Male Germline Recombination of a Conditional Allele by the Widely Used Dermo1-Cre (Twist2-Cre) Transgene

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Abstract

Conditional gene knockout using the Cre/loxP system is instrumental in advancing our understanding of the function of genes in a wide range of disciplines. It is becoming increasingly apparent in the literature that some Cre transgenes mediated recombination occurs in unexpected tissues. Dermo1-Cre (Twist2-Cre) has been widely used to target skeletal lineage cells as well as other mesoderm-derived cells. Here we report that Dermo1-Cre exhibits spontaneous male germline recombination activity leading to a Cre-mediated recombination of a floxed *Ptk2* (Protein tyrosine kinase 2, also known as *Fak* [Focal adhesion kinase]) allele but not a floxed *Rb1cc1* (RB1 inducible coiled-coil 1, also known as *Fip200* [FAK-family Interacting Protein of 200 kDa]) allele at high frequency. This ectopic germline activity of Dermo1-Cre occurred in all or none manner in a given litter. We demonstrated that the occurrence of germline recombination activity of Dermo1-Cre transgene can be avoided by using female mice as parental Dermo1-Cre carriers.

Key words: Cre-loxP; conditional knockout; *Fak*; *Fip200*; Dermo1-Cre; Twist2-Cre; germline

Dermo1 (also named *Twist2*) is highly expressed in condensed mesenchyme during skeletal development and later in perichondrial and periosteal cells surrounding cartilage (Li *et al.*, 1995). Similar to the *Dermo1* expression pattern, Cre-recombinase activity in Dermo1-Cre mice was detected as early as E9.5 in mesodermal tissues. During endochondral ossification, Dermo1-Cre recombinase activity is detected in condensed mesenchyme from which both osteoblasts and chondrocytes are derived (Yu *et al.*, 2003). Thus, Dermo1-Cre has been widely used as a tool to target skeletal lineage cells (Elefteriou and Yang, 2011). In addition, Dermo1-Cre has also been frequently used to target other mesenchymal lineage cells (Cornett *et al.*, 2013; Geske *et al.*, 2008; Lavine *et al.*, 2008; Lin *et al.*, 2008; Yin *et al.*, 2008).

FAK (Focal adhesion kinase) is an intracellular non-receptor tyrosine kinase and a major mediator of signal transduction by integrins (Guan and Shalloway, 1992). Disruption of *Fak* gene in mice resulted in an early embryonic lethal phenotype, which precludes the thorough examination of tissue-specific phenotypes in postnatal life (Ilic *et al.*, 1995). We have used the Cre-loxP recombination system to circumvent the early embryonic lethality by targeting the *Fak* gene disruption to the tissue of interest (Nagy *et al.*, 2007; Peng *et al.*, 2008; Shen *et al.*, 2005; Sun *et al.*, 2016). Recently, we reported that *Fak* deletion in osteoblast progenitor cells leads to osteopenia in mice (Sun *et al.*, 2016). In order to elucidate the role of *Fak* in mesenchymal and osteochondrogenitor cells, we are employing the Dermo1-Cre transgenic mouse line (Yu *et al.*, 2003). *Fak* floxed mice were bred with Dermo1-Cre mice to generate *Fak* conditional knockout mice. Cre-mediated recombination of the floxed allele inactivates the *Fak* function in Dermo1-Cre expressing cells and their descendants. The Cre transgene, wild type (WT or +), floxed (Flox), and Cre-recombined floxed *Fak* alleles (FAK^R) were detected by PCR analysis of tail-tip genomic DNA with allele specific primers (Figure 1). To generate the conditional knockout mice,

male $FAK^{F/+}$;Dermo1-Cre/+ mice were bred with female $FAK^{F/F}$ mice. Tail-tip DNA was used to perform PCR to genotype the offspring. In this mating scheme, 4 genotypes ($FAK^{F/F}$;Dermo1-Cre, $FAK^{F/F}$, $FAK^{F/+}$;Dermo1-Cre, and $FAK^{F/+}$) were expected at 25% ratio for each (Figure 2A, top numbers). The Cre-mediated recombination should only occur in offspring carrying Dermo1-Cre transgene but not the offspring without Dermo1-Cre transgene including $FAK^{F/F}$ and $FAK^{F/+}$. We obtained a total of 121 mice from 16 litters of the offspring. Surprisingly, thirteen percent (16/121) of offspring had unexpected genotype in which there was the absence of wild type allele and Dermo1-Cre transgene but the presence of *Fak* floxed allele and a Cre-recombined allele (designated as $FAK^{F/R}$). There was 15% of offspring had the expected genotype of $FAK^{F/F}$. Thus, 47% (16/34) of the offspring whose genotype showed the presence of *Fak* floxed allele and absence of both wild type allele and Dermo1-Cre transgene (thus genotypically "homozygous" for *Fak* floxed allele) showed the presence of the Cre-recombined allele (FAK^{R}) (Figure 2a and 2b). Noticeably, whenever there was $FAK^{F/F}$ offspring in one litter, there was no $FAK^{F/R}$ mouse and vice versa (Table I), suggesting this unexpected recombination occurred in all or none manner.

The recombination of floxed allele in the absence of Cre transgene has been reported in using different promoters to drive Cre (Cochrane *et al.*, 2007; Hayashi *et al.*, 2003; Lallemand *et al.*, 1998; Ramirez *et al.*, 2004; Sakai and Miyazaki, 1997; Vincent and Robertson, 2003; Zhang *et al.*, 2013). To determine whether this unexpected Dermo1-Cre transgene independent DNA recombination occurred at specific tissues or a more global manner, e.g. germline level, female $FAK^{F/R}$ mice were mated with male WT mice (Figure 3a). About 50% (22/45) of the offspring have $FAK^{F/+}$ genotype, and the other 50% (23/45) of the offspring have $FAK^{R/+}$ genotype (Fig 3). Thus, the recombined *Fak* allele can be transmitted to the offspring in Mendelian ratio,

indicating a global floxed *Fak* allele recombination in $FAK^{F/R}$ mice. These results suggest that the unexpected recombination occurs either during spermatogenesis or soon after fertilization.

To determine whether Cre mRNA and/or protein carried by sperms that are genotypically negative for Dermo1-Cre can cause the recombination at or after zygote stage, male Dermo1-Cre/+ mice were mated with female $FAK^{F/F}$ mice (Figure 4). In this experimental model, all offspring showed expected genotypes and there was no unexpected recombination in Cre-negative mice (Fig 4). Thus, we concluded that the unexpected *Fak* allele recombination only occurred before zygote stage. To further support this conclusion, there was no offspring with $FAK^{+/R}$ genotype shown in Fig 2. Because $FAK^{+/R}$ genotype can only occur when the maternally contributed floxed *Fak* allele is recombined, the absence of $FAK^{+/R}$ genotype suggests that the recombination did not occur at or after the fertilization, otherwise the maternally contributed floxed *Fak* allele has the equal opportunity as paternally contributed floxed *Fak* allele to be recombined in zygotes and consequently this should lead to the occurrence of $FAK^{+/R}$ genotype.

We identified the unexpected *Fak* floxed allele recombination occurs in male germline cells, next we investigated whether this unfavorable recombination can be avoided by using female mice as parental Cre carriers. Female $FAK^{F/+}$;Dermo1-Cre/+ mice were mated with male $FAK^{F/F}$ mice and we evaluated F1 progeny for recombination of the inherited Flox allele in Cre-negative mice (Figure 5). Our data demonstrated that there was no recombination of the *Fak* allele occurred in Cre-negative progeny in this mating scheme (Fig 5).

To determine whether Dermo1-Cre causes universal male germline recombination of floxed alleles, we examined the possible ectopic Dermo1-Cre recombination event in mouse with floxed allele of *Fip200* (FAK-family Interacting Protein of 200 kDa) gene, whose product was identified as a FAK interacting protein (Ueda *et al.*, 2000). In this experimental model, male

FIP200$^{F/+}$;Dermo1-Cre/+ mice were bred with female FIP200$^{F/F}$ mice using a similar breeding scheme shown in Fig. 2, and we evaluated F1 progeny for recombination of the inherited floxed allele in Cre-negative mice. In contrast to *Fak* floxed alleles, no recombination of the *Fip200* allele occurred in Cre-negative progeny (0/48). This result indicates that the germline recombination of floxed alleles by Dermo1-Cre is not universal and only some genes may be susceptible to the amount of Cre recombinase produced. Dermo1-Cre mice were generated by inserting the Cre transgene within the first exon of *Dermo1* gene (Yu *et al.*, 2003). The germline *Fak* floxed allele recombination identified in the offspring of the breeding using male mice to carry Dermo1-Cre suggests that *Dermo1* gene may be expressed in male germline cells. The absence of floxed *Fip200* allele recombination suggests that the *Fip200* floxed allele is not susceptible to the amount of Cre protein produced by Dermo1-Cre transgene in germline cells. *Fak* gene is located at mouse chromosome 15 (Fiedorek and Kay, 1995) and it is expressed during spermatogenesis (Gungor-Ordueri *et al.*, 2014). This may indicate a more "open" chromatin structure where *Fak* gene locates, which may make *Fak* floxed alleles more susceptible to Cre recombinase at this developmental stage.

It is often reported that the frequency of Cre transgene-independent recombination occurs more often when female mice are used to carry Cre transgene (Cochrane *et al.*, 2007; Zhang *et al.*, 2013) or only "maternal inheritance" but not "paternal inheritance" occurs (Hayashi *et al.*, 2003). Our data showed that female mice may be the preferred maternal Cre carrier when Dermo1-Cre is used. However, it takes more effort to maintain mating units using female as Cre carrier especially for embryonic studies. If germline recombination of floxed allele does occur when male mice are used as paternal Dermo1-Cre carrier, PCR analysis with tail tip DNA in progenies without Dermo1-Cre transgene can be employed to effectively identify the mice

having germline recombination. However, since germline deletion may also occur in the presence of Dermo1-Cre, $FAK^{F/F}$;Dermo1-Cre/+ mice could be indeed $FAK^{F/R}$;Dermo1-Cre/+ mice but it is not possible to distinguish them by PCR analysis using the tail-tip DNA because rearranged *Fak* allele is expected in the tail tissue of both genotypes. Intriguingly, our data showed that the unexpected germline recombination of *Fak* floxed allele was an "all or none" event evidenced by the exclusive presence of either $FAK^{F/F}$ or $FAK^{F/R}$ genotype when male mice were used as paternal Dermo1-Cre carrier. This suggests that the *Fak* floxed allele recombination either happened or not happened to all the sperms used to produce one particular litter from the $FAK^{F/+}$;Dermo1-Cre/+ mice.

Due to the limitation and scope of current report, neither the exact timing of male germline *Fak* allele recombination nor the mechanism of the "all or none" phenomenon is known. However, our data have two important implications. First, our data calls for the necessity to examine potential germline recombination when Dermo1-Cre is carried by male mice to target any other genes of interest. Second, we demonstrated that using female mice as Dermo1-Cre carriers can avoid the germline recombination of floxed alleles.

Materials and Methods

Animals

The floxed *Fak* ($FAK^{F/F}$) mice and floxed *Fip200* ($FIP200^{F/F}$) mice were generated by us previously (Gan *et al.*, 2006; Shen *et al.*, 2005). Generation of transgenic mice was described previously (Yu *et al.*, 2003) and they were obtained from Jackson laboratory (Bar Harbor, ME, strain 008712). All mice were backcrossed for at least 8 generations onto a C57BL/6NCrl background. Mice were housed under pathogen-free conditions at 22 ± 2 °C on a 12:12-h

light/dark cycle, fed with 5001 or 5008 (for breeding pairs) rodent diet (LabDiet). All animal handling protocols were approved by IACUC at the University of Michigan.

Genotype Analysis by PCR

Genomic DNA from tail tip was prepared as described previously (Liu *et al.*, 2013; Liu *et al.*, 2010). DNA extracts were amplified by PCR using primer pairs to detect the Cre transgenes, wild type, floxed, and Cre-recombined *Fak* or *Fip200* alleles as we previously described (Gan *et al.*, 2006; Shen *et al.*, 2005). PCR products were electrophoresed on agarose gels, stained with ethidium bromide, and imaged using UV light. Cre transgenes were amplified and identified as a 696-bp band using the Cre 1 (5'-GAGTGATGAGGTTCGCAAGA-3') and Cre 2 (5' CTACACCAGAGACGGAAATC 3'). As an internal DNA control, primers Alk2-5 (5'-ATGCTAGACCTGGGCAGCCATA-3') and Alk2-3 (5'-CATGCTAGCAGCTCGGAGAAAC-3') were applied simultaneously with Cre primers, generating a 371-bp amplicon. The reaction cycles for Cre and internal control are: 94^{o}C, 1 min; 67 ^{o}C, 1 min; 72 ^{o}C, 1min; 25 cycles. *Fak* alleles were identified with primer set: P1 (5′-GCTGATGTCCCAAGCTATTCC-3′) and P2 (5′-TGGCCTGCTATGGATTTCGC-3′) using reaction cycles: 94^{o}C, 1 min; 67 ^{o}C, 1 min; 72 ^{o}C, 2min; 32 cycles. The wild type and floxed *Fak* alleles were detected as 800-bp and 1000-bp products, respectively. To detect Cre-mediated recombination of the floxed *Fak* allele, primes P1 and P3 (5′-AGGGCTGGTCTGCGCTGACAGG-3′) were used under the same conditions. The rearranged *Fak* allele was detected as a 550-bp product. *Fip200* alleles were identified with primer set: FP2 (5'-CAAAGAACAACGAGTGGCAGTAG -3') and FP3 (5'-CATCAGATACACTAGAGCTGG-3') using reaction cycles: 3 cycles at 94°C for 3 min, 60°C for 1 min, and 72°C for 2 min, followed by 33 cycles at 94°C for 1 min, 60°C for 1 min, and

72°C for 2 min, and 1 cycle at 94°C for 1 min, 60°C for 1 min, and 72°C for 10 min. The wild type and floxed *Fip200* alleles were detected as 262-bp and 225-bp products, respectively. To detect Cre-mediated recombination of the floxed *Fip200* allele, primes FP1 (5′-GGAACCACGCTGACATTTGACACTG-3′) and FP3 were used under the same conditions. The recombined *Fip200* allele was detected as an 800-bp product.

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Figure Legends

Figure 1. *Fak* floxed locus and rearrangement (a) Schematic of mouse *Fak* gene and targeted allele. *Fak* gene is composed of FERM, Kinase, PXXP, and FAT domains from N terminal to C terminal. Exon3 locating at FERM domain was flanked by LoxP sites. Primers used to identify the wild type (WT), floxed (Flox) and rearranged (FAK^R) alleles are shown as solid arrows: primer pair P1/P2 amplifies a 800-bp WT and 1000-bp Flox bands; primer pair P1/P3 amplifies a 550-bp rearranged floxed *Fak* allele (FAK^R). **(b)** Genomic DNA was extracted from mouse tail and analyzed by PCR using P1, P2, and P3 primers to distinguish different types of alleles with or without the rearrangement of *Fak* floxed allele.

Figure 2. Unexpected *Fak* allele recombination in the absence of Dermo1-Cre transgene (a) Schematic showing breeding strategies and *Fak* gene deletion using male mice as paternal Dermo1-Cre carrier to generate *Fak* conditional knockout mice. Male mice heterozygous for both the *Fak* floxed allele and the Dermo1-Cre transgene were mated with female mice homozygous for *Fak* floxed allele. Offspring exhibiting *Fak* deletion without Dermo1-Cre transgene was observed (indicated by broken line). The table below the scheme shows the expected as well as the observed genotype distribution. The numbers of animals per total number

of animals (n=121) is shown in parentheses. **(b)** Representative PCR reaction showing genotyping result of the mice whose genotype showed the presence of *Fak* floxed allele and absence of both wild type allele and Dermo1-Cre transgene. Primer pair Cre 1/Cre 2 was used to amplify Dermo1-Cre transgene as a 696-bp band and Primer pair Alk2-5/Alk2-3 was used to amplify Alk2 gene as an internal DNA control (upper panel). Primer pair P1/P2 was used to amplify the floxed *Fak* allele (Flox) as 1000-bp band (middle panel). Primer pair P1/P3 was used to amplify a 550-bp rearranged floxed *Fak* allele (FAK^R) (lower panel). Asterisks at the bottom of the gel indicate progeny with unexpected rearrangement of *Fak* floxed allele (FAK^R).

Figure 3. Schematic showing breeding strategies to determine whether the occurrence of *Fak* floxed allele recombination is at germline level (a) Female $FAK^{F/R}$ mice were mated with male wild type mice. The table below the scheme shows the observed genotype distribution. The numbers of animals per total number of animals (n=45) is shown in parentheses. **(b)** Representative PCR reaction showing genotyping result of the offspring of female $FAK^{F/R}$ mice and male wild type mice. Primer pair P1/P2 was used to amplify WT and floxed *Fak* allele (Flox) as 800-bp and 1000-bp bands, respectively. Primer pair P1/P3 was used to amplify a 550-bp rearranged floxed *Fak* allele (FAK^R).

Figure 4. Schematic showing breeding strategies to determine whether the occurrence of *Fak* floxed allele recombination is at or after zygote stage. Male mice heterozygous for Dermo1-Cre transgene were mated with female mice homozygous for *Fak* floxed allele. The table below the scheme shows the expected as well as the observed genotype distribution. The numbers of animals per total number of animals (n=26) is shown in parentheses.

Figure 5. Schematic showing breeding strategies and *Fak* gene deletion using female mice as Dermo1-Cre carrier. Female mice heterozygous for both the *Fak* floxed allele and the Dermo1-Cre transgene were mated with male mice homozygous for *Fak* floxed allele. The table below the scheme shows the expected as well as the observed genotype distribution. The numbers of animals per total number of animals (n=123) is shown in parentheses. The data were obtained from 9 different female breeders.

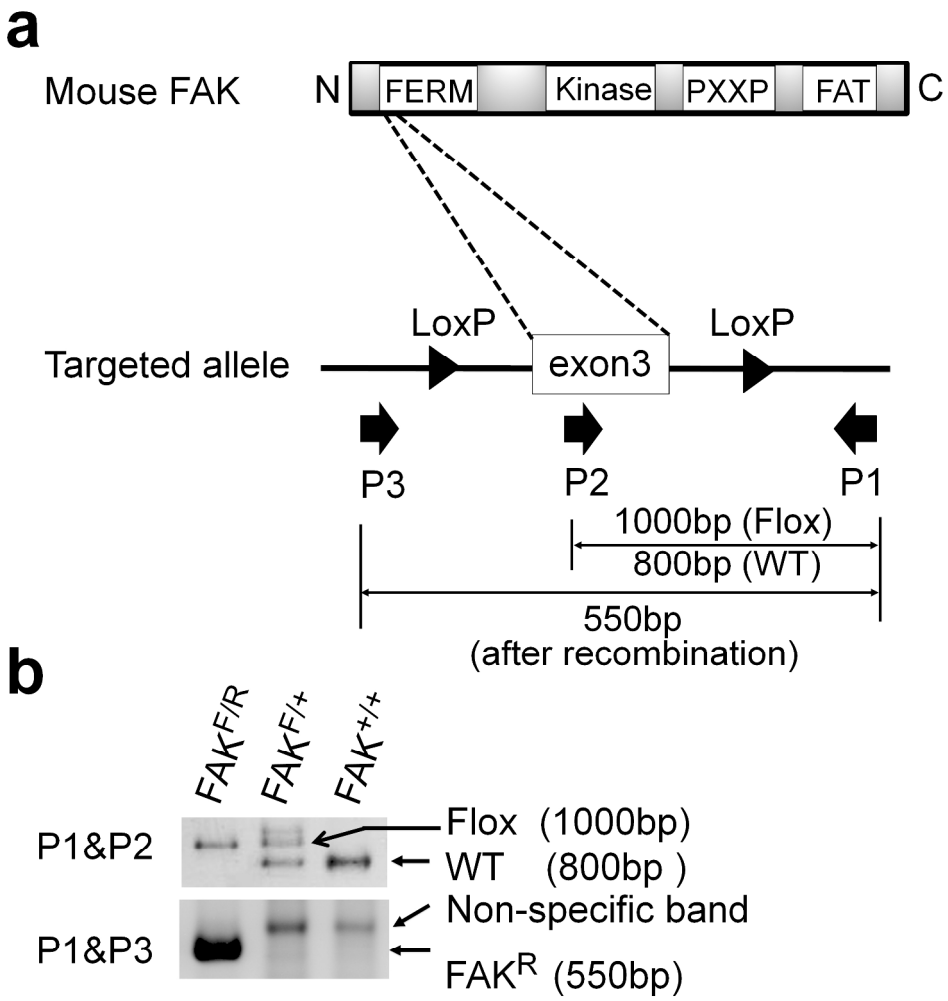

Fig 1

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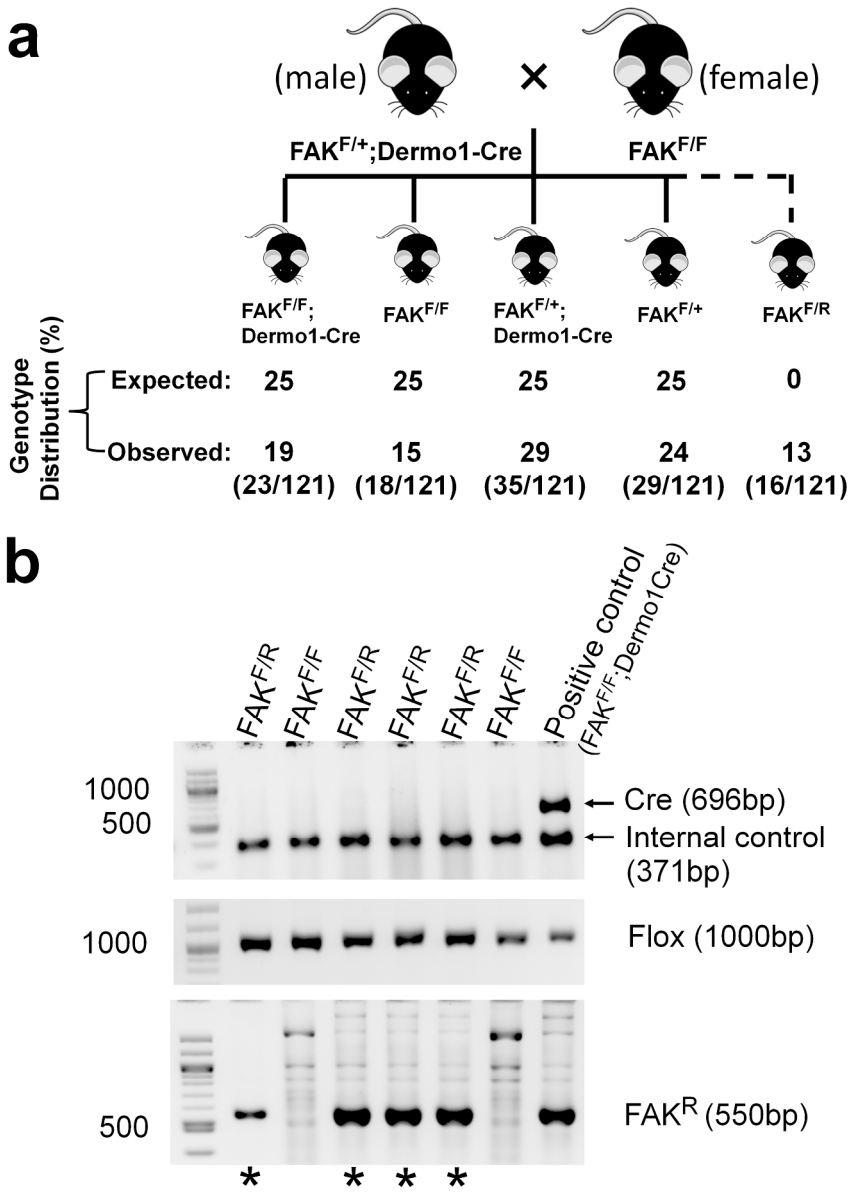

Fig 2

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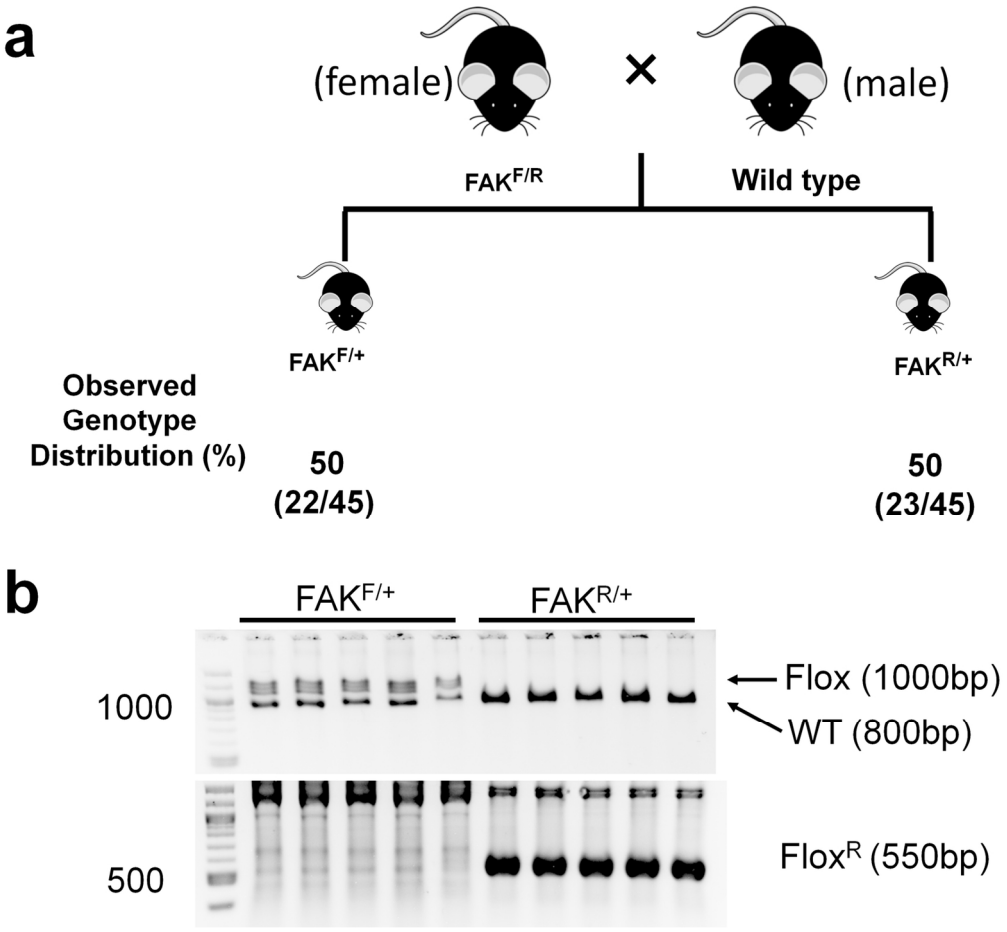

Fig 3

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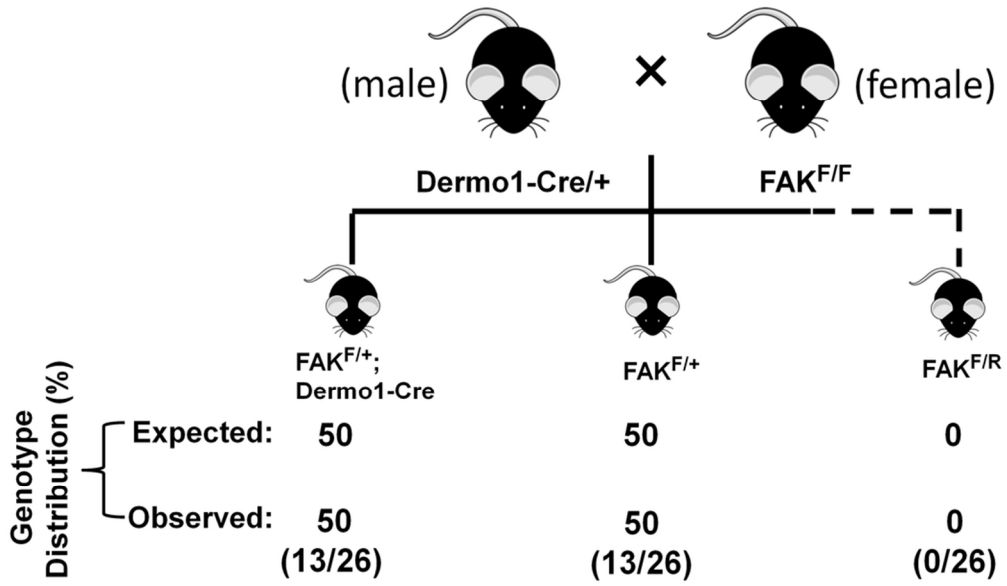

Fig 4

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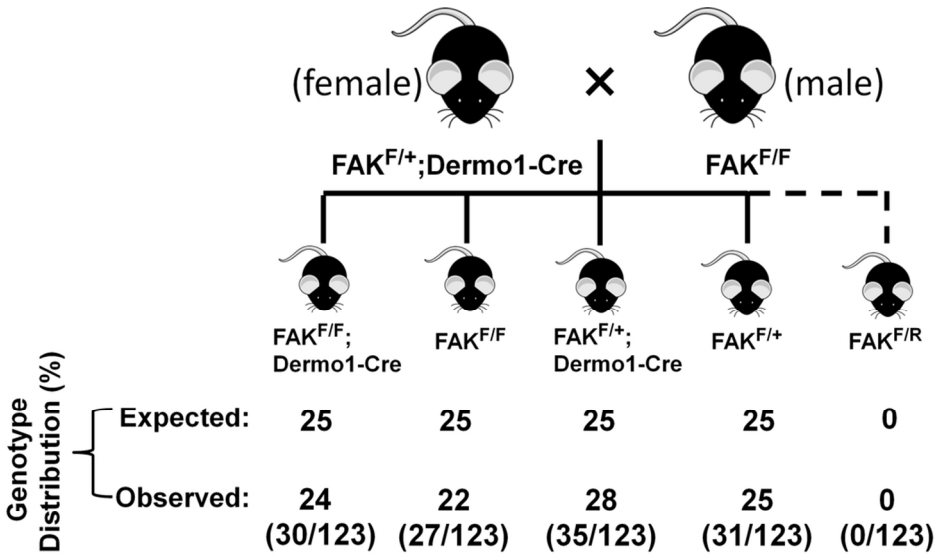

Fig 5

100x60mm (300 x 300 DPI)

Table I. Distribution of $FAK^{F/F}$ and $FAK^{F/R}$ mice in different litters from three breeding pairs between male $FAK^{F/+}$;Dermo1-Cre and female $FAK^{F/F}$ mice

Breeding Male	Litter Number	Litter Size	Number of $FAK^{F/F}$ mice	Number of $FAK^{F/R}$ mice
Male #1	#1	13	4	0
Male #1	#2	7	2	0
Male #1	#3	9	2	0
Male #1	#4	8	1	0
Male #1	#5	9	3	0
Male #1	#6	12	3	0
Male #2	#7	5	1	0
Male #3	#8	3	1	0
Male #3	#9	7	1	0
Male #1	#10	2	0	1
Male #2	#11	9	0	3
Male #2	#12	8	0	2
Male #2	#13	7	0	2
Male #2	#14	8	0	3
Male #2	#15	8	0	3
Male #2	#16	6	0	2