Generation of targeted overexpressing models by CRISPR/Cas9 and need of careful validation of your knock-in line obtained by nuclease genome editing

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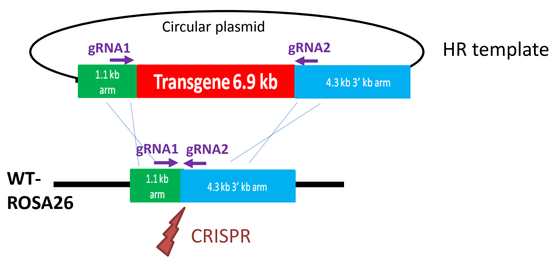
Introduction:

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) protein 9 system has been described as a robust and multiplexable genome editing tool, enabling researchers to precisely manipulate specific genomic elements, and facilitating the elucidation of target gene function in biology and diseases. It opens new possibilities for gene modifications and will likely start a new era for genetics. However, CRISPR/Cas9 gene editing is a very young method. The range of possibilities for genome modification and at the opposite the technical limits & bias of CRISPR/Cas9 still need to be better assessed.

1a. Knock-in of a 6.9 kb transgene into the *Rosa*26 locus using CRISPR/Cas

Classical *Rosa26* HR arms: 15 kb circular targeting vector (6.9 kb Tg Knock-In)
Cas9 (WT or nickase) mRNAs + guide RNAs + circular plasmid template for Homologous recombination (HR)

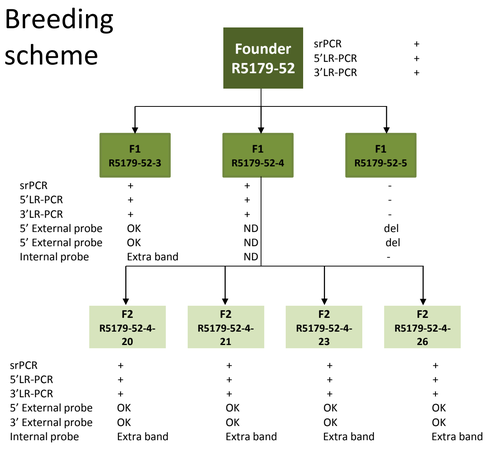


- Microinjection in C57BL/6N fertilized oocytes
  - 990 eggs microinjected & 90 mice born
- High frequency of NHEJ events
- Germ line transmission achieved with low frequency of homologous recombination (1.1 %)

One founder obtained **Founder R5179-52**

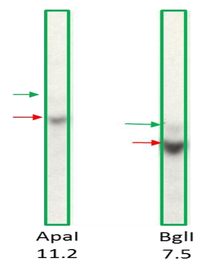
1b. Multiple transgene insertion

Breeding scheme



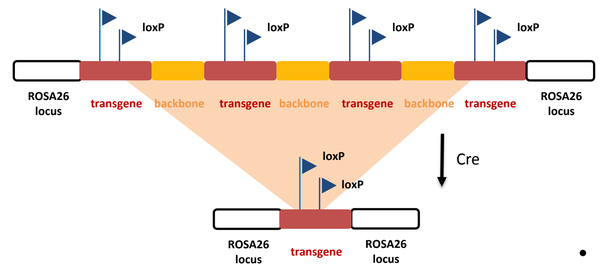
Southern Blot with a GFP internal probe

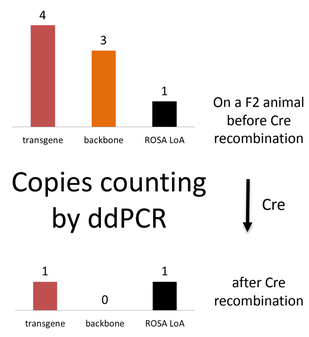
- Extra band not segregated by breeding
- Extra band = multicopy
- Multicopy corresponding to the restriction pattern of the complete vector



1c. At the target site

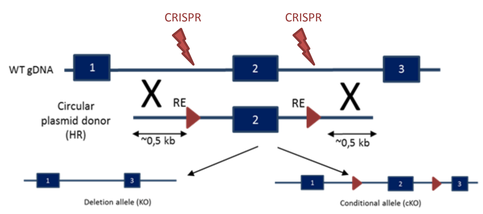
Tandem integration of the 6.9 kb transgene

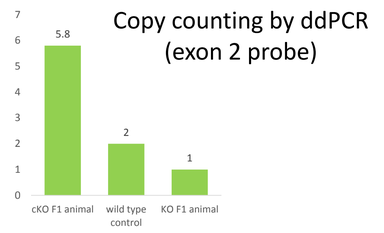




- Copy counting by ddPCR show removal of the floxed sequence

2. Same results for generation of conditional knock-out model by CRISPR/Cas9

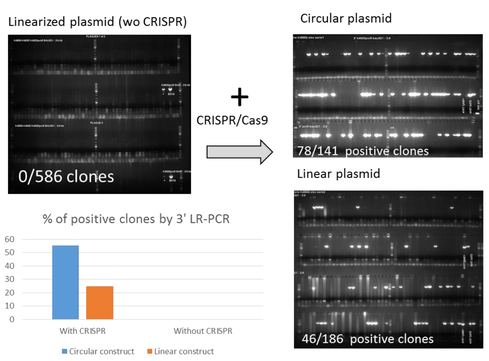




3a. CRISPR/Cas highly improves homologous recombination *in vitro* in ES

Rescue of failed project with the help of CRISPR/cas

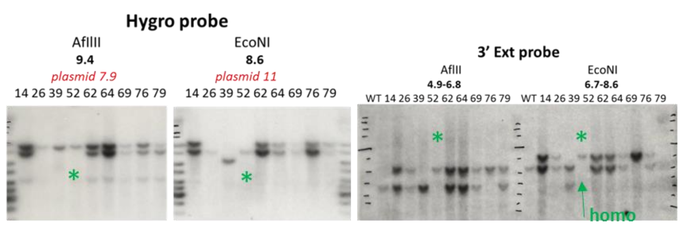
Project	Nb of clones screened w/o CRISPR/cas	Nb of clones screened with CRISPR/cas	Nb of positives clones	% positive clones by LR-PCR	
1	0/543	372	16	4,3	Linearized construct
2	0/586	141	78	55,3	
3	0/586	186	46	24,7	
4	0/1560	158	26	16,5	
5	0/85	186	165	88,7	Long arm
6	ND	159	131	82,4	Short arm
7	ND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



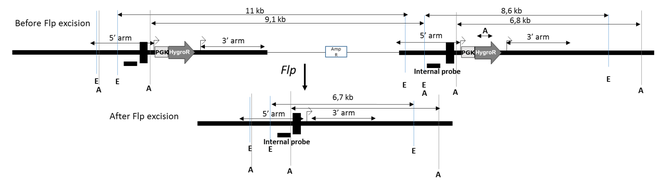
- Co-electroporation of a standard targeting vector and a vector expressing a specific guide RNA and the Cas9 dramatically improves the homologous recombination efficiency (see beside)
- 100% of our unsuccessful projects have been rescued

3b. But concatemers are also observed

Southern Blot analyses on mutant ES clones

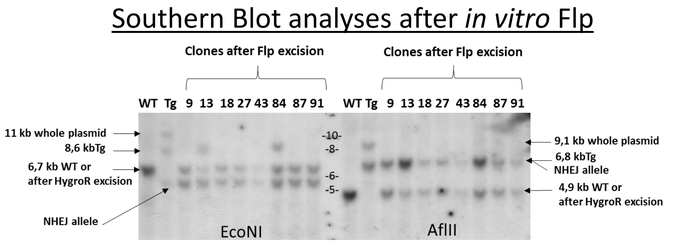


- The different constructs electroporated as circular template were integrated as 'concatemers' at the target site for more than 50% of the tested mutant ES cells



- It is possible to get rid of these extra copies if loxP or FRT sites are located in the targeting construct (see figure on the right hand side)

Southern Blot analyses after *in vitro* Flp



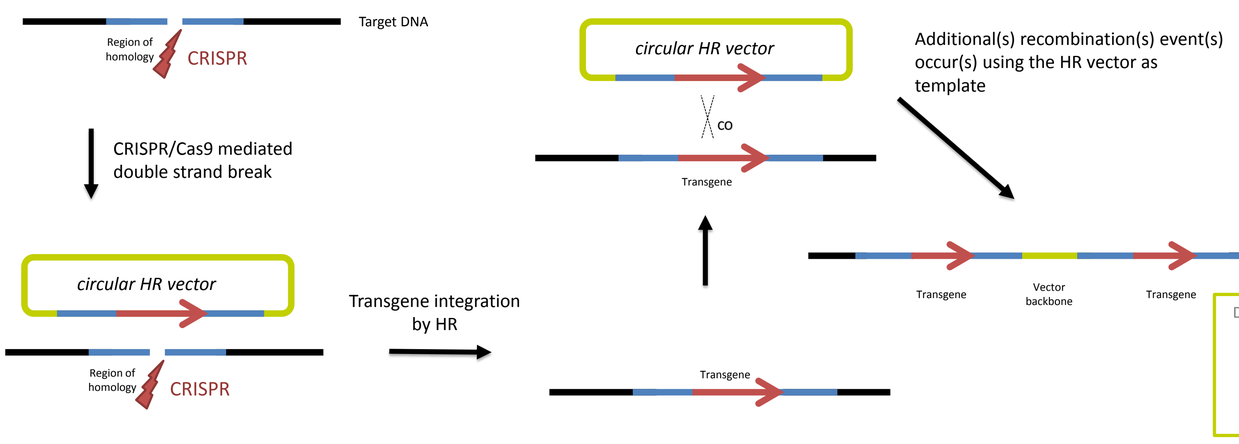
Conclusions & recommendations

Injection of a circular DNA template with both Cas9 and a target specific gRNA is a powerful method for generation of overexpressing models.
Transgene will be integrated in multiple copies, all in the same orientation and at the target locus.

Models obtained by CRISPR/Cas9 by homologous recombination should be carefully validated. The number of copies integrated at the locus should by verified by Southern Blot, qPCR or ddPCR - with an internal probe or simply by a short PCR that detects the vector backbone.

Proposed mechanism

Integration of the transgene by homologous recombination (HR) followed by multiple integration of the vector by one crossing over.



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