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Generation of Mice with a Conditional Allele for *Ift172*

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Abstract

Ift172 encodes a gene product that is part of a complex that mediates intraflagellar transport (IFT), a process necessary for the genesis and maintenance of cilia. Genetic studies in mice have offered evidence that *Ift172* also plays a role in hedgehog signaling. Disruption of *Ift172* in mice is associated with lethality at about embryonic day 11, limiting studies to understand the role for *Ift172* in later development and the adult. To further our understanding of the later roles of *Ift172*, we have generated mice with a conditional allele for *Ift172*. We have confirmed the phenotype of the disrupted allele by using CRE expression directed by the *prx1* enhancer to disrupt the conditional *Ift172* allele in the developing limb.

Keywords

Intraflagellar transport; Hedgehog; Limb Development; CRE recombinase

The intraflagellar transport protein, IFT172, is a member of a complex of proteins that are required for genesis and maintenance of primary cilia (Eggenchwil and Anderson 2007). Disruption of intraflagellar transport by mutation of *Ift* genes is associated with several human pathologies including polycystic kidney disease (Yoder 2007). Mutation of the genes for the IFT proteins IFT88 or IFT172 also blocks hedgehog signaling (Huangfu et al. 2003). Subsequent studies have demonstrated that many hedgehog signaling components, including the Gli transcriptional regulators, must localize to the primary cilium for proper signaling to occur (Eggenchwil and Anderson 2007). We originally cloned the cDNA encoding IFT172 in a yeast two-hybrid screen for proteins that interact with the LIM domains of the LIM-homeodomain transcription factor Lhx3 (Howard and Maurer 2000). Disruption of *Ift172* causes lethality in mice at about embryonic day 11 (Huangfu et al. 2003; Gorivodsky et al. 2009). To further our understanding of the possible involvement of *Ift172* in Lhx3-mediated pituitary signaling we have generated mice harboring a conditional allele for *Ift172*. This mouse model circumvents the embryonic lethality of the *Ift172* null by allowing the tissue-specific or temporal disruption of *Ift172*.

Materials and methods

Construction of the *Ift172* gene targeting vector

Genomic DNA containing the *Ift172* gene was obtained by screening a λ GT-10 library of 129/SvJ genomic DNA. A targeting vector in which exons 2 and 3 are flanked by *loxP* sites and which also contains neomycin and chloramphenicol resistance cassettes flanked by *frt* sites

was prepared by homologous recombination in *E. coli* (Lee et al. 2001). Details of the construction of the targeting vector are available on request.

The targeting vector was tested for proper design and function by subjecting it to FLP-mediated recombination in the bacterial strain EL250 and CRE mediated recombination in the bacterial strain EL350 (Lee et al. 2001).

Generation of Mice Carrying the Conditional and Disrupted *Ift172* Alleles

The completed targeting vector was linearized and electroporated into 129/SvJ embryonic stem cells and recombinant cells were selected with G418. Recombinant embryonic stem cell colonies were screened by restriction digest and Southern blot analysis. Properly recombined colonies were injected into C57Bl/6 blastocysts which were then implanted into pseudo-pregnant C57Bl/6 females. Resulting chimeric animals were bred with C57Bl/6 mice. Embryonic stem cell work, injections, and implanting was performed by the Oregon Health Sciences University Transgenic/Gene Targeting Core Facility. 129S4/SvJaeSor-Gt(ROSA)26Sor^{tm1(FLP1)}Dym/J mice constitutively expressing FLP1 recombinase and BALB/c-TgN (CMV-Cre)1Cgn mice constitutively expressing CRE recombinase were obtained from the Jackson Laboratory, Bar Harbor, Maine. Prx1cre transgenic mice were obtained by Ronen Schweitzer at the Shriners' Hospital for Children, Portland, OR.

PCR Genotyping

Genomic DNA was isolated from the ear punch tissue and genotyped by polymerase chain reaction. Three sets of primer pairs were used for genotyping: set#1, TTGGTGCCAGGCATGAGAAATGCC and ACTGGCCTTCCACTCATTCTTCCA; set#2, ACCTGAAGCCAGTCTGGGTTACAC and TCTGAAGGAGATGTGAGTGGGTGC; set#3, AGGAGGTAGGAGATTGTGATGGAG and ACCGGCAAACGGACAGAAGCATTT. Primer set#1 yielded products of 175 bp with *ift172*^{wt} and 381 bp with *ift172*^f. Primer set#2 yielded products of 998 bp with *ift172*^{wt}, 1237 bp with *ift172*^f and 331 bp with *ift172*^Δ. Primer set#3 produced a product of 300 bp with the *prx1cre* transgene.

Immunoblot analysis of IFT172 expression

Embryos were obtained from a cross of *Ift172*^{f/Δ} X *Ift172*^{f/Δ} mice at embryonic day 11. A portion of the embryo tail was removed for isolation of genomic DNA and genotyping as described above. The remaining portion of the embryo was disrupted by pipetting repeatedly in lysis buffer consisting of 100 mM NaPO₄, pH 7.8, 0.5% NP40, and Complete protease inhibitor cocktail from Roche. The lysates were frozen twice in a dry ice and ethanol bath and then quickly thawed in a 37°C water bath. The lysates were then centrifuged in a microfuge at full speed for two minutes and the supernatants were assayed for protein concentration. 12.5 μg of total protein was analyzed using a denaturing polyacrylamide gel. For immunoblotting, proteins were transferred to polyvinylidene difluoride membranes (Millipore). The antiserum to IFT172 was produced by immunizing rabbits with a fusion protein containing glutathione S-transferase fused to residues 1214 to 1749 of rat IFT172. The GST-IFT172 fusion protein was produced in *Escherichia coli* and purified by affinity chromatography as described (Smith and Johnson 1988). Following a blocking reaction, the membrane was incubated with a 1:5,000 dilution of antiserum to IFT172 or ERK1 (Santa Cruz) followed by a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit (Santa Cruz) and with chemiluminescent reagent (Amersham Renaissance) using conditions recommended by the suppliers.

Analysis of Mutant Embryos and Skeletal Staining with Alizarin Red and Alcian Blue

For analysis of mutant embryos pregnant mice were euthanized at embryonic day 12 and embryos isolated. The embryos were genotyped as described above. For skeletal staining mice at weaning age were euthanized by CO₂ asphyxiation. Skin and viscera were removed. Carcasses were fixed in 95% ethanol for 4 days and then stained in 0.015% alcian blue 8GS and 0.005% alizarin red S in 5% acetic acid and 70% ethanol. After two days the skeletons were rinsed in water and then immersed in 1% KOH for two days. Skeletons were then further cleared in 1% KOH in 20% glycerol, followed by sequential immersion in 50%, then 80%, and finally 100% glycerol.

Results and discussion

Our initial strategy to preparing a conditional allele for *Ift172* was to flank exons 2–6 with *loxP* sites. In addition to the two *loxP* sites, the targeting vector contains a neomycin resistance cassette for selection in embryonic stem cells. The targeting vector also contains a bacterial expression cassette for chloramphenicol resistance which was used for construction of the target vector using bacterial homologous recombination in *E. coli*. The neomycin/chloramphenicol cassettes are flanked by *frt* sites permitting later removal of this cassette using the bacterial FLP recombinase. This targeting vector was transfected into 129/SvJ embryonic stem cells and neomycin-resistant colonies isolated. Only one correctly targeted colony out of 192 screened colonies was identified. An additional single colony was identified that contained only one *loxP* site. Embryonic stem cells from the one appropriately recombined colony were injected into C57Bl/6J blastocysts. This produced a highly chimeric mouse but the floxed allele was not transmitted to progeny. We observed very poor efficiency of homologous recombination in *E. coli* while making the targeting construct. On the basis of the poor recombination in *E. coli* and the very limited number of correctly targeted embryonic stem cells that were obtained, we suspect that there is a block to recombination somewhere in the region of exons 4–6. Therefore the targeting vector was redesigned so that *loxP* sites would flank exons 2 and 3 (Fig. 1, panel A). Deletion of exons 2 and 3 is predicted to delete amino acids 14–98, which should remove about a third of a WD40 repeat domain and cause a frameshift resulting in a stop codon. Transfection of this targeting vector into 129/SvJ embryonic stem cells resulted in 13 correctly targeted colonies. Two colonies were selected and injected into C57Bl/6 blastocysts and implanted into pseudo-pregnant C57Bl/6 females. One highly chimeric male mouse harbored the targeted allele and stably passed it on to progeny. Mice with the targeted allele were bred with mice expressing the FLP recombinase driven by the constitutive *Gt(ROSA)26Sor* promoter (Farley et al. 2000) to remove the neomycin/chloramphenicol expression cassette. Mice with appropriate recombination that deleted the neomycin and chloramphenicol expression cassettes were identified and are designated *ift172^f*. The *ift172^f* mice were bred to remove the FLP transgene and then further bred to be homozygous for the floxed *ift172* allele (*ift172^{f/f}*). Mice heterozygous for the floxed *ift172* allele were also bred with mice producing CRE recombinase under control of the cytomegalovirus promoter (Schwenk et al. 1995) to produce the disrupted allele designated *ift^Δ*. Polymerase chain reaction was used to detect the wild type, floxed and disrupted alleles (Fig 1B). *Ift172^{f/f}* and *ift172^{f/Δ}* mice appear normal and are fertile.

To determine if deletion of *Ift172* exons 2 and 3 yields a loss of function phenotype, we generated embryos carrying *Ift172^{Δ/Δ}* alleles. *Ift172^{wt/f}* mice were bred with mice carrying a constitutive, germline-expressing CRE recombinase (Schwenk et al. 1995). The resulting *Ift172^{wt/Δ}* mice were then interbred. No mice were born with the *Ift172^{Δ/Δ}* genotype, consistent with the possibility of embryonic lethality as described previously (Huangfu et al. 2003; Gorivodsky et al. 2009). Analysis of embryos at E12 led to the identification of embryos with the *Ift172^{Δ/Δ}* genotype. However the embryos with homozygous disruption of *Ift172* were

severely malformed with neural tube defects (Fig. 2). Overall these findings are consistent with previously published results for other disrupted *Ift172* alleles (Huangfu et al. 2003; Gorivodsky et al. 2009) and suggests that CRE-mediated excision of *Ift172* exons 2 and 3 results in loss of function. To further test if CRE-mediated excision of exons 2 and 3 of *Ift172* alters expression of the IFT172 protein, whole embryo extracts were assayed by immunoblotting (Fig. 3). Excision of exons 2 and 3 of *Ift172* deletes the coding sequence for residues 14 to 98 followed by a frame shift resulting in a stop codon. The IFT172 antibody that was used for the IFT172 immunoblot was generated using an antigen representing a large carboxy-terminal region of IFT172, residues 1214 to 1749. Therefore this antibody should be able to determine if there is expression of *Ift172* exons downstream of the deleted exons. The immunoblot analysis demonstrated that the IFT172 protein could be readily detected in *Ift172^{f/f}* or *Ift172^{f/Δ}* mice, but was undetectable in *Ift172^{Δ/Δ}* mice. These findings provide evidence that CRE-mediated excision of exons 2 and 3 abrogates expression of IFT172 consistent with severe loss of function phenotype observed in the *Ift172^{Δ/Δ}* mice. Following CRE-mediated deletion of exon 2 and 3, splicing of exon 1 to exon 4 should lead to a frame shift. The alternate reading frame of exon 4 includes a stop codon. Therefore, excision of exon 2 and 3 is predicted to lead to expression of the first 13 amino acids of IFT172 encoded by exon 1 fused to 41 amino acids unrelated to IFT172 encoded by the alternate reading frame of exon 4. It seems unlikely that the first 13 amino acids of IFT172 would have any biological activity. Indeed, as the phenotype of germ line disruption of exons 2 and 3 as observed in the present study is similar to the phenotype following disruption of exons 1–3 of IFT172 (Gorivodsky et al. 2009) and a loss of function *Ift172* mutant (Huangfu et al. 2003), it seems very likely that all of these alleles lead to profound loss of function.

To examine tissue-specific disruption of the floxed *Ift172* allele we utilized mice expressing CRE recombinase driven by the *prx1* enhancer (Logan et al. 2002). This enhancer drives expression in the developing limb bud mesenchyme. Previous studies have used this CRE transgene to disrupt *ift88*, a gene whose product is part the same IFT complex as IFT172. It was found that *prx1cre*-induced disruption of *ift88* results in severe polydactyly (Haycraft et al. 2007). As previously seen with *Ift88^{f/Δ}; prx1cre* mice, *Ift172^{f/Δ}; prx1cre* mice have eight digits on each forelimb and a single extra digit on each hindlimb (Fig. 4). The initial report describing the *prx1cre* transgenic mouse noted partially penetrant germline CRE mediated recombination in F2 offspring of female transgenic mice. It was also noted that the penetrance of germline recombination varied depending on nature of the particular locus flanked by the *loxP* sites (Logan et al. 2002). In a mating typical for a cross of *Ift172^{wt/f}; prx1cre* X *Ift172^{wt/f}; prx1cre* we found 2 pups with *Ift172^{wt/wt}*, 6 pups with *Ift172^{wt/Δ}*, and 1 pup with *Ift172^{f/Δ}* genotypes. Only the pup with the *Ift172^{f/Δ}* genotype displayed severe polydactyly presumably due to CRE-induced disruption of the floxed allele in the limb bud. It appears that germline recombination and disruption of the floxed *Ift172* allele occurred very frequently so that the disrupted allele was usually transmitted to pups and the floxed *Ift172* allele was found only rarely. Germline recombination of the floxed *Ift172* allele occurred only in the presence of the *prx1cre* transgene. Mice homozygous for the *Ift172^{f/f}* allele are normal and stably pass the floxed allele to their progeny. Importantly, the severe polydactyly observed with the *Ift172^{f/Δ}* genotype in the presence of the *prx1cre* transgene demonstrates the usefulness of the floxed *Ift172* allele for analysis of the tissue-specific function of the gene product.

In summary, we have created a mouse with a conditional allele of *Ift172*. We have shown that the disruption of the allele obtained by expression of CRE recombinase results in an embryonic lethal phenotype, similar to other *Ift172* null alleles. We have also shown the appropriate and efficient tissue-specific recombination of this allele by using a tissue specific CRE expressing transgene. This mouse should be useful for further studies of the contribution of *Ift172* and intraflagellar transport to tissue-specific development and function.

Acknowledgments

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Fig. 1. *Ift172* targeting vector and polymerase chain amplification analysis of genotypes. **A.** Schematic diagram indicating the structure of the first 6 exons of *Ift172* and the altered structure after recombination with the targeting vector, after FLP mediated recombination to generate *Ift172^f* and after CRE-mediated disruption of the conditional allele in vivo to generate *Ift172^Δ*. **B** Gel electrophoresis analysis of polymerase chain reaction products produced from wild type and modified *Ift172* alleles. The wild type allele, *Ift172^{wt}*, produces a 998 bp amplification product, the floxed allele, *Ift172^f*, produces a 1,237 bp product and the disrupted allele, *Ift172^Δ*, produces a 311 bp fragment.

Fig. 2.
 Embryos with homozygous disruption of *Ift172* are deformed with neural tube defects. Embryos with wild type alleles, *Ift172*^{wt/wt} (wt/wt) or heterozygous for wild type and disrupted alleles, *Ift172*^{wt/Δ} appeared normal while embryos with homozygous disruption of *Ift172* (Δ/Δ) were smaller and evidence of neural tube defects and other development issues can be seen. Same relative magnification for all embryos.

Fig. 3.

Analysis of IFT172 protein expression in embryos with homozygous disruption of *Ift172*. Embryos were dissected at embryonic day 11 and genomic DNA and whole protein extracts were prepared. Embryos were genotyped by polymerase chain reaction as in figure 1B. The embryo extracts (12.5 μg protein) were resolved on a denaturing polyacrylamide gel and then transferred to polyvinylidene difluoride membrane. IFT172 was visualized by immunoblotting with a polyclonal rabbit serum against a carboxy-terminal region of IFT172. IFT172 protein is apparent in both mice with one floxed allele and one disrupted allele, *Ift172^{f/Δ}* (f/Δ) and mice with two floxed alleles, *Ift172^{f/f}* (f/f). No IFT172 proteins is detected in mice with two disrupted alleles, *Ift172^{Δ/Δ}* (Δ/Δ). The membrane was then stripped and probed with anti-serum to ERK1 as a loading control (lower panel).

Fig. 4.

CRE-mediated disruption of *Ifit172* in developing limb buds leads to polydactyly and severely shortened limbs. Limb skeletons were assessed in mice 12 days after birth. All mice that were assessed for limb development carried the *prx1cre* transgene directing CRE recombinase expression to the limb bud. As described in the text, due to high frequency CRE-mediated recombination in germ cells, only a few mice were born with a floxed allele, *Ifit172^f*. Mice with either one or two wild type alleles, *Ifit172^{wt/wt}* (*wt/wt*) or *Ifit172^{wt/Δ}* (*wt/Δ*) had normal appearing limb skeletons. Mice with one floxed allele and one disrupted allele, *Ifit172^{f/Δ}* (*f/Δ*) can be seen to be polydactyl and have shortened limbs, presumably due to CRE-mediated disruption of the floxed allele in the developing limb bud.