
# CRISPR/Cas9 genome editing in rodents : *In vivo* and *in vitro* applications

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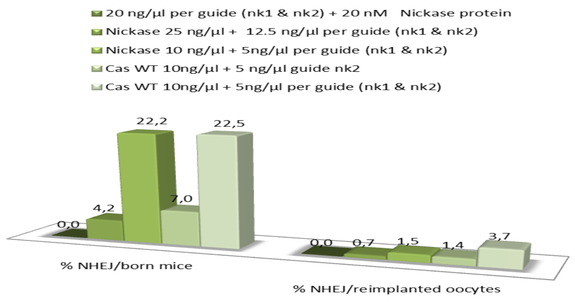
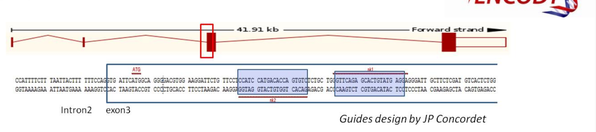
**Introduction :** The iCS is producing more than 150 new mouse lines per year and most of them are generated by conventional homologous recombination in ES cells (a well defined and very efficient workflow). Most of these lines are 'à la carte' targeted mutant. Different projects have been undertaken in the last 2 years using nucleases (ZFN, TALE and CRISPR/Cas). We describe here some of our CRISPR/Cas projects (*in vivo* and *in vitro* approaches)

## 1. Generation of Klhl15 KO using CRISPR/cas approach

- Microinjection guides and Cas9 mRNAs into the pronucleus of C57Bl/6N fertilized eggs
- Founders with double stand breaks (DSB) are often mosaic (up to 4 different alleles observed in F1 mice)
- Frequency of DSB increased with the mRNA concentration
- KO to be confirmed by Western blot




*Guides design by JP Concordet*

**Founder -68 : 4 differents lines (alleles)**

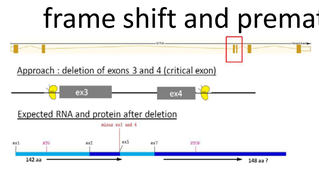

Line R5068-68 (del1 A or T)

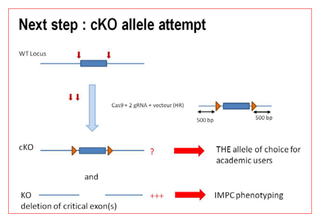

Line R5068-68 (del79)

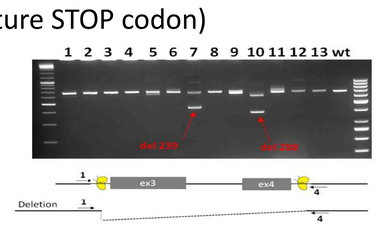

ATG removed after CRISPR/cas genome editing but another 'in frame ATG' might be recognized as an initiation ATG

## 2. The best strategy to obtain KO (quick and efficient)

- Microinjection in C57Bl/6N fertilized oocytes
- Deletion of one or more 'critical 'exon' (generation of a frame shift and premature STOP codon)




⇒Deletion of both exons 3 and 4 confirmed by sequencing tails PCR product for mice 7 and 10 (15 % of born mouse)

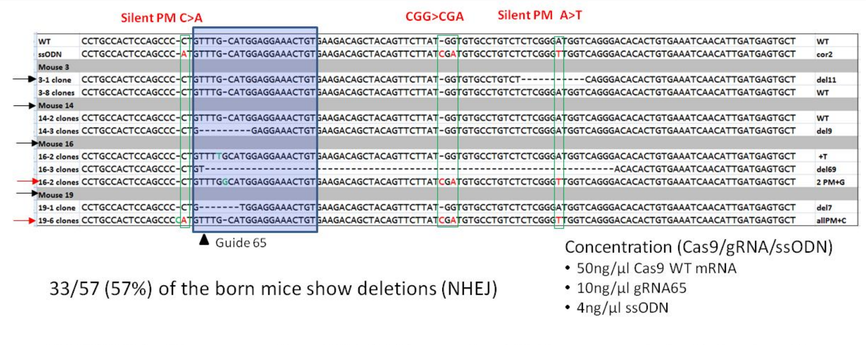
⇒Real KO allele

⇒Validation of the IMPC strategy for genes with no available ES cell clones (http://www.mousephenotype.org/)

## 3. Correction of the Crb1$^{rd8}$ in C57BL/6N Mice

- Fertilized C57Bl/6N oocytes were co-injected with one or 2 guide RNAs, Cas9 mRNAs and a ssODN bearing the desired mutation plus 3 silent diagnostic mutations.
- Different injection conditions were tested
- Homology-directed repair was observed in 3.5 % (2/57) of live-born animals and showed minimal illegitimate recombination of donor DNA.
- Extensive founder mosaicism was observed, emphasizing the need to analyze founder's offsprings.
- Two founders carried the corrected allele, but they also carried a secondary nearby mutation in Crb1 nullifying the functional rd8 correction (as observed in Low et al (2014) Invest Ophthalmol Vis Sci.2014;55:387–395 and and previous *in house* experiments with TALE nucleases).

**Founders genotyping results after PCR subcloning and sequencing**




33/57 (57%) of the born mice show deletions (NHEJ)

Concentration (Cas9/gRNA/ssODN)
- 50ng/µl Cas9 WT mRNA
- 10ng/µl gRNA65
- 4ng/µl ssODN

2/57 (3.5%) of the mouse show HDR (Rd8 correction) BUT accompanied with the insertion of a supplementary nucleotide at the site of clivage of the guide RNA

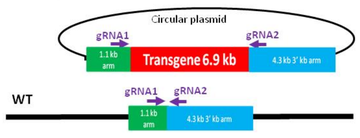
## 4. Knock-in of a 6.9 kb transgene into the *Rosa*26 locus using CRISPR/Cas

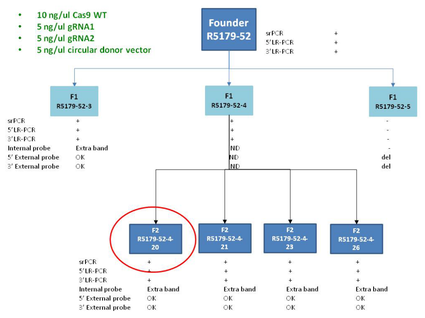
*Collaboration with Romeo Ricci's lab (IGBMC, Illkirch, France),*

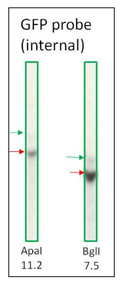
- Microinjection in C57Bl/6N fertilized oocytes
- High frequency of NHEJ events
- Low frequency of homologous recombination (1.1 %)
- Efficiency of HR varies between microinjectionnists
- Germ line transmission achieved

Classical *Rosa* HR arms: 15 kb circular targeting vector (1.5 and 3.5 kb HR arms, 6,9 kb Tg Knock-In)
- Cas9 WT et nickase mRNAs
- Guide RNAs
- Circular plasmid

Microinjection into C57BL/6N pronucleus

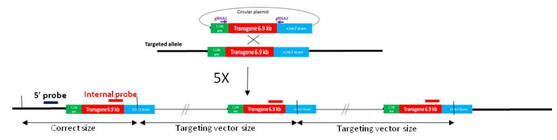


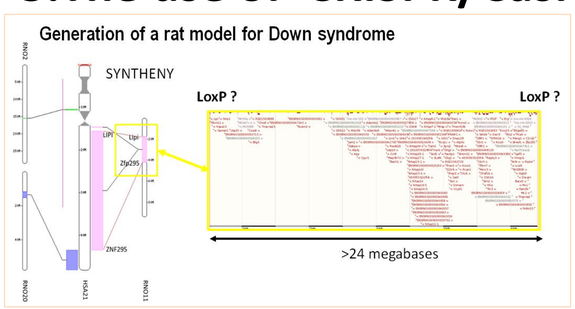




NUMBERS
- About 1000 eggs microinjected
- >700 eggs reimplanted
- 90 mice born and analyzed

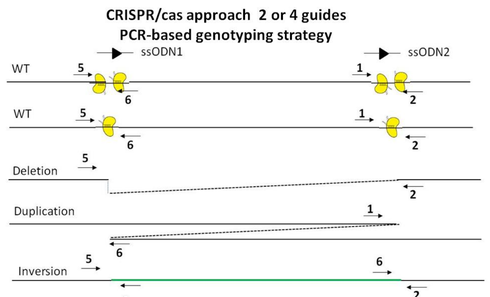
- Only one HR event with contamere at the locus
  - Hypothesis : one crossing over event in the targeted construct (supplementary copies at the locus)



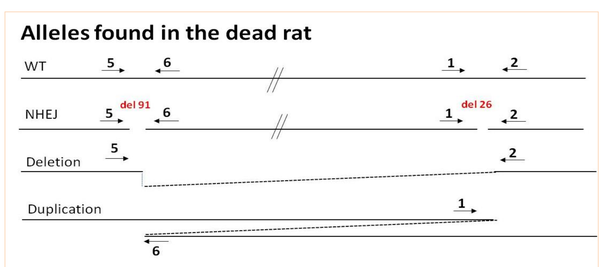

## 5.The use of CRISPR/Casfor big genomic rearrangements in rat



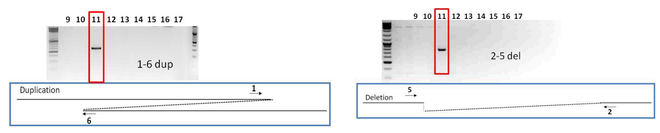

- 20 rat pups analyzed
- 1 rat found dead at birth




Four oligonucleotides: two amplifying the first CRISPR target site and two others the second one are needed to detect the different re-arrangements.



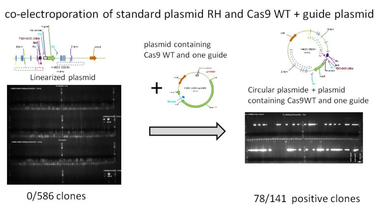

*Del and dup confirmed by sequencing*




- Generation of cells carrying partial monosomy and trisomy in a diploid context
- **> 24 Mb del-dup observed**
- Mosaicism
- In human, complete monosomy 21 leads to premature death of the patient
- Very fast to obtain deletions and duplications compared to classical route using the Cre/LoxP system (3-4 months vs 3-4 years)

## 6. CRISPR/Cas highly improves homologous recombination *in vitro* in ES Cells

- Co-electroporation of a standard targeting vector and a vector expressing a specific guide RNA and the Cas9 dramatically improves the homologous recombination efficiency
- We managed to rescue 100% of the unsuccessful project
- Insertion of supplementary copies (concatemeres) has been observed at the locus (see 4.) when a circular targeting construct is electroporated




Rescue of failed project with the help of CRISPR/cas

| Project | Nb of clones screened w/o CRISPR/cas | Nb of clones screened with CRISPR/cas | Nb of positives clones | % positive clones by LR-PCR | |
|---|---|---|---|---|---|
| 1 | 0/543 | 372 | 16 | **4,3** | Linearized construct |
| 2 | 0/586 | 141 | 78 | **55,3** | |
| 3 | 0/586 | 186 | 46 | **24,7** | |
| 4 | 0/1560 | 158 | 26 | **16,5** | |
| 5 | 0/85 | 186 | 165 | **88,7** | Long arm |
| 6 | ND | 159 | 131 | **82,4** | Short arm |
| 7 | ND | 93 | 44 | **47,3** | |
| 8 | ND | 163 | 154 | **94,5** | |

**Conclusion :** The CRISPR/Cas technology is a powerful tool for the generation of genetically engineered rodents. The generation of KO lines is straight forward, deletions and duplications work well. The efficiency for the generation of complicated alleles (ie Knock-In) needs still to be improved and off-targets events need to be assessed.