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Fltp^{T2AiCre}: A new knock-in mouse line for conditional gene targeting in distinct mono- and multiciliated tissues

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ABSTRACT

We recently identified *Flattop* (*Fltp*; *1700009p17Rik*) in a screen for potential Foxa2 target and novel mouse organizer genes. Besides its expression in the embryonic node, we found that *Fltp* is active in other monociliated tissues such as the sensory organs of the inner ear, duct and islets of the pancreas as well as in testis. Additionally, *Fltp* mRNA is expressed in multiciliated epithelial cells of the lung and of the choroid plexi in the brain. To genetically lineage trace these cells during development and injury as well as to conditionally inactivate genes in these tissues, we generated a Cre recombinase knock-in mouse line using the *Fltp* gene locus. By homologous recombination we have fused the *Fltp* open-reading frame to a tandem affinity purification (TAP) tag followed by an intervening viral T2A sequence for co-translational cleavage and an improved Cre recombinase (iCre). This strategy allows both the analysis of the tagged Fltp-TAP-T2A protein and the usage of the iCre recombinase for conditional targeting approaches. Using the ROSA26 reporter mouse line we show that *Fltp*^{T2AiCre} is first active in the monociliated cells of the node, notochord, floorplate and prechordal plate, consistent with the Fltp-TAP-T2A protein production in the node progenitor cells. Furthermore iCre recombinase activity is detected in multiciliated tissues such as choroid plexi of the brain and epithelial cells of the lung with the onset at E10.5 and E13.5, respectively. In the pancreas, β-galactosidase activity is seen in the monociliated cells of the pancreatic duct and islet of Langerhans. Intercrossing *Fltp*^{T2AiCre} mice with the CAG-CAT-EGFP reporter mouse line further confirms iCre activity in multiciliated cells of the lung and brain on a cellular level. Thus, the *Fltp*^{T2AiCre} line is a powerful tool to conditionally inactivate genes in distinct mono- and multiciliated tissues and to analyze the tagged Fltp protein *in vivo*.

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1. Introduction

Cilia, the antenna-like structures of mammalian cells are important organelles regulating diverse functions including sensation, cell signaling, sperm motility or fluid flow. Architecturally, these microtubule (MT)-based structures are classified into two categories: immotile primary cilia (PC; 9+0 axonemal structure) and motile cilia (9+2 axonemal structure). PC are found on the apical surface of the majority of cells and were thought to be evolutionary relicts. However, recently it was shown that PC sense extracellular signals due to high density of receptors and channels in the ciliary membrane and as such play a crucial role in Ca^{2+}, Hedgehog, PDGFRα, Wnt/β-catenin and planar cell polarity (PCP) signaling (Corbit et al., 2005, 2008; Eggenschwiler and Anderson, 2007; Gerdes et al., 2007; Huangfu et al., 2003; McGrath et al., 2003; Schneider et al., 2005). Motile cilia are present in large numbers on the apical surface of epithelial linings of the trachea, lung, oviduct or ventricles of the brain and generate a fluid flow across the cell surface to transport mucus, liquid or solid material (Ishikawa and Marshall, 2011). Ciliary dysfunction can cause pleiotropic human syndromes with clinical features ranging from blindness, polycystic kidney disease, hydrocephalus, chronic bronchitis, obesity and possibly also cancer and diabetes, making cilia an interesting and highly relevant research topic (Hildebrandt et al., 2011).

Recently we discovered *Flattop* (*Fltp*; *1700009p17Rik*) in a microarray-based screen to identify potential Foxa2 target genes and novel mouse organizer genes (Tamplin et al., 2008). *Pitchfork* (*Pifo*), another node expressed gene identified in the same screen is essential for cilia disassembly (Kinzel et al., 2010). Interestingly, both genes were also found in a screen for mRNAs abundant in tissues that are rich in highly ciliated cells, such as the olfactory epithelium, testis and lung, suggesting that both *Pifo* and *Fltp* encode for ciliary proteins (McClintock et al., 2008). To investigate

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the expression and function of *Fltp* in more detail, we have generated a knock-out (data not shown) and an iCre recombinase knock-in mouse line. Here we describe the generation of the *Fltp*^{T2AiCre} mouse line and the analysis of the iCre recombinase activity pattern during development and in adulthood.

2. Material and methods

2.1. Generation of the targeting vector

The knock-in construct was designed as shown in Fig. 1A. 5′ and 3′ homology regions (HR) for the *Fltp* gene were amplified by PCR (EP 449 fwd 5′ HR *Asc*I; EP 450 rev 5′ HR *Hind*III, *Spe*I; EP 451 fwd 3′ HR *Hind*III; EP 452 rev 3′ HR *BamH*I) using BAC RP23-333P11 clone of C57BL/6J origin as a template. These 2 PCR products were subcloned into the vector pL254 using *Asc*I and *BamH*I. The vector L125 containing the PCR products was cut with *Hind*III and *Spe*I and electroporated into electrocompetent EL350 bacteria containing the *Fltp* BAC clone resulting in vector pL254.

For cloning of the knock-in cassette into pBluescript KS-(pBKS-) 5′ and 3′ HR for the knock-in into exon 6 of Fltp were generated by PCR (EP 457 fwd 5′ HR *Sac*II; EP 458 rev 5′ HR *Sal*I, *Not*I; EP 455 fwd 3′ HR *Sal*I; EP 456 rev 3′ HR *Kpn*I) using the previously mentioned BAC as a template and subcloned into pBKS—using the introduced restriction sites, resulting in L127 pBKS-*Ex6-HR*.

The iCre sequence was amplified and subcloned into pBKS—using primers carrying a *Not*I site and a perfect Kozak sequence 5′ and a *Spe*I site plus a translational stop codon 3′ (iCre_fwd *Not*I; iCre_rev *Spe*I) using pBlue.iCre (Shimshek et al., 2002) as a template, resulting in a plasmid named pBKS-*KozakiCre*.

The T2A sequence from *Thosea asigna* virus was introduced into the *Not*I site of the iCre by annealing the following oligos, that created a *Not*I compatible overhang: (2A_fwd; 2A_rev). In the next step the PGK promoter driven neomycin resistance gene flanked by FRT sites was cut out with *Eco*RI and *Sma*I from pL451ΔloxP (Liu et al., 2003) and subcloned into pBKS-*T2AiCre* digested with *Eco*RI and *Eco*RV resulting in pBKS-*T2AiCre-FRT-Neo-FRT* (L140). The SF-TAP-tag (Gloeckner et al., 2009) was amplified from vector L143 by PCR (EP 489 fwd; EP 490 rev), cut with *Not*I and *Asc*I and cloned into L140 which was cut with *Not*I and *Mlu*I resulting in pBKS-*TAP-tag-T2AiCre-FRT-Neo-FRT* (L144).

pBKS-*Ex6-HR* (L127) containing the homology arms and pBKS-*TAP-TAG-T2AiCre-FRT-Neo-FRT* (L144) were cut with *Not*I and *Sal*I. The product (L158) was cut with *Not*I and filled up with Klenow resulting in the targeting construct pBKS-*homArms-M-TAP-tag-T2A-iCre-FRT-Neo-FRT* (L159) Table 1.

2.2. Cell culture and homologous recombination in ES cells

Mouse embryonic stem (ES) cells were cultured on a murine embryonic feeder (MEF) layer in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Paisley, UK) containing 15% fetal calf serum (FCS, PAN, Invitrogen), 2 mM L-glutamine (Invitrogen, 200 mM), 1 × non essential amino acids (Invitrogen; 100 ×), 100 μM β-mercaptoethanol (Invitrogen, 50 mM) and 1.500 U/ml leukemia inhibitory factor (LIF Chemicon, 10^7 U/ml). Cells were split every 2 days using trypsin (0.05% trypsin, 0.53 mM EDTA_4Na; Invitrogen). IDG3.2 cells (Hitz et al., 2007) were electroporated with the *Asc*I-linearized *Fltp*^{T2AiCre} targeting vector and neomycin resistant clones were selected using 300 μg/ml G418 (Invitrogen). Homologous recombination at the *Fltp* locus was confirmed by Southern blot analysis of *Xho*I-digested genomic DNA using the Fltp 5′-probe (620 bp) located outside of the targeting vector (forward primer: 5′-*NNNctcgagGAGCCCTTACGCACACTTAAG*-3′, including *Xho*I site; reverse primer: 5′-*NNNtctagaCGGGACATTAACTGCATCTTATCT GAGGTTG*-3′, including *Xba*I site) amplified from the BAC clone RP23-333P11. Germline chimeras were generated by CD1 morula aggregation and the *FRT*-flanked *neo* selection cassette was subsequently removed in the germline by intercrossing with Flp-e mice (Dymecki, 1996).

2.3. Genotyping

Mice were genotyped by PCR analysis of tail tip DNA. Genotyping of the *Fltp*$^{T2AiCre/+}$ intercrosses (after Flp-e mediated excision of the *neomycin* selection cassette) was performed using a forward primer in exon 6 of the *Fltp* locus (EP 566) and a forward primer in the iCre (EP 568) in combination with the reverse primer in the 3′ UTR (EP 565). Using 35 cycles at an annealing temperature of 57 °C, this PCR yields products of 317 bp and 229 bp for the wild-type (wt) and for the targeted allele, respectively. Genotyping of R26R Cre-reporter strain (background C57Bl/6) was performed by PCR as described (Soriano, 1999).

2.4. Generation of Fltp and T2A antibody

A rabbit polyclonal antibody against mouse Fltp has been generated using the peptide sequence: DNPDEPQSSHPSAGHTC (Pineda, Berlin, Germany).

A peptide comprising 17 amino acids EGRGSLLTCGDVEENPG from *Thosea asigna* T2A protein was synthesized and coupled to BSA and OVA (PSL, Heidelberg, Germany). Rats were immunized subcutaneously and intraperitoneally with a mixture of 50 μg peptide-OVA, 5 nmol CPG oligonucleotide (Tib Molbiol, Berlin),

Table 1
PCR primer sequences.

EP 449	5′-NNNGGCGCGCCAGTCAGGAAGTGGAAGAGAAGAACACAG-3′
EP 450	5′-NNNAAGCTTACTAGTGTGGTGGAGTGCCTGTCTACATGTG-3′
EP 451	5′-NNNAAGCTTCACGACAGTCAAAGCTGCAATAGAAC-3′
EP 452	5′-NNNGGATCCGGTAATTTGGCAATTATAGAACTCAGGC-3′
EP 457	5′-NNNCCGCGGGTTGGATTCTGAGGCTGACTGGGAACAATC-3′
EP 458	5′-NNNGTCGACGCGGCCGCTTGGTGCTCTTACAAGGGCTCGGAGG-3′
EP 455	5′-NNNGTCGACGCGGCCGCTTGGTGCTCTTACAAGGGCTCGGAGG-3′
EP 456	5′-NNNGGTACCATGCTGTGGGAGTCACTGACATTCTTG-3′
iCre_fwd	5′-NNNgcggccgcGCCACCATGGTGCCCAAGAAGAAGAGGAAAG-3′
iCre_rev	5′-NNNactagtTCAGTCCCCATCCTCGAGCAGCCTCAC-3′
2A_fwd	5′-GGCCGCACGCGTTTGAAGGTAGAGGCTCTTTACTAACATGCGGCGACGTTGAGGAAAACCCAGGACC-3′
2A_rev	5′-GGCCTGGTCCTGGGTTTTCCTCAACGTCGCCGCATGTTAGTAAAGAGCCTCTACCTTCAAACGCGTGC-3′
EP 489	5′-NNNGCGGCCGCCAGCTGGACCC-3′
EP 490	5′-NNNGGCGCGCCTTTATCATCATCATCTTTATAATCCTCTCCGC-3′
EP 566	5′-GAGGCTGACTGGGAACAATC-3′
EP 568	5′-GCTGGTGGCTGGACCAATGTG-3′
EP 565	5′-CAGCATGGCATAGATCTGGAC-3′

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