HHS Public Access

Author manuscript

Dev Biol. Author manuscript; available in PMC 2018 October 01.

Published in final edited form as:

Dev Biol. 2017 October 01; 430(1): 156–165. doi:10.1016/j.ydbio.2017.08.003.

Three Tctn proteins are functionally conserved in the regulation of neural tube patterning and Gli3 processing but not ciliogenesis and Hedgehog signaling in the mouse

Chengbing Wang[1,†], **Jia Li**[3,†], **Qing Meng**[3,*], and **Baolin Wang**[1,2,*]

[1]Department of Genetic Medicine, Weill Medical College of Cornell University, 1300 York Avenue, W404, New York, NY 10065, USA

[2]Department of Cell and Developmental Biology Weill Medical College of Cornell University, 1300 York Avenue, W404, New York, NY 10065, USA

[3]Institute of Biological Sciences and Biotechnology, Donghua University, Shanghai 201620, China

Abstract

Tctn1, Tctn2, and Tctn3 are membrane proteins that localize at the transition zone of primary cilia. *Tctn1* and *Tctn2* mutations have been reported in both humans and mice, but *Tctn3* mutations have been reported only in humans. It is also not clear whether the three Tctn proteins are functionally conserved with respect to ciliogenesis and Hedgehog (Hh) signaling. In the present study, we report that loss of *Tctn3* gene function in mice results in a decrease in ciliogenesis and Hh signaling. Consistent with this, *Tctn3* mutant mice exhibit holoprosencephaly and randomized heart looping and lack the floor plate in the neural tube, the phenotypes similar to those of *Tctn1* and *Tctn2* mutants. We also show that overexpression of Tctn3, but not Tctn1 or Tctn2, can rescue ciliogenesis in *Tctn3* mutant cells. Similarly, replacement of *Tctn3* with *Tctn1* or *Tctn2* in the *Tctn3* gene locus results in reduced ciliogenesis and Hh signaling, holoprosencephaly, and randomized heart looping. Surprisingly, however, the neural tube patterning and the proteolytic processing of Gli3 (a transcription regulator for Hh signaling) into a repressor, both of which are usually impaired in ciliary gene mutants, are normal. These results suggest that Tctn1, Tctn2, and Tctn3 are functionally divergent with respect to their role in ciliogenesis and Hh signaling but conserved in neural tube patterning and Gli3 processing.

Keywords

Tctn1; Tctn2; Tctn3; Hedgehog; Gli2; Gli3; cilia

[*]Corresponding Author: Baolin Wang, 1300 York Avenue, W404, New York, NY 10065, Phone: 212-746-5357, Fax: 212-746-8318, baw2001@med.cornell.edu, Qing Meng, mengqing@dhu.edu.cn.

[†]These authors contributed equally.

1. Introduction

Primary cilium is a microtubule-based structure that protrudes from the cell surface. It is found in almost all cell types and tissues in vertebrates with one per cell. Ciliogenesis begins with the docking of the mother centriole (also known as basal body) to a vesicle (Sorokin, 1962). Following the docking, the basal body extends an axoneme that invaginates the vesicle. The fusion between the vesicle and plasma membrane exposes the cilia axoneme to extracellular environment. The cilia assembly requires vesicle trafficking that brings lipids and membrane proteins to the cilia membrane.

Once transported to basal body, the cilia-bound transmembrane proteins must cross the transition zone, a small compartment between the basal body and the ciliary axoneme. The transition zone is believed to serve as a gatekeeper to selectively allow only ciliary proteins to enter the cilia, thus controlling the protein composition in cilia (Garcia-Gonzalo and Reiter, 2017). How ciliary proteins are selectively transported into cilia remains poorly understood. Nevertheless, the integrity of the protein complexes localizing to this compartment appears to be essential, as several recent studies show that loss of each single protein component within a protein complex all results in defects in membrane protein composition in cilia (Chih et al., 2011; Garcia-Gonzalo et al., 2011; Roberson et al., 2015; Sang et al., 2011).

Ciliary dysfunction underlies a broad spectrum of human diseases, collectively termed ciliopathies (Gerdes et al., 2009). They include Joubert syndrome (JBTS), Meckel syndrome (MKS), nephronophthisis (NPHP), and Bardet-Biedl syndrome (BBS), etc. Some of the manifestations among these different syndromes are overlapping, suggesting that they may affect signaling pathways that control the same developmental process.

Defective ciliary structure and function impact several signaling pathways, one of which is Hedgehog (Hh) signaling (Goetz and Anderson, 2010). Consistent with this, Patched 1 (Ptch1), a twelve-pass membrane receptor for Hh, is localized to cilia in the absence of Hh signaling. In contrast, Hh signaling leads to the exit of Ptch1 from cilia and subsequently the accumulation of Smoothened (Smo)(Corbit et al., 2005; Rohatgi et al., 2007), a seven-transmembrane G-protein coupled receptor (GPCR) that transduces Hh signals. Similarly, Gli2 and Gli3 transcription regulators that mediate Hh signaling also accumulate in cilia (Chen et al., 2009; Haycraft et al., 2005; Wen et al., 2010). So far, all known ciliary gene mutations disrupt Hh signaling between Smo and Gli2/Gli3, and the vast majority of the mutations reduce Gli3 processing that generates a C-terminally truncated transcriptional repressor.

The family of Tectonic (Tctn) proteins consists of one membrane-bound (Tctn1) and two single-pass membrane proteins (Tctn2 and Tctn3) (Reiter and Skarnes, 2006). Tctn proteins localize to the transition zone of the cilia and form a large complex with several known ciliopathy-associated proteins (Chih et al., 2011; Garcia-Gonzalo et al., 2011; Sang et al., 2011). *Tctn1* and *Tctn2* mouse mutants have been previously reported (Reiter and Skarnes, 2006; Sang et al., 2011). Mutations in each of the three human *Tctn* genes also result in defects in ciliary biogenesis and function (Alazami et al., 2012; Shaheen et al., 2011;

Thomas et al., 2012). As a consequence, different *Tctn* mutants exhibit diverse phenotypes, such as loss of the floor plate in the neural tube, defect in eye development, polydactyly, and lack of left-right asymmetry. Most of these phenotypes are due to consequent dysfunction of Hh signaling. Given that Tctn1, Tctn2, and Tctn3 share high levels of sequence identity, an interesting question is whether they are functionally conserved with regard to ciliogenesis and Hh signaling.

In the present study, we show that ablation of the mouse *Tctn3* gene results in loss of the floor plate in the neural tube, holoprosencephaly, polydactyly, and randomized heart looping, the phenotypes similar to *Tctn1* and *Tctn2* mutants (Reiter and Skarnes, 2006; Sang et al., 2011). In *Tctn3* mutant embryos, both cilia number and Gli3 processing are reduced. Overexpression of Tctn3, but not Tctn1 or Tctn2, rescues ciliogenesis in *Tctn3* mutant cells. Similarly, substitution of Tctn1 or Tctn2 for Tctn3 in mice results in reduced ciliogenesis and Hh signaling, holoprosencephaly, and randomized heart looping. Surprising, however, neural tube patterning and Gli3 processing are not affected. These results suggest that Tctn proteins are functionally divergent with respect to their role in ciliogenesis and Hh signaling but conserved in neural tube patterning and Gli3 processing.

2. Results

2.1. Ablation of the mouse Tctn3 gene results in defect in Hh-dependent embryonic development

To investigate whether loss of *Tctn3* gene function in the mouse affects Hh signaling, we generated a *Tctn3* conditional knockout allele and subsequently a *Tctn3* mutant allele by deleting exon 3 of the gene (Fig. 1A and B). The deletion of this exon is expected to cause a reading frame shift, even if exon 2 were spliced to any one of exons 4–11, thus likely inactivating most, if not all, of the gene function. Mouse embryos homozygous for the *Tctn3* mutation died between embryonic day 14.5 (E14.5) and E16.5 (n = 68). Occasionally, pups were born but died within one day (P0). *Tctn3* mutant embryos exhibited holoprosencephaly as evidenced by incomplete separation of prosencephalon (Fig. 1C and D), a defect associated with reduced Hh signaling. On the other hand, *Tctn3* mutant embryos also displayed polydactyly (Fig. 1E), a defect often associated with reduced Gli3 repressor levels. Given that the Hh pathway activation is dependent on Gli2 and Gli3 transcriptional activators, these observations suggest that *Tctn3* mutation affects both Gli2/Gli3 activator and repressor function. In addition, some of the mutants developed with hearts that turn in a rightward (n = 8/28) rather than leftward orientation normally seen in wild type embryos (n = 37/37) (Fig. 1C). Furthermore, *Tctn3* mutant embryos exhibited edema in the back of upper body (Fig. 1E). These defects are typically associated with defective ciliogenesis.

To further determine whether *Tctn3* mutation affects Hh signaling, we examined the neural tube patterning along the anterior and posterior of the mutant embryos. Foxa2 expression in the floor plate was normally induced by a high level of Sonic Hedgehog (Shh) (Briscoe et al., 2000), but missing in the *Tctn3* mutant. Nkx2.2 positive V3 progenitors were specified, but its expression domain shifted ventrally due to the loss of the floor plate. However, the expression and patterning of Hb9, a motoneuron marker, appeared to be normal in the mutant. Similarly, the expression of Pax6 and Pax7 dorsal markers, which is ventrally

restricted by low Shh signaling, was also unaffected (Fig. 1F). Thus, a high level of Hh signaling is compromised in *Tctn3* mutant. In support of this conclusion, RT-qPCR (reverse transcription-quantitative polymerization chain reaction) results showed that the expression of *Gli1* and *Ptch1* RNA, which is directly regulated by Hh signaling, was significantly reduced in *Tctn3* mutant as compared to wild type (Fig. 1G).

To understand the molecular basis underlying the impaired Hh signaling in *Tctn3* mutant, the extent of Gli3 processing was compared between wild type and the mutant. Immunoblotting with a Gli3 N-terminal antibody, which recognizes both $Gli3^{FL}$ and $Gli3^{Rep}$, showed that Gli3 processing was clearly reduced in the mutant as compared to wild type. Similarly, $Gli2^{FL}$ levels in the mutant were also higher than those in wild type (Fig. 1H). Since loss of the floor plate is associated with reduced $Gli2^{FL}$/$Gli3^{FL}$ activators, yet both $Gli2^{FL}$ and $Gli3^{FL}$ levels are actually increased, and $Gli3^{Rep}$ levels are reduced in *Tctn3* mutant, these data indicate that *Tctn3* mutation results in reduced Gli2/Gli3 activator and repressor function.

2.2. Tctn3 mutation results in defective ciliogenesis

To investigate how *Tctn3* mutation affects Hh signaling in embryonic development, we first examined Tctn3 subcellular localization. Wild type mouse embryonic fibroblasts (MEFs) were immunostained for Tctn3, acetylated tubulin (labeling cilia axoneme), and gamma tubulin (a marker for basal body). The result showed that Tctn3 protein localized between cilia axoneme and basal body. The staining is specific, as no staining was detectable in *Tctn3* mutant MEFs (Fig. 2A). This observation is consistent with the previous studies showing that the Tctn protein complex localizes at the transition zone of cilia (Garcia-Gonzalo et al., 2011).

We next assessed ciliogenesis in *Tctn3* mutant embryos. Immunostaining of embryonic sections for Arl13b and acetylated tubulin, two cilia markers, showed that cilia number was significantly reduced in all the tissues examined, including neural epithelia in the neural tube and mesencephalic vesicle, perineural tube area, and notochord in the mutant as compared to those in wild type. Similarly, cilia number in the mutant MEFs was also markedly lower than that in wild type cells (Fig. 2B and C). Thus, as *Tctn1* and *Tctn2* mutation, *Tctn3* mutation disrupts ciliogenesis and Hh signaling.

2.3. Tctn3 is required for the trafficking of ciliary membrane proteins into cilia but not the recruitment of appendage proteins and IFTs

The transition zone of cilia serves as a gatekeeper for the entry of ciliary proteins into cilia. Consistent with this, a previous study showed that Tctn1 and Tctn2 are required for the membrane protein composition in cilia (Garcia-Gonzalo et al., 2011). As in *Tctn1* and *Tctn2* mutant cells, cilia number in *Tctn3* mutant cells was significantly lower than that in wild type (Fig. 2B and C). To determine whether *Tctn3* mutation affects the membrane protein composition in cilia, we compared ciliary localization of three known membrane proteins in the mutant cells to that in wild type cells. Immunostaining showed that adenylyl cyclase type 3 (AC3) was not detectable in the cilia of *Tctn3* mutant MEFs, though it was readily detected in the cilia of wild type cells. Similarly Smo was barely detectable in the cilia of

Tctn3 mutant MEFs that were treated with ShhN, while it was markedly enriched in cilia of wild type cells. In addition, although Arl13b was still detectable in the mutant cilia, its levels were significantly reduced (Fig. 3A). Thus, like Tctn1 and Tctn2, Tctn3 is also required for the trafficking or efficient trafficking of ciliary membrane proteins into cilia.

We were also curious about the question whether *Tctn3* mutation affects the basal body integrity and the recruitment of IFT proteins to the basal body. To this end, wild type and *Tctn3* mutant cells were stained for three basal body appendage proteins (Cep164, Odf2, and Ninein) (Graser et al., 2007; Lange and Gull, 1995; Mogensen et al., 2000; Nakagawa et al., 2001) and IFT88 and IFT140 (subunits for IFT-B and IFT-A complexes, respectively) (Pedersen and Rosenbaum, 2008). None of these markers' localization to the basal body was affected in the mutant cells (Fig. 3B), suggesting that Tctn3 is not essential for the assembly of basal body appendages and recruitment of IFT complexes.

2.4. Tctn proteins interact with one another and some known transition-zone-localized proteins

Previous studies showed that three Tctn proteins interact with one another and some of the known proteins localizing at the transition zone (Chih et al., 2011; Garcia-Gonzalo et al., 2011; Roberson et al., 2015; Sang et al., 2011). To confirm those studies, we performed coimmunoprecipitation using HEK293 cells overexpressing various transition-zone-localized proteins. The results showed that GFP-tagged Tctn1 interacted with Tctn2 and Tctn3 and that Tctn proteins also interacted with Tmem231, Mks1, and B9d2, three known transition-zone-localized proteins (Fig. 4). It should be noted that Mks1-GFP was coimmunoprecipitated with Tctn1-FH or Tctn2-FH, but not Tctn3-FH (Fig.4, third panel from the left), suggesting that Mks1 does not directly interact with Tctn3. Our results are in agreement with the previous reports.

2.5. Overexpression of Tctn3, but not Tctn1 or Tctn2, rescues ciliogenesis in Tctn3 mutant cells

Tctn1, Tctn2, and Tctn3 share significant levels of sequence identity (Reiter and Skarnes, 2006). This raises an interesting question: whether they are functionally conserved with regard to ciliogenesis. To answer this question, *Tctn3* mutant MEFs were transduced with murine retrovirus carrying Tctn1-FH, Tctn2-FH, or Tctn3-FH expression constructs (FH, FLAG and HA double tags). After serum starved, the cells were then immunostained for acetylated tubulin. Quantitative analysis showed that overexpression of Tctn3-FH, but not Tctn1-FH or Tctn2-FH, significantly increased cilia number from average about 10% to more than 30% (Fig. 5A–F). Since Western blot showed that the three proteins were expressed at comparable levels (Fig. 5G), the failed rescue of ciliogenesis by Tctn1 and Tctn2 overexpression was due to the difference in protein function between Tctn1 or Tctn2 and Tctn3. Thus, Tctn1, Tctn2, and Tctn3 are not functionally conserved with regard to their role in ciliogenesis.

2.6. Substitution of Tctn1 or Tctn2 for Tctn3 results in defects in ciliogenesis and Hh signaling but not neural tube patterning and Gli3 processing in the mouse

To determine whether Tctn1, Tctn2, and Tctn3 are functionally conserved *in vivo*, we asked whether Tctn1 or Tctn2 could functionally replace Tctn3. To this end, FH-tagged Tctn1 or Tctn2 cDNA was inserted immediately after the initiation codon of Tctn3 in the *Tctn3* locus using targeted gene homologous recombination approach. The resulting knockin alleles are designated as $Tctn3^{1ki}$ and $Tctn3^{2ki}$, respectively (Fig. 6A–B). To assess whether the knockin alleles expressed Tctn1-FH and Tctn2-FH proteins, the protein lysates prepared from E10.5 mouse embryos were subjected to immunoblotting with FLAG antibody. As expected, both Tcnt1-FH and Tctn2-FH were expressed (Fig. 5C).

Mice heterozygous for either $Tctn3^{1ki}$ or $Tctn3^{2ki}$ were normal. Crosses between heterozygotes, i.e. $Tctn3^{+/1ki}\times Tctn3^{+/1ki}$ or $Tctn3^{+/2ki}\times Tctn3^{+/2ki}$, did not produce any $Tctn3^{1ki/1ki}$ or $Tctn3^{2ki/2ki}$ pups (n=84 and 82, respectively), indicating that the knockin mice died during gestation. To investigate when the knockin mouse embryos died, embryos at different gestation stages were dissected and genotyped. At E13.5, neither $Tctn3^{1ki/1ki}$ nor $Tctn3^{2ki/2ki}$ live embryos were recovered (n = 43 and 45). However, at E10.5, live mutant embryos were about one quarter of the total embryos collected (72/309 and 23/105). Therefore, $Tctn3^{1ki}$ and $Tctn3^{2ki}$ homozygous embryos die at E11.5 and/or E12.5, which is earlier than E14.5–16.5 at which *Tctn3* mutants die.

Mouse embryos homozygous for $Tctn3^{1ki}$ or $Tctn3^{2ki}$ exhibited holoprosencephaly. Some of them (17/41 and 6/9, respectively) developed with hearts that looped in the rightward direction, opposite of normal heart looping (Fig. 6D). This suggests a defect in the patterning of left-right asymmetry.

To determine whether substitution of Tctn1 or Tctn2 for Tctn3 in mice impairs Hh signaling, we next examined the neural tube patterning of the knockin embryos. Of the ventral markers examined that are normally specified by Shh (Briscoe et al., 2000), Foxa2, Nkx2.2, Hb9, and Isl1, which mark the floor plate, V3 interneuron, and motoneuron, respectively, all appeared to be normally specified and patterned along the anterior and posterior regions of the neural tube. Consistent with this, as in wild type, dorsal marker Pax6 expression remained ventrally restricted in the neural tube of the knockin embryos (Fig. 6E, data not shown). Thus, the neural tube patterning is unaffected in the $Tctn3^{1ki/1ki}$ and $Tctn3^{2ki/2ki}$ mice.

One of the hallmarks of ciliary gene mutants is an increase in $Gli2^{FL}/Gli3^{FL}$ levels and a decrease in $Gli3^{Rep}$ levels (Goetz and Anderson, 2010). To determine whether this is the case in $Tctn3^{1ki/1ki}$ and $Tctn3^{2ki/2ki}$ embryos, immunoblotting was performed using protein lysates prepared from wild type and the knockin E10.5 mouse embryos. The results showed that the levels of $Gli2^{FL}$, $Gli3^{FL}$, and $Gli3^{Rep}$ were comparable in $Tctn3^{1ki/1ki}$, $Tctn3^{2ki/2ki}$, and wild type embryos (Fig. 6F). Thus, $Gli2^{FL}/Gli3^{FL}$ protein stability and processing were also unaffected in the knockin mice.

To more directly determine whether Hh signaling is impacted in $Tctn3^{1ki/1ki}$ and $Tctn3^{2ki/2ki}$ mouse embryos, the expression levels of Gli1 and Ptch1, two direct targets of Hh signaling, were examined by real time PCR. The results showed that *Gli1* and *Ptch1* RNA expression

levels in the knockin mouse embryos were significantly lower than those in wild type (Fig. 7A). Thus, Hh signaling is compromised in the knockin mice.

Given that Tctn proteins are required for cilia biogenesis and function, we then wanted to know whether cilia formed normally in *Tctn3*$^{1ki/1ki}$ and *Tctn3*$^{2ki/2ki}$ embryos. The neural tube sections of wild type and the knockin embryos were immunostained for both Arl13b and acetylated tubulin. The results showed that cilia density in *Tctn3*$^{1ki/1ki}$ and *Tctn3*$^{2ki/2ki}$ neural tube was significantly lower than that in wild type neural tube (Fig. 7B), indicating that ciliogenesis is defective in the knockin embryos. Similarly, the percentage of ciliated cells in *Tctn3*$^{1ki/1ki}$ and *Tctn3*$^{2ki/2ki}$ primary MEFs (pMEFs) was significantly lower than that in wild type pMEFs. Therefore, Tctn1 or Tctn2 was unable to functionally replace Tctn3 in ciliogenesis *in vivo*.

3. Discussion

In the present study, we show that like *Tctn1* or *Tctn2* mutations, *Tctn3* mutation results in defects in Hh signaling and ciliogenesis in the mouse. We also demonstrate that Tctn1, Tctn2, and Tctn3 are functionally divergent with respect to their role in ciliogenesis and Hh signaling but conserved in neural tube patterning and Gli3 processing.

The family of Tctn proteins consists of three membrane proteins that were recently discovered to be associated with ciliogenesis. Tctn1 is a membrane-bound protein without a transmembrane domain, while Tctn2 and Tctn3 are single-pass membrane proteins (Reiter and Skarnes, 2006). Tctn proteins predominantly localize at the transition zone of cilia. They interact with one another and form a protein complex with several known transition-zone-localized proteins, including Cc2d2a, B9d1, B9d2, Tmem216, Tmem231, Tmem67, Mks1 (Chih et al., 2011; Garcia-Gonzalo et al., 2011; Sang et al., 2011). Consistent with this, loss of function of any one of these proteins result in very similar phenotypes. Cilia number in either *Tctn1* or *Tctn2* mutants is significantly lower than that in wild type. Both *Tctn1* and *Tctn2* mutants exhibit holoprosencephaly, randomized heart looping, polydactyly, and failed specification of the floor plate and V3 interneuron in the developing neural tube. *Tctn1* and *Tctn2* mutations also result in a reduced Gli3 processing. Consistent with the idea that Tctn1 and Tctn2 serve as a gatekeeper at the transition zone for ciliary membrane proteins, several ciliary membranes proteins are either excluded from cilia or partially blocked to enter cilia in Tctn1 and Tctn2 mutant cells. In the present study, we show that *Tctn3* mutant mostly displays phenotypes similar to those of *Tctn1* and *Tctn2* mutants. This further confirms that Tctn proteins function as a protein complex at the transition zone. Since holoprosencephaly and failed specification of the floor plate and V3 interneuron are usually associated with reduced Gli2/Gli3 activator activities, while polydactyly is the result of reduced Gli3 repressor level, loss of Tctn1, Tctn2, or Tctn3 affects both Gli2/Gli3 activator and repressor function.

It is worth noting that unlike *Tctn1* and *Tctn2* mutations (Reiter and Skarnes, 2006; Sang et al., 2011), *Tctn3* mutation does not affect Nkx2.2 positive V3 interneuron specification (Fig. 1F). This difference may be due to the mouse genetic background used in this and previous studies. It may also indicate a slightly different role of Tctn3 in ciliogenesis and Hh

signaling. In addition, it is theoretically possible that the *Tctn3* mutant allele may still retain residual function. However, we think that this is unlikely, given that the deletion of exon 3 in the *Tctn3* gene is expected to cause a reading frame shift, even if exon 2 were spliced to any one of exons 4–11. In this case, the resulting mutant protein would retain signal peptide but lack most of sequence including the transmembrane domain. Thus, if any, it would be secreted rather than integrated in plasma membrane.

Three Tctn proteins share a high level of sequence identity. This raises an interesting question of whether they are functionally conserved. In the present study, we show that overexpression of Tctn3, but not Tctn1 or Tctn2, restores ciliogenesis in *Tctn3* mutant cells (Fig. 5). We also find that expression of Tctn1 or Tctn2 in the *Tctn3* gene locus (*Tctn3*$^{1ki/1ki}$ and *Tctn3*$^{2ki/2ki}$), which simultaneously inactivates *Tctn3*, significantly reduces cilia number in the mouse (Fig. 7B, C). Similarly, Hh signaling is also somewhat impaired in the knockin mice, as evidenced by reduced *Gli1* and *Ptcth1* RNA expression (Figs. 7A). Thus, three Tctn proteins are functionally divergent with respect to their role in ciliogenesis and Hh signaling. It should be mentioned that targeted gene knockin approach potentially has a caveat, though it is probably so far the best method to test if two proteins are functionally conserved *in vivo*. Given that Tctn1 or Tctn2 cDNA, but not genomic sequence, was inserted into the Tctn3 locus, the expression levels of Tctn1 or Tctn2 might not fully recapitulate those of Tctn3.

Given that both cilia number and Hh signaling are reduced in *Tctn3*$^{1ki/1ki}$ and *Tctn3*$^{2ki/2ki}$ embryos, it is not surprising that the knockin mice display holoprosencephaly and randomized heart looping (Fig. 6D), which are also seen in *Tctn3* mutant (Fig. 1C, D). However, it is unexpected that *Tctn3*$^{1ki/1ki}$ and *Tctn3*$^{2ki/2ki}$ mice exhibit some phenotypes distinct from *Tctn3* mutant. First, the knockin mouse embryos die at E11.5–12.5, which is markedly earlier than *Tctn3* mutant embryos at E14.5–16.5. Second, all ventral neuronal markers are specified normally in *Tctn3*$^{1ki/1ki}$ and *Tctn3*$^{2ki/2ki}$ developing neural tube (Fig. 6E), while the floor plate is missing in *Tctn3* mutant (Fig. 1F). Third, consistent with this, Gli2^{FL}, Gli3^{FL}, and Gli3^{Rep} levels in the knockins, but not in *Tctn3* mutant, are comparable with those in wild type mice (Figs. 1F, 6F). Given that the neural tube patterning is largely dependent on Gli2^{FL}/Gli3^{FL} activators (Bai et al., 2004; Matise et al., 1998) and that Gli3^{Rep} level is not reduced in the knockin mice, together, these results suggest that Gli2/Gli3 activator function is not noticeably affected in the neural tube of the knockin embryos. This seems to contradict with the observation that Hh signaling is reduced in the knockin embryos, as evidenced by a decrease in *Gli1* and *Ptch1* RNA expression (Fig. 7A). One possible explanation for the difference is that Gli2/Gli3 activator function in the neural tube is actually reduced, but the reduction is too small to affect the neural tube patterning. The other is that Tctn1 and Tctn2 expression in *Tctn3* locus may affect Gli2/Gli3 activator function differently in neural tube and other tissues. Given that ciliogenesis is reduced in the neural tube of the knockin mouse embryos (Fig. 7B) and that so far all known cilia gene mutations impair Hh signaling, the first possibility is more likely. Nevertheless, the difference in neural tube patterning and Gli3 processing between *Tctn3* mutant and *Tctn3*$^{1ki/2ki}$ mice suggests that Tctn1 and Tctn2 can rescue part of Tctn3 function in Hh signaling. On the other hand, the fact that *Tctn3*^{1ki} and *Tctn3*^{2ki} fail to restore ciliogenesis and fully Hh signaling reveals that the three Tctn proteins are also functionally divergent.

4. Materials and Methods

Mouse strains and the generation of Tctn3 mutant, Tctn3^{1ki}, and Tctn3^{2ki} mice

A BAC clone containing mouse *Tctn3* genomic DNA sequences was purchased from the BACPAC Resources Center (Oakland, CA, USA) and used to create *Tctn3* conditional, *Tctn3*^{1ki}, or *Tctn3*^{2ki} targeting constructs. The *Tctn3* targeting construct was engineered by inserting the Frt-pGKneo-Frt-loxP cassette into the intron between exons 2 and 3 and another loxP site right after exon3 (Fig. 1A). The *Tctn3*^{1ki} and *Tctn3*^{2ki} targeting constructs were generated by inserting Tctn1-FH-pA or Tctn2-FH-pA (FH, FLAG and HA double tags; pA, polyadenylation addition sequence) into the first ATG in the first exon of the *Tctn3* locus and a loxP-pGKneo-loxP cassette into the intron after exon 3 (Fig. 6A). The linearized constructs were electroporated into W4 ES cells. The targeted *Tctn3* mutant ES cell clones were identified by digestion of ES cell genomic DNA with EcoRI (5' homologous arm) or HindIII (3' homologous arm), followed by a Southern blot analysis using two probes as indicated (Fig. 1A–B). The targeted *Tctn3*$^{+/1ki}$ and *Tctn3*$^{+/2ki}$ mutant ES cell clones were determined by digestion of genomic DNA with BglII, followed by Southern blot analysis (Fig. 6B). Two for each of *Tctn3, Tctn3*^{1ki}*, Tctn3*^{2ki} targeted ES cell clones were injected into C57BL/6 blastocysts to generate chimeric founders, which were then bred with C57BL/6 to establish F1 heterozygotes. *Tctn3* mutant allele was created by breeding the *Tctn3* conditional mutant with actin-Flpe (Stock No. 005703, Jackson labs) and subsequently actin-Cre (stock No. 003376, Jackson lab) transgenic mice. Heterozygotes for each of the three mutant lines were maintained in 129/SVE, C57BL/6, and SW mixed background. PCR (polymerization chain reaction) analysis was used for routine genotyping *Tctn3* wt and mutant with the following primers: forward primer BW948F, 5'-TCCGGTGGAGGAGTAAGAGTG -3' and reverse primer BW949, 5'-TCAATGCAGATTGCCAGGCTA-3' for wt (500 bp) and mutant (250 bp). *Tctn3*^{1ki} and *Tctn3*^{2ki} were genotyped with primers: BW1218F, 5'-CGACTCTTCTTCCCAGGGCTC -3', and BW1218R2, 5'-GTCTCAGAAAGCATCAGGACCA-3' for wt (150 bp); primers: BW1219F, 5'-TTGTGTCCGTGTGAACAACTG-3', and CW649loxP-R, 5'-CGAAGTTATATTAAGGGTTCCG -3' for the mutant (270 bp). The animal work was approved by Institutional Animal Care and Use Committee at Weill Medical College of Cornell University.

Cell lines and cell Culture

Wild type and *Tctn3* mutant primary mouse embryonic fibroblasts (pMEFs) for figure 2B were prepared from E12.5 mouse embryos. Wild type and *Tctn3*$^{1ki/1ki}$ and *Tctn3*$^{2ki/2ki}$ pMEFs for figure 6C were prepared from E10.5 mouse embryos. The pMEFs and HEK293 cells were cultured in DMEM supplemented with 10% FBS (fetal bovine serum), penicillin, and streptomycin.

cDNA Constructs, Cloning, and transfection

The mouse *Tctn1, Tctn2, Tctn3* full-length cDNA clones were purchased from Open Biosystem Inc. The mouse Tmem231, Mks1, and B9d2 cDNAs were amplified by PCR from a mouse cDNA library and cloned into designated vectors. Murine retroviral pLNCX-Tctn1-FH, pLNCX-Tctn2-FH, and pLNCX-Tctn3-FH constructs were created by inserting

the cDNA fragments into pLNCX-FH retroviral vector by PCR and general cloning techniques. pLNCX-FH was engineered by inserting FH, FLAG and HA double tags, into pLNCX (Clontech). The expression constructs pRK-Tmem231-myc and pRK-B9d2-myc were created by inserting the cDNA fragments into a CMV-based pRK-myc vector. pEGFP-Tctn1 and pEGFP-Mks1 were generated by cloning the cDNA fragments into pEGFP-N1 (Clontech). The constructs were verified by DNA sequencing. Virus carrying Tctn1-FH, Tctn2-FH, and Tctn3-FH was generated by cotransfection of Phoenix-Eco cells (ATCC CRL-3214) with each of the viral constructs and a pEco packaging construct using the calcium phosphate precipitation method (Wang et al., 2013).

Reverse transcription quantitative PCR (RT-qPCR)

Total RNA was isolated from E10.5 wild type, *Tctn3*$^{-/-}$, *Tctn3*$^{1ki/1ki}$, *Tctn3*$^{2ki/2ki}$ mouse embryos by using TRIzol solution according to manufacturer's instruction (Thermo Fisher scientific). Five micrograms of each of the isolated total RNA were used to synthesize first stranded cDNA using SuperScript II reverse transcriptase according to the manufacturer's instruction (Thermo Fisher Scientific). Quantitative PCR was performed using a qPCR kit (MasterMix-R, Applied Biological Materials, Inc., Canada) and following primers: Gli1, BW1905F, 5'-CCAAGCCAACTTTATGTCAGGG -3' and BW1905R, 5'-AGCCCGCTTCTTTGTTAATTTGA -3'; Ptch1, BW1906F, 5'-AAAGAACTGCGGCAAGTTTTTG -3' and BW1906R, 5'-CTTCTCCTATCTTCTGACGGGT -3'; GAPDH (control), BW1907F, 5'-AGGTCGGTGTGAACGGATTTG -3' and BW1907R, 5'-TGTAGACCATGTAGTTGAGGTCA -3'. For a 20μl reaction, 0.15ul of the first stranded cDNA was used.

Embryo section immunofluorescence and Hematoxylin/Eozin staining

For immunofluorescence of neural tube sections, mouse embryos at 10.5 days post coitus (E10.5) were dissected, fixed in 4% paraformaldehyde (PFA)/PBS for 1 hr at 4°C, equilibrated in 30% sucrose/PBS overnight at 4°C, and embedded in OCT. The frozen embryos were transversely cryosectioned from forelimb to hind limb regions (10 μm/section). Tissue sections were immunostained with antibodies against Foxa2 (concentrated), Nkx2.2, HB9, Isl1, Pax6, and Pax7 (Developmental Study Hybridoma Bank (DSHB), Iowa) as described (Pan et al., 2009). Coronal frozen sections of E12.5 brains were stained with Hematoxylin/Eosin (Sigma) with a standard method.

Immunofluorescence and Microscopy

For cell ciliation studies, cells were plated on coverslips coated with 0.1% gelatin for at least overnight and serum starved with 0.1 % FBS for 24 hrs to arrest the cells. For detection of Smo in cilia, cells were incubated with ShhN conditioned medium (a 1:10 dilution) overnight before immunostaining. For centrosome staining, cells were fixed in −20°C cold methanol for 5 mins. For the cytoplasmic and cilia staining, cells were fixed in 4% PFA/PBS for 15 mins. After washed with PBS, the cells were incubated with blocking solution (PBS/0.2% Triton X-100/4% heat inactivated calf serum) for 20 mins. The cells were then incubated with primary antibodies in blocking solution for 1 hr at room temperature. After washed with PBS 3 times, the cells were incubated with secondary antibodies in blocking

solution for 1 hr at room temperature. After washed three times with PBS, the coverslips were mounted to glass slides with Vectashield mounting fluid with DAPI (Vector Labs). The staining was visualized using a Zeiss Axiovert fluorescent microscope.

Antibodies

Tctn3 antibody was generated by Covance. Rabbits were immunized with His-tagged mouse His-Tctn3(297-E) fragment purified from bacteria. This and other antibodies (1:1000 dilution, unless otherwise indicated) include: Gli2 N-terminal, Gli3 N-terminal (Pan et al., 2006; Wang et al., 2000), ARL13B (Caspary et al., 2007), Smo (Rohatgi et al., 2007), IFT88 and IFT140 (Pazour et al., 2002), Cep164, Odf2, Ninein (Wu et al., 2014), AC3, Myc (sc-1701, sc-789, Santa Cruz Biotechnology), acetylated tubulin, FLAG (T6793, F9291, Sigma). Secondary antibodies Alexa Fluor 488-conjugated goat anti-rabbit IgG (111—545-144) and Cy3-conjugated goat anti-mouse IgG (115-165-146) were purchased from Jackson Immunoresearch, Inc.

Immunoblot and coimmunoprecipitation

E10.5 wild type, *Tctn3*$^{-/-}$, *Tctn3*$^{1ki/1ki}$, and *Tctn3*$^{2ki/2ki}$ embryos were lysed in RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1%Triton X-100, 1% sodium deoxycholate, 0.1% SDS, protease inhibitors). The protein lysates were subjected to immunoblotting with antibodies indicated in figures. Transfected HEK293 cells used for co-immunoprecipitation were lysed in lysis buffer (50 mM HEPES (pH7.4), 150 mM NaCl, 10% glycerol, 1% NP40, protease inhibitors). Immunoblot and coimmunoprecipitation were performed as described (Wang et al., 2000).

Acknowledgments

– We thank Dr. Gregory Pazour for IFT88 and IFT140 antibodies, Dr. Tamara Caspary for Arl13b antibody, and Dr. Rajat Rohatgi for Smo antibody. Monoclonal antibodies Nkx2.2, Foxa2, Isl1, Hb9, Pax6, and Pax7 were purchased from the Developmental Studies Hybridoma Bank maintained by the University of Iowa, Department of Biological Sciences, Iowa City, Iowa 52242, under contract NO1-HD-7-3263 from the NICHD. This study was supported by National Institutes of Health (USA) (R01GM114429) to B.W., Chinese Natural Science Foundation (31570721), Chinese Ministry for Foreign Experts (GDW20143100069), and Shanghai Ministry for Science (14521100700 and 14520720200) to Q.M. J.L. is a recipient of Scholarship from Chinese Scholarship Council.

References

Alazami AM, Alshammari MJ, Salih MA, Alzahrani F, Hijazi H, Seidahmed MZ, Abu Safieh L, Aldosary M, Khan AO, Alkuraya FS. Molecular characterization of Joubert syndrome in Saudi Arabia. Human mutation. 2012; 33:1423–1428. [PubMed: 22693042]

Bai CB, Stephen D, Joyner AL. All mouse ventral spinal cord patterning by hedgehog is Gli dependent and involves an activator function of Gli3. Dev Cell. 2004; 6:103–115. [PubMed: 14723851]

Briscoe J, Pierani A, Jessell TM, Ericson J. A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. Cell. 2000; 101:435–445. [PubMed: 10830170]

Caspary T, Larkins CE, Anderson KV. The graded response to Sonic Hedgehog depends on cilia architecture. Dev Cell. 2007; 12:767–778. [PubMed: 17488627]

Chen MH, Wilson CW, Li YJ, Law KK, Lu CS, Gacayan R, Zhang X, Hui CC, Chuang PT. Cilium-independent regulation of Gli protein function by Sufu in Hedgehog signaling is evolutionarily conserved. Genes Dev. 2009; 23:1910–1928. [PubMed: 19684112]

Chih B, Liu P, Chinn Y, Chalouni C, Komuves LG, Hass PE, Sandoval W, Peterson AS. A ciliopathy complex at the transition zone protects the cilia as a privileged membrane domain. Nat Cell Biol. 2011; 14:61–72. [PubMed: 22179047]

Corbit KC, Aanstad P, Singla V, Norman AR, Stainier DY, Reiter JF. Vertebrate Smoothened functions at the primary cilium. Nature. 2005; 437:1018–1021. [PubMed: 16136078]

Garcia-Gonzalo FR, Corbit KC, Sirerol-Piquer MS, Ramaswami G, Otto EA, Noriega TR, Seol AD, Robinson JF, Bennett CL, Josifova DJ, Garcia-Verdugo JM, Katsanis N, Hildebrandt F, Reiter JF. A transition zone complex regulates mammalian ciliogenesis and ciliary membrane composition. Nat Genet. 2011; 43:776–784. [PubMed: 21725307]

Garcia-Gonzalo FR, Reiter JF. Open Sesame: How Transition Fibers and the Transition Zone Control Ciliary Composition. Cold Spring Harbor perspectives in biology. 2017; 9

Gerdes JM, Davis EE, Katsanis N. The vertebrate primary cilium in development, homeostasis, and disease. Cell. 2009; 137:32–45. [PubMed: 19345185]

Goetz SC, Anderson KV. The primary cilium: a signalling centre during vertebrate development. Nat Rev Genet. 2010; 11:331–344. [PubMed: 20395968]

Graser S, Stierhof YD, Lavoie SB, Gassner OS, Lamla S, Le Clech M, Nigg EA. Cep164, a novel centriole appendage protein required for primary cilium formation. The Journal of cell biology. 2007; 179:321–330. [PubMed: 17954613]

Haycraft CJ, Banizs B, Aydin-Son Y, Zhang Q, Michaud EJ, Yoder BK. Gli2 and Gli3 localize to cilia and require the intraflagellar transport protein polaris for processing and function. PLoS Genet. 2005; 1:e53. [PubMed: 16254602]

Lange BM, Gull K. A molecular marker for centriole maturation in the mammalian cell cycle. The Journal of cell biology. 1995; 130:919–927. [PubMed: 7642707]

Matise MP, Epstein DJ, Park HL, Platt KA, Joyner AL. Gli2 is required for induction of floor plate and adjacent cells, but not most ventral neurons in the mouse central nervous system. Development. 1998; 125:2759–2770. [PubMed: 9655799]

Mogensen MM, Malik A, Piel M, Bouckson-Castaing V, Bornens M. Microtubule minus-end anchorage at centrosomal and non-centrosomal sites: the role of ninein. J Cell Sci. 2000; 113(Pt 17):3013–3023. [PubMed: 10934040]

Nakagawa Y, Yamane Y, Okanoue T, Tsukita S, Tsukita S. Outer dense fiber 2 is a widespread centrosome scaffold component preferentially associated with mother centrioles: its identification from isolated centrosomes. Mol Biol Cell. 2001; 12:1687–1697. [PubMed: 11408577]

Pan Y, Bai CB, Joyner AL, Wang B. Sonic hedgehog signaling regulates Gli2 transcriptional activity by suppressing its processing and degradation. Mol Cell Biol. 2006; 26:3365–3377. [PubMed: 16611981]

Pan Y, Wang C, Wang B. Phosphorylation of Gli2 by protein kinase A is required for Gli2 processing and degradation and the Sonic Hedgehog-regulated mouse development. Dev Biol. 2009; 326:177–189. [PubMed: 19056373]

Pazour GJ, Baker SA, Deane JA, Cole DG, Dickert BL, Rosenbaum JL, Witman GB, Besharse JC. The intraflagellar transport protein, IFT88, is essential for vertebrate photoreceptor assembly and maintenance. The Journal of cell biology. 2002; 157:103–113. [PubMed: 11916979]

Pedersen LB, Rosenbaum JL. Intraflagellar transport (IFT) role in ciliary assembly, resorption and signalling. Curr Top Dev Biol. 2008; 85:23–61. [PubMed: 19147001]

Reiter JF, Skarnes WC. Tectonic, a novel regulator of the Hedgehog pathway required for both activation and inhibition. Genes Dev. 2006; 20:22–27. [PubMed: 16357211]

Roberson EC, Dowdle WE, Ozanturk A, Garcia-Gonzalo FR, Li C, Halbritter J, Elkhartoufi N, Porath JD, Cope H, Ashley-Koch A, Gregory S, Thomas S, Sayer JA, Saunier S, Otto EA, Katsanis N, Davis EE, Attie-Bitach T, Hildebrandt F, Leroux MR, Reiter JF. TMEM231, mutated in orofaciodigital and Meckel syndromes, organizes the ciliary transition zone. The Journal of cell biology. 2015; 209:129–142. [PubMed: 25869670]

Rohatgi R, Milenkovic L, Scott MP. Patched1 regulates hedgehog signaling at the primary cilium. Science. 2007; 317:372–376. [PubMed: 17641202]

Sang L, Miller JJ, Corbit KC, Giles RH, Brauer MJ, Otto EA, Baye LM, Wen X, Scales SJ, Kwong M, Huntzicker EG, Sfakianos MK, Sandoval W, Bazan JF, Kulkarni P, Garcia-Gonzalo FR, Seol AD,

O'Toole JF, Held S, Reutter HM, Lane WS, Rafiq MA, Noor A, Ansar M, Devi AR, Sheffield VC, Slusarski DC, Vincent JB, Doherty DA, Hildebrandt F, Reiter JF, Jackson PK. Mapping the NPHP-JBTS-MKS protein network reveals ciliopathy disease genes and pathways. Cell. 2011; 145:513–528. [PubMed: 21565611]

Shaheen R, Faqeih E, Seidahmed MZ, Sunker A, Alali FE, AlQahtani K, Alkuraya FS. A TCTN2 mutation defines a novel Meckel Gruber syndrome locus. Human mutation. 2011; 32:573–578. [PubMed: 21462283]

Sorokin S. Centrioles and the formation of rudimentary cilia by fibroblasts and smooth muscle cells. The Journal of cell biology. 1962; 15:363–377. [PubMed: 13978319]

Thomas S, Legendre M, Saunier S, Bessieres B, Alby C, Bonniere M, Toutain A, Loeuillet L, Szymanska K, Jossic F, Gaillard D, Yacoubi MT, Mougou-Zerelli S, David A, Barthez MA, Ville Y, Bole-Feysot C, Nitschke P, Lyonnet S, Munnich A, Johnson CA, Encha-Razavi F, Cormier-Daire V, Thauvin-Robinet C, Vekemans M, Attie-Bitach T. TCTN3 mutations cause Mohr-Majewski syndrome. American journal of human genetics. 2012; 91:372–378. [PubMed: 22883145]

Wang B, Fallon JF, Beachy PA. Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb. Cell. 2000; 100:423–434. [PubMed: 10693759]

Wen X, Lai CK, Evangelista M, Hongo JA, de Sauvage FJ, Scales SJ. Kinetics of hedgehog-dependent full-length Gli3 accumulation in primary cilia and subsequent degradation. Mol Cell Biol. 2010; 30:1910–1922. [PubMed: 20154143]

Wu C, Yang M, Li J, Wang C, Cao T, Tao K, Wang B. Talpid3-binding centrosomal protein Cep120 is required for centriole duplication and proliferation of cerebellar granule neuron progenitors. PLOS ONE. 2014 in press.

Highlights

- Loss of Tctn3 gene results in defects in ciliogenesis and Hedgehog signaling.
- Overexpression of Tctn3, but not Tctn1 or Tctn2, can rescue ciliogenesis in Tctn3 mutant cells.
- Replacement of Tctn3 with Tctn1 or Tctn2 in the Tctn3 locus leads to reduced ciliogenesis and Hedgehog signaling but does not affect neural tube patterning and Gli3 processing.

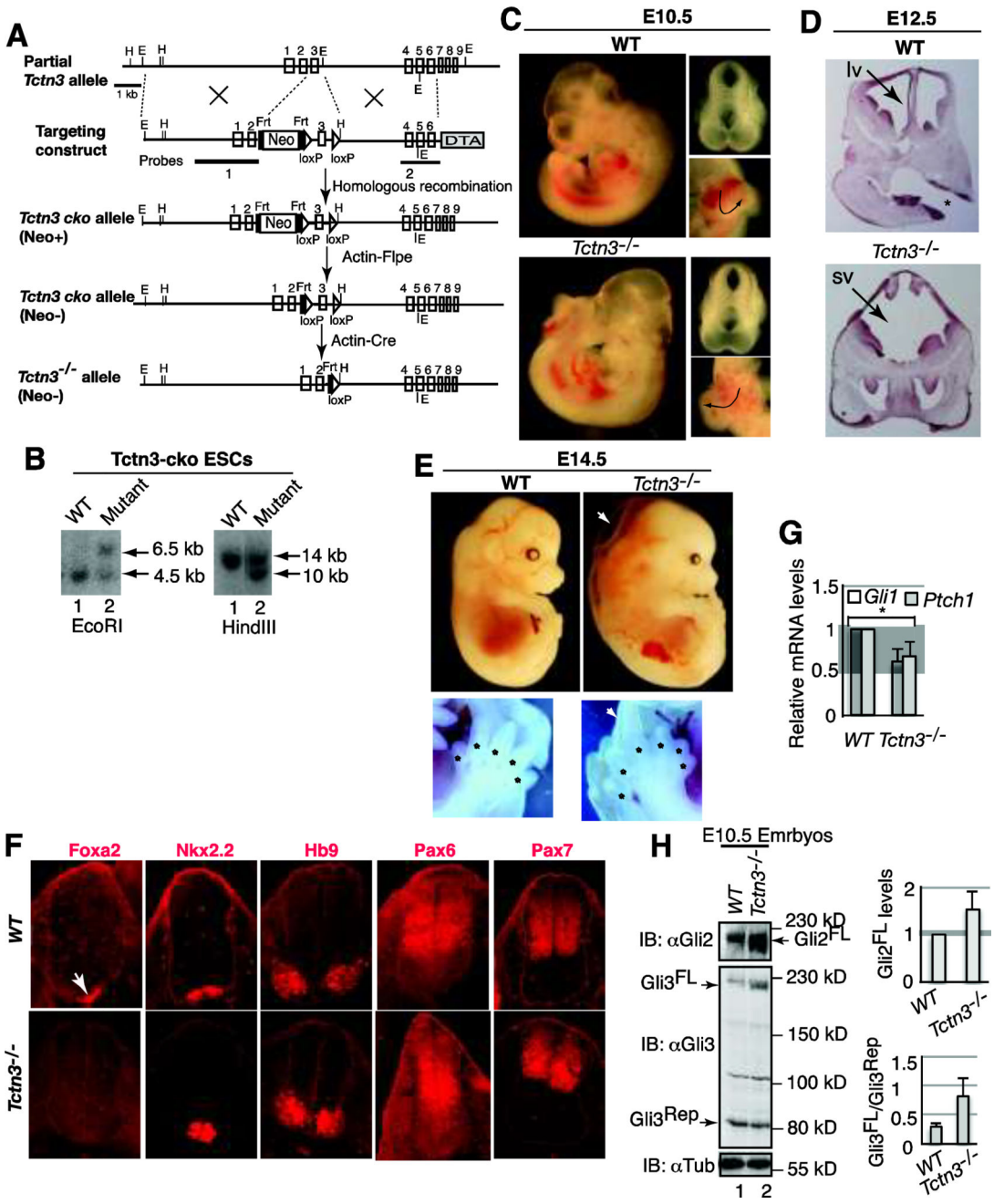

Figure 1.

Inactivation of *Tctn3* results in reduced Hh signaling in mice. (**A**) The gene targeting strategy used to create a mouse *Tctn3* mutant allele. Open rectangles are referred to as exons and lines as introns. Probe and restriction sites (H, HindIII; E, EcoRI) used for Southern blot are shown. Triangle, loxP site; solid rectangles, Frt site; Neo, pGK-neomycin; DTA, diphtheria toxin A. (**B**) Southern blot of representative wild type (wt) and mutant (heterozygous) ES cell clones (n= 1 experiment). (**C**) The morphology of wt and *Tctn3* mutant embryos at stage E10.5. Prosencephalon is indicated by arrowhead and heart loop by arrow. Note holoprosencephaly (unsplit prosencephalon) (n = 28/28) and rightward heart

looping in the mutant (n = 8/28). (**D**) Coronal sections of wild type and *Tctn3* mutant forebrain region at E12.5, stained with Hematoxylin and Eosin. Note that the lateral ventricles (lv) formed in wt but not mutant (single ventricle (sv)) (n = 2/2 embryos). An asterisk points to a part of section broken. (**E**) *Tctn3* mutant embryos exhibit edema (an arrowhead in upper right panel) and polydactyly (asterisks mark digits; arrowhead indicates tail in lower panels) (n = 10/15). (**F**) The floor plate is missing in the *Tctn3* mutant neural tube. The transverse sections of wt and *Tctn3* mutant neural tube at E10.5 were immunostained for neuronal markers as indicated. Note that Foxa2 is missing in the *Tctn3* mutant (n = 3/3 embryos each). (**G**) RT-qPCR showing relative *Gli1* and *Ptch1* RNA expression levels in E10.5 wt and *Tctn3* mutant embryos from four independent experiments (2-tailed Student t-test p-value = 0.012 for *Gli1*, 0.024 for *Ptch1*). An asterisk indicates that the significant difference in *Gli1* and *Ptch1* mRNA levels between wt and mutant. (**H**) Immunoblots showing $Gli2^{FL}$, $Gli3^{FL}$, and $Gli3^{Rep}$ levels in wt and *Tctn3* mutant embryos. Bar graphs showing relative $Gli2^{FL}$ levels and $Gli3^{FL}$ to $Gli3^{Rep}$ ratio from two independent experiments.

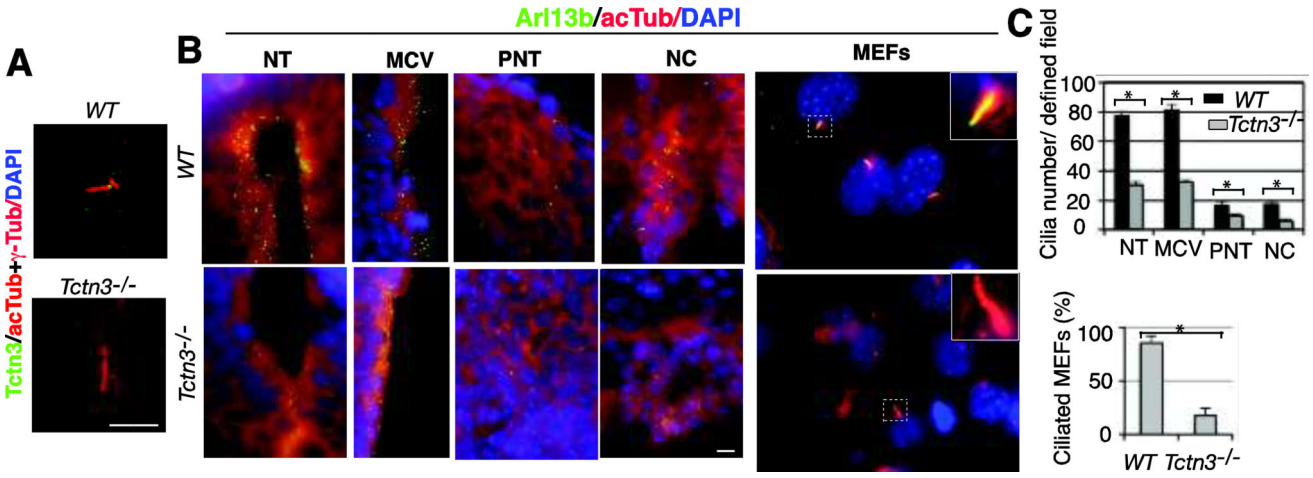

Figure 2.

Inactivation of *Tctn3* results in reduced cilia number in mouse embryo and MEFs. (**A**) Tctn3 localizes to the transition zone of cilia. Wt and *Tctn3* mutant MEFs were stained for Tctn3, acetylated tubulin (acTub), and γ-tubulin (γ-Tub). The cells were counterstained with DAPI for nucleus. (**B**) Cilia number is significantly reduced in various tissues of *Tctn3* mutant embryos and pMEFs. The transverse sections of E10.5 wt and mutant embryos and MEFs were immunostained for cilia markers, Arl13b and acetylated tubulin (acTub), and nucleus (DAPI). NT, neural tube; MCV, mesencephalic vesicle; PNT, perineural tube; NC, notochord. (**C**) Graphs show cilia number in each defined microscopic area (NT and MCV, 93 μm long NT lumen; PNT, 93.3 μm × 120 μm; NC, entire notochord) of embryo sections (n = 2 embryos) (upper graph) and the percentage of ciliated pMEFs (n = 110–120 randomly selected cells)(lower graph). The results are derived from three independent experiments. Two-tailed Student t-test shows p-value ≤ 0.006 and = 0.00001 for upper and lower graphs, respectively.

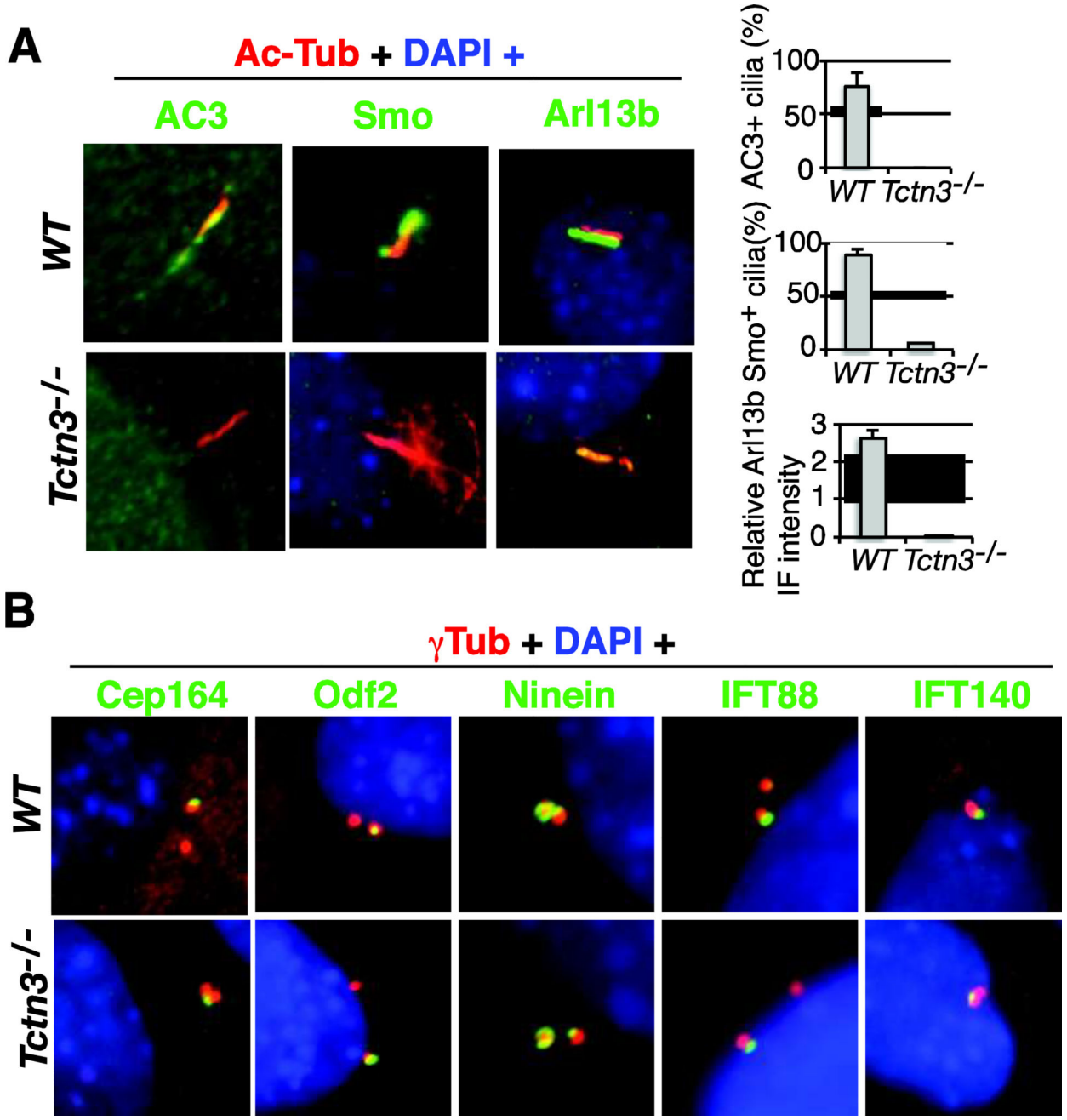

Figure 3.

Tctn3 mutation disrupts the ciliary membrane protein composition in cilia. (**A**) Wt and *Tctn3* mutant pMEFs were stained for adenylyl cyclase type 3 (AC3), Smo, or Arl13b together with acetylated tubulin (Ac-Tub) and the nucleus (DAPI). Bar graphs show the quantitative data derived from three independent experiments. Two-tailed Student t-test shows p-values ≤0.0004, thus significant. IF, immunofluorescence. (**B**) Loss of Tctn3 does not impact the localization of appendage markers and recruitment of IFT88 and IFT140 to the basal body (n = two independent experiments). Wt and *Tctn3* mutant MEFs were stained for indicated proteins, γ-tubulin, and the nucleus (DAPI). Cep164, a distal appendage

marker; Odf2 and Ninein, subdistal appendage proteins; IFT88, a subunit of IFT-B; IFT140, a subunit of IFT-A.

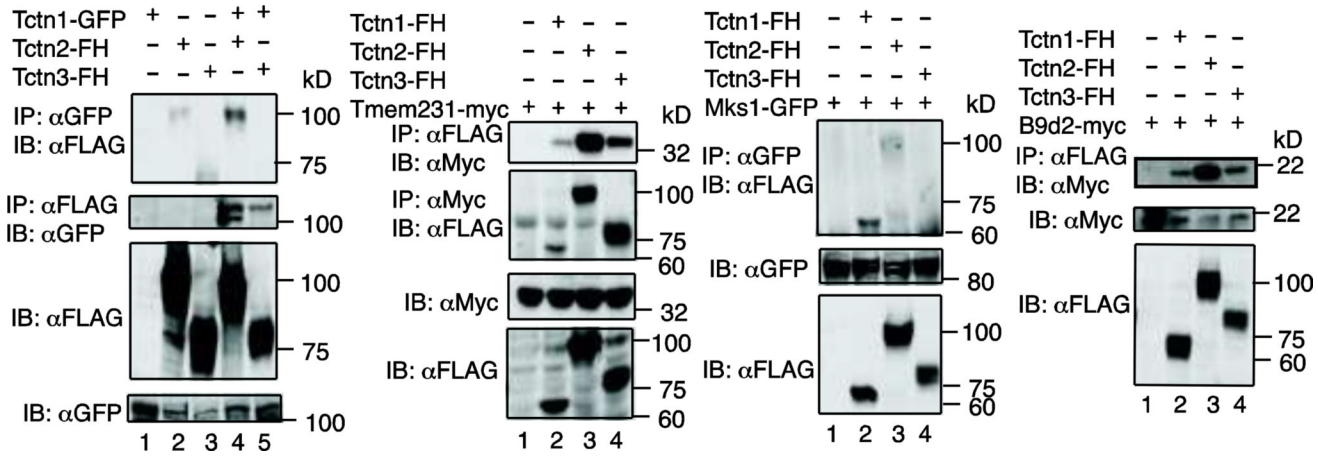

Figure 4.

Tctn proteins interact with one another and some of the known transition-zone-localized proteins. Coimmunoprecipitation shows protein-protein interaction among different components of the Tctn protein complex. Upper panels are coimmunoprecipitation data, and lower panels are immunoblots of input proteins. Note that some proteins fail to interact, suggesting that the interaction may be indirect. For some proteins, coimmunoprecipitation worked only with one antibody but not the other, owing to non-specific background. Proteins were overexpressed in HEK293 cells; two independent experiments were performed. IP, immunoprecipitation; IB, immunoblot; FH, FLAG and HA double tags. A background band migrating slightly faster than Tctn1-GFP in the left bottom panel is labeled with asterisks. Molecular weight markers (kD) are to the right of each panel.

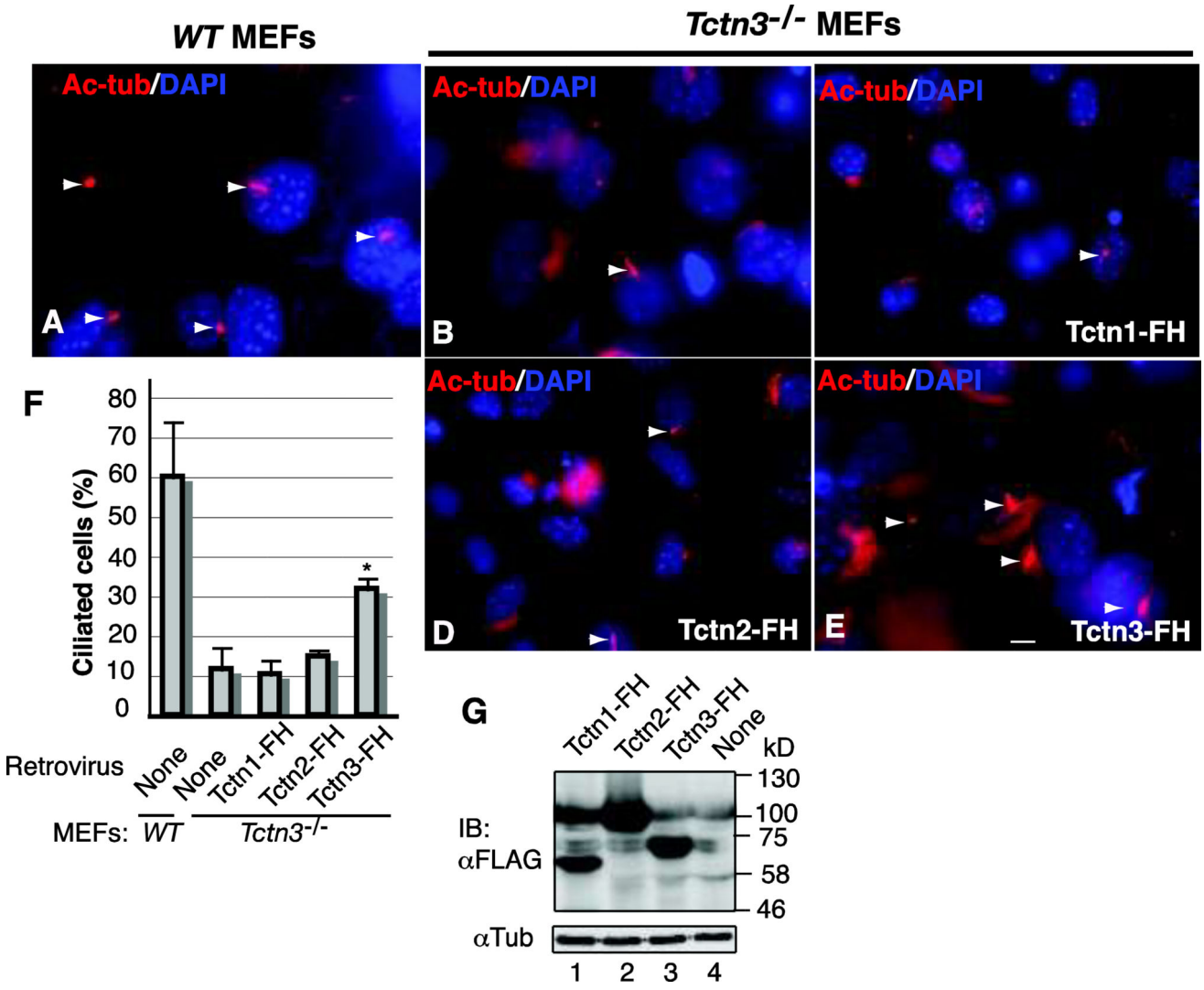

Figure 5.

Expression of Tctn3-FH, but not Tctn1-FH or Tctn2-FH, can rescue ciliogenesis in *Tctn3* mutant MEFs. *Tctn3* mutant MEFs were transduced either without (**B**) or with Tctn1-FH (**C**), Tctn2-FH (**D**), and Tctn3-FH (**E**) retrovirus and then stained for acetylated tubulin (Ac-Tub) and nuclei (DAPI). Arrowheads indicate cilia. (**F**) A graph shows the percentage of ciliated cells. The data are derived from three independent experiments. Two-tailed Student t-test p-values are 0.78, 0.44, and 0.006 for Tctn1-FH, Tctn2-FH, and Tctn3-FH, respectively. An asterisk indicates significant rescue. (**G**) Western blot showing that Tctn proteins were expressed at similar levels. FH, FLAG and HA double tags.

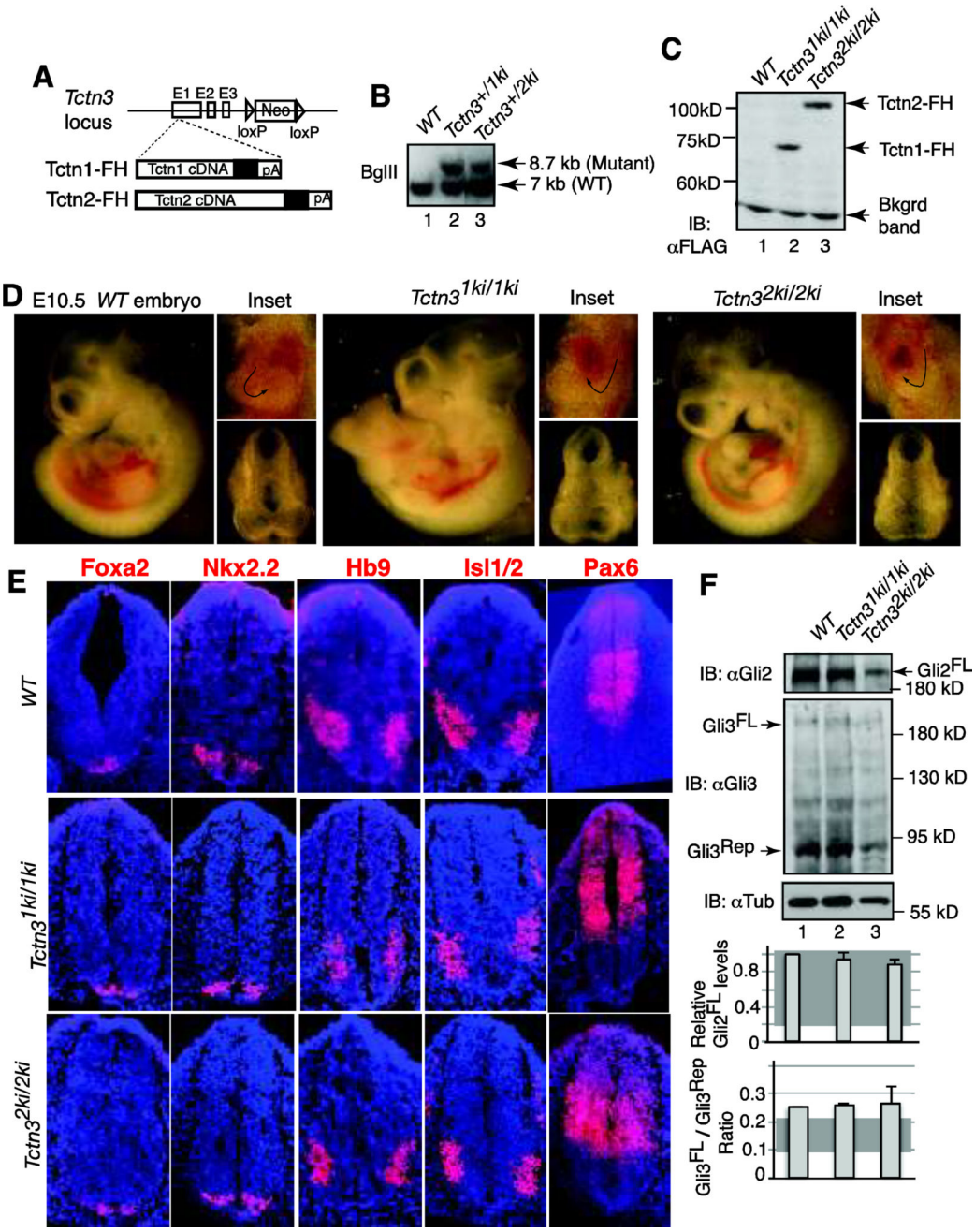

Figure 6.

Tctn3^{1ki} and *Tctn3*^{2ki} homozygous mice exhibit holoprosencephaly and randomized heart loop but normal neural tube patterning and Gli3 processing. (**A**) Diagram showing the strategy to generate *Tctn3*^{1ki} and *Tctn3*^{2ki} alleles. FH, FLAG and HA double tags; pA, polyadenylation signal. (**B**) Southern blot of representative wt and knockin (heterozygous) ES cell clones (n = 1 experiment). (**C**) An immunoblot showing Tctn1-FH and Tctn2-FH expression in E10.5 knockin embryos (n = 2 experiments). A background band (bkgrd) serves as a loading control. (**D**) Morphology of wt, *Tctn3*$^{1ki/1ki}$, *Tctn3*$^{2ki/2ki}$ E10.5 embryos. Insets show the heart looping orientation (black arrows, leftward for wt, rightward for the

mutants) (17/41 and 6/9 for *Tctn3*$^{1ki/1ki}$ and *Tctn3*$^{2ki/2ki}$ respectively) and holoprosencephaly (incomplete separation of prosencephalon in the mutants, white arrows) (41/41 and 9/9 for *Tctn3*$^{1ki/1ki}$ and *Tctn3*$^{2ki/2ki}$, respectively). (**E**) Transverse neural tube sections of E10.5 embryos with indicated genotypes were immunostained for neuronal markers shown at the top. Blue, DAPI for nucleus. Note that all neural tube markers examined are specified. (**F**) Immunoblots showing Gli2^{FL}, Gli3^{FL}, and Gli3^{Rep} levels in wt, *Tctn3*$^{1ki/1ki}$, and *Tctn3*$^{2ki/2ki}$ mouse embryos (E10.5). Graphs showing the relative average Gli2^{FL} levels and Gli3^{FL}/Gli3^{Rep} ratio from two independent experiments after normalized against tubulin loading control.

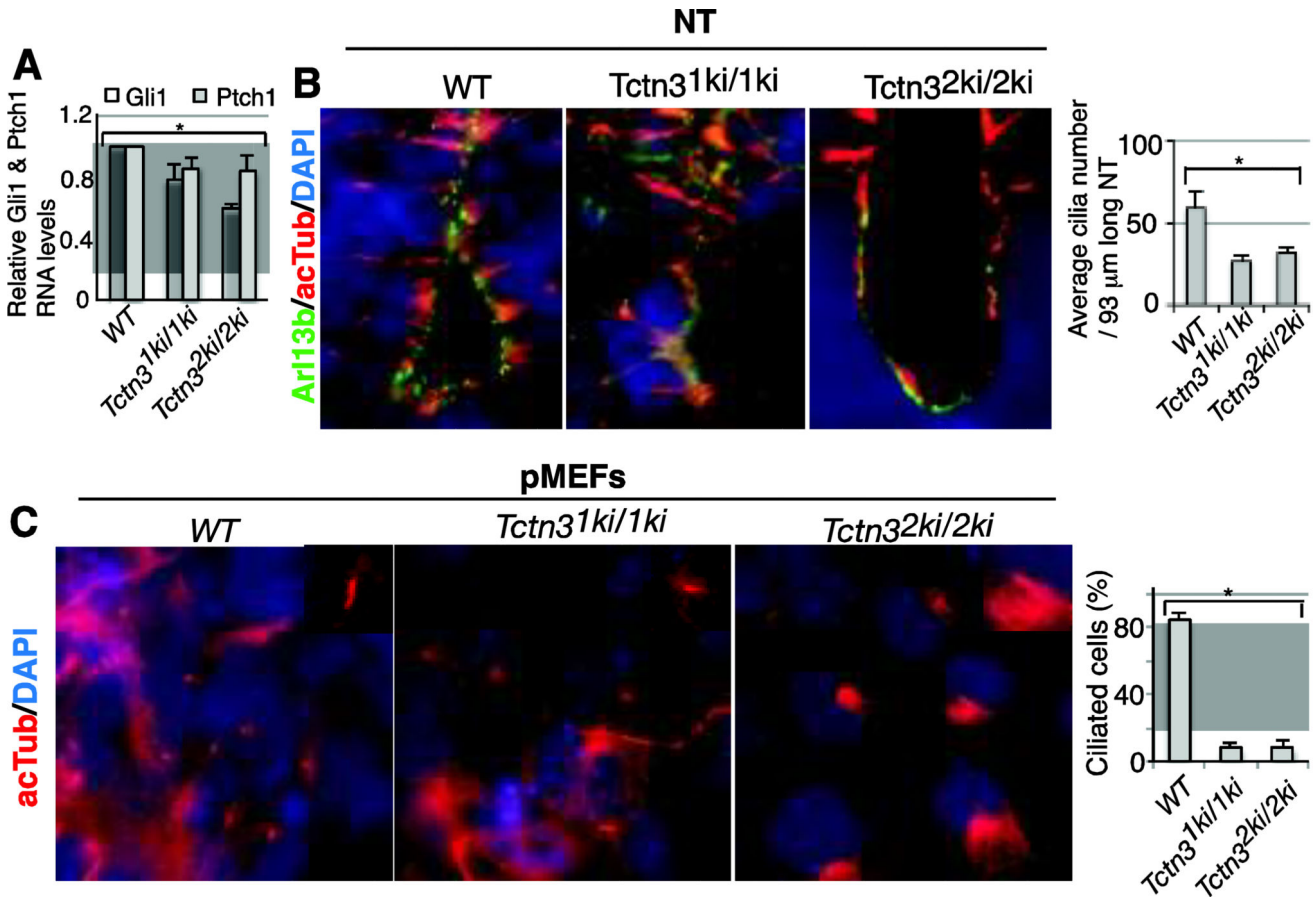

Figure 7.

Hh signaling and cilia number are reduced in *Tctn3*$^{1ki/1ki}$ and *Tctn3*$^{2ki/2ki}$ mouse embryos and pMEFs. (**A**) RT-qPCR results showing that *Gli1* and *Ptch1* RNA expression levels in E10.5 *Tctn3*$^{1ki/1ki}$ and *Tctn3*$^{2ki/2ki}$ are lower than those in wt littermates. Two-tailed Student t-test p-values ≤ 0.019 for Gli1 and 0.04 for Ptch1, thus significant (n = 4 independent experiments). (**B**) Neural tube sections of E10.5 embryos were stained for cilia markers, Arl13b and acetylated (acTub). Blue, DAPI for nucleus. Dash lines trace the neural tube lumen but are shifted to right in parallel to avoid overlapping with the immunostaining. Cilia number in a 93 μm long neural tube lumen is quantified as a bar graph to the right. Two-tailed Student t-test p-values ≤ 0.01 (n = 6 sections from 2 embryos), thus significant. (**C**) Cilia number in *Tctn3*$^{1ki/1ki}$ and *Tctn3*$^{2ki/2ki}$ pMEFs is significantly reduced. pMEFs were immunostained for acetylated tubulin (ac-Tub) and nuclei (DAPI). The graph to the right shows the percentage of ciliated cells (n = 140 – 259 cells). Two-tailed Student t-test p-values ≤ 0.0001, thus significant.