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THM1 negatively modulates mouse sonic hedgehog signal transduction and affects retrograde intraflagellar transport in cilia

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Abstract

Characterization of previously described intraflagellar transport (IFT) mouse mutants has led to the proposition that normal primary cilia are required for mammalian cells to respond to the sonic hedgehog (SHH) signal. Here we describe an *N*-ethyl-*N*-nitrosourea-induced mutant mouse, *alien* (*aln*), which has abnormal primary cilia and shows overactivation of the SHH pathway. The *aln* locus encodes a novel protein, THM1 (tetra-ricopeptide repeat-containing hedgehog modulator-1), which localizes to cilia. *aln*-mutant cilia have bulb-like structures at their tips in which IFT proteins (such as IFT88) are sequestered, characteristic of *Chlamydomonas reinhardtii* and *Caenorhabditis elegans* retrograde IFT mutants. RNA-interference knockdown of *Ttc21b* (which we call *Thm1* and which encodes THM1) in mouse inner medullary collecting duct cells

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AUTHOR CONTRIBUTIONS P.V.T. carried out most experiments. C.J.H. carried out immunostaining of IFT88 on limb cryosections and GLI localization to *aln* cilia. T.Y.B. generated the m368-2 cell line and performed live-cell imaging experiments with P.V.T. A.T.-D. carried out immunofluorescence analysis of neural tube sections and whole-mount *in situ* hybridization experiments. B.J.H. generated the *aln* mutant and mapped the *aln* locus to chromosome 2. A.L.C. generated the antibody to THM1, and H.Q. conducted resequencing analysis of candidate loci for *aln*. P.J.S. did the initial GLI3 western blot on *aln* tissue. D.R.B., B.K.Y., J.V.S., C.J.H. and P.V.T. designed the experiments. R.W.S. provided thoughtful discussions and was instrumental throughout the study. P.V.T., R.W.S. and D.R.B. wrote the manuscript. J.V.S., B.K.Y., C.J.H. and T.Y.B. critically commented on the manuscript.

expressing an IFT88-enhanced yellow fluorescent protein fusion recapitulated the *aln*-mutant ciliary phenotype, and live imaging of these cells revealed impaired retrograde IFT. In contrast to previously described IFT mutants, Smoothed and full-length glioblastoma (GLI) proteins localize to *aln*-mutant cilia. We hypothesize that the *aln* retrograde IFT defect causes sequestration of IFT proteins in *aln*-mutant cilia and leads to the overactivated SHH signaling phenotype. Specifically, the *aln* mutation uncouples the roles of anterograde and retrograde transport in SHH signaling, suggesting that anterograde IFT is required for GLI activation and that retrograde IFT modulates this event.

The SHH signaling pathway has essential and diverse roles in development and stem cell renewal¹. Dysregulation of the pathway in the human embryo causes severe developmental anomalies, and inappropriate regulation in the adult leads to several cancers². Signaling initiates when SHH ligand binds to a 12-transmembrane receptor, Patched (PTC), releasing its repression of the 7-transmembrane signal transducer Smoothed (SMO) and resulting in modulation of the GLI transcriptional regulators. In the presence of ligand, full-length GLI proteins activate transcription of target genes, with GLI2 being the primary effector of the pathway. Conversely, in the absence of ligand, full-length GLI3 protein (GLI3A) is cleaved to form a transcriptional repressor (GLI3R)³.

Recent studies have established a crucial role for primary cilia in mediating mammalian SHH signaling⁴⁻⁷. Vertebrate primary cilia, which are similar to flagella of *C. reinhardtii*, are microtubule-based extensions of the plasma membrane that may function as sensory organelles. During ciliogenesis, proteins are transported along the microtubules from the base to the tip of the cilium by anterograde IFT, which is powered by the kinesin motor, and back to the base by retrograde transport, which is mediated by a cytoplasmic dynein. *C. reinhardtii* IFT proteins can be separated biochemically into complexes B or A, which are involved in anterograde and retrograde transport, respectively⁸. To date, mice carrying mutations affecting the complex B proteins (IFT52, IFT57, IFT88 and IFT172) and subunits of the kinesin and dynein motors (KIF3A and DYNC2H1, respectively) have been characterized^{4-7,9,10}. All of these mutants show disruption of SHH pathway activation; these mice do not develop a floor plate in the neural tube, suggesting that the GLI2 transcriptional activator is not functional. In addition, GLI3 proteolytic processing in these mutants is markedly reduced, resulting in lower GLI3R expression and a higher GLI3A:GLI3R ratio^{5,9,10}. Genetic analyses indicate that cilia regulate mammalian SHH signaling downstream of SMO and upstream of the GLI factors, yet delineating ciliary functions essential to pathway activation or repression downstream of SMO has not been possible.

In mammalian cells, data suggest that exit of SHH-bound PTC out of cilia is succeeded by translocation of SMO to cilia and transduction of the SHH signal¹¹. A mutation in *Smo* that inhibits the entry of SMO into cilia also impedes activation of a SHH reporter¹². In the *Dync2h1* mutant, SMO does not localize to nodal cilia¹⁰ and the pathway is not activated^{9,10}, consistent with the hypothesis that ciliary translocation of SMO is required for pathway activation. Full-length GLI proteins also localize to the distal tips of primary cilia⁷. In *Ift88*-mutant cells, overexpression of full-length GLI proteins leads to their proper stabilization and nuclear import, yet GLI2 remains inactive. Cilia are absent in these mutant

cells; thus, ciliary translocation of the GLI proteins cannot occur⁷. This suggests that activation of the full-length GLI transcriptional factors, but not their stabilization nor nuclear import, is contingent on ciliary or IFT function.

RESULTS

aln is a THM1-null mutant

In an *N*-ethyl-*N*-nitrosourea–induced mutagenesis screen for recessive mutations affecting late embryogenesis in mice, we identified *aln*, whose mutation results in preaxial polydactyly, split and fused ribs, cortical layering abnormalities, delayed eye and forebrain development and neural tube defects¹³. We mapped the *aln* locus to a 2-Mb interval on chromosome 2 between microsatellite markers *D2Mit90* and *D2Mit325* using recombination analysis in intraspecific and interspecific crosses. Sequence analysis of candidate genes revealed an A→C mutation near the N-terminus of the novel gene *Ttc21b* (Fig. 1a). This mutation converts a highly conserved glutamine to a proline at residue 15 (Fig. 1). *Ttc21b* encodes THM1, a 1,317-amino acid protein of ~ 150 kDa, predicted to contain 11 tetratricopeptide repeat (TPR) domains. Western blot analysis using antibodies to an 80-amino acid THM1 peptide revealed a reduction or absence of THM1 protein in heterozygous and *aln* extracts, respectively (Fig. 1b), indicating that *aln* is a THM1-null mutant.

In a BLAST homology search, we identified a paralog of *Ttc21b*, *Ttc21a* (which we named *Thm2*). *Ttc21a*, situated on chromosome 9, encodes a protein that is 49% identical to THM1 and has a similar predicted protein structure (Supplementary Fig. 1a online). The region encompassing the *aln* mutation in THM1 is also conserved in THM2 (Supplementary Fig. 1b), underscoring the presumptive functional importance of this motif. At embryonic (E) day 10.5, *Ttc21b* and *Ttc21a* are widely expressed, with more intense expression in the maxillary prominence, branchial arches, limb buds, somites and spinal cord (Supplementary Fig. 1c).

SHH pathway is inappropriately activated in *aln* mutants

The phenotype caused by the *aln* mutation includes abnormalities of the skeleton, limbs, craniofacial region and nervous system, and is very similar to that caused by mutation of *Rab23*, a negative modulator of SHH signaling¹⁴. We analyzed *Ptch1*, which is a transcriptional target of SHH¹⁵, and observed more intense expression in E9.5 *aln* mutants, particularly in the maxilla, first branchial arches and somites (Fig. 2a–d). *Ptch1* was also expressed throughout the entire *aln*-mutant limb, rather than being restricted to the posterior region as in wild-type embryos (Fig. 2c–f). This ectopic *Ptch1* expression indicates that the SHH pathway is upregulated in *aln* mutants.

In the ventral neural tube, SHH ligand forms a ventral-to-dorsal decreasing gradient from its source in the notochord and floorplate and specifies neuronal cell fates in a concentration-dependent manner¹⁶. Mutations in negative regulators of SHH signaling, such as *Ptch1*, *Rab23*, *Prkaca* or *Gli3*, result in dorsal expansion of ventral cell types^{14,17,18}. In *aln* mutants, the caudal neural tube showed a consistently abnormal morphology at E9.5 (Fig. 2) and E10.5 (Fig. 3), similar to that observed in *Rab23* mutants¹⁴. In the *aln*-mutant caudal neural tube, we observed moderate dorsal expansion of the floor plate marker FOXA2 and the V3

interneuron progenitor marker NKX2.2 (Fig. 2j,l). The most notable expansion, however, was observed with the motoneuron progenitor marker *Olig2* at E9.5 (Fig. 2h) and the motoneuron marker MNR2 at E10.5 (Fig. 3m). NKX6.1, a marker of V3, motoneuron and V2 interneurons, was also expanded dorsally at E9.5 (Fig. 2n). Also at E9.5, the FOXA2- and NKX2.2-expressing cells were scattered in expanded dorsal regions and were likely to be interspersed with the NKX6.1-expressing cells, revealing that the normal sharp boundary between these progenitor subtypes was lost (Fig. 2j,l,n). At E10.5, cells expressing PAX6, a marker of motoneurons, V2–V0 interneurons and dorsal neuronal progenitors, were shifted dorsally (Fig. 3q). Finally, the expression domain of dorsal marker MSX2 was shifted dorsally and was smaller in *aln* mutants than in wild-type embryos (Figs. 2p and 3u), suggesting the loss of dorsal neurons in *aln* mutants.

To quantitatively evaluate the dorsal expansion of neuronal cells, we calculated the number of nuclei expressing a given neuronal marker divided by the total number of DAPI-stained nuclei in the neural tube. At E9.5, a larger proportion of cells in the *aln*-mutant neural tube expressed FOXA2, NKX2.2 and NKX6.1 ($P < 0.05$) and a smaller proportion of cells expressed PAX6 and MSX2 ($P < 0.05$) compared to wild-type neural tubes (Supplementary Fig. 2 online). These data show that ventral cell types of the *aln*-mutant neural tube, particularly the motoneurons, are expanded at the expense of dorsal neuronal cells. This ventralization is consistent with a requirement for THM1 in the restriction of SHH signaling.

***Ttc21b* is epistatic to *Smo* and restricts GLI2 activity**

To determine the position of THM1 in the SHH signaling cascade, we carried out a series of epistasis analyses using crosses between mice mutant for *aln* and mice mutant for *Shh* or *Smo*. The homozygous *Shh*-null mutant has holoprosencephaly, cyclopia and truncated limbs with a single digit^{19,20}. In contrast, the *aln Shh* double mutant resembles the homozygous *aln* mutant (Fig. 3a), with partially intact head structures, two eyes and extended limbs with polydactyly, indicating that the *aln* mutation restores activity to the signaling pathway in the absence of SHH. Mice homozygous for a null mutation of *Smo* die shortly after E9.5, when the mutant embryo has not yet turned and its heart has not yet looped²¹. However, mice homozygous for both *aln* and *Smo* mutations show some turning of the body, have more advanced cranial development than does the single *Smo* mutant, and survive to E10.5 (Fig. 3b). The evidence that the *aln* mutation can partially rescue the defects in these mice suggests that THM1 functions as a negative regulator downstream of both the SHH ligand and the SMO signal transducer.

We further tested whether THM1 acts upstream of the GLI transcription factors by analyzing mice mutated in both *aln* and *Gli2*. At E10.5, homozygous *Gli2*-null mice have well-developed telencephalic vesicles²², which are poorly defined in *aln* mutants (Fig. 3c). However, the telencephalic vesicles in the *aln Gli2* double mutants are well developed, suggesting that THM1 acts upstream of GLI2. To evaluate this in more detail, we examined dorsoventral patterning of the double-mutant lumbar neural tube (Fig. 3d–w). GLI2 is required for specification of the floor plate and the majority of V3 interneurons; in mice lacking *Gli2* only, these cells are absent, and motoneurons and lateral and dorsal cell types are shifted ventrally²³ (Fig. 3f,j,n,r,v). In marked contrast to *aln* mutants, in which the

ventral cell types are expanded dorsally (Fig. 3e,i,m), the double-mutant neural tube resembles the dorsalized *Gli2*-mutant neural tube (Fig. 3g,k,o,s,w). These data confirm that THM1 acts upstream of GLI2. The double-mutant phenotype further indicates that the ventralization of the *aln*-mutant neural tube is mediated to a large extent by active GLI2. The dorsal expansion of floor plate cells in *aln* mutants, in particular, reveals that GLI2 is overactivated.

GLI3A:GLI3R ratios are elevated in *aln* mutants

Because our data indicate that THM1 is an antagonist of SHH signaling that functions downstream of SMO and upstream of GLI2, we investigated whether THM1 also affects proteolytic processing of GLI3. SHH expression in the posterior region of the developing limb creates an increasing GLI3A:GLI3R gradient across the anterior-posterior axis^{3,24,25}; this ratio is crucial for determining digit number and identity²⁶. We found significantly higher levels of GLI3A in E10.5 *aln*-mutant anterior limb buds relative to wild-type anterior limb buds (Fig. 4a), increasing the GLI3A:GLI3R ratio by tenfold (Supplementary Fig. 3 online). However, GLI3R levels were also elevated in *aln* mutants, indicating overall higher GLI3 expression. To determine whether this upregulation occurs at the transcriptional level, we performed whole-mount *in situ* hybridization for *Gli3* on E10.5 embryos. *Gli3* expression was more intense in *aln* mutants, particularly in the branchial arches and in the limb (Fig. 4b). Thus, loss of THM1 increases *Gli3* transcription and, in turn, GLI3 protein expression. GLI3 protein is efficiently processed in the *aln*-mutant limb, generating slightly higher GLI3R expression, but the simultaneous excess of GLI3A markedly increases the GLI3A:GLI3R ratio, resulting in preaxial polydactyly.

Cilia in *aln* mutants have abnormal morphology and accumulation of IFT proteins

The *C. reinhardtii* ortholog of THM1 is flagellar-associated protein-60 (Fig. 1c), suggesting a role for THM1 in primary cilia physiology. In polarized epithelial inner medullary collecting duct (IMCD) cells and in the nodes of E8.0 mice, THM1 colocalized with acetylated α -tubulin in cilia and was expressed in a punctate manner throughout the axoneme from the ciliary base to the tip (Fig. 5a). This expression pattern is reminiscent of that of ciliary proteins such as IFT88, IFT57 and IFT20 (ref. 27). To study trafficking in *aln*-mutant cilia, we conducted immunofluorescence analyses using antibodies to acetylated α -tubulin, which stains the axoneme, and Polaris/IFT88, which normally localizes to the proximal and distal ends of cilia and in a punctate pattern throughout the axoneme⁷. In contrast, in cilia of *aln*-mutant primary limb cells (Fig. 5b) and on *aln*-mutant limb cryosections (Supplementary Fig. 4 online), IFT88 was remarkably more intense at the distal ends of the cilia compared to wild-type cilia, and was undetectable at the proximal ends. Axonemes stained by acetylated α -tubulin also appeared shorter in *aln* mutants than in wild-type cilia. Viewed under scanning electron microscopy, cilia in E11.5 limbs of *aln* mutants had bulb-like structures at their distal tips (Fig. 5c); these structures were never observed on wild-type cilia. The dimensions of the *aln*-mutant ciliary tips differed significantly from wild-type: *aln*-mutant cilia at their widest points measured $0.229 \pm 0.038 \mu\text{m}$ (s.d.), whereas wild-type cilia measured $0.176 \pm 0.023 \mu\text{m}$ ($P < 0.00013$). Together, these results suggest that IFT88 accumulates at the distal ends of *aln*-mutant cilia in the bulb-like structures.

SMO and full-length GLI proteins traffic into cilia of *aln* mutants

Because ciliary translocation of SMO is required to activate a SHH reporter¹², we evaluated whether the SHH pathway activation we observed in *aln* mutants is associated with SMO localization to cilia. Using whole-mount immunofluorescence with antibodies to SMO, we observed punctate patterns of SMO protein along the axonemes of nodal cilia in both wild-type and *aln*-mutant embryos (Fig. 6a). Because full-length GLI proteins localize to the distal tips of cilia⁷, we further examined whether the trafficking of GLI proteins also occurs in *aln*-mutant cilia. Overexpression of the GLI proteins in *aln*-mutant mouse embryonic fibroblasts (MEFs) revealed that the full-length GLI1, GLI2 and GLI3 proteins localize at the distal ends of the mutant cilia, whereas GLI3R localizes to the nuclei (Fig. 6b and Supplementary Fig. 5 online). These results show that translocation of SMO and full-length GLI proteins into cilia is not abrogated in the *aln* mutant.

Loss of THM1 impairs retrograde intraflagellar transport

In *C. elegans*, IFT mutants are classified morphologically as complex B, complex A or dynein mutants²⁸. The complex B class, which also includes kinesin mutants, has very short or absent cilia as a result of defective anterograde IFT. Complex A mutants have shortened ciliary axonemes with bulges at their distal tips, where IFT protein particles accumulate. This accumulation is a result of partially defective retrograde IFT, whereas anterograde transport into the cilia is unaffected^{28,29}. Given the similarity of the *aln*-mutant ciliary phenotype to that described for complex A mutants, we tested the possibility that the accumulation of IFT88 in *aln*-mutant cilia is caused by a trafficking defect. This was done by analyzing anterograde and retrograde velocities of an IFT88-enhanced yellow fluorescent protein (EYFP) fusion using live imaging of cilia of THM1-deficient cells. We generated a clonal IMCD cell line that stably expresses IFT88-EYFP (m368-2; Fig. 7a) and infected these cells with lentiviruses expressing an shRNA targeted against *Ttc21b*. Colonies from this infection were clonally expanded and analyzed for THM1 expression. Western blot analysis of the clonally derived cell lines R1-4, R1-2 and R1-5 revealed that they expressed 12.5%, 11.5% and 73.0%, respectively, of the THM1 protein expressed by the clonal control cell line EV4 (Fig. 7b). R1-4 and R1-2 cells had shortened cilia, many of which had accumulations of IFT88-EYFP at their distal ends, recapitulating the *aln*-mutant phenotype (Fig. 7). Notably, this finding confirms that the loss of *Ttc21b* expression is the cause of the phenotype seen in the *aln*-mutant mouse. In R1-4 and R1-2 cells, mean anterograde velocities of IFT88-EYFP ($0.420 \pm 0.129 \mu\text{m/s}$ and $0.495 \pm 0.121 \mu\text{m/s}$, respectively) were not significantly different from those measured in EV4 cells ($0.382 \pm 0.054 \mu\text{m/s}$; Fig. 7c). In contrast, the mean retrograde velocities in R1-4 and R1-2 cells were significantly lower ($0.413 \pm 0.062 \mu\text{m/s}$ and $0.402 \pm 0.061 \mu\text{m/s}$, respectively) than those of EV4 cells ($0.559 \pm 0.085 \mu\text{m/s}$; $P < 0.002$; Fig. 7c and Supplementary Videos 1 and 2 online). R1-5 cells, which showed minimal THM1 knockdown, had similar velocities to those of EV4 cells (Fig. 7c). These data reveal that loss of THM1 impairs retrograde IFT, which explains the abnormal accumulation of IFT88 at the distal ends of *aln*-mutant cilia.

DISCUSSION

In this report, we describe the characterization of the *aln* mutant, which has defective cilia that show overactivation of the SHH pathway. In *aln* mutants, the neural tube is ventralized and *Ptch1* expression is increased. Overactivation of the pathway in most tissues is ligand independent, as indicated by the *aln Shh* double-mutant phenotype. Because GLI3R levels are not reduced in *aln* mutants, the increased SHH signaling activity is likely to be the result of overactivation of GLI2 and GLI3A. In support of this hypothesis, FOXA2-expressing cells, which are generated only by GLI2, are dorsally expanded in the *aln*-mutant neural tube. Like the *Gli2*-mutant neural tube, the *aln Gli2* double-mutant neural tube is dorsalized, indicating that ventralization of the *aln*-mutant neural tube is mediated to a large extent by GLI2.

Interestingly, ectopic *Ptch1* expression in the E9.5 *aln*-mutant limb is similar to the *Gli1* expression pattern in the E10.5 limb of the *Gli3^{P1-4/P1-4}* mouse, which expresses only the full-length form of GLI3 and can activate SHH target gene expression in the limb in the absence of SHH ligand²⁶. Thus, ectopic *Ptch1* expression in the *aln*-mutant anterior limb suggests that GLI3A is also active in *aln* mutants. Given that *aln* mutants have normal levels of GLI3R, in contrast to the absence of GLI3R in *Gli3^{P1-4/P1-4}* mutants, the ectopic *Ptch1* expression in *aln* mutants further suggests that, like GLI2, GLI3A is also inappropriately activated. The fact that these full-length transcriptional factors are functional makes the *aln* mutant unique among characterized cilia mutants. All mouse cilia mutants characterized to date lack fully activated GLI2, as indicated by the absence of a floor plate^{4-6,9,10,30,31}, dorsalization of the neural tube^{4-6,9,10,30} or significant downregulation of *Ptch1* expression in the posterior limb^{5,10}.

The distribution of THM1 protein in cilia is reminiscent of other IFT proteins, such as IFT88, IFT57 and IFT20, which are similarly localized in a punctate manner in the axoneme from the cilia base to the tip²⁷. In addition, the predicted protein structure of THM1 reveals the presence of 11 TPR domains, which are thought to mediate transient protein interactions and are enriched in cilia and centrosomal proteins, including IFT88 (ref. 32), BBS4 (ref. 33) and BBS8 (ref. 34). Notably, the cilia phenotype of *aln* mutants is very similar to the phenotypes of *C. reinhardtii* and *C. elegans* complex A mutants, in which retrograde IFT is partially defective, resulting in accumulation of complex B proteins in the axonemes^{28,29,35-37}. In the *C. reinhardtii* complex A mutants *fla15*, *fla16* and *fla17*, retrograde velocities were 2.6 $\mu\text{m/s}$, 2.2 $\mu\text{m/s}$ and 2.0 $\mu\text{m/s}$, respectively, whereas that of control strain *pf15* was 3.1 $\mu\text{m/s}$ (ref. 36). This 27% reduction in retrograde IFT velocity is the same as the reduction we observed in THM1-deficient cell lines compared to wild-type cells. Consistent with these experimental observations, the sequence of the *C. reinhardtii* Complex A component IFT139 has been reported to be the same as that of the THM1 ortholog flagellar-associated protein-60 in a recent GenBank entry (ABU95018).

Like the *aln* mutant, the *Dync2h1* mutant is a presumptive retrograde mutant, yet its SHH phenotype is markedly different from that of the *aln* mutant¹⁰. In the organisms in which they have been studied in detail, dynein mutants are generally more severe than complex A mutants, with cilia axonemes that are even shorter, and with larger bulges at their distal tips.

As discussed above, the *C. reinhardtii* complex A mutants show a reduction in retrograde IFT velocity. In contrast, in the *C. reinhardtii* LC8 mutant, which harbors a mutation in a dynein subunit, retrograde IFT is absent³⁸. This is also seen in *C. elegans*, in which complex A and B proteins cannot exit the cilia in the *xbx-1* dynein mutant; however, retrograde transport of the dynein complex is still evident when analyzed in a complex A mutant³⁹. It is likely that, in contrast to the partially defective retrograde IFT in *aln*-mutant mice, retrograde IFT is absent in *Dync2h1*-mutant mice as it is in the LC8 mutant of *C. reinhardtii*³⁸. Perhaps more important is the observation that SMO cannot localize to cilia in the *Dync2h1* mutant but does so in the *aln* mutant. This may account for the difference in SHH pathway activation in mutants that both have retrograde IFT defects.

Activation of the GLI proteins is poorly understood. In *Drosophila melanogaster*, stabilization, nuclear import and activation of cubitus interruptus (ci), the *D. melanogaster* ortholog of the GLI proteins, are independent events, and activation of ci alone seems to be dependent on translocation of SMO to the plasma membrane⁴⁰. In mammalian cells, activation of a SHH reporter requires translocation of SMO to cilia, an event proposed to be analogous to the SMO translocation step in *D. melanogaster*^{11,12}. In the mouse *Dync2h1* mutant, pathway activation does not occur, and SMO does not localize to the nodal cilia¹⁰. In the *Ift88* mutant, which lacks cilia, GLI proteins are present in the nucleus, but GLI2 is inactive⁷. In contrast, both SMO and the full-length GLI proteins are able to translocate to *aln*-mutant cilia, and full pathway activation occurs.

aln is the first cilial mutant in which the roles of anterograde and retrograde IFT are uncoupled in SHH signaling. Given that anterograde IFT is functional in *aln* mutants, we propose that anterograde IFT is required for GLI activation. Anterograde IFT might colocalize all the components necessary for the activation of the GLI proteins, which might occur at the cilial tips. Flagellar tips (the functional equivalents to the distal portions of cilia) are specialized compartments in which numerous functions essential to flagella synthesis and maintenance are carried out⁴¹. However, since GLI2 is inappropriately activated in *aln* mutants and retrograde IFT is defective, we further propose that retrograde IFT is required for the correct modulation of this activation step. Our identification of a mutant in which this abnormal activation occurs should facilitate the biochemical characterization of the underlying process.

METHODS

N-ethyl-*N*-nitrosourea mutagenesis and mapping and cloning of *Ttc21b*

N-ethyl-*N*-nitrosourea mutagenesis and mapping of the *aln* locus to chromosome 2 were done as described¹³. We used intraspecific crosses between A/J and FVB inbred mouse strains and interspecific crosses between A/J and *Mus castaneus* (Jackson Laboratory) to narrow the interval to 4.5 Mb between microsatellite markers *D2Mit205* and *D2Mit524*. We generated an additional intraspecific cross between A/J and DBA strains, as A/J and *M. castaneus* crosses were not providing further recombinants within the 4.5 Mb interval. From the A/J × DBA crosses, we obtained a recombinant carrier female that narrowed the interval containing *aln* to 2 Mb between *D2Mit90* and *D2Mit325*. Four genes in this interval—*Galnt3* and *Birc5l* and the novel genes *Xirp2* (also known as *Xin2*) and *Ttc21b*—were fully

sequenced. Initial characterization of *Ttc21b* was incomplete because the predicted gene sequence did not include the first exon. However, the expression pattern of *Ttc21b* suggested it as the candidate for *aln*, and our further analysis revealed the correct full-length sequence and uncovered the mutation described in Results. This sequence change was confirmed in both genomic and cDNA sequences and is not present in the parental A/J DNA.

Genotyping of mice

Before cloning the *aln* mutation, we generated microsatellite PCR markers for mutants bred on *M. castaneus* and DBA backgrounds. Approximately 130-bp, 140-bp and 150-bp bands were amplified from *M. castaneus*, A/J and DBA alleles, respectively, using primers Scn9aF and Scn9aR. Other microsatellite PCR markers were generated for mutants bred on an FVB background. Primers 65MbDF and 65MbDR amplified approximately 205-bp and 230-bp products in FVB and A/J strains, respectively. Once the mutation was identified, we generated diagnostic primers alndiagF and alndiagR, which produce an amplicon of 109 bp. PCR amplifications were followed by *Ava*II digestion, which cleaves the *aln* allele into 90-bp and 19-bp products. All amplification products were separated on a 4% MetaPhor agarose gel (Cambrex). Primer sequences are provided in Supplementary Table 1 online.

Genetic analysis

Mice heterozygous for *aln* were crossed with mice heterozygous for *Shh*¹⁹, *Smo*²¹ or *Gli2* (ref. 22). Double-heterozygous mice were identified and intercrossed. Intercross progeny were examined and genotyped at E18.5 (for *aln* × *Shh* crosses) or E10.5 (for *aln* × *Smo* and *aln* × *Gli2* crosses). *Shh* alleles were genotyped using primers ShhwtB5, ShhKOB5 and ShhC3 (Supplementary Table 1). *Smo* and *Gli2* alleles were genotyped using a modified protocol²¹ and a previously described protocol²², respectively.

Immunofluorescence on spinal cord sections and *in situ* hybridization

Immunofluorescence and whole-mount *in situ* hybridization were done using established protocols. For immunofluorescence, E9.5 and E10.5 embryos were fixed in 4% paraformaldehyde for 20 min and 30 min, respectively, at 4 °C, infused in 30% sucrose at 4 °C for 7 d and embedded in OCT compound (Tissue Tek). 10-μm cryosections of E9.5 and E10.5 lumbar spinal cords were incubated with primary antibodies obtained from the Developmental Studies Hybridoma Bank and Cy3-tagged antibody to mouse (Jackson Immuno-Research). For whole-mount *in situ* hybridization, E9.5 and E10.5 embryos were fixed in 4% paraformaldehyde overnight. Riboprobes were transcribed from plasmids carrying cDNAs of *Ptch1* and *Olig2* (ref. 42). Riboprobes for *Ttc21b* and *Rab23* corresponded to the first 800 bp of coding sequences. The *Ttc21a* riboprobe consisted of a 1.4-kb fragment comprising the last 800 bp of coding sequence and 600 bp of 3'-UTR sequence. The *Gli3* riboprobe corresponded to a 705-bp region of exon 15 (Supplementary Table 1). All cDNAs were amplified, TA-cloned into pCR2.1 vector (Invitrogen) and sequence-verified. After hybridization with a riboprobe, embryos were placed into the BioLane HT1 *in situ* hybridization machine for all remaining steps of the procedure. After whole-mount *in situ* hybridization, embryos were fixed in 10% formalin at 4 °C for several days, rinsed in PBS, infused in 30% sucrose at 4 °C overnight, embedded in OCT compound and cryosectioned at 20 μm thickness.

Generation of antibody to THM1

We generated an antibody to the last 80 amino acids of mouse THM1. *Ttc21b* DNA sequence was PCR-amplified and ligated into the pET-30c expression vector. The peptide was induced and expressed in BL21 cells, affinity-purified and sent to ProSci for polyclonal antibody production. THM1 antiserum was affinity-purified before use.

Western blot analysis

Western blots were done using established protocols⁴³. For the GLI3 western blot, E10.5 limb buds were bisected into anterior and posterior regions as described³. Anterior and posterior regions were pooled from at least three mice for protein extraction. Protein (100 µg) was loaded on a 7% acrylamide gel, and the western blot was performed using antiserum to GLI3 as described³. DM1A (Sigma), a mouse monoclonal antibody to α -tubulin, was used for all loading controls.

Scanning electron microscopy analysis

E11.5 embryos were dissected in PBS, fixed for 1 h in 2.5% glutaraldehyde, washed three times for 15 min each in 0.1 M sodium cacodylate buffer, postfixed in osmium tetroxide for 1 h and rinsed three times for 5 min each in 0.1 M sodium cacodylate buffer. Embryos were then dehydrated in an ethanol series, dried in a Samdri PVT-3B Critical Point Dryer (Tousimis), mounted onto metal stubs and coated in the Hummer V Sputter Coater (Anatech). Primary cilia of limb ectoderm were visualized using a Leo 1450VP scanning electron microscope (Carl Zeiss SMT).

Primary MEF and limb cell cultures and immunostaining of cilia

MEFs and limb cells were generated from E11.5 mice. To isolate MEFs, embryos were eviscerated, and limbs and heads were removed. Eviscerated bodies and pooled limbs were trypsinized (0.25%) for 10–15 min at room temperature before addition of 10% FBS to stop the reaction. Primary cells were centrifuged at 2,000 rpm for 3 min, resuspended and cultured in medium containing 20% FBS. Infection of cells and localization of GLI-GFP fusion proteins were done as described⁷. Whole-mount immunofluorescence on E8.0 embryos was done as described¹² using primary antibodies to SMO¹² (LifeSpan Biosciences), acetylated α -tubulin (Sigma) and THM1 and secondary antibodies tagged with Alexa Fluor 488 and Alexa Fluor 594 (Invitrogen).

Generation of IFT88-EYFP expressing cell line

Mouse IMCD cells were infected by vesicular stomatitis virus-G-pseudotyped murine Moloney leukemia retrovirus constructed from a modified pBABE vector⁴⁴ with a blasticidin resistance cassette and harboring a C-terminal-tagged IFT88-EYFP fusion cDNA. After infection, colonies were isolated after selection in 2 µg/ml of blasticidin and expanded to create monoclonal cell lines.

Live-cell imaging

m368-2 cells (clonal line of mouse IMCD cells expressing IFT88-EYFP) were cultured on 18-mm poly-L-lysine-coated coverslips in DMEM with 10% FBS to ~100% confluency.

Cells were examined for IFT88 movement 3–4 d later on a Nikon TE2000 E2 inverted microscope system heated with an enclosed chamber (In Vivo Scientific). Time-lapse images were captured using a PlanApo 60× NA 1.4 oil objective, and images were captured by IPLab software to a cooled charge-coupled device camera (COOLSNAP-HQ, Roper Instruments). Before being placed on the microscope, cells on cover slips were rinsed in 1× PBS, then immersed in live-cell medium (Leibovitz's L-15 medium; phenol red free; 10% FBS, 2 mM L-glutamine, 1 mM penicillin and streptomycin, 1 mM sodium pyruvate and MEM nonessential amino acids). Cover slips were then placed face down on a 35 × 50 mm microscope cover glass (Fisher Scientific) and sealed with vacuum grease (Dow Corning). Cells were kept at 37 °C during analysis. For a selected field, 50–100 images were taken at 0.75-s intervals with exposure time of 400 ms at 2 × 2 binning. To determine the velocities of IFT88-EYFP particles, image sequences were analyzed and assembled into kymographs using the ImageJ software package and analyzed blind to the specific experimental condition. For each cell line, 7–11 cilia and three to five IFT88-EYFP particles for each cilium were analyzed.

RNA interference

The 5'-phosphorylated oligonucleotide Thm1 R1 (Supplementary Table 1), corresponding to the 5' end of the *Ttc21b* transcript, and the 5'-phosphorylated complementary strand were annealed and cloned into the AgeI/EcoRI sites of the pLKO.1 lentiviral vector, generating plasmid pLKO.1-R1. Viruses were made by transfecting 293T cells with plasmids VSVG, delta8.2 and pLKO.1-R1 (pLKO.1 served as the empty control vector) for 48 h using FuGENE (Roche). After transfection, medium containing the lentiviruses was collected and filtered (0.45 µm pore size). Target m368-2 cells were then infected with lentiviruses expressing either empty control vector or shRNA R1 using Polybrene. Upon confluency, cells were trypsinized and selected by replating in medium containing puromycin. To create clonal cell lines, confluent cells were plated at very low densities; single colonies were isolated using sterile cloning disks (Fisher) and expanded.

Statistical methods

Student's *t*-tests were used to measure differences between control and mutant samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. *Ttc21b* is mutated in *aln*-mutant mice. (a) An A→C mutation that converts a glutamine to a proline at residue 15 of THM1 was identified in the *aln* mutant. (b) Western blot analysis of THM1 on E10.5 wild-type (WT), heterozygous (het) and *aln*-mutant head extracts revealed an absence of THM1 protein in *aln* mutant extracts. (c) THM1 sequence encompassing the *aln* mutation is highly conserved across species. In the *C. elegans* and *C. reinhardtii* orthologs, the substitution of glutamine by arginine is predicted to be a structurally conservative amino acid substitution⁴⁵. The start methionine is shown for *Mus musculus*, *Bos taurus*, *Homo sapiens* and *Gallus gallus* orthologs. For the other species listed, the presumptive first exon is absent from the current annotations. Asterisks denote site of *aln* mutation.

Figure 2.

SHH signaling targets are misexpressed in *aln* mutants. (a–h) Whole-mount *in situ* hybridization for *Ptch1* on E9.5 wild-type (WT) and *aln*-mutant embryos. In *aln* mutants, *Ptch1* was expressed at greater intensities in the region of the maxilla and first branchial arches (brackets) and in the somites (arrowheads). *Ptch1* was also expressed ectopically in the anterior region of the *aln*-mutant limb (arrows). (g–p) Analysis of dorsoventral patterning of the *aln*-mutant caudal neural tube at E9.5 using *in situ* hybridization (g,h) and immunofluorescence (i–p). Normal expression patterns were observed in wild-type mice. In contrast, the expression domains of ventral markers FOXA2, NKX2.2, NKX6.1 and *Olig2* were expanded dorsally by the *aln* mutation, whereas dorsal marker MSX2 was shifted dorsally and confined to a smaller domain than in wild-type embryos, indicating that the *aln*-mutant neural tube is ventralized. Normal dorsoventral patterning was observed at rostral levels of the *aln*-mutant neural tube (data not shown).

Figure 3.

Genetic analyses of epistasis between mice mutant for *aln* and mice mutant for *Shh*, *Smo* or *Gli2*. (a) Frontal view of a *Shh* mutant at postnatal day 1.0 shows truncated limbs (asterisks). In contrast, limbs and certain head structures were partially restored in the *aln Shh* double mutant. (b) At E10.5, *Smo* mutants are arrested and unturned, with poor cranial development. In contrast, the *aln Smo* double-mutant embryo has partially turned and has more developed head structures. These double mutant phenotypes indicate that THM1 acts as a negative regulator downstream of both SHH and SMO. (c) At E10.5, *Gli2* mutants have well-developed telencephalic vesicles, which is not the case in *aln* mutants. However, *aln Gli2* double-mutant mice also show well-developed telencephalic vesicles, indicating that THM1 acts upstream of GLI2. Arrows point to a groove normally present caudal to the telencephalic vesicles, which is absent in *aln* mutants. (d–w) Dorsoventral patterning of the caudal spinal cord of E10.5 wild-type (WT), *aln*, *Gli2* and *aln Gli2* double-mutant embryos. Normal patterning is observed in wild-type embryos. In *aln* mutants, the neural tube is ventralized (see also Fig. 2j,l,n,p). In contrast, the *aln Gli2* double-mutant spinal cord is dorsalized, resembling that of the *Gli2* mutant; FOXA2 is absent and MNR2 is expanded to the most ventral regions of the spinal cord, whereas PAX6 and MSX2 are shifted ventrally. These analyses indicate that the ventralization of the *aln*-mutant caudal neural tube is largely mediated by GLI2, which is inappropriately activated in *aln* mutants.

Figure 4.

GLI3 protein and transcript levels are elevated in *aln* mutants. **(a)** Western blot analysis of GLI3 in extracts of anterior (A) and posterior (P) limb buds of E10.5 wild-type (WT) and *aln*-mutant mice. *aln*-mutant anterior limb buds show increased levels of GLI3A and GLI3R proteins relative to wild type. **(b)** Whole-mount *in situ* hybridization for *Gli3* on E10.5 embryos shows more intense expression in *aln* mutants, particularly in the branchial arches (brackets) and the limb (arrows). In the limb, the *Gli3* expression domain appears less graded in *aln* mutants than in wild-type mice.

Figure 5.

IFT88 is sequestered in *aln*-mutant primary cilia. **(a)** Immunofluorescence analysis of THM1 (green) and acetylated α -tubulin (red) in polarized IMCD cells and in nodal cilia of E8.0 embryos. THM1 colocalizes with acetylated α -tubulin in the ciliary axoneme and is expressed in a punctate pattern from the base to the tip of the cilium. **(b)** Immunostaining for IFT88 (green) and acetylated α -tubulin (red) on cultured primary limb cells showed much more intense staining of IFT88 at the distal ends of *aln*-mutant cilia relative to wild type. Identical exposure times were used to image wild-type and *aln*-mutant cells. In *aln* mutants, IFT88 is visualized adjacent to, but not overlapping with, γ -tubulin, which stains the base of cilia (data not shown). Axonemes stained by acetylated α -tubulin also appeared shorter in *aln* mutants than in wild type. **(c)** Scanning electron microscopy analysis of wild-type and *aln*-mutant limb ectodermal primary cilia revealed bulb-like structures at the distal tips of *aln*-mutant cilia. Scale bar represents 1 μ m.

Figure 6.

SMO and GLI proteins translocate to *aln*-mutant cilia. **(a)** Whole-mount immunofluorescence analysis of SMO in wild-type (WT) and *aln*-mutant embryos showed colocalization of SMO (red) with acetylated α -tubulin (green) in nodal cilia. **(b)** Overexpression of GLI proteins in *aln*-mutant MEFs. *aln*-mutant MEFs were cultured in serum-free medium to promote the growth of cilia, then infected with adenoviruses expressing green fluorescent protein (GFP)-fused GLI1 and immunostained for IFT88. Full-length GLI1-GFP (green) fusion proteins co-localized with IFT88 (red), which stains the distal tips of *aln*-mutant cilia (see Fig. 5 and Supplementary Fig. 4).

Figure 7.

Retrograde IFT is impaired in cilia of THM1-deficient IMCD cells. **(a)** Western blot analysis of IFT8-EYFP in wild-type (mIMCD) and m368-2 cell extracts. IFT88 and IFT88-EYFP bands represent endogenously and exogenously expressed protein, respectively. **(b)** Western blot analysis of THM1 in EV4, R1-4, R1-2 and R1-5 cell extracts. Expression of THM1 in R1-4, R1-2 and R1-5 cell lines was about 12.5%, 11.5% and 73% of that in control EV4 cells. **(c)** Anterograde and retrograde IFT velocities (mean $\pm$ s.d.) of IFT88-EYFP in cilia of EV4, R1-4, R1-2 and R1-5 cell lines. Retrograde IFT velocities are significantly lower in R1-4 and R1-2 cells (*, $P < 0.002$) than in EV4 and R1-5 cells. **(d)** Live-cell images of EV4 and R1-4 cell lines (top and bottom left panels) revealed punctate distribution of IFT88-EYFP from the base to the tip of cilia. R1-4 cell line showed a much higher accumulation of IFT88 at the tip of the cilium (white arrows, top panels). White arrows on bottom left panels indicate tips of cilia. Broken and solid black lines on both EV4 and R1-4 kymographs represent anterograde and retrograde IFT, respectively (bottom right panels). Scale bars, 5 μ m.