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A more efficient method to generate null mutants using Hprt-Cre with floxed alleles

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Abstract

Purpose—The generation of non-viable homozygous null mouse embryos from heterozygote null/+ breedings can be highly resource consuming, with only 25% of the embryos in the litter being null mutants. We hypothesized that 1) we could double the number of homozygous null mouse embryos in a litter without reducing litter size using Hypoxanthine-guanine phosphoribosyltransferase-Cre (Hprt-Cre) (which is active in the female germ line at the time of fertilization) and 2) these homozygous null mutants would be identical to mutants generated through traditional null/+ breedings.

Methods—To test this hypothesis we used a conditional allele *Fgfr2IIIb*^{flox}. This allele when recombined is identical to the *Fgfr2IIIb*^{null} allele. An F1 generation of *Fgfr2IIIb*$^{rec/+}$; *Hprt*$^{Cre/+}$ females was created by mating *Fgfr2IIIb*$^{+/+}$; *Hprt*$^{cre/cre}$ females to a *Fgfr2IIIb*$^{flox/flox}$ male. The F1 females were then mated to a *Fgfr2IIIb*$^{flox/flox}$ male. F2 embryos were genotyped and the morphology and histology of the lungs, intestine, limbs and brain was analyzed.

Results—The Hprt-Cre mating strategy results in 51% of pups being genotypic homozygous null embryos (85/166) versus 23% for the standard null/+ approach (38/167). These embryos did not express the *Fgfr2IIIb* transcript and were phenotypically identical to null embryos generated through standard null/+ breedings.

Conclusions—The Hprt-Cre mating strategy increases the number of homozygous mutant embryos in a litter without decreasing litter size. Embryos generated through this approach are phenotypically identical to those from standard heterozygous breedings. We recommend this

approach to investigators using an model system that relies on the generation of homozygous null embryos.

Keywords

Hprt-Cre; cre loxp system; breeding strategy; homozygous null mutants; efficiency; generation of mutants; knockout

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Use of murine homozygous null mutant embryos has been critical for modeling a number congenital defects in humans. With this approach animal models for imperforate anus and cloaca [1], intestinal atresia [2,3], diaphragmatic hernia [4], abdominal wall defects [5–7], limb defects [8], and cleft palate [9,10] have been described. Generation of homozygous null embryos through null/+ (n/+) heterozygous breedings can be highly resource consuming. When employing the n/+ breedings for one gene, a single homozygous null (n/n) embryo is generated for every 4 embryos. Thus, a litter of 10 will yield 2 or 3 homozygous null mutant embryos. Generating compound homozygous null mutants for 2 or 3 genes with this process becomes even less efficient as the frequency of homozygous null mutants decreases to 1 in 16 embryos and 1 in 64 embryos, respectively. Researchers also face increasing pressure to minimize the number of animals used while maintaining, or even increasing, the quality of basic science research. This pressure is driven in part by both the animal rights movement [11,12] and increasing animal care and housing costs at research universities. Strategies that can reduce the number of mice required to generate homozygous null embryos have the potential to significantly cut research costs for scientists in many fields.

We hypothesized that Hprt Cre [13] could be used to circumvent standard heterozygote breeding strategy inefficiencies. In these mice, a *Cre* gene under a cytomegalovirus enhancer/chicken β-globin promoter has been inserted into the X-linked locus for hypoxanthine-guanine phosphoribosyltransferase yielding Cre protein in the female germ line prior to the generation of oocytes. Thus, in a female heterozygous for *Hprt-Cre*, all ova receive the active Cre protein regardless of inheritance of the *Hprt Cre* gene. Once the oocyte with the Cre protein is fertilized with sperm, the flox allele derived from the male genome should be recombined by the Cre protein at the 1 or 2 cell stage. Hypothetically if a male homozygous for a conditional allele is mated to a female who is carrying a single copy of the *Hprt-Cre* gene and a copy of the recombined allele of interest then half of the progeny should be homozygous for the recombined allele. If the recombined allele is identical to the null allele for the same gene, this method should increase the number of null mutants in a litter as the active ova-derived Cre protein recombines a sperm-derived floxed allele at fertilization.

The novel Hprt-Cre approach depends on having a conditional floxed allele of the gene of interest (a hypothetical *Gene* A^{flox}). Ideally, following Cre dependent recombination of *Gene* A^{flox}, the *Gene* A^{rec} should be genetically identical to a *Gene* A^{null} allele. Thereafter, the strategy is simple: First, generate *Gene* $A^{rec/+}$; $Hprt^{Cre/+}$ female progeny (F1) from a homozygous $Hprt^{Cre/Cre}$ to a homozygous *Gene* $A^{flox/flox}$ mating. Next, breed the F1 *Gene* $A^{rec/+}$; $Hprt^{Cre/+}$ females to a *Gene* $A^{flox/flox}$ male to generate homozygous mutants (F2). Half of the ova from the F1 females will carry the *Gene* A^{rec} allele and all the ova will have an active Cre protein. Sperm-derived *Gene* A^{flox} alleles will be recombined upon fertilizing these ova. Presumably, all of the *Gene* $A^{rec/rec}$ embryos will be phenotypically equivalent to *Gene* $A^{null/null}$ embryos. Thus, half of the F2 embryos generated with this strategy should be *Gene* $A^{rec/rec}$ homozygotes and half should be *Gene* $A^{rec/+}$ heterozygotes.

To test the efficiency of the Hprt-Cre strategy, we determined the number of phenotypically null embryos in a litter as compared to the standard heterozygous null/+ breeding strategy.

We utilized the floxed *Fgfr2IIIb* allele [14,15] which, when recombined, is genetically identical to the *Fgfr2IIIb* null allele [2]. The $Fgfr2IIIb^{null/null}$ embryos have been well characterized with defects in a number of organ systems including, but not limited to, the limbs, lungs, intestines, pituitary gland and eyes [2,14,16]. Here we report that $Fgfr2IIIb^{rec/rec}$ embryos generated with Hprt-Cre methodology are identical to previously characterized $Fgfr2IIIb^{null/null}$ embryos and that the Hprt-Cre strategy increases the frequency of null embryos in a given litter from 1-in-4 to 1-in-2 without reduction in litter size.

1. Methods

1.1 Animals

IACUC approval for these studies was obtained from the University of Wisconsin School of Medicine and Public health (P.F.N. protocol # M02258). All animals were maintained in a clean facility with *ad libitum* access to fresh food and water, and kept on a 12 hour alternating light/dark cycle. For animals generated with *Hprt-Cre*, the recombined *Fgfr2IIIb* floxed allele is genetically identical to that of the *Fgfr2IIIb null* allele [14,15]. Five $Fgfr2IIIb^{+/+}$; $Hprt^{Cre/Cre}$ females were bred to $Fgfr2IIIb^{flox/flox}$ males generating a total of 40 pups. Male pups were sacrificed and female pups were genotyped by toe clipping before P10 for *Hprt-Cre, Fgfr2IIIb wild-type, null* and *floxed* alleles [14,15]. The genotype of all female pups was $Fgfr2IIIb^{rec/+}$; $Hprt^{Cre/+}$. $Fgfr2IIIb^{rec/rec}$ embryos were generated by breeding mature $Fgfr2IIIb^{rec/+}$; $Hprt^{Cre/+}$ females to $Fgfr2IIIb^{flox/flox}$ males. $Fgfr2IIIb^{null/null}$ embryos were generated through traditional heterozygous crossing of $Fgfr2IIIb^{null/+}$ females to $Fgfr2IIIb^{null/+}$ males. Genotyping of embryos for *Fgfr2IIIb* alleles was performed as described previously [14,15]. Ratios of $Fgfr2IIIb^{rec/rec}$ and $Fgfr2IIIb^{null/null}$ embryos were determined.

1.2 RT-PCR

Total RNA was prepared from fetal tissues on Embryonic day (E)18.5 using the GeneJet RNA extraction kit (Fermentas Inc., Glen Burnie, MD), according to the manufacturer's protocol. 0.5 μg of RNA was used with the RevertAid cDNA Synthesis Kit (Fermentas Inc., Glen Burnie, MD), according to the manufacturer's protocol. The following cDNA-specific primers were used; 5′ *Fgfr2IIIb*, ttc aat gtg acg gag atg; 3′ *Fgfr2IIIb*, ctg ttt ggg cag gac agt g; 5′ *Gapdh*, caa ggt cat cca tga caa ctt tg; 3′ *Gapdh*, gtc cac cac cct gtt gct gta g. Thermocycler conditions for *Fgfr2IIIb* were: 94°C/30 sec, 58°C/30 sec, 72°C/45 sec, for 35 cycles then 72°C/7 minutes for one cycle. Thermocycler conditions for *Gapdh* were: 94°C/30 sec, 58°C/30 sec, 72°C/45 sec, for 35 cycles then 72°C/5 minutes. PCR products were run on a 1.5% agarose gel and visualized by ethidium bromide staining.

1.3 Morphologic studies

Embryos generated using the *Hprt-Cre* strategy were harvested at either (E) 10.5, E14.5 or E18.5. $Fgfr2IIIb^{null/null}$ embryos generated from $Fgfr2IIIb^{null/+}$ heterozygous crossings were harvested at E18.5. Whole mount photographs were taken of E18.5 embryos under a stereoscopic dissecting microscope. The thorax was opened and the embryos were fixed overnight in 4% paraformaldehyde at 4°C. The intestines were dissected out and photographs of the duodenal, cecal and colonic regions were obtained. Incidence of duodenal, colonic and cecal defects for all mutants was determined.

1.4 Histological studies

E10.5 and E14.5 embryos were dissected free of the yolk sac and embryos were fixed overnight at 4°C in 4% paraformaldehyde. They were then dehydrated through a series of

escalating Methanol/PBST washes, isopropyl alcohol and xylene. They were washed 3 times in paraffin at 60 °C in a vacuum oven and then embedded head up in paraffin. Sections were taken a 5μm thickness. Sections were stained for standard H&E or TUNEL staining and then photographed under either a light microscope or a fluorescent microscope.

1.5 Three-dimensional reconstructions

Photomicrographs of transverse sections of the head region of E10.5 embryos were uploaded sequentially into Amira™ (Visage Imaging, Inc., San Diego, CA) and aligned using the auto align tool. They were labeled using the segmentation editor. Following this, the three dimensional structures of Rathke's pouch and the Buccal cavity along with the Diencephalon were generated using the surface generator tool. A final image was generated as a JPEG file at 600 pixels per square inch and imported into Adobe Photoshop (San Jose, CA) for labeling.

Apoptosis studies

Embryos heterozygous and homozygous for the *Fgfr2IIIb*^{rec} allele were harvested at E10.5, fixed and embedded in paraffin as described above. Transverse sections were generated at 5 μm and processed for detection of apoptosis. The DeadEnd Fluorometric TUNEL System (Promega, Madison, WI) was used per supplied instructions with appropriate positive and negative controls. Slides were cover slipped and processed sections were then photographed with a fluorescent microscope.

2. Results

Comparative efficiencies of Hprt-Cre and null/+ breedings in generating homozygous mutant embryos

We tested whether the Hprt-Cre strategy could double the number of homozygous mutant embryos compared to the standard heterozygous null/+ breeding strategy using the *Fgfr2IIIb*^{flox} allele. Eighteen litters were generated by mating *Fgfr2IIIb*$^{rec/+}$*; Hprt*$^{Cre/+}$ females to *Fgfr2IIIb*$^{flox/flox}$ males and 18 litters were generated through standard *Fgfr2IIIb*$^{null/+}$ heterozygous matings. The 18 *Hprt-Cre* litters yielded 166 embryos (average of 9.22/litter), where fifty-one percent were homozygous mutants. The 18 standard heterozygous null/+ strategy litters yielded a total 167 embryos (average of 9.28/litter) of which 23% were homozygous mutants (Table 1). The results indicated that the Hprt-Cre doubles the number of homozygous mutant embryos within a litter without a reduction in total litter size.

2.2 Expression of the *Fgfr2IIIb* exon in *Fgfr2IIIb*$^{rec/rec}$ embryos

To confirm Hprt-Cre removes all detectable expression of the conditional allele of interest, expression of the *Fgfr2IIIb* exon in *Fgfr2IIIb*$^{rec/rec}$ embryos was assessed by RT-PCR. By E18.5, there were no detectable levels of *Fgfr2IIIb* in the *Fgfr2IIIb*$^{rec/rec}$ embryos (Figure 1A). In contrast, levels of *Gapdh* (a standard housekeeping gene) in the wild-type embryos was identical to that of *Fgfr2IIIb*$^{rec/rec}$ embryos (Figure 1B). This indicated that *Fgfr2IIIb*$^{rec/rec}$ embryos generated with Hprt-Cre were genotypically and transcriptionally equivalent to *Fgfr2IIIb*$^{null/null}$ embryos.

2.3 External appearance, limb and pituitary development

To determine whether *Fgfr2IIIb*$^{rec/rec}$ embryos are phenotypically equivalent to *Fgfr2IIIb*$^{null/null}$ embryos, we conducted morphological and histological studies of *Fgfr2IIIb*$^{rec/rec}$ embryos in several organ systems and compared these results to the published phenotype for *Fgfr2IIIb*$^{null/null}$ embryos.

Fgfr2IIIb$^{rec/rec}$ embryos appeared externally identical to *Fgfr2IIIb*$^{null/null}$ embryos [14]. At E18.5, all *Fgfr2IIIb*$^{rec/rec}$ mutants exhibited a curved tail and an eyelid defect (Figure 2A–C) with absent or significantly attenuated limbs (Figure 2A). Consistent with this, at E10.5 *Fgfr2IIIb*$^{rec/rec}$ embryos had diminished limb bud mesenchyme in comparison to heterozygous littermates (Figure 2D and E). *Fgfr2IIIb*$^{rec/rec}$ embryos were also smaller at E18.5 than heterozygous littermates (Figure 2A). Not surprisingly, these embryos also exhibited early defects in pituitary development at E10.5. Rathke's pouch was narrowed along its dorsal-ventral axis and shortened along the rostral-caudal axis such that it was not in continuity with the buccal cavity (Figure 2F–I). These findings are equivalent to *Fgfr2IIIb*$^{null/null}$ embryos at this stage [14].

2.4 Lungs

Fgfr2IIIb$^{rec/rec}$ embryos also had a lung defect that was identical to that described in *Fgfr2IIIb*$^{null/null}$ embryos [14]. At day 10.5 a tracheal bud formed, but only minimal secondary branching occurred with bronchial buds terminating blindly in the early lung mesenchyme (Figure 3A and B, yellow arrow). Examination of *Fgfr2IIIb*$^{rec/rec}$ embryos at day 14.5 demonstrated the absence of lungs in the pleural cavity (Figure 3C and D).

2.5 Intestine

The morphology of the gastrointestinal tract of *Fgfr2IIIb*$^{rec/rec}$ embryos at E18.5 was identical to that reported for *Fgfr2IIIb*$^{null/null}$ embryos. The *Fgfr2IIIb*$^{rec/rec}$ embryos had a hypomorphic stomach in comparison to heterozygous litter mates (Figure 4A). Forty-two percent of *Fgfr2IIIb*$^{rec/rec}$ embryos developed a type III duodenal atresia (Figure 4A, white arrow). The penetrance of this defect was equivalent to that reported for *Fgfr2IIIb*$^{null/null}$ embryos (Table 2) [2]. Additionally, all *Fgfr2IIIb*$^{rec/rec}$ embryos had a cecal defect (Figure 4B and C, white arrow) and a distal colonic atresia (Figure 4E, white arrow), both morphologically identical to that reported in *Fgfr2IIIb*$^{null/null}$ embryos [16, 17]. The incidence of the colonic and cecal defects (Table 2) was also identical to that reported for *Fgfr2IIIb*$^{null/null}$ embryos [16, 17]. Finally, the colonic endoderm of *Fgfr2IIIb*$^{rec/rec}$ embryos demonstrated significant apoptosis at E10.5 (Figure 4G). Apoptosis in the colonic endoderm occurs in *Fgfr2IIIb*$^{null/null}$ embryos and is the leading event in the formation of colonic atresia [3]. These morphological and histological findings indicated that *Fgfr2IIIb*$^{rec/rec}$ embryos derived from the Hprt-Cre system are phenotypically identical to *Fgfr2IIIb*$^{null/null}$ embryos for all structures examined.

3. Discussion

We hypothesized that Hprt-Cre could double the number of homozygous knockout embryos in a litter without decreasing the litter size. To test this we chose Fgfr2IIIb mutants because they have defects in many organs. Embryos homozygous for the recombined allele had defects of the limbs, eyes, lungs and gut that are identical in both penetrance and appearance to that published for *Fgfr2IIIb*$^{null/null}$ embryos [2,14,16,17]. This strongly suggests that deletion of flox allele occurred in all cells and no mosaicism with non-recombined cells. We also demonstrated that *Hprt-Cre* in the maternal germ line effectively removes *Fgfr2IIIb* expression in *Fgfr2IIIb*$^{rec/rec}$ embryos. Thus rendering the *Fgfr2IIIb*$^{rec/rec}$ embryos genetically and phenotypically equivalent to *Fgfr2IIIb*$^{null/null}$ embryos.

Use of Hprt-Cre to generate homozygous mutant embryos has been reported previously [18]. However, a detailed comparison between homozygous mutant embryos generated from heterozygous null/+ breedings to those generated with Hprt-Cre has not been published. Furthermore, this is the first comprehensive, quantitative demonstration that this method doubles the number of null embryos in a litter. The use of *Hprt-Cre* to generate null embryos

should double the efficiency by which investigators can generate null embryos for a single gene, while simultaneously reducing the cost.

We would caution the investigator to characterize *rec/rec* embryos of their gene of interest to confirm they are phenotypically equivalent to a homozygous null embryos generated by a standard heterozygous breeding strategy. It is also possible that the recombination of the gene of interest by the Cre-recombinase can vary based on the genomic structure thus investigators should confirm that there is no mosacism as well. There is also an additional drawback that no wild type embryos can be obtained from this cross. Nevertheless this method is far more efficient than standard heterozygous breedings.

To illustrate the increased efficiency of *Hprt-Cre* in generating functional null embryos we conducted a comparison to the Standard Approach of a heterozygous mating strategy using a hypothetical *Gene A*. The Standard Approach One begins with one heterozygote for *Gene* $A^{null/+}$ male and one *Gene* $A^{+/+}$ female (Figure 5A). The *Hprt-Cre* Approach begins with one $Hprt^{Cre/Cre}$ female and one *Gene* $A^{flox/flox}$ male (Figure 5B). For purpose of comparison, the litter size of the F1 generation in each Approach will be 10 pups. The Standard Approach F1 progeny must be genotyped to identify those heterozygous for *Gene* $A^{null/+}$, of which there will be 5. For the sake of argument, three heterozygous pups will be females and two will be males, the remaining wild-type pups are sacrificed. By contrast, all of the F1 pups in the *Hprt-Cre* Approach will have the genotype *Gene* $A^{rec/+;}$ $Hprt^{Cre/+}$. These pups are sexed, the females retained and the males are sacrificed (Figure 5B). When the F1 females of both approaches are greater than mature, they are bred to males. In the case of the *Hprt-Cre* Approach, the *Gene* $A^{rec/+;}$ $Hprt^{Cre/+}$ females are bred to the original *Gene* $A^{flox/flox}$ males. In the Standard Approach, the *Gene* $A^{null/+}$ females are bred to a *Gene* $A^{null/+}$ male. Each female becomes pregnant (5 F1 females in the Hprt-Cre Approach and 3 in the Standard Approach). Assuming a litter size of 10 embryos, the 5 *Gene* $A^{rec/+;}$ $Hprt^{Cre/+}$ females will generate 50 embryos half of which (25) are *Gene* $A^{rec/rec;}$ and half (25) are *Gene* $A^{rec/+}$. In the Standard Approach, the 3 *Gene* $A^{null/+}$ females will generate 30 pups of which 25% (7 or 8) will be *Gene* $A^{null/null}$ embryos. Keeping the number of cages equal, the cost of generating a mutant with the *Hprt-Cre* Approach will be about 1/3 that of the Standard Approach. This calculation excludes the time and money required for genotyping the Standard Approach F1 generation, a step not required in the *Hprt-Cre* Approach.

Differences in efficiency between the heterozygous mating strategy and the *Hprt-Cre* Approach increase dramatically when dealing with compound mutants. For example, one breeding pair of compound heterozygotes ($A^{null/+}$; $B^{null/+}$ X $A^{null/+}$; $B^{null/+}$) will generate a single double homozygous embryo for every 16 embryos (Figure 5C). In contrast, an $A^{rec/+}$; $B^{rec/+}$; $Hprt^{Cre/+}$ X $A^{flox/flox}$; $B^{flox/flox}$ breeding will generate double homozygous embryos at a frequency of 1 in 4 (Figure 5D). The real advantage, however, comes in generating the F1 progeny from an $A^{+/+}$; $B^{+/+}$; $Hprt^{Cre/Cre}$ X $A^{flox/flox}$; $B^{flox/flox}$ mating which will yield 5 $A^{rec/+}$; $B^{rec/+}$; $Hprt^{Cre/+;}$ females from a single breeding pair. In contrast, a single mating pair of $A^{null/+}$;$B^{+/+}$ X $A^{+/+}$; $B^{null/+}$ will generate both a single female and male $A^{null/+}$; $B^{null/+}$ in the F1 generation only under ideal circumstances. Due to a 1 in 16 chance of generating an $A^{null/null}$; $B^{null/null}$ double homozygote from an $A^{null/+}$; $B^{null/+}$X $A^{null/+}$; $B^{null/+}$ breeding, a second $A^{null/+}$; $B^{null/+}$ female is required. In the end, a single $A^{null/null}$; $B^{null/null}$ mutant will be generated from these $A^{null/+}$; $B^{null/+}$ breedings. That is in stark contrast to the 12 or 13 $A^{rec/rec}$; $B^{rec/rec}$ embryos that 5 F1 $A^{rec/+}$; $B^{rec/+}$; $Hprt^{Cre/+;}$ females should generate. The *Hprt-Cre* Approach yields a 12-fold increase in efficiency. This approach could be very useful when generating embryos that require homozygous mutations in multiple genes, such in the Msx1/Msx2 double knock-out model of omphalocele.

Existing *Hprt-Cre* technology represents an efficiency improvement for generating mutant murine animal models of human conditions. We believe this strategy will be useful to many investigators in helping to lower animal housing costs, reducing the time spent performing genotyping, and increasing the efficiency in generating null embryos.

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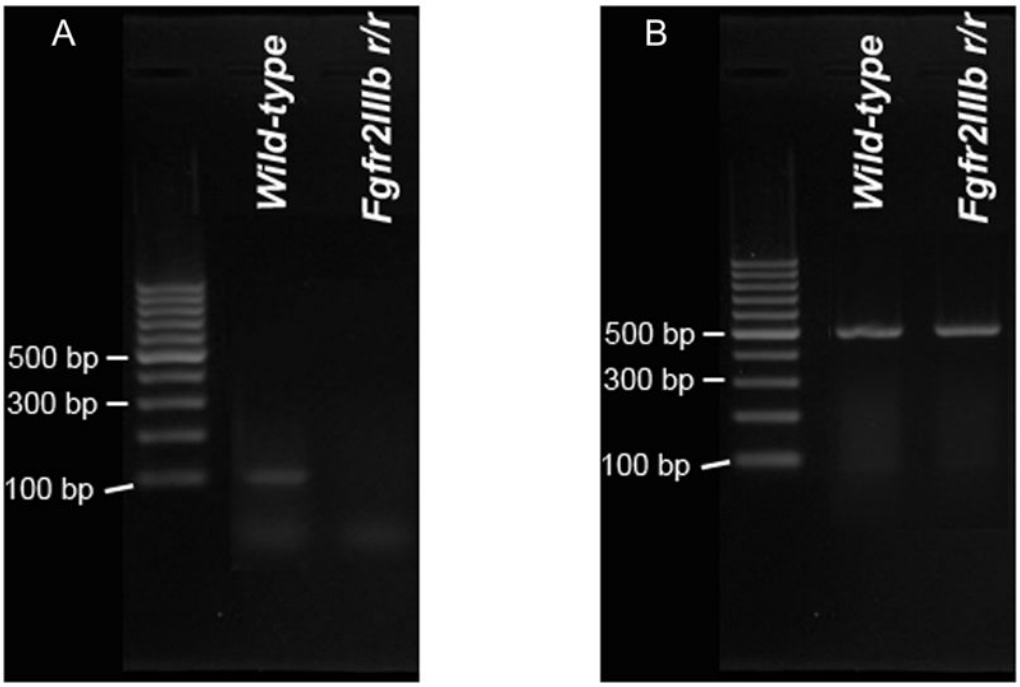


Figure 1. *Fgfr2IIIb*$^{rec/rec}$ mutants do not express the Fgfr2IIIb exon

RT-PCR was used to assess expression of the *Fgfr2IIIb* exon in wild-type embryos and embryos homozygous for the *Fgfr2IIIb*^{rec} allele. RNA was isolated from whole embryos at E18.5 and converted to cDNA. RT-PCR was performed with primers that hybridize within exon *IIIb* of *Fgfr2* **(A)** or with primers for *Gapdh* **(B).** The product size for the *Fgfr2IIIb* exon is 102 bp and 496 bp for *Gapdh*. Note the lack of expression of the *Fgfr2IIIb* exon in the embryos homozygous for the *Fgfr2IIIb*^{rec} allele.

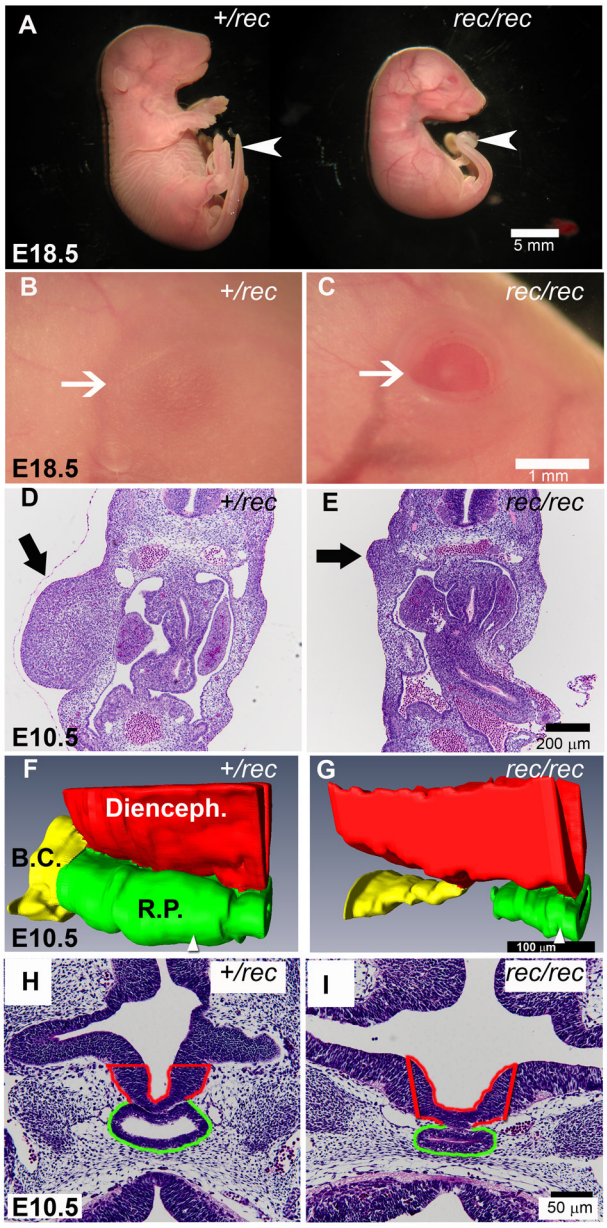


Figure 2. Morphology of *Fgfr2IIIb*$^{rec/rec}$ mutants

(A) Low magnification views of *Fgfr2IIIb*$^{rec/+}$ and *Fgfr2IIIb*$^{rec/rec}$ embryos. At E18.5, the *Fgfr2IIIb*$^{rec/rec}$ mutant lacks limbs, is smaller than its heterozygous littermate, and also has a curved tail (white arrow). **(B and C)** Higher magnification of the eye (white arrow) from embryos shown in **(A)**. The *Fgfr2IIIb*$^{rec/rec}$ embryo has an eyelid defect. **(D and E)** Transverse sections through the limb region at E10.5. The *Fgfr2IIIb*$^{rec/rec}$ mutant **(E,** black arrow**)** exhibits defects in the limb mesenchyme. **(F–I)** Pituitary development at E 10.5. **(F and G)** 3-dimensional reconstructions of Rathke's pouch (RP), the Buccal cavity (BC), and the diencephalon (Di) generated from transverse sections. **(H and I)** Representative sections from the specimens reconstructed in **F** and **G**. Position of the sections **(H and I)** within each reconstruction are indicated by the white arrowhead at the bottom of panels **F** and **G**. In the *Fgfr2IIIb*$^{rec/rec}$ mutant, Rathke's pouch is narrower in the dorsal-ventral axis and no longer continuous with the Buccal cavity.

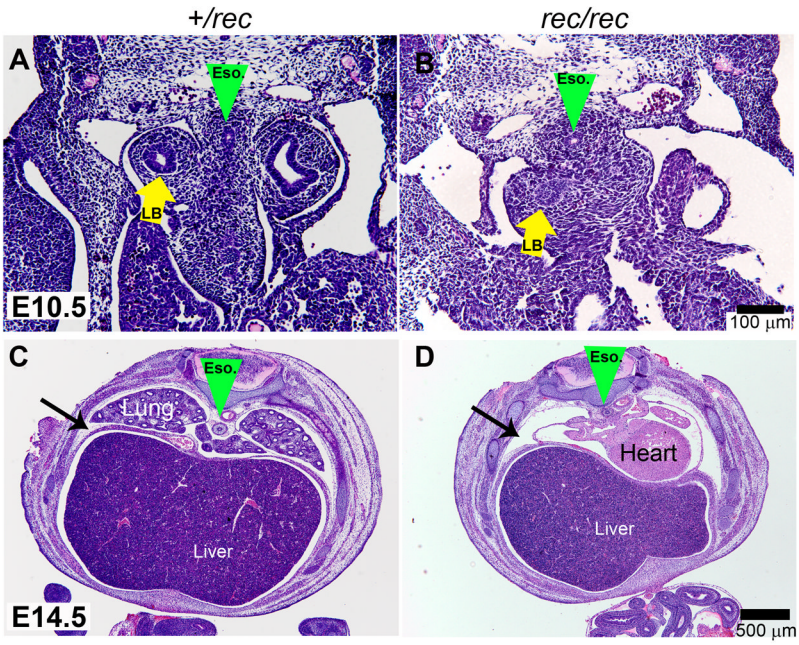


Figure 3. Lung development in *Fgfr2IIIb*$^{rec/rec}$ mutants

(A and B) Transverse sections through the thoracic region at E10.5. Green arrowhead indicates position of the esophagus. Yellow arrows indicate lung buds. **(B)** Note that *Fgfr2IIIb*$^{rec/rec}$ mutant lung bud has terminated into the mesenchyme. **(C and D)** Low magnification views of transverse sections through the thoracic cavity at E 14.5.. In the *Fgfr2IIIb*$^{rec/rec}$ mutant, lungs are absent from the thoracic cavity which is indicated by the black arrows.

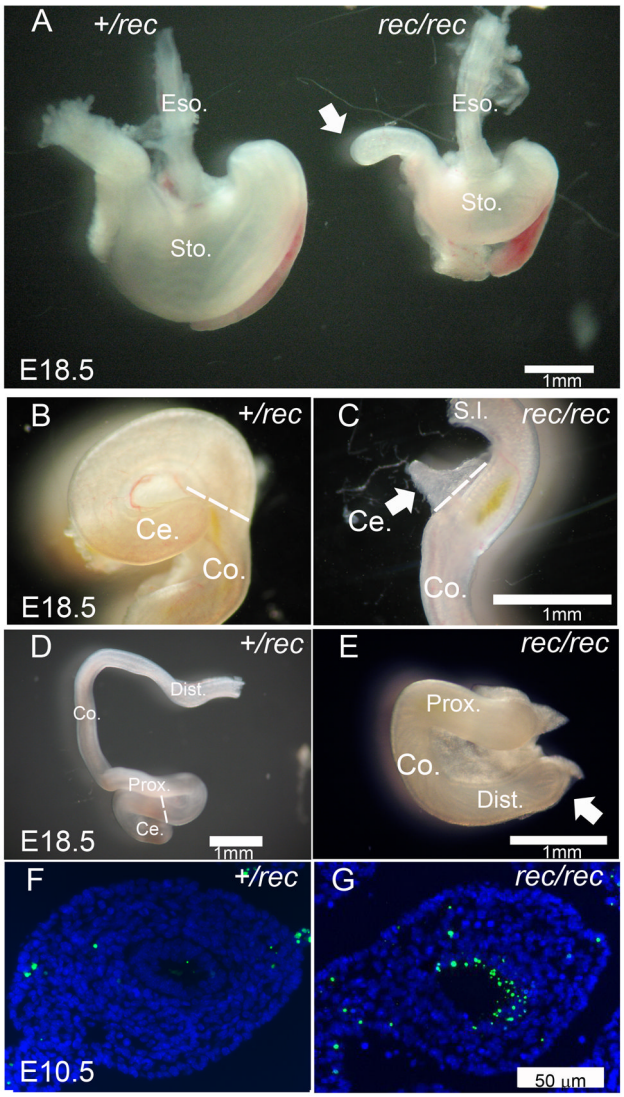
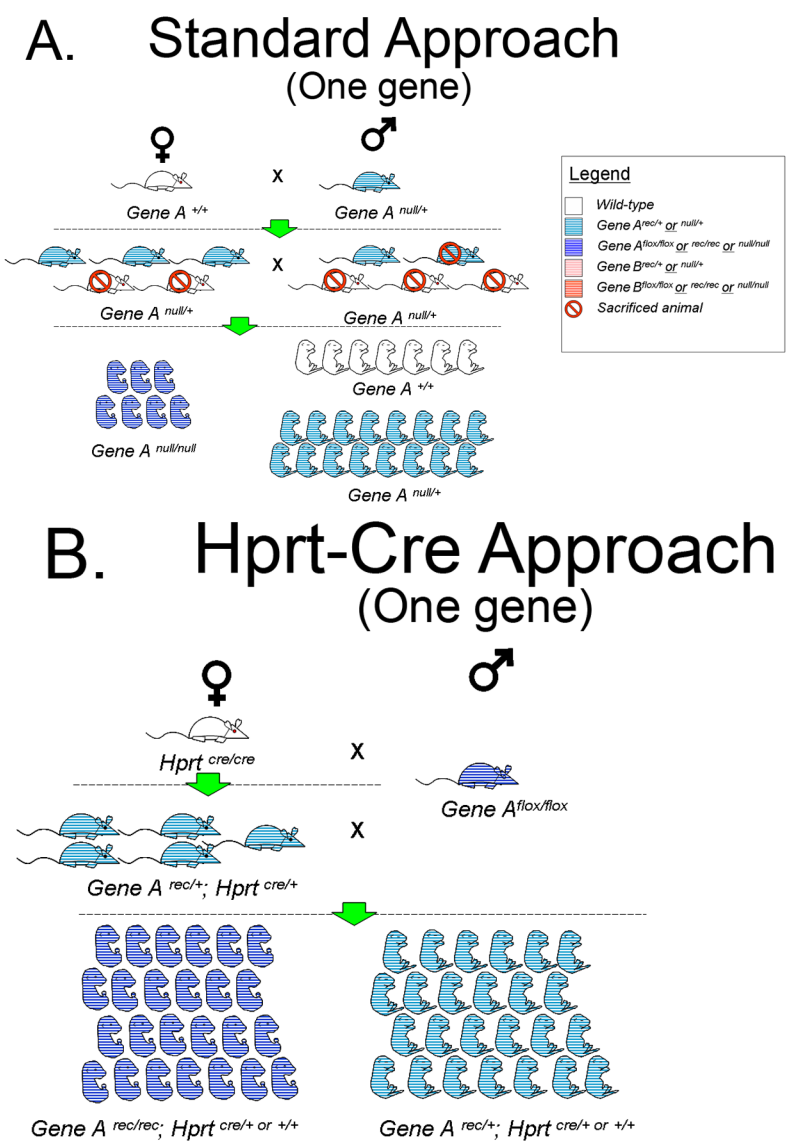


Figure 4. Alimentary track development in *Fgfr2IIIb*$^{rec/rec}$ mutants

(A) Stomach and duodenum of *Fgfr2IIIb*$^{rec/+}$ and *Fgfr2IIIb*$^{rec/rec}$ embryos at E18.5 **(B and C)** the cecum, **(D and E)** and the colon. White arrows in **A** and **E** indicate atresias. White arrow in **C** indicates malformed cecum. Broken white line in **B, C**, and **D** indicates boundary between cecum and colon. TUNEL staining of transverse sections of colon in **(F)** *Fgfr2IIIb*$^{rec/+}$ and **(G)** *Fgfr2IIIb*$^{rec/rec}$ embryos at E10.5.



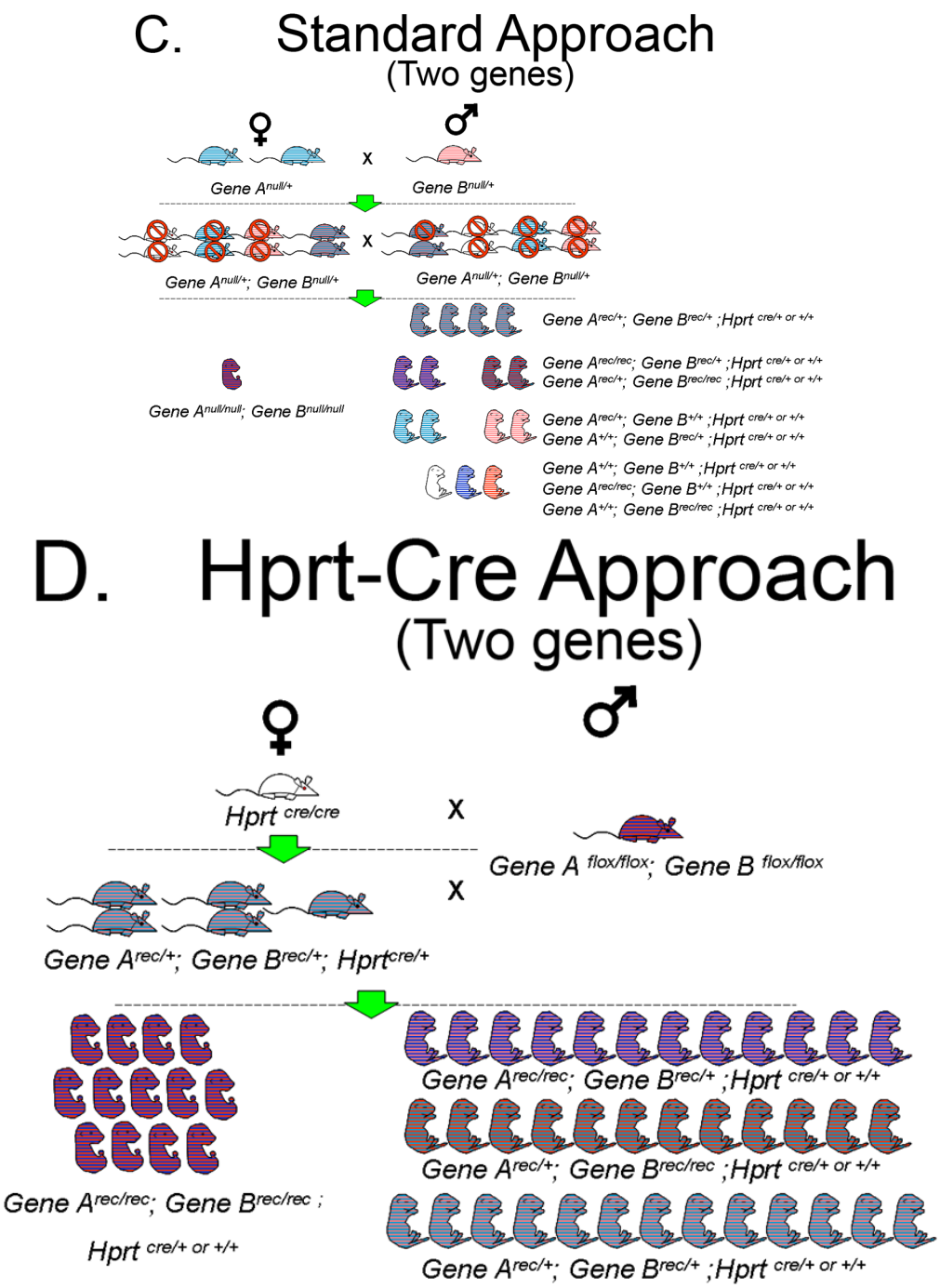


Figure 5. Comparison of the efficiency of a standard heterozygous null/+ breeding strategy to the Hprt-Cre strategy

A. Generation of homozygous null embryos for a single gene with the Standard Approach, a heterozygous null/+ breeding strategy. With this strategy the 3 F1 heterozygous females and single F1 heterozygous male will yield 7 to 8 null embryos. Thus, 25% of embryos in the F2 generation will be homozygous null embryos.

B. Generation of homozygous null embryos for a single gene with the *Hprt-Cre* Approach. With this strategy 50% of embryos in the F2 generation will be homozygous for the recombined allele.

C. Generation of homozygous null embryos for two genes with the Standard Approach, a heterozygous null/+ breeding strategy. With this strategy multiple females carrying the null allele are needed and 2 F1 double heterozygous females will be required to generate a single embryo. Only 6.25% (1/16) of embryos in the F2 generation will be homozygous null embryos for both genes.

D. Generation of homozygous null embryos for two genes with the *Hprt-Cre* Approach. With this strategy 5 F1 double heterozygous females will yield a total of 50 embryos, of which 12 or 13 will be homozygous null embryos for both genes. Therefore, 25% of embryos in the F2 generation will be homozygous for the recombined allele.

Table 1

Relative efficiencies of generating mutant embryos with Hprt-Cre vs. Traditional heterozygous *null*/+ breedings

Breeding	Normal (n)	Mutant (n)	% Mutants	Litters	Embryos/litter(n)
Fgfr2IIIb$^{rec/+}$; *Hprt*$^{Cre/+}$ ^{X} *Fgfr2IIIb* $^{flox/flox}$	81	85	51.20%	18	9.22
Fgfr2IIIb$^{null/+}$ ^{X} *Fgfr2IIIb*$^{null/+}$	129	38	22.75%	18	9.28

Table 2

incidence of duodenal atresia in *Fgfr2IIIb*$^{rec/rec}$ emrbyos generated with Hprt-Cre

Mutants (n)	Colonic Atresia	Cecal Atresia	Duod. Atresia
45	45 (100%)	45 (100%)	19 (42%)