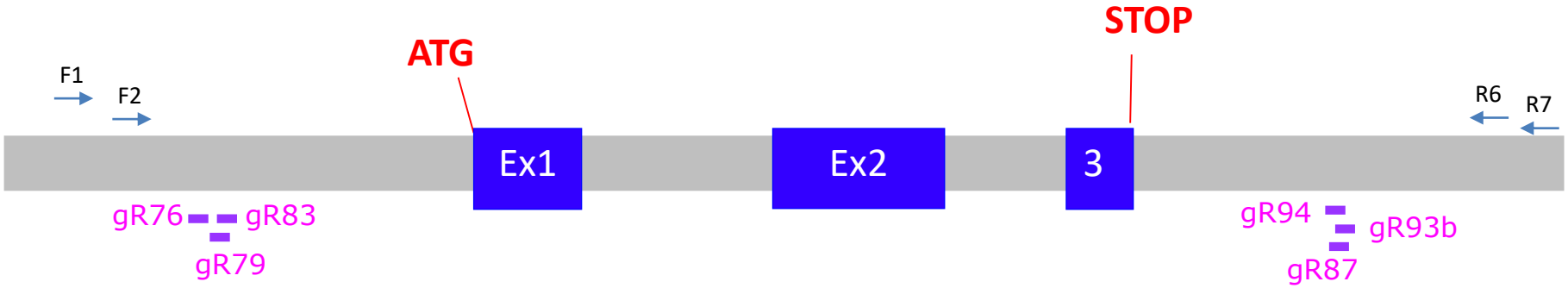
Strategy 1: Deletion of the whole Mif gene

Approach proposed: KO by CRISPR/Cas9

Accepted the 17/09/18



Position of the sgRNA target sequence (in yellow, PAM are in purple)

```
AAGTCCGTCG ATCCTACCCA CTGGCAGGAG AGATAAGGCC AACCTACCGG TCCCATCAAT GGCTTAAGTT TCCTCTACTT GGTACAAATC TCTCAGACCT GAACTTGCTC CTACTAATAC GGTTAATCTG TACAGCATCT ACTTGCAATG
TTCAGGCAGC TAGGATGGGT GACCGTCCTC TCTATTCCGG TTGGATGGCC AGGGTAGTTA CCGAATTCAA AGGAGATGAA CCATGTTTAG AGAGTCTGGA CTTGAACGAG GATGATTATG CCAATTAGAC ATGTCGTAGA TGAACGTTAC

                                                gR76                                                gR79
TCTCGACGAA CCTAATCGCT AGAGTCAAGT CCTCACTACC TAGCTTATTA AATGAGGCAT CCTCCGTTTC TATCTTAGGA AACAGAGAGC CAATGTAATA TTTTCTAGAG GCACAGCAAG ACCTCGGCAG AAACAGCGCG CTGGAGCGTA
AGAGCTGCTT GGATTAGCGA TCTCAGTTCA GGAGTGATGG ATCGAATAAT TTACTCCGTA GGNGGCAAAG ATAGAATCCT TTGTCTCTCG GTTACATTAT AAAAGATCTC CGTGTCGTTC TGGAGCCGTC TTTGTCGCGC GACCTCGCAT
                                                                                                    gR83

GTCACCGCCC CCTTTGGGAC GTGGCCTGAC GTCAGCGGAG GCGTAGCGAG GGGAGGAGCA GCAGCCGGCT TGGGGCGGTC CTGAGCTGGG TCACGTAGCT CAGGTCCCAG ACTTGGGTCA CACCGCGCTT TACACCGTCC TCCGGCCGTC
CAGTGGCGGG GGAAACCCTG CACCGGACTG CAGTCGCCTC CGCATCGCTC CCCTCCTCGT CGTCGGCCGA ACCCCGCCAG GACTCGACCC AGTGCATCGA GTCCAGGGTC TGAACCCAGT GTGGCGCGAA ATGTGGCAGG AGGCCGGCAG

                ATG                                                 Ex1
GCTCGCAGTC TCTCCGCCAC CATGCCTATG TTCATCGTGA ACACCAATGT TCCCCGCGCC TCCGTGCCAG AGGGGTTTCT CTCCGAGCTC ACCCAGCAGC TGGCGCAGGC CACCGGCAAG CCGGCACAGG TTTGCAGGGA GGGCACAGGA
CGAGCGTCAG AGAGGCGGTG GTACGGATAC AAGTAGCACT TGTGGTTACA AGGGGCGCGG AGGCACGGTC TCCCCAAAGA GAGGCTCGAG TGGGTCGTCG ACCGCGTCCG GTGGCCGTTC GGCCGTGTCC AAACGTCCCT CCCGTGTCCT
AGAGAGTAGG GTGGGGTGGG CCGGCCGGAC GTGTGAGGAG GAATGGGGCT GGAAGCCAAG GCGGGCCGGC GGGTGGCGGC TGGAGCTTTC TGCAAGACCT GCGGCCCTGT AACCACGGGC GGGCTAACCG CGTGTCTGTC CCTCCCGCTC
TCTCTCATCC CACCCCACCC GGCCGGCCTG CACACTCCTC CTTACCCCGA CCTTCGGTTC CGCCCGGCCG CCCACCGCCG ACCTCGAAAG ACGTTCTGGA CGCCGGGACA TTGGTGCCCG CCCGATTGGC GCACAGACAG GGAGGGCGAG

                                                    Ex2
TCCGCCCCTC CCCCCACACA GTACATCGCA GTGCACGTGG TCCCGGACCA GCTCATGACT TTTAGTGGCA CGAGCGACCC CTGCGCCCTC TGCAGCCTGC ACAGCATCGG CAAGATCGGT GGCGCCCAGA ACCGCAACTA CAGCAAGCTG
AGGCGGGGAG GGGGGTGTGT CATGTAGCGT CACGTGCACC AGGGCCTGGT CGAGTACTGA AAATCACCGT GCTCGCTGGG GACGCGGGAG ACGTCGGACG TGTCGTAGCC GTTCTAGCCA CCGCGGGTCT TGGCGTTGAT GTCGTTCGAC
                Ex2
CTGTGCGGCC TGCTGTCCGA TCGCCTGCAC ATCAGCCCGG ACCGGTGCGT GGGGGTTGAG GGGCACTGGG AGGTCGGCTG GCTTGGGGGC GTTCGGTGGG CACCGGCACG CAGCACGCCC TCCGAGACCA CGTGCTTTAG CTCAGTAGAC
GACACGCCGG ACGACAGGCT AGCGGACGTG TAGTCGGGCC TGGCCACGCA CCCCCAACTC CCCGTGACCC TCCAGCCGAC CGAACCCCCG CAAGCCACCC GTGGCCGTGC GTCGTGCGGG AGGCTCTGGT GCACGAAATC GAGTCATCTG
                                                                STOP
                    Ex3
CCTTTCATTC TCTCAGGGTC TACATCAACT ATTACGACAT GAACGCAGCC AACGTGGGCT GGAACGGTTC CACCTTCGCT TGAGCCCGGG CCTCACTTAC CTGCACCGCT GTTCTTCGAG TCTTGCTGCA CGCCCCGTTC TGTGTTTATC
GGAAAGTAAG AGAGTCCCAG ATGTAGTTGA TAATGCTGTA CTTGCGTCGG TTGCACCCGA CCTTGCCAAG GTGGAAGCGA ACTCGGGCCC GGAGTGAATG GACGTGGCGA CAAGAAGCTC AGAACGACGT GCGGGGCAAG ACACAAATAG
                                                                                                                                                    gR93b
                                                                                                                                                gR94
CACCCGTAAT GATGGCCACC TTCCGGTCGG GAGAAATAAA TGGTTTGAGA CCATGGTTGC CTCCGCTTCT GCTTCCTTGG CTTGCTTGCG AGGGGTTGGG TGCAGGGCGG GGACCTTGAA TGGAAATCCA GGCTGGGAAC GTGCGATGGG
GTGGGCATTA CTACCGGTGG AAGGCCAGCC CTCTTTATTT ACCAAACTCT GGTACCAACG GAGGCGAAGA CGAAGGAACC GAACGAACGC TCCCCAACCC ACGTCCCGCC CCTGGAACTT ACCTTTAGGT CCGACCCTTG CACGCTACGC
                                                                                                                                                            gR87
gR93b
GCGTGATGGC AGACTTGAAA TTGGCCAGTT TGGTGGTTTA CATAGTCTTT GCTTGGGCTT TCTCTAAACT GTGCTGGCCT TACGTCCAAA AGAAGCCGAC TTAGTAAACT GGTCAGGAGT TGTATCAGAA CTTCCCGGGC AGTTGTTGCA
CGCACTACCG TCTGAACTTT AACCGGTCAA ACCACCAAAT GTATCAGAAA CGAACCCGAA AGAGATTTGA CACGACCGGA ATGCAGGTTT TCTTCGGCTG AATCATTTGA CCAGTCCTCA ACATAGTCTT GAAGGGCCCG TCAACAACGT
```

Strategy 1: Deletion of the whole Mif gene

PROS

- The whole Mif gene will be deleted (only 1.1 kb)
- This deletion will lead to a knock-out

CONS

- /

Milestones

- The guide RNAs will be synthesized and validated *in vitro.* A pair of sgRNAs for both extremities will selected for microinjection
- Microinjection in Sprague Dawley (rat) fertilized oocytes
- F0 founders genotyping
  - ✓ PCR + sequencing (estimation of the deleted sequence)
- F0 founders of interest will be bred to obtain germ line transmission
- F1 validated animals will characterized
  - ✓ By PCR + sequencing
- Fully validated lines (F1 ou F2 animals) will be delivered

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Data

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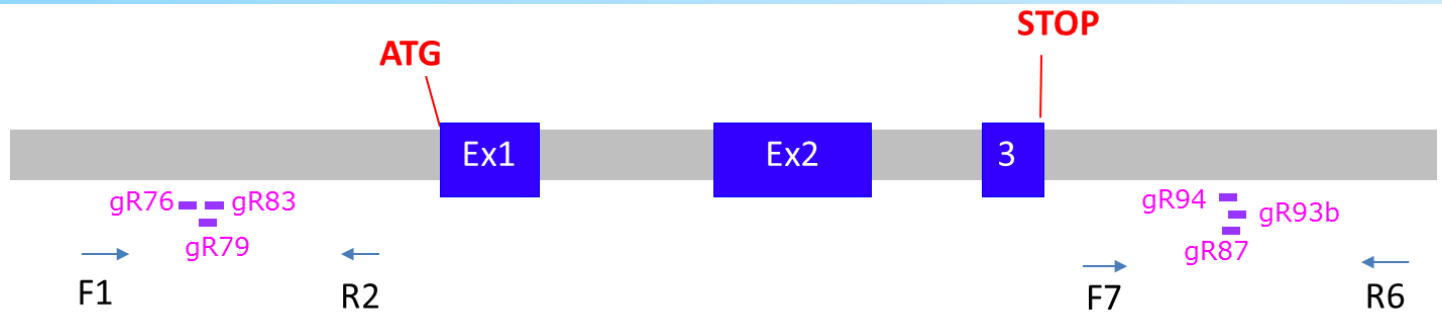
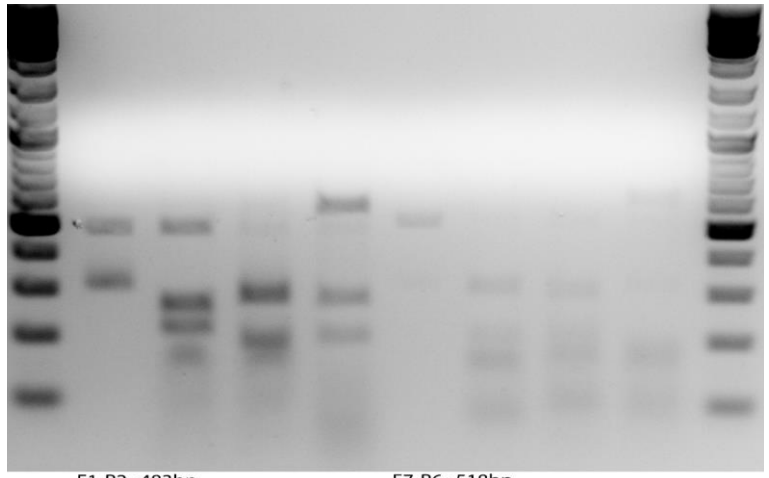
In vitro efficiency assay



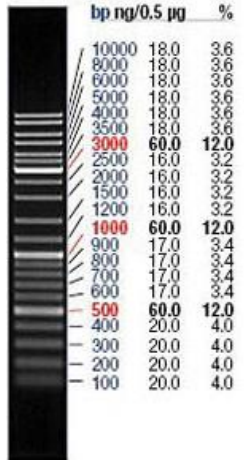
F1-R2 +gR76 +gR79 +gR83 F7-R6 +gR94 +gR87 +gR93b

F1-R2 (483bps)



F1-R2=483bp
+gR76=216+267
+gR79=194+289
+gR83=187+296

F7-R6=518bp
+gR94=208+310
+gR87=205+313
+gR93b=198+320



CRISPR guide efficiency was tested *in vitro* using Sureguide kit (Agilent Technologies 5190-7716).

In presence of the Cas9 protein, the PCR product including the region of interest should be cut. A guide is validated if it cuts the target PCR fragment. The amount of DNA cut does not mean anything and the cut does not guarantee the *in vivo* efficiency of the guides.

The pair gR76 and gR79 (5') and the pair gR94 and gR87(3') were choosen for microinjection.

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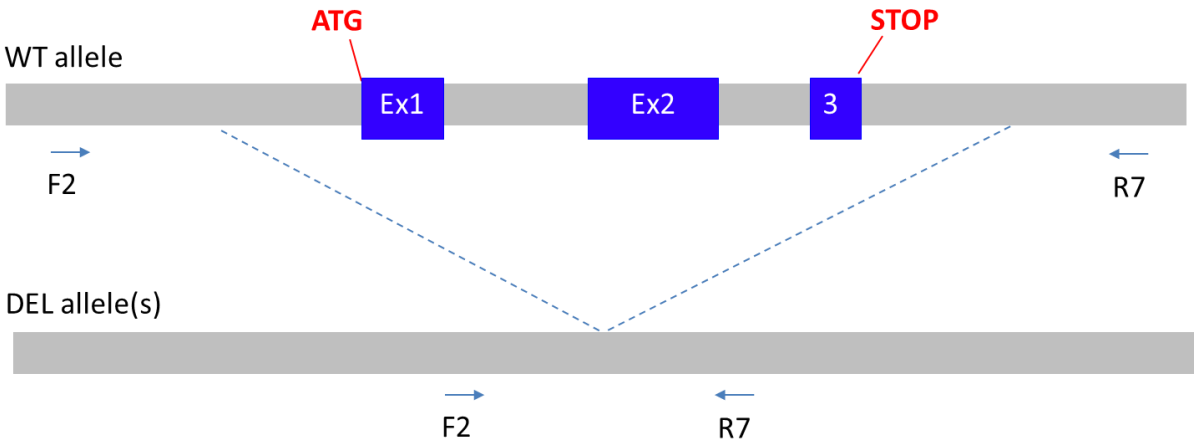
Microinjection in C57BL/6N fertilized eggs

Concentration ADN/ARN (ng/µl)	Nb oocysts reimplanted	Nb pups born	Number of founders with a deletion allele
25 ng/µl Cas9 mRNA + 12 ng/µl gR76 + 12 ng/µl gR79 + 12 ng/µl gR94 + 12 ng/µl gR87	115	14	2
10 ng/µl Cas9 mRNA + 10 ng/µl gR76 + 10 ng/µl gR79 + 10 ng/µl gR94 + 10 ng/µl gR87	57	8	3
Total	**172**	**22**	**5**

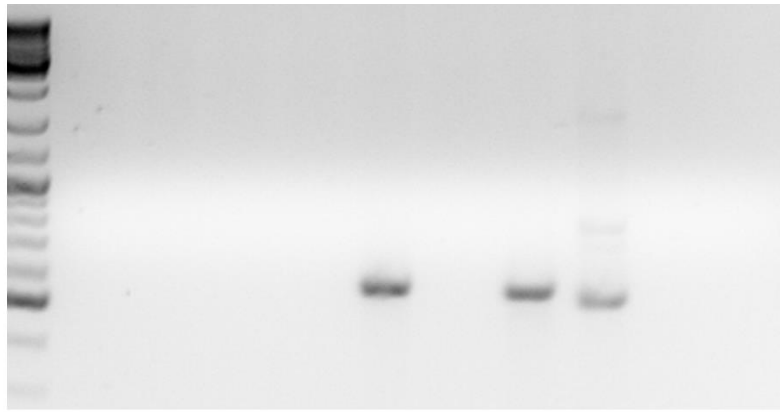
PCR genotyping for the characterization of founders

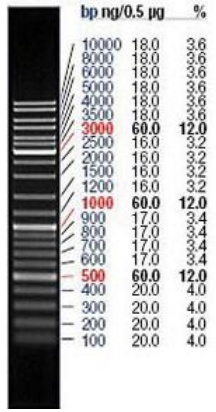


F0 generation characterization

MW 6975 6976 6977 6978 6979 6980 6981 6982 WT H_20

Expected DEL alleles size





PCR F2-R7 was used to screen the pups.
The figure shows the genotype of the first litters.

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Next step

- Three founders (F0-6979, F0-6981 and F0-6982) were bred at 8 weeks (mid-february 2019) with wild type animals. The next slide will describe the genotyping of some F1.
- New lines are now established.

Kur7103-6979-DEL

Line established

Oligo F2 in orange
Oligo R7 in green
Ins C in red

ttcgctaaagtccgtcgatcctaccCactggcaggagagataaggccaacctaccggtcccatcaat
ggcttaagtttcctctacttggtacaaatctctcagacctgaacttgctcctactaatacggttaatctgt
acagcatctacttgcaatgtctcgacgaacctaatcgctagagtcaagtcctcactacctagcttatta
atatgaggcatcctccgtttc----------JUNCTION DEL----------
ccgtgcgatggggcgtgatggcagacttgaaattggccagtttggtggtttacatagtctttgcttggg
ctttctctaaactgtgctggccttacgtccaaaagaagccgacttagtaaactggtcaggagttgtatc
agaacttcccgggcagttgttgcatcctgagctaggttcttccgcctgggcgggaatcctgaattgtgc
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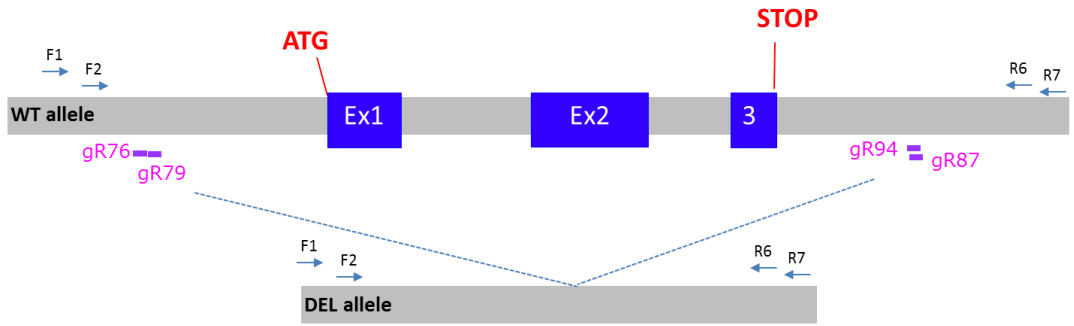
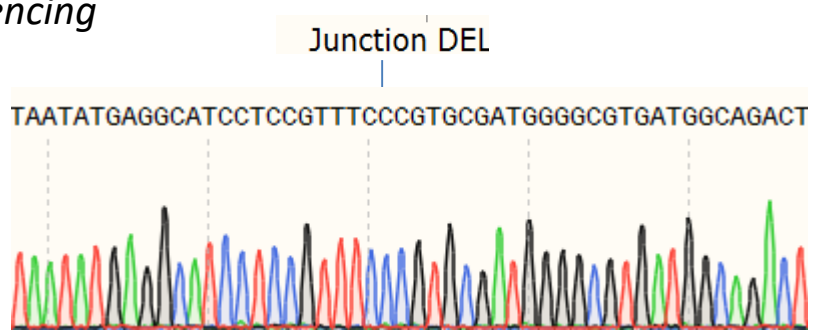
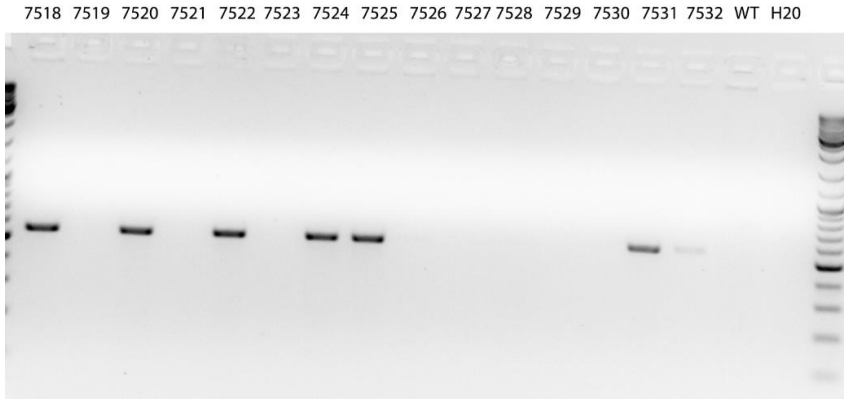


Sanger sequencing



F1 genotyping

F2-R7



DEL
PCR size
540bps

FO	Nb F1 born	F1 heterozygote DEL (del)	
		F	M
Kur7103-6979	25	6	6

Kur7103-6979-DEL line is established. This line was cryopreserved (Mif^{em1Ics}).

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Kur7103-6981-DEL

Line established

Oligo F2 in orange
Oligo R7 in green
Insertion in red

ttcgctaaagtccgtcgatcctaccCACTGGCAGGAGAGATAAGGCCAACCTACCGGTCCCATCAATG
GCTTAAGTTTCCTCTACTTGGTACAAATCTCTCAGACCTGAACTTGCTCCTACTAATACGGTTAATCTGTA
CAGCATCTACTTGCAATGTCTCGACGAACCTAATCGCTAGAGTCAAGTCCTCACTACCTAGCTTATTAA-
----------JUNCTION DEL----------
cgTTCCATGCACGTAACGatggggcgtgatggcagacttgaaattggccagtttggtggtttac
atagtctttgcttgggctttctctaaactgtgctggccttacgtccaaaagaagccgacttagtaaactg
gtcaggagttgtatcagaacttcccgggcagttgttgcatcctgagctaggttcttccgcctgggcggg
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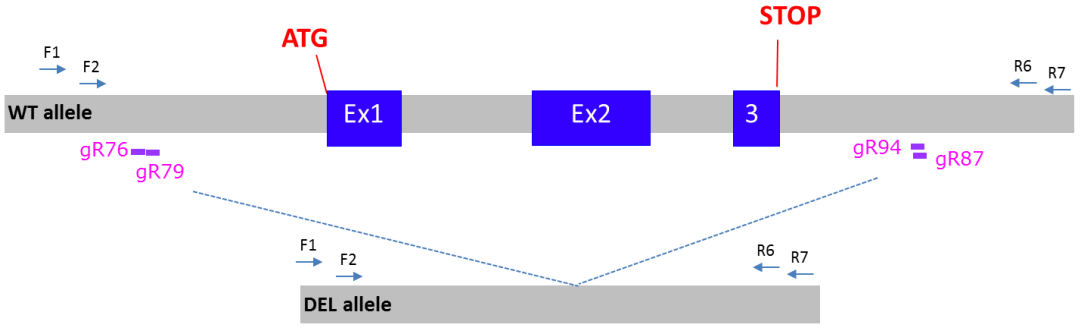
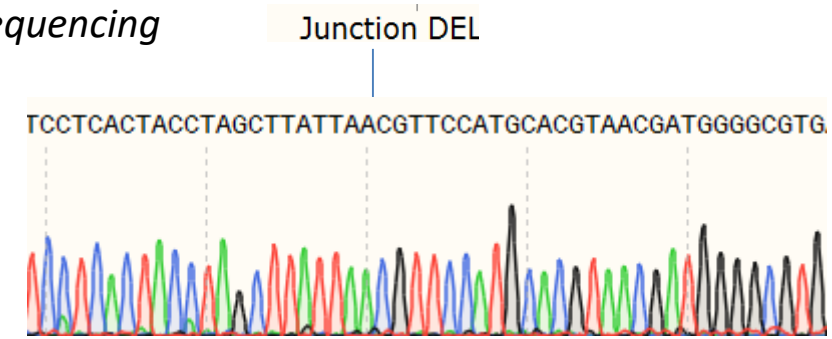


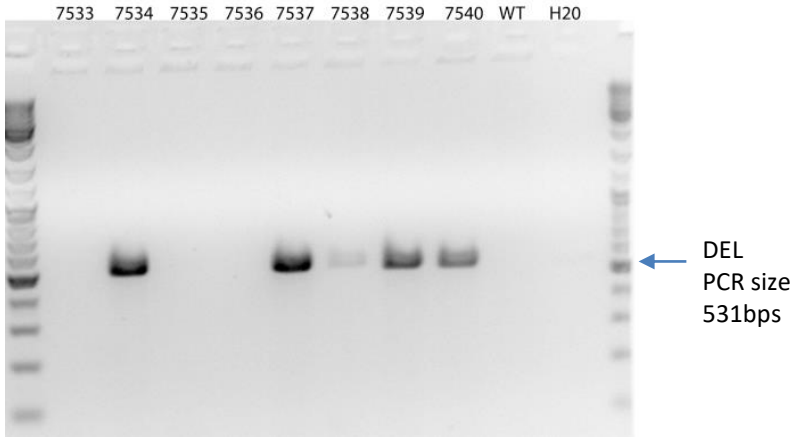
Sanger sequencing



FO	Nb F1 born	F1 heterozygote DEL (del1142)	
		F	M
Kur7103-6981	39	13	12

F1 genotyping

F2-R7



Kur7103-6981-DEL line was established but not cryopreserved.

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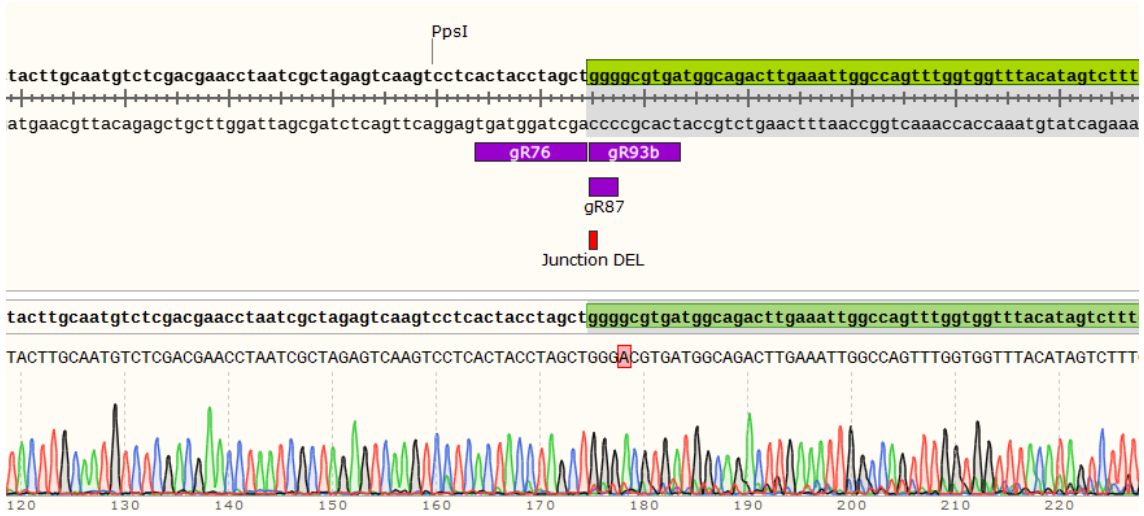
Kur7103-6982

Sequence with F2-R7
Oligo FWD in orange
Oligo REV in green

ttcgctaaagtccgtcgatcctaccCACTGGCAGGAGAGATAAGGCCAACCTACCGGTCCCATCAATGGCTTAAGTTTCCTC
TACTTGGTACAAATCTCTCAGACCTGAACTTGCTCCTACTAATACGGTTAATCTGTACAGCATCTACTTGCAATGTCTCGACG
AACCTAATCGCTAGAGTCAAGTCCTCACTACCTAGCT----------JUNCTION DEL----------
GGGGCGTGATGGCAGACTTGAAATTGGCCAGTTTGGTGGTTTACATAGTCTTTGCTTGGGCTTTCTCTAAACTGTGCTGGCCTT
ACGTCCAAAAGAAGCCGACTTAGTAAACTGGTCAGGAGTTGTATCAGAACTTCCCGGGCAGTTGTTGCATCCTGAGCTAGG
TTCTTCCGCCTGGGCGGGAATCCTGAATTGTGCCCTTCCTTACCTCTGTGGCAAATGGAAGGGGAGTTGAGTGGGCAAAAGT
ATAGGGATTAAATTAATAGTGCGGCAGCTGGgcctgtatctttctcagccccattt

Sanger sequencing



Founder 6982 (born 12/12/2018) breeding was stopped of germ line transmission was obtained by breeding 2 other founders

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INVESTISSEMENTS D'AVENIR

Genotyping protocol

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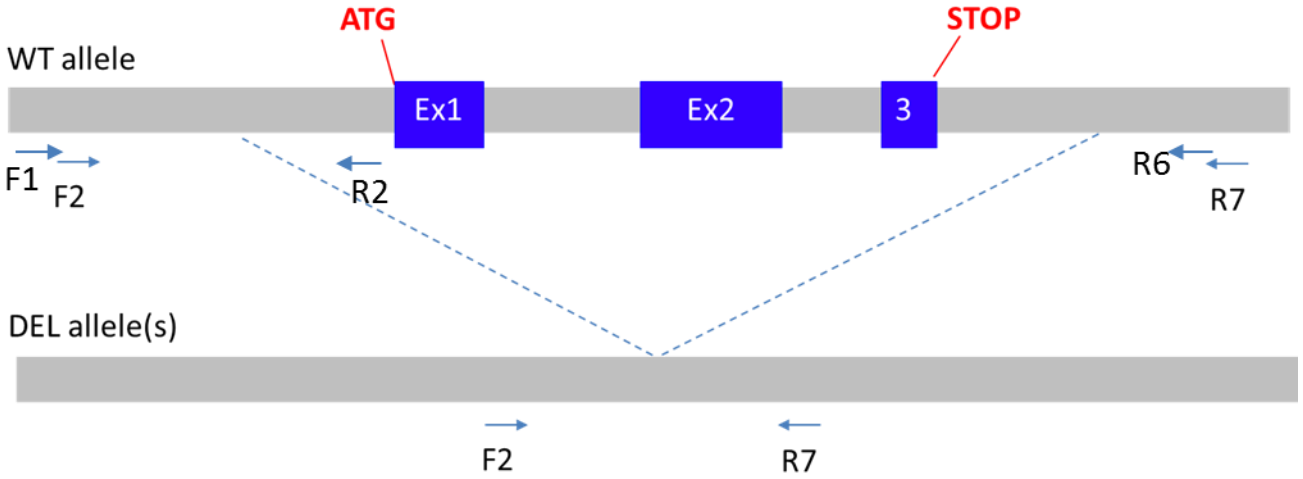
Université de Strasbourg

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

GENOTYPING INSTRUCTIONS



PCR genotyping strategy

Primer ref.	Sequence	Amplification product size WT	Amplification product size KO
F2	TTCGCTAAAGTCCGTCGATCCTACC	1567 bps*	~500 bps
R7	AAATGGGGCTGAGAAAGATACAGGC		
F1	GATTGTTCTCCAAGTACAAGCCATC	483 bps	NA
R2	CCCAAGTCTGGGACCTGAGCTACGT		
F7	GCTTTAGCTCAGTAGACCCTTTCAT	518 bps	NA
R6	GGAAGGGCACAATTCAGGATTCCCG		

This PCR fragment might not be amplified on tails/ear lysat (too large)

F1-R2 and/or F7-R6 can be used to genotype the wild type allele

GENOTYPING INSTRUCTIONS

PCR Protocol

This section describes the composition of the mix and the cycling conditions used for genotyping F0.

Reagents:	Volume (per sample):
- Phusion HS (Thermo Scientific) 5X Buffer	4 µl
- 10mM dNTP	0.4µl
- 5’ primer (100 µM)	0.1µl
- 3’ primer (100 µM)	0.1µl
- DNA (lysate 1/10)	2µl
- Phusion Hot Start II	0.2µl
- Sterile H2O	up to 20 µl

Cycling conditions

Temp	Time	#Cycles
96°C	5min	1
96°C	8s	
62°C	10s	30
68°C	45s	
68°C	5min	1
12°C	5min	1

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