Title: A pancreas specific *Ptf1a*-driven Cre mouse line causes paternally transmitted germline recombination

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Abstract:
Cre-*lox*P recombination system is a commonly used tool to achieve site-specific genetic manipulation in genome. For multiple Cre driver mouse lines, parental transmissions of recombined flox alleles are reported. *Ptf1a*-driven Cre lines are widely used to achieve genetic manipulation in a pancreas specific manner. Herein, we report germline recombination in breedings when *Cre* allele is retained paternally in *Ptf1a*$^{tm1(cre)Hnak}$. The germline recombination frequency changed depending on the target allele. Therefore, unless the reporter allele is on the target gene, the reporter activity is to be validated. Overall, we highlight that *Ptf1a-Cre* mouse lines should be genotyped for possible germline recombination and we advise the maternal transmission of the *Cre* to progeny.

Introduction:
Identified firstly in 1981 (Sternberg and Hamilton), "Causing recombination" (Cre) is isolated from P1 bacteriophage, which is shown to recombine "*locus of crossing over (x1),* P1*"* (loxp) sites [1]. Since its discovery, use of Cre-*lox*P system is highly utilized in mouse models. Depending on the approach, it can be used to provide precision in both cell type and temporal control of the genetic manipulation.

Although the method is powerful, recent evidence suggests certain limitations. Cre-mediated cytotoxicity is reported for various cell types both *in vitro* and *in vivo* [2–11]. In these studies, postulated reasons are DNA damage induced by Cre-mediated genome-wide non-specific recombination events and altered cell signaling pathways (e.g. PKA) [12]. Additionally, organ-specific Cre expression is highly problematic due to limitations in identification of individual proteins getting expressed in specific cell types.

Multiple Cre lines have been developed in order to achieve pancreas-specific recombination in mouse models. Among many, one of the most used models is the line in which *Cre* is knocked in *Pancreas associated transcription factor 1a* (*Ptf1a*, a.k.a. *p48*) locus [13]. *Ptf1a* has an essential role during pancreatic epithelium generation. Further differentiation of sub-pancreatic compartments like acini, endocrine, and ducts are also regulated by *Ptf1a* [14–19].

In order to study *Ptf1a* function in the developing mouse, Nakhai et al. generated a model in which *Cre*-coding sequence was knocked in the Exon-1 of *Ptf1a* via homologous recombination ($Ptf1a^{tm1(cre)Hnak}$). Several studies showed PTF1A expression in developing pancreas [16], neural tube [20], and cerebellum [21,22]. With the use of $Ptf1a^{tm1(cre)Hnak}$ mouse model, PTF1A was shown to be expressed also in the mouse retina in addition to the previously identified locations [13]. Interestingly, a recent study proposed a regulatory function of *Ptf1a* in hypothalamus for sexual development during embryonic stage [23]. Although there are many studies for the function of *Ptf1a* in the developing mouse, the ones focusing specifically on the reproductive system of adult mice are in scarce.

Here, we provide evidence for paternally driven germline recombination mediated by Cre expression in $Ptf1a^{tm1(cre)Hnak}$ mouse line. The recombination event was observed in multiple floxed alleles, while the frequency varied depending on the target gene. Additionally, *Cre* allele transmission into progeny was not necessary for the germline recombination. Recent other reports also support parental transmission of germline recombination in many different Cre driver lines [24–34]. Therefore, precaution needs to be considered for the experimental design of the mouse studies. Here in, we propose that the *Cre* transmission should be carried out only through females in which we observed no germline recombination in the progeny.

Results:

In order to study the function of *RelB* in pancreatic cancer, we aimed to generate a mouse model, which expresses $KRAS^{G12D}$ and knocks out *RelB* in the pancreas. Therefore, using mouse models $Relb^{tm1Fwei}$, $Ptf1a^{tm1(cre)Hnak}$, and $Kras^{tm4Tyj}$ we followed a breeding scheme as provided in Figure 1A. Our genotyping primers for *RelB* were already designed to recognize all wt, flox, and recombined alleles (kindly provided by Marc Reimann), which made it possible for us to

track-down the recombination in every pup. Initially, in F0 generation a *Relb*$^{fl/fl}$ male was put into breeding with 2 separate females either with a *Ptf1a-Cre/+* or *Kras*$^{fl/+}$. In the F1 generation, females with either *Relb*$^{fl/+}$; *Ptf1a-Cre/+* or *Relb*$^{fl/+t}$;*Kras*$^{fl/+}$ were put into breeding with *Relb*$^{fl/fl}$ males in order to obtain homozygous *Relb*$^{fl/fl}$ allele either with *Ptf1a-Cre* or *Kras*. None of the progeny from these breedings had a recombined *Relb*^{fl} allele (data not shown). And finally, in the F2 generation, we put a *Relb*$^{fl/fl}$; *Ptf1a-Cre/+* male into breeding with a *Relb*$^{fl/fl}$; *Kras*$^{fl/+}$ female. Interestingly, 28 of 28 pups had one recombined and one unrecombined flox alleles observed upon ear-genotyping (Figure 1B). The absence of germline recombination in F2 generation was validated with post-genotyping (Figure 1C). The germline recombination occurrence was irrespective of their *Cre* status (Figure 1B). This implies *Cre* transmission is not required for germline recombination in the descendants. To validate the whole-body recombination, mice with heterozygous germline recombination were sacrificed and their various organs were harvested for DNA extraction and PCR genotyping. The results show that all of the organs contained one recombined and one unrecombined *Relb* allele, while no *Kras* germline recombination was observed in the respective pups (Figure 1D).
The genotyping results of the pancreatic DNA implies a difference in the recombination efficiency in different floxed genes. While the recombination efficiency of the single-unrecombined floxed *RelB* allele is quite strong, *Kras* recombination is not that efficient. Immunoblot analysis of the various organs also showed a reduction in RelB expression (Figure 1E). However, since none of the alleles had a reporter activity, we couldn't test additional measurements.

To see if the same recombination event occurred in different alleles we used two different mouse lines: *Rel*^{tm1Ukl} and *GFP-Rel*. *Rel*^{tm1Ukl} is a knock-in mouse model in which endogenous *Rel* Exon-1 locus is flanked by 2 *loxp* sequences. Upon recombination, exon 1 is recombined and a promoterless *GFP* gene meets with a *PGK* promoter. This way, *Rel* recombination can be efficiently traced down by GFP expression. GFP-Rel is a transgenic mouse model (kindly provided by Marc Schmidt-Supprian) in which a BAC construct with a *PGK* promoter controlled, N' *GFP* tagged *Rel* is integrated into genome. Simplified maps along with the locations of the primers recognizing the recombined and unrecombined floxed alleles are shown in Supplementary Figure 1.

Breedings with *Rel*^{fl} (*Rel*^{tm1Ukl}) and *Ptf1a-Cre* carrying males indicated similar results as in *RelB* mice. In a retrospective PCR analysis of 3 different breeding pairs with *Rel*^{fl}; *Ptf1a-Cre/+*

fathers, 36/45 pups (80%) had recombined *Rel* allele, while 9 of them (20%) retained their unrecombined floxed *Rel* (for pups where 1 Rel^{fl} but not *wt* allele is paternally transferred) (Table 1). PCR from the different organs collected from various recombined mice also supported the germline recombination presence (Figure 2A). Immunoblot analysis from the heterozygous recombined mice also indicated eGFP expression only in the germline recombined mice and *Ptf1a-Cre/+* pancreas along with a reduced c-Rel expression (Figure 2B). Of note, breeding pairs with 6 different females with Rel^{tm1Ukl}; *Ptf1a-Cre/+* showed no recombination in their progeny (0/93 pups, 0%) (Data not shown).

In order to analyze if there was recombination in reproductive system of the males which were not germ-line recombined, we isolated DNA from testes and pancreas. PCR analysis showed recombination of *Rel* in both of the testes along with pancreas, although none of the mice had germline recombination. Interestingly, despite *Rel* was recombined, *Kras* was not (Figure 2C). *Tp53* is a very commonly used gene in mouse models to study pancreatic cancer. In order to test possible *Tp53* germline recombination in $Trp53^{tm1Brn}$ model, we genotyped 5 different breeding pairs in which Cre was paternally transmitted. We observed no *Tp53* germline recombination in these mice (data not shown). Interestingly, the male reproductive system still showed recombined *Tp53* allele (Figure 2D).

To eliminate the possibility of mosaic Cre recombination in the tail and ear biopsies, we took advantage of the *GFP-Rel* mouse model. Because it is a transgenic allele, it has been kept always in a heterozygous state, and always bred with a wildtype mate. To test possible mosaic recombination, a male with $GFP\text{-}Rel^{tg/+}$; *Ptf1a-Cre/+* was bred with $Kras^{fl/+}$ females. A new set of primers were designed to recognize both unrecombined and recombined alleles (Supplementary Figure 1). Among 40 pups, 22 of them have *GFP-Rel* allele. 14 of 22 were re-genotyped for *GFP-Rel* germ line recombination. 6/14 mice had *GFP-Rel* recombination in tail PCRs irrespective of *Cre* transmission, and there was no unrecombined *GFP-Rel* allele in these mice (Table 2) (Figure 3). This eliminates the possibility of a mosaic recombination.

Discussion:

In this work, we provided evidence that paternal transmission of *Cre* in $Ptf1a^{tm1(cre)Hnak}$ mouse model can cause germline recombination in the progeny. For the germline recombination, *Cre* transmission to progeny was not necessary. The germline recombination frequency changed depending on the target floxed allele. Neither *Kras* nor *Trp53* showed germline recombination

while *RelB, Rel* and *GFP-Rel* showed in their respective mouse models with varying frequencies. No *Kras* recombination in the germline was observed. We postulate two possible reasons for this: recombination happens in the father germ cells before the fertilization occurs and the recombination efficiency of *Kras* is very low. Unlike other alleles, we didn't observe any *Kras* recombination in the testes of the *Kras*$^{fl/+}$*; Ptf1a-Cre/+* mice. Additionally, a difference in the *RelB* and *Kras* recombination efficiency can be observed in the pancreas PCRs (Figure 1D). While almost all of the floxed *RelB* allele was recombined in the *Ptf1a-Cre/+* pancreata, a big portion of *Kras* is still unrecombined.

Additionally, we observed recombination of the *Tp53* allele in the testes but not in the germline in *Trp53*^{tm1Brn} model. A possible explanation is the acquired disadvantage for fertilization in the *Tp53* knock out sperms. Although *Tp53*$^{-/-}$ males are fertile, germ cell quality was reported to be regulated by *Tp53* [35–39]. *Trp53*^{tm2Tyj} mouse model (LSL-Trp53^{R172H}) has been highly used in pancreatic cancer research, especially in therapeutics [40]. Therefore, it is important to analyze whether or not it is recombined, as it can influence the drug response.

Depending on the gene, homozygous germline recombination might lead to embryonic lethality. However, many essential genes might still persist recombined heterozygously in the whole body which may or may not show any impact phenotypically. Especially in preclinical studies focusing on pancreatic pathology such as cancer and pancreatitis, a gross crosstalk is present between pancreatic and other cells (immune cells, neurons, blood vessels, stellate cells). Therefore, even if the heterozygous recombination of a protein is absent under normal circumstances, it may still impact the phenotype under pathological conditions.

Another highly used *Ptf1a*-driven mouse line is *Ptf1a*$^{tm1.1(Cre)Cvw}$ in which the entire *Ptf1a*-coding sequence was replaced with *Cre* [16], whereas in *Ptf1a*$^{tm1(cre)Hnak}$ Exon 1 was replaced [13]. In both of the mouse lines transcriptional regulatory elements before the coding sequence were retained. While we tested paternal recombination transmission only in *Ptf1a*$^{tm1(cre)Hnak}$, we anticipate such an event to occur also in *Ptf1a*$^{tm1.1(Cre)Cvw}$. In support of this, in JAX, paternal germline recombination transmission was already reported for an inducible *Ptf1a*$^{tm2(cre/ESR1)Cvw}$ model in which Exon-1 and 2 were replaced by *Cre-ERTM* [41].

Overall, we propose a necessity of genotyping for wt, floxed, and recombined alleles in all mice during regular mouse maintenance in all *Ptf1a-Cre* driven mouse lines. Accordingly, additional

information regarding the genotyping protocols of the mouse models should be asked and provided by journals for good scientific practice. Unfortunately, for many of the mouse models there are no available primer sequences for the detection of recombined allele. Likewise, the maps and sequences of the targeted constructs are also in most of the cases not available for the individual design of primers. With this observation, we alert scientists, journals and reviewers to take immediate precautions for future studies.

Materials and methods:

Mouse models

The mouse models used are *Ptf1a*$^{tm1(cre)Hnak}$ [13]; *Kras*^{tm4Tyj} [42]; *Relb*^{tm1Fwei} [43]; *Rel*^{tm1Ukl} [44]; *GFP-Rel* (manuscript in revision, Marc Schmidt Supprian); *Trp53*^{tm1Brn}[45]. Mice were kept in an animal room at room temperature (20-22°C) with light:dark cycle of 12:12 hours (light period: 06:00 am–06:00 pm) in groups of 2–4 animals in type III cages with bedding and nesting material. All animals were provided ad libitum with the standard food (No. 1324 – 10 mm pellets, Altromin, Lage, Germany) and water. All animals housed under specific pathogen-free conditions in accordance with the European Directive 2010/63/EU. All animal experiments were approved and conducted in accordance with the federal German guidelines for ethical animal treatment (Regierung von Oberbayern).

Sample lysis and PCR:

Collected samples are lysed in 200ul DirectPCR Lysis Reagent (Viagen) supplemented with 10ul PCR grade Proteinase K (Sigma) at 55°C. Once the samples are lysed, proteinase K is inactivated at 85°C for 1 hour. For PCR 2ul from the samples are directly used as template. For all of the PCRs, GoTaq® Green Master Mix (Promega) is used. For each reaction, 7.5ul 2X GoTaq master mix, 0.6ul primer mix (each 10uM) and 4.9ul nuclease free water are mixed. The cycling protocol is 95°C 1 min, (95°C 30 sec, 58°C 30 sec, 72°C 1.5 min) x 40 cycles, 72°C 10 min.

Sample lysis and western blot:

Tissues are homogenized and sonicated in NP-40 RIPA buffer (150mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, 25mM Tris pH 7.4) with fresh protease and phosphatase inhibitors (Serva). 20-30µg per sample is run in 10% SDS-PAGE gel and transferred to 0.45µM PVDF membrane, blocked in 5% milk 1hour RT and incubated overnight with primary antibodies diluted in milk. Next day they are incubated with HRP conjugated anti-rabbit secondary antibody for 1 hour at room temperature. Primary antibodies and their concentrations

are as given: RelB sc-226 (C-19) Santa Cruz Biotechnology 1:500, GFP (ab183734) 1:1000 Abcam, c-Rel 12707 (D4Y6M) Cell Signaling Biotechnology 1:1000.

Primer sequences:

Name		Sequence	Expected sizes
Generic Cre	01	ACC AGC CAG CTA TCA ACT CG	wt: 350bp Cre/+: 200bp
	02	TTA CAT TGG TCC AGC CAC C	
	03	CTA GGC CAC AGA ATT GAA AGA TCT	
	04	GTA GGT GGA AAT TCT AGC ATC ATC C	
Kras	01	GTC TTT CCC CAG CAC AGT G	wt: 622bp flox: 500bp recombined: 650bp
	02	CTC TTG CCT ACG CCA CCA GCT C	
	03	AGC TAG CCA CCA TGG CTT GAG TAA GTC TGC A	
RelB	01	AGG TTG ATG GTA ACT TTG GAT G	wt: 230bp flox: 270bp recombined: 186bp
	02	TCC AAA AAA ACC AAA CCA ACA AC	
	03	GTT TTC CCT GCT TGG TTC TGC	
Rel	01	CCA GCC AGG GTA AGT CTT CA	wt: 400bp flox: 266bp
	02	GCT AGG GGA AGG TGT CAC TGT	
	03	TCC AAA CTC ATC GAA GTT CCT	
	A	CAGGATGTTGCCGTCCTCCTTG	A+B recombined: 470bp A+C unrecombined: 470bp
	B	CCTGCAGCCAATAAGCTTATAAC	
	C	TATCATGTCTGGATCAATTCATAAC	
GFP-Rel	01	CTG CTG CAA GAT TGT GGT AAG A	wt: 290bp flox: 358bp
	02	AAT AGT GAG AGT GTG CGG GA	
	A	TGG GCA ACG TGC TGG TTA TT	flox: 385bp recombined: 584bp
	B	TTC TTC TGC TTG TCG GCC AT	
	C	TGC CTG CTT GCC GAA TAT CA	
p53	01	CAC AAA AAC AGG TTA AAC CCA G	wt: 288bp flox: 370bp
	02	AGC ACA TAG GAG GCA GAG AC	
	A	CAC AAA AAC AGG TTA AAC CCA G	recombined: 612bp
	B	GAA GAC AGA AAA GGG GAG GG	

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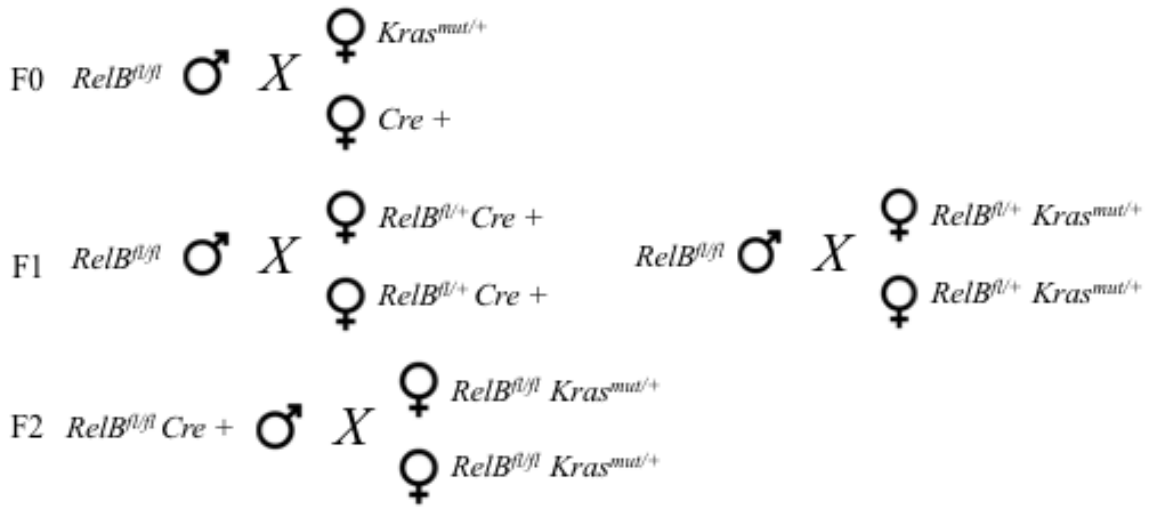
Figures and Figure Legends:

A



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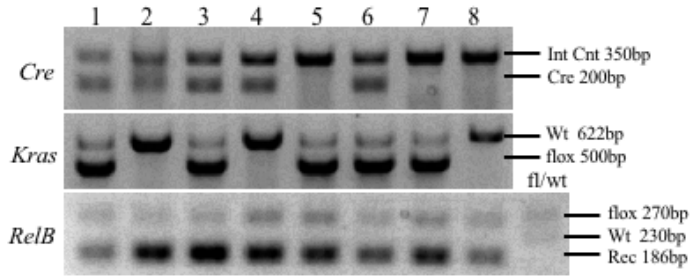

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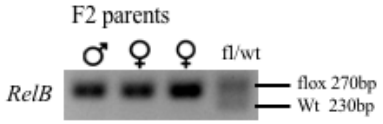

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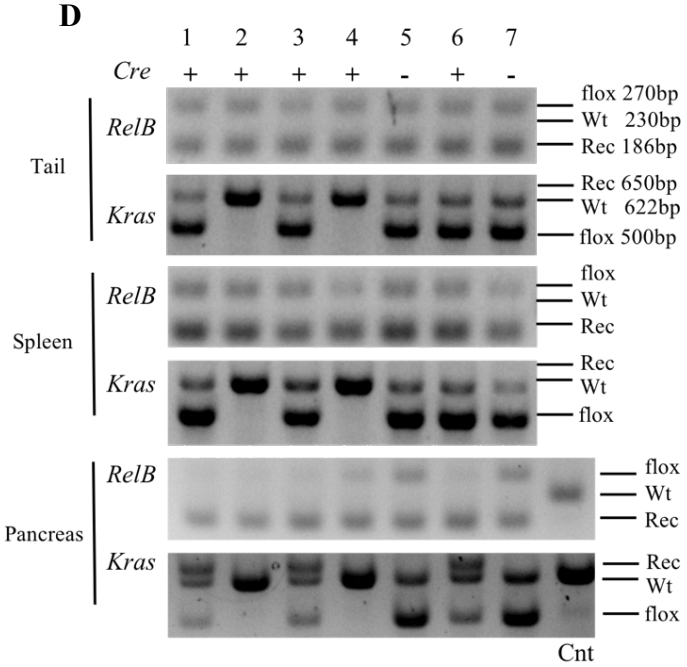

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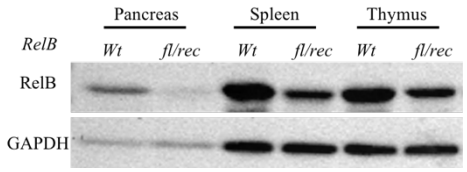


Figure 1: Germline recombination observed in *RelB*^{fl} mice. Names of the primer pairs are indicated on the left. Lengths of the respective alleles are indicated on the right. A) Breeding scheme to obtain *Relb*$^{fl/f}$; *Ptf1a-Cre/*+; *Kras*$^{fl/+}$ mice. B) Representative Genotyping PCR results of the pups obtained from F2 generation. C) Genotypes of the mice which were in F2 breeding. D) PCR results from tail, spleen and pancreas recognizing both the recombined and unrecombined alleles. D) RelB western blot results of thymus, spleen and pancreas in *RelB* germline recombined and *wt* tissues.

Rec: recombined floxed allele. **Int Cnt**: internal control

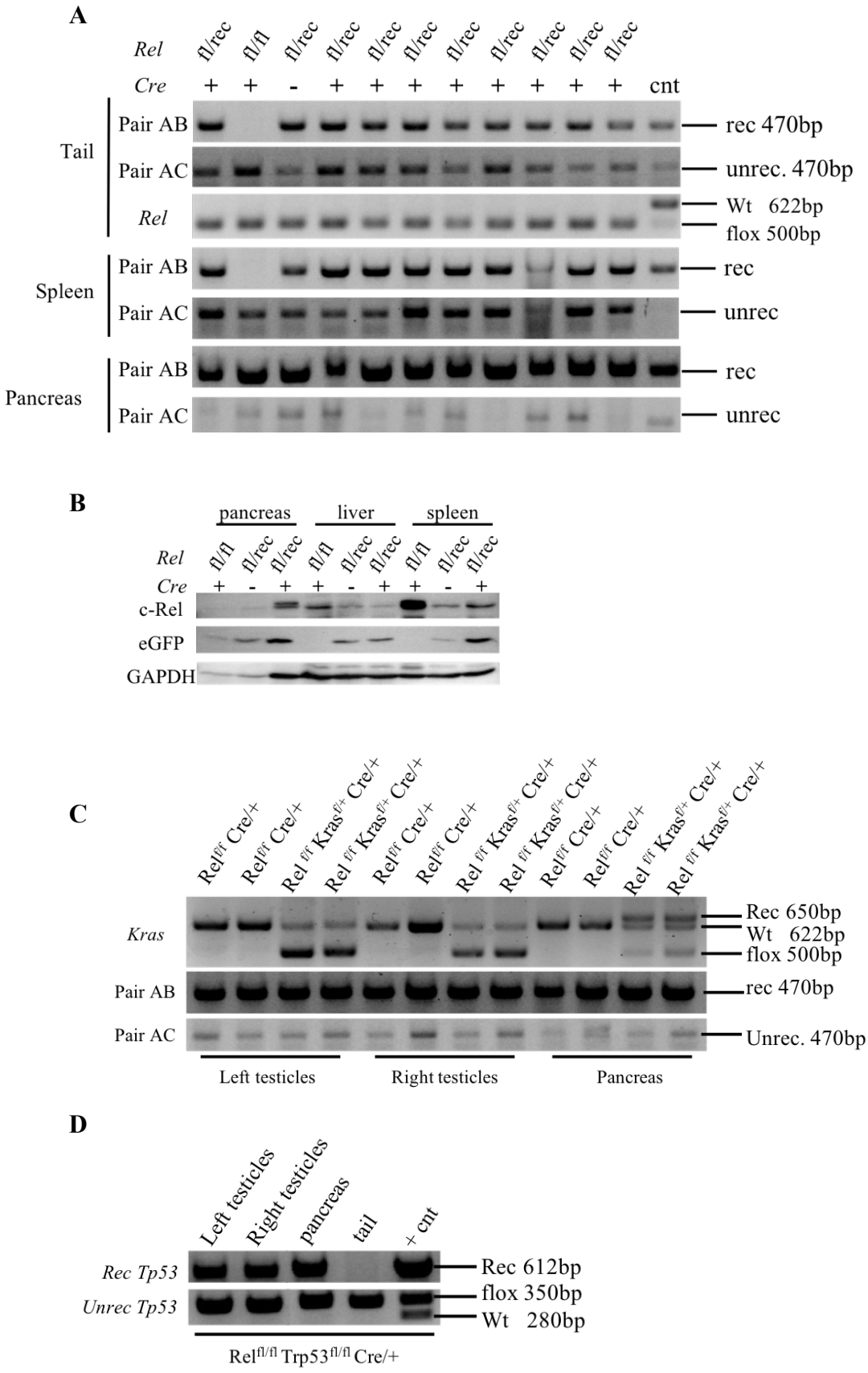

A
Rel
fl/rec
fl/fl
fl/rec
fl/rec
fl/rec
fl/rec
fl/rec
fl/rec
fl/rec
fl/rec
fl/rec
Cre
+ + - + + + + + + + + cnt
Tail
Pair AB
Pair AC
Rel
rec 470bp
unrec. 470bp
Wt 622bp
flox 500bp
Spleen
Pair AB
Pair AC
rec
unrec
Pancreas
Pair AB
Pair AC
rec
unrec
B
pancreas
liver
spleen
Rel
fl/fl
fl/rec
fl/rec
fl/fl
fl/rec
fl/rec
fl/fl
fl/rec
fl/rec
Cre
+ - + + - + + - +
c-Rel
eGFP
GAPDH
C
Rel f/f Cre/+
Rel f/f Cre/+
Rel f/f Kras f/+ Cre/+
Rel f/f Kras f/+ Cre/+
Rel f/f Cre/+
Rel f/f Cre/+
Rel f/f Kras f/+ Cre/+
Rel f/f Kras f/+ Cre/+
Rel f/f Cre/+
Rel f/f Cre/+
Rel f/f Kras f/+ Cre/+
Rel f/f Kras f/+ Cre/+
Kras
Rec 650bp
Wt 622bp
flox 500bp
Pair AB
rec 470bp
Pair AC
Unrec. 470bp
Left testicles
Right testicles
Pancreas
D
Left testicles
Right testicles
pancreas
tail
+ cnt
Rec Tp53
Unrec Tp53
Rec 612bp
flox 350bp
Wt 280bp
Rel fl/fl Trp53 fl/fl Cre/+

Figure 2: Germline recombination observed in *Rel*^{fl} mice. A) Primer pairs A+B recognize the recombined allele, while the A+C recognizes the unrecombined floxed allele. *Rel* is the genotyping primer mix recognizing either floxed or wt alleles which can't differentiate recombination. B) eGFP and c-Rel western blot results of pancreas, liver and spleen in *Rel* germline recombined and unrecombined tissues. C) PCR results for *Kras* and *Rel* recombination in testes and pancreas. D) PCR results for Tp53 recombination in testes pancreas and tail.

Rec: recombined floxed allele. **Unrec**: unrecombined floxed allele.

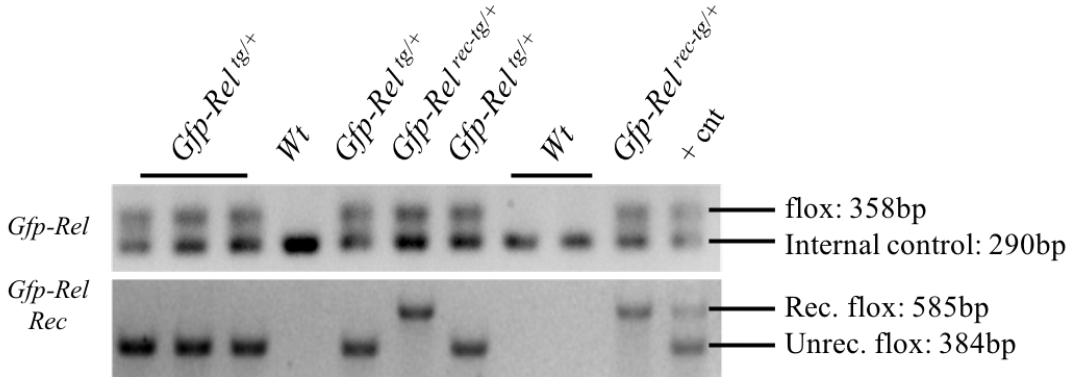


Figure 3: Representative PCR results obtained from GFP-Rel^{tg} mice breedings. A) Primer mix *Gfp-Rel* recognize the floxed allele along with an internal control. *Gfp-Rel Rec* primer mix gives band only if there is a *GFP-Rel* allele which can be recombined and unrecombined.

Rec: recombined floxed allele. **Unrec**: unrecombined floxed allele. **rec-tg**: Recombined transgenic allele

Tables and table legends:

Table 1: Number of mice obtained from *Rel* breeding

	Rel fl/+ Cre/+ Rel fl/fl	***Rel fl/+ Cre/+ Rel fl/fl***	***Rel fl/fl Cre/+ Rel fl/+***
Rel status of pups	number of pups	number of pups	number of pups
Rel fl/fl	3	2	3
Rel fl/+	9	12	1
Rel rec/fl	9	14	5
Rel rec/+	0	0	8
total	21	28	17

	When flox Rel allele is paternally transferred	
	∑ number of pups	Mendelian prediction
pups with recombined Rel	36	80%
pups without recombined Rel	9	20%
total	45	

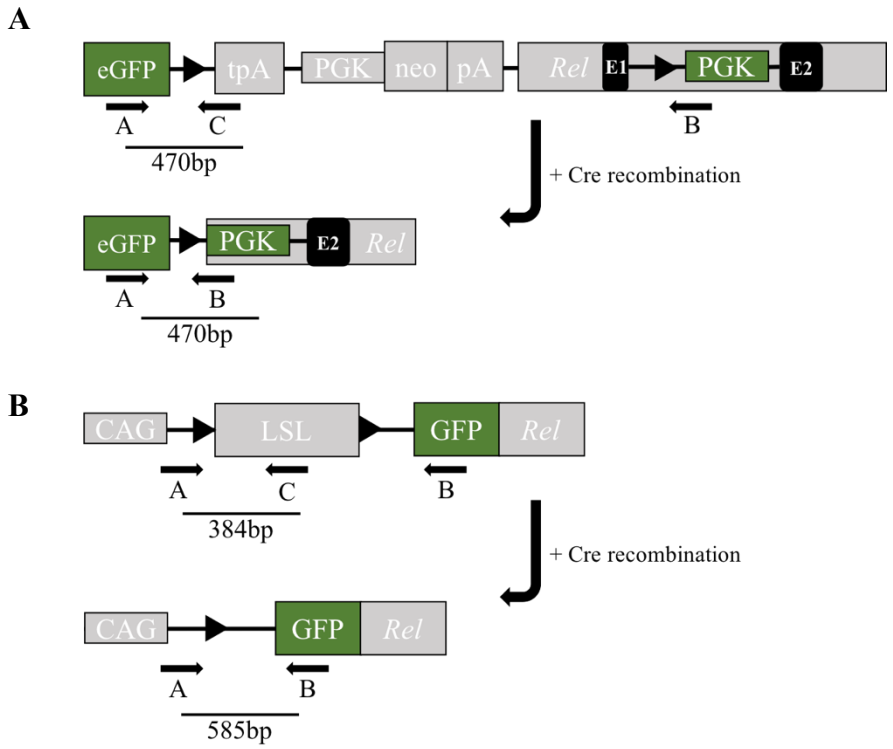
These pups are not included in the mendelian prediciton as only the paternal Wt Rel allele is transferred to pups

Table 2: Number of mice obtained from GFP-Rel breeding

	Gfp-Rel tg/+ Cre/+ Kras fl/+
Gfp-Rel status of pups	number of pups
tg/+	22
wt	18
total	40

	When Gfp-Rel allele is paternally transferred	
	∑ number of pups	Mendelian prediction
pups with recombined Rel	6	43%
pups without recombined Rel	8	57%
total	14	

Supplementary Figures:



Supplementary Figure 1: Schematics of the mouse lines with or without Cre recombination. A) A simplified map for Rel^{tm1Ukl} model. Exon1 of *Rel* is removed upon Cre mediated recombination, which brings together eGFP and PGK. Primer pair A+C recognizes the unrecombined allele with a size of 470bp. The distance between A-B pairs is too long to be amplified in unrecombined status. If there is recombination, A+B pair gives a band also at 470 bp. Primers sequences are kindly provided by Ulf Klein upon personal communication. B) A simplified map for GFP-Rel model. Upon Cre mediated recombination, constitutively active CAG promoter meets with N' GFP-tagged *Rel*. As in Rel^{tm1Ukl} model, pair A+C recognizes unrecombined, and A+B recognizes recombined alleles with the given sizes. Primer sequences are designed based on the sequence map kindly provided by Marc Schmidt-Supprian.