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RFX2 is broadly required for ciliogenesis during vertebrate development

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ABSTRACT

In *Caenorhabditis elegans*, the RFX (Daf19) transcription factor is a major regulator of ciliogenesis, controlling the expression of the many essential genes required for making cilia. In vertebrates, however, seven RFX genes have been identified. Bioinformatic analysis suggests that Rfx2 is the closest homologue of the Daf19. We therefore hypothesize that Rfx2 broadly controls ciliogenesis during vertebrate development. Indeed, here we show that Rfx2 in *Xenopus* is expressed preferentially in ciliated tissues, including neural tube, gastrocoel roof plate, epidermal multi-ciliated cells, otic vesicles, and kidneys. Knockdown of Rfx2 results in cilia-defective embryonic phenotypes and fewer or truncated cilia are observed in Rfx2 morphants. These results indicate that Rfx2 is broadly required for ciliogenesis in vertebrates. Furthermore, we show that Rfx2 is essential for expression of several ciliogenic genes, including TTC25, which we show here is required for ciliogenesis, HH signaling, and left–right patterning.

Introduction

Cilia are microtubule-based organelles that project from the surface of most vertebrate cells and have evolved to play diverse roles in signaling, motility, and sensory reception (Eggenschwiler and Anderson, 2007; Marshall and Nonaka, 2006; Pedersen et al., 2008). Cilia can be generally categorized as either primary cilia or motile cilia. Primary cilia are short, immotile, have widespread distribution, and play essential roles in signal transduction. Motile cilia, on the other hand, are typically much longer and are responsible for generating directional fluid flows. Therefore, motile cilia are present in more restricted tissues, such as the ventricles of brain, the airways, the oviducts, and in a specialized region of the notochord (Gerdes et al., 2009; Goetz and Anderson, 2010; Roy, 2009).

Widespread essential roles for cilia during vertebrate development were first identified by forward genetic screens in the mouse, which showed that cilia are required for embryonic patterning (Huangfu et al., 2003). Substantial studies have now revealed that defects in cilia structure or function lie at the root of a wide range of human diseases, such as primary ciliary dyskinesia, polycystic kidney disease, and Bardet–Biedl and Meckel–Gruber syndromes (Baker and Beales, 2009; Hildebrandt et al., 2011; Singla and Reiter, 2006; Zariwala et al., 2007). Numerous studies using genetic and biochemical approaches have begun to unravel the protein machinery underlying cilia structure and function (Gherman et al., 2006; Hayes et al., 2007; Inglis et al., 2006). By contrast, very little is yet known about the transcriptional programs that regulate ciliogenesis.

The RFX transcription factor (called Daf19 in *Caenorhabditis elegans*) has been identified as an essential regulator of ciliogenesis in *C. elegans* and *Drosophila* (Dubruille et al., 2002; Swoboda et al., 2000). In vertebrates, seven distinct RFX genes (RFX 1–7) have been identified based on the highly conserved DNA binding domain (Aftab et al., 2008; Emery et al., 1996), but it is not until recently that studies have linked any of these RFX genes to ciliogenesis. Unlike Daf-19, the reported role for RFX4 is quite circumscribed, modulating Shh signaling by controlling ciliogenesis, but only in the neural tube (Ashique et al., 2009). By contrast, RFX3 is more broadly required, governing nodal ciliogenesis and left–right asymmetry (Bonnafe et al., 2004), ciliogenesis in pancreatic endocrine cells (Ait-Lounis et al., 2007), and ciliogenesis of motile cilia in the brain (Baas et al., 2006; El Zein et al., 2009). Quite curiously, while loss of RFX3 reduces the number of cilia in some multi-ciliated cell types in the brain, it

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actually *increases* the number of cilia on other cell types, suggesting a complex role for RFX proteins (Baas et al., 2006). Like RFX3, RFX6 has been recently shown to be involved in pancreatic development. Surprisingly however, RFX6 appears not to be required for ciliogenesis in pancreas (Smith et al., 2010; Soyer et al., 2010). Finally, while RFX1 has recently been shown to activate expression of a known ciliary gene (Purvis et al., 2010), neither RFX1 nor RFX5 is generally associated with cilia. Rather, these function instead in the immune system (Reith and Mach, 2001; Steimle et al., 1995; Zhao et al.).

RFX2 is a crucial factor required for spermatogenesis, but the exact role for RFX2 in the control of cilia assembly is poorly understood (Grimes et al., 2005; Horvath et al., 2004; Horvath et al., 2009; Kistler et al., 2009; Liu et al., 2007; VanWert et al., 2008; Wolfe et al., 2004; Wolfe et al., 2006; Yu et al., 2008). Nonetheless, RFX2 has been shown to be expressed in some ciliated tissues (Hellman et al., 2010; Liu et al., 2007; Ma and Jiang, 2007) and its expression was reported to be controlled by another ciliogenic transcription factor, FoxJ1 (Yu et al., 2008).

Here we report that Rfx2 in *Xenopus* is expressed preferentially in tissues containing ciliated cells, including neural tube, gastrocoel roof plate, epidermal multi-ciliated cells, and kidneys. Knockdown of Rfx2 results in phenotypes associated with defective cilia, such as disruption of neural tube closure and left–right asymmetry. Moreover, fewer or truncated cilia were observed in Rfx2 morphants, indicating that Rfx2 is indeed essential for ciliogenesis in vertebrates. Finally, we found that Rfx2 is essential for the expression of several ciliogenic genes, including TTC25, which we show here is required for ciliogenesis, HH signaling, and left–right patterning.

Materials and methods

Bioinformatics

We identified human orthologs of daf-19 as in McGary et al. (2010) and confirmed the predicted ortholog relationships using the tree-based database TreeFam, accession TF321340 (Ruan et al., 2008).

We further re-analyzed the TreeFam sequences to rebuild the evolutionary tree of RFX homologs in vertebrates using maximum likelihood (Supp. Fig. 1). Sequences were aligned using MAFFT (Katoh et al., 2005) with default settings, plus the local pair option and 1000 maximum iterations, for increased accuracy. This alignment was then trimmed to remove excess gaps using trimAl (Capella-Gutierrez et al., 2009) with default settings and the gt option set to 0.7. The maximum likelihood tree was inferred using raxmlHPC (Stamatakis et al., 2008) using default options, with the model set to PROTCATWAG. We include a supplemental file treedata.tar.gz, which includes the input sequences, the alignment and the ML tree with branch lengths.

Morpholino and RNA injection

Capped mRNA was synthesized using mMESSAGE mMACHINE (Ambion). mRNA and antisense morpholino were injected into ventral blastomeres at the 4-cell stage to target the epidermis and into dorsal blastomeres to target the neural tissues (Moody, 1987). Embryos were incubated until appropriate stages according to Nieuwkoop and Faber (1994) and were fixed in MEMFA (Davidson

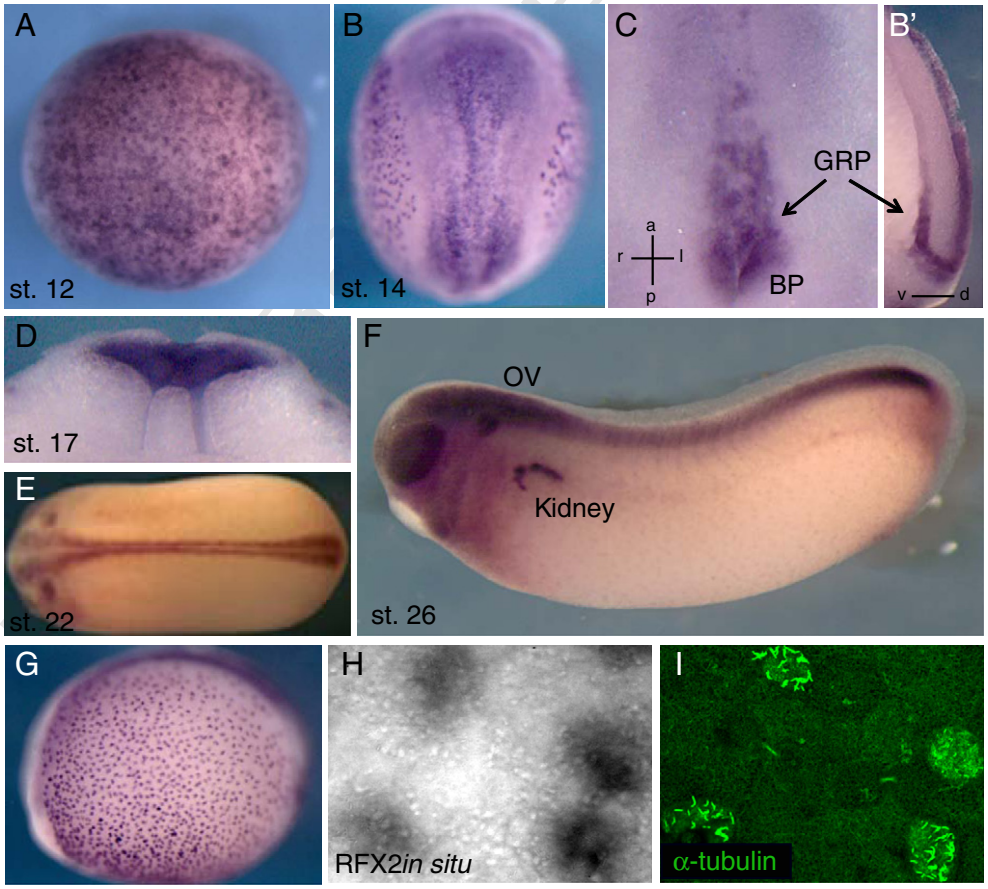

Fig. 1. Rfx2 is expressed in ciliated tissues. (A) Early expression of *Rfx2* in epidermis. (B) *Rfx2* is expressed in the neural plate. Dorsal view. (B′) (C) Sagittal sections showed that *Rfx2* is expressed in the gastrocoel roof plate (GRP) (arrows). BP: blastopore. (D) Transverse section view revealed robust *Rfx2* expression in the neural tube. (E) *Rfx2* is expressed in the neural tube. Dorsal view. (F) *Rfx2* is expressed in otic vesicles and kidneys. (G) Punctate expression of *Rfx2* in the epidermis. (H) Closer view of epidermal *Rfx2* expression in (G). (I) Epidermal ciliated cells are observed by staining with α-tubulin. Ciliated cells are co-localized with *Rfx2 in situ* pattern in (H).

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and Wallingford, 2005). Embryos were embedded in 2% agarose for thick (250–300 μm) sections or in 4% low-melt agarose for thin (50–100 μm) sections, which were cut with a Vibratome series 1000 (Davidson and Wallingford, 2005). The Rfx2 morpholino sequence is:

AATTCTGCATACTGGTTTCTCCGTC

This oligonucleotide was compared to all *Xenopus* RFX transcription factor sequences and is predicted to bind only the Rfx2 mRNA. The TTC25 morpholino sequence is reported (Hayes et al., 2007) and was injected at 15 ng.

Immunohistochemistry

Immunostaining was performed as described in Lee et al. (2008). Briefly, fixed embryos were dehydrated completely in methanol at −20 °C overnight and were bleached in 10% hydrogen peroxide/67% methanol for 3 h and rehydrated consecutively with TBS (155 mM NaCl, 10 mM Tris–Cl, pH 7.4). To reduce autofluorescence of yolk platelets, the embryos were incubated with 100 mM $NaBH_4$ in TBS for 4 h at room temperature or overnight at 4 °C and rinsed in TBST (0.1% Triton X-100 in TBS). Primary antibodies used were: monoclonal anti-α-tubulin antibody (1:500 dilution, clone DM1A, Sigma), rabbit anti-GFP antibody (1:500 dilution, Invitrogen), mouse anti-acetylated-α-tubulin (1:500, clone 6-11B-1, Sigma), and rabbit anti- Arl13b (1:500, gift of T. Caspary). Antibodies were diluted in fetal bovine serum (FBS) solution (TBS containing 10% FBS and 5% DMSO). Primary antibodies were detected with Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes) and Alexa-555 goat anti-rabbit IgG (Molecular Probes), 1:500.

Embryos were prepared for confocal imaging as described (Wallingford, 2010). Embryos were cleared in Murray's Clear solution (benzyl benzoate:benzyl alcohol = 2:1), and images were obtained using a Zeiss LSM5 Pascal confocal microscope. Cilia lengths were measured with LSM5 Pascal or ImageJ software. Images used throughout this paper have been enhanced using the Unsharp Mask filter in Adobe Photoshop.

In situ hybridization

In situ hybridization was performed as described previously (Sive et al., 2000). Bright field and low magnification fluorescence images were captured on a fluorescent stereomicroscope, Leica MZ16FA. To observe multi-ciliated cells on the epidermis, embryos were then immunostained with α-tubulin and imaged as mentioned above.

Animal cap explants and reverse transcription PCR

Rfx2 morpholino (10 ng) was injected into the animal pole at the 4-cell stage. Animal cap explants were dissected at stage 8 and cultured as described previously (Sive et al., 2000). Fifty explants of each sample were collected for the preparation of cDNA.

PCR was performed with the following primers (5′ to 3′, forward and reverse):

Ef1α, CAGATTGGTGCTGGATATGC and ACTGCCTTGATGACTCCTAG; *α-tubulin 1b*, AGATGCCCAGTGACAAGACC and GGGCTCCATCAAATCGTAGA; *Ift122*, CCGAAACCTACATGAAGATCG and CGCAGACCTTGTAGCCTCTC; *Ift172*, GGAAATATGGCCAGAGCAAA and TCCTGTTGCTTCTGTTGCAC; *TTC25*, AGAATGTGCCCTGAAGGATG and GCGTGTCCAGGTACAGGATT; *WDPCP*, TGGCGATTTTATAACGTCATTC and TCTTCCTTTTGGTCCTGGAA.

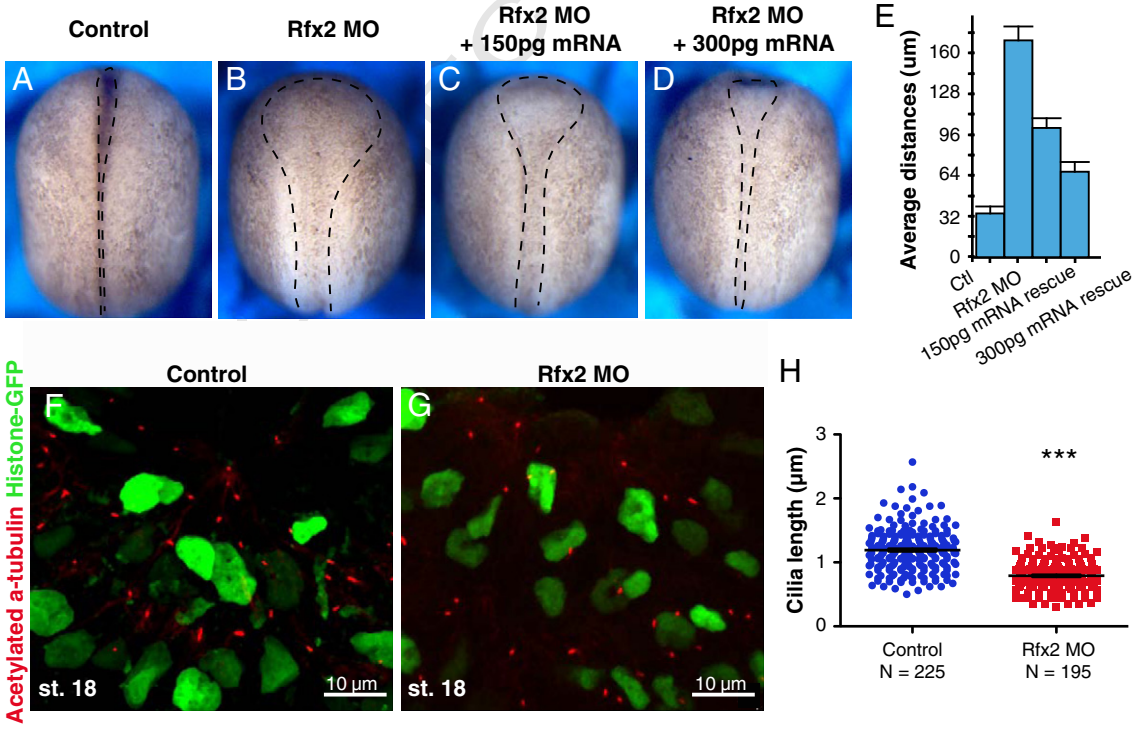

Fig. 2. Rfx2 is required for neural tube closure and proper neural cilia formation. (A–D) Stage 19 embryos, dorsal view, anterior top. Black dashed lines outline the distance between the neural folds. (A) Stage 19 control embryo. The neural tube is almost closed. (B) Embryo injected with Rfx2 morpholino dorsally. The neural tube closure defects are shown. The neural tube closure defects caused by disruption of Rfx2 can be partially rescued by co-injection with 150 pg GFP-Rfx2 mRNA (C) and 300 pg GFP-RFX2 mRNA (D). The average distance between the neural folds is shown in (E). (F) Transverse section view of neural plate of stage 18 control embryos. Cilia are stained with acetylated α-tubulin. (G) Transverse section view of neural plate of Rfx2 morphants. Shorter cilia are observed. (H) Cilia were measured in 15 μm projection confocal images. The average length of control cilia is 1.19 ± 0.021 μm (mean ± SEM, n = 225). However, the average length significantly reduces to 0.79 ± 0.016 μm in Rfx2 morphants (mean ± SEM, n = 195). Horizontal lines indicate the mean, vertical lines SEM, *** $p<0.0001$ Mann–Whitney test.

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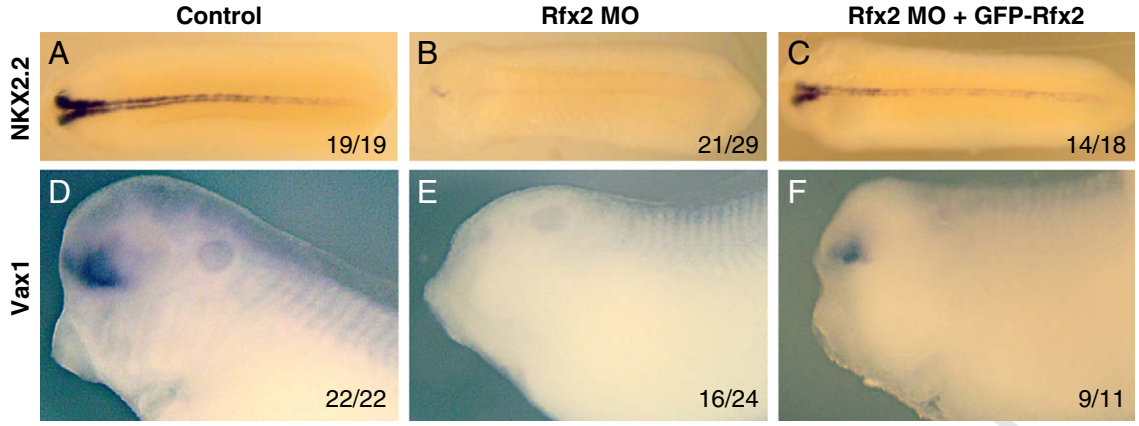

Fig. 3. Rfx2 is required for Shh signaling. (A–C) *In situ* hybridization of a Shh downstream gene, *Nkx2.2*.Dorsal view, anterior left. Stage 24. Expression of *Nkx2.2* in the ventral neural tube (A) is dramatically reduced in the spinal cord of Rfx2 morphants (21/29) (B). The reduced expression of *Nkx2.2* can be rescued by co-injection of 300 pg GFP-Rfx2 mRNA (C). (D–F) *In situ* hybridization of a Shh downstream gene, *Vax1*. Lateral view, anterior left. Stage 35.Expression of *Vax1* in the ventral forebrain (D) is robustly reduced in Rfx2 morphants (16/24) (E) and can be rescued (F).

Results

Phylogeny of vertebrate RFX proteins

Since the *daf-19* transcription factor is a central regulator of ciliogenesis in *C. elegans*, we reasoned that its orthologs would be excellent candidates for control of vertebrate ciliogenesis.

In vertebrates, the RFX transcription factor family includes three phylogenetic sub-groups (Aftab et al., 2008); a tree including mammalian and non-mammalian vertebrate RFX proteins is presented in Supp. Fig. 1). RFX5 and RFX7 are the most distantly related to *daf-19*, and so it is notable that neither gene has so far been associated with ciliogenesis. Curiously, RFX4 and RFX6 are closely related, yet only RFX4 has a role in ciliogenesis (Ashique et al., 2009).

Finally, RFX1, RFX2, and RFX 3 are co-orthologs of *daf-19* (Supp. Fig. 1; (Aftab et al., 2008)). Given this phylogenetic relationship, it is these three factors which would be predicted to play the most central roles in governing ciliogenesis. However, while Rfx3 plays a broad role in ciliogenesis, the role for RFX1 appears minimal (Ait-Lounis et al., 2007; Baas et al., 2006; Bonnafe et al., 2004; El Zein et al., 2009; Purvis et al., 2010). Given its close phylogenetic relationship to *daf-19*, we hypothesized that RFX2 may be a broadly required regulator of ciliogenesis. However, no loss-of-function analysis of RFX2 has yet been reported.

Rfx2 is expressed in tissues containing ciliated cells

To test the prediction of a broad role for Rfx2 in ciliogenesis, we first examined its expression pattern by *in situ* hybridization in *Xenopus*. We observed that *Rfx2* was expressed preferentially in tissues containing ciliated cells. For instance, *Rfx2* expression in the neural plate was detected from the early neurula stage to the later tadpole stage (Figs. 1B, D–F). At late gastrula and neurula stages, *Rfx2* was also expressed in the gastrocoel roof plate (GRP), where motile cilia are responsible for generating directional flow to regulate left–right asymmetry (Figs. 1B′, C). Moreover, punctate expression of *Rfx2* was observed in the epidermis from the late gastrula stages (Figs. 1A, G). The *in situ* staining of *Rfx2* on the epidermis co-localized with α-tubulin immunostaining, indicating that *Rfx2* was expressed specifically in epidermal multi-ciliated cells (Figs. 1H–I). Expression of *Rfx2* was also observed in otic vesicles and kidney, where cilia are required for the proper tissue functions (Fig. 1F). Rfx2 is broadly expressed in ciliated tissues, suggesting that Rfx2 may be essential for ciliogenesis in vertebrates.

To ask if Rfx2 may act in coordination with other ciliogenic Rfx genes, we also examined the expression patterns of *Rfx1*, *Rfx3*, *Rfx4*, and *Rfx5* in *Xenopus*. *In situ* hybridization showed that all Rfx genes were expressed in the neural tube (Supp. Figs. 2B, E, I, L), suggesting potentially redundant roles for these genes in the control of the neural tube ciliogenesis. *Rfx3* shared the most similar pattern with *Rfx2*, as they were both expressed in epidermal ciliated cells and GRP (Fig. 1 and Supp. Figs. 2D, F). However, while the expression of *Rfx2* in ciliated epidermal cells was observed as early as stage 12, the low level transcription of *Rfx3* was only observed beginning at stage 14. This temporal difference of expression might suggest that Rfx2 plays a higher hierarchic role than Rfx3 in regulating epidermal cilia development. Moreover, while Rfx2 was robustly expressed in GRP (Figs. 1B′, C), only weak staining of Rfx3 was observed (Supp. Fig. 2F). *Rfx4* was robustly expressed in the central nervous system (Supp. Figs. 2H–J), consistent with its role in controlling Shh signaling in the mouse spinal cord (Ashique et al., 2009). Notably, *Rfx4* expression was never observed in gastrocoel roof plate or epidermal ciliated cells, suggesting a tissue-specific role in regulating cilia formation.

Rfx2 is essential for neural tube closure and neural ciliogenesis

Many genes associated with ciliogenesis are also required for neural tube closure, though the mechanistic link between cilia and the morphogenetic cell movement in the developing neural tube remains obscure (Murdoch and Copp, 2010). To ask if Rfx2 is required for neural tube ciliogenesis, we first examined the effect of Rfx2 knockdown on neural tube closure. We designed antisense morpholino oligonucleotides (MO) to block Rfx2 translation, and we used targeted micro-injection to deliver these specifically to the dorsal tissues (Moody, 1987). At the end of neurulation (stage 19), the neural folds are apposed and begin to fuse (Fig. 2A). At an equivalent time point in Rfx2 morphants, the neural folds failed to close (Fig. 2B), with the distance between neural folds being five-fold greater in Rfx2 morphants than in control embryos at stage 20 (Fig. 2E). We confirmed the specificity of our MO by rescue of the neural tube closure phenotype with a GFP-tagged form of Rfx2 mRNA that is not recognized by Rfx2 morpholino (Figs. 2C–E).

Neural tube defects (NTDs) in mammals represent a complex spectrum of phenotypes, including “open” defects, in which the neural tissues remain externally exposed, and “closed” defects in which the neural tissue is dysmorphic but nonetheless covered by epidermis. Defects in ciliogenesis in mouse models are generally associated with exencephaly (an open NTD; (Murdoch and Copp, 2010). However, some cilia-related mouse models develop encephalocoel, a closed

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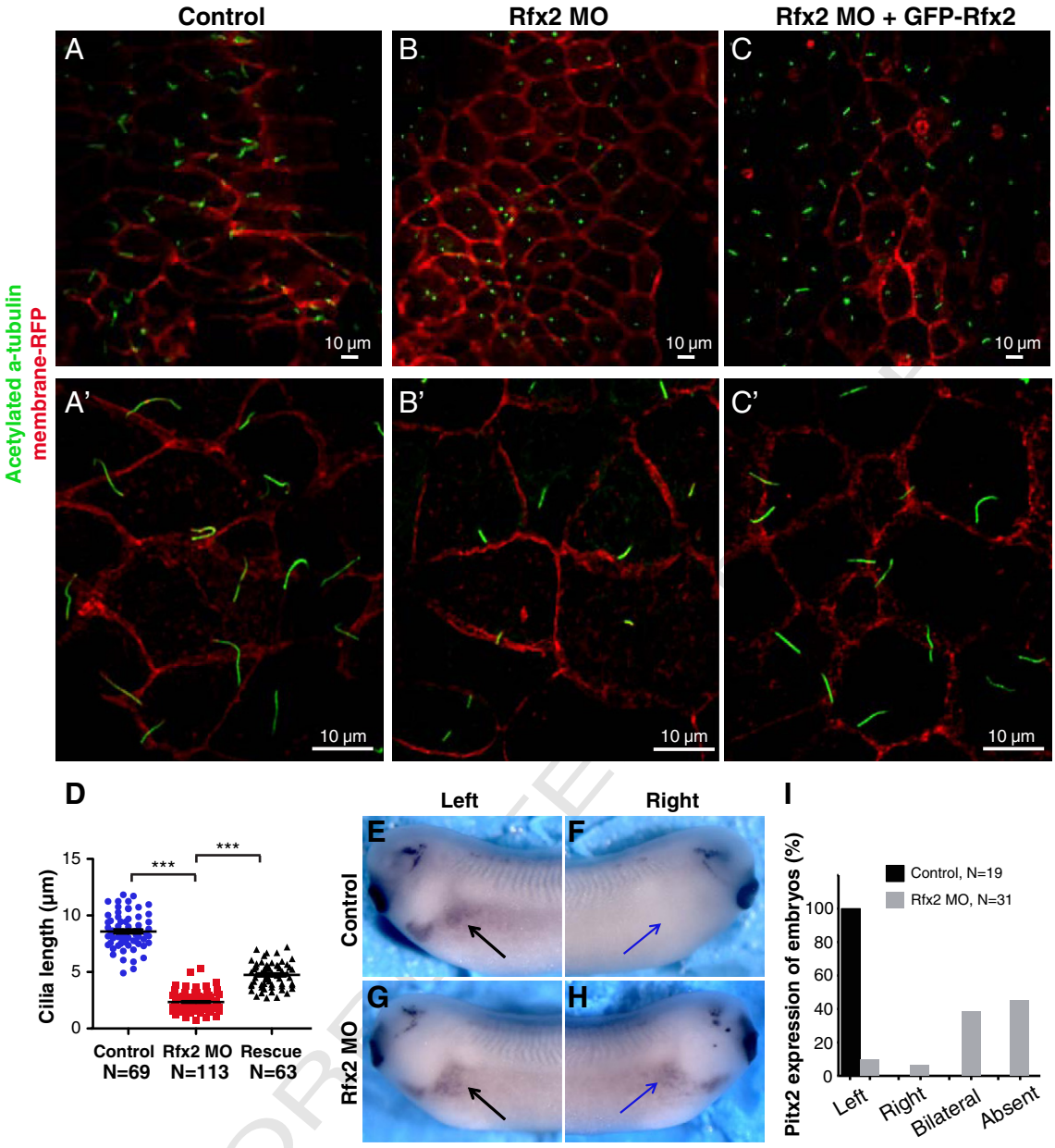

Fig. 4. Rfx2 is required for GRP cilia assembly and the left–right asymmetry pattern. (A) GRP tissue of a membrane RFP-injected control embryo. Acetylated <α-tubulin labels cilia (green) and RFP labeled cell boundary (red). (B) GRP tissue of a Rfx2 morphant. Membrane-RFP is co-injected as a tracer. (C) GRP tissue of an embryo injected with Rfx2 morpholino and GFP-Rfx2 mRNA. (A′), (B′), (C′) are zoom in view of (A), (B), (C), respectively. (D) Average length of GRP cilia. While the average length of control GRP cilia is 8.59 ± 0.18 μm (mean ± SEM, n = 69), it's significantly reduced to 2.34 ± 0.07 μm in Rfx2 morphants (mean ± SEM, n = 113). The shorter cilia phenotype is partially rescued to 4.74 ± 0.14 μm (mean ± SEM, n = 63) by co-injecting with GFP-Rfx2 mRNA. Horizontal lines indicate the mean, vertical lines SEM, *** $p < 0.0001$ Mann–Whitney test. (E–F, I) *Pitx2c* expression at stage 26, lateral view. In control embryos, *Pitx2c* is expressed in the left LPM (black arrow) but not the right LPM (blue arrow). (G–H) In Rfx2 morphants, bilateral LPM of *Pitx2c* expression is observed. (blue arrow). (I) Quantification of *Pitx2c* expression patterns in control embryos and Rfx2 morphants.

NTD (Gray et al., 2009), and patients with the ciliopathic Meckel–Gruber syndrome also present with encephalocoel (Smith et al., 2006). We therefore further characterized the NTDs in our Rfx2 morphant embryos.

In most morphant embryos, the neural tube remained open even many stages after normal tube closure. In some cases, however, closure did eventually occur. Notably, when these embryos were examined in cross-section, we found that while dorsal epidermis had fused over the neural tissue, the neural epithelium had not fused into a tube (Supp. Fig. 3). As such, manipulations of Rfx2 are associated with both open and closed NTD in *Xenopus*.

We next asked whether neural tube closure phenotypes were correlated with ciliogenesis defects in Rfx2 morphants by visualizing cilia in the neural plate with acetylated α-tubulin immunostaining. At neurula stages, we observed small cilia throughout the neural plate (which is bilayered at this stage in *Xenopus*), and the length of these cilia was significantly reduced by the RFX2 MO (Figs. 2F–H). At later stages, longer cilia are visible, projecting into the neural tube lumen; these cilia were also fewer in number and shorter than in Rfx2 morphants as compared to controls (Supp. Fig. 4A–C). Together, these data demonstrate that Rfx2 is required for normal neural tube cilia formation and for neural tube closure.

Rfx2 is required for Sonic Hedgehog signaling in the developing central nervous system

Previous studies have shown that primary cilia in the neural tube are essential for Hedgehog signaling (Eggenschwiler and Anderson,

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2007; Goetz et al., 2009; Huangfu et al., 2003). We therefore examined whether Rfx2 morphants exhibit phenotypes consistent with disrupted Hedgehog signaling. Indeed, Rfx2 morphants developed with craniofacial defects including close-set eyes and cyclopia in some cases (data not shown). Moreover, the expression level of the Hedgehog target gene, *Nkx2.2*, in the ventral neural tube was dramatically downregulated in morphants, and the reduced expression could be rescued by co-injection of GFP-tagged Rfx2 mRNA. (Figs. 3A–C and Supp. Fig. 4D–H). The MO phenotype was specific to the ventral neural tissue, as *Sox3*, a marker of general neural tissue, was not affected (data now shown). Likewise, the expression of a Hedgehog-responsive gene in the brain, *Vax1*, was also strongly reduced in Rfx2 morphants and the reduced expression could be rescued (Figs. 3D–F). Together, these data suggest that Rfx2 is essential for Hedgehog signaling through regulation of primary cilia development in neural tissue.

Rfx2 is required for gastrocoel roof plate ciliogenesis and left/right patterning

Rfx2 mRNA is strongly expressed in the GRP by *in situ* hybridization (Figs. 1B′, C), where motile cilia generate a directional fluid flow that is required for left–right (LR) asymmetry (Blum et al., 2009). The GRP cilia are thus the functional equivalents of amniote nodal cilia and Kupffer's vesicle cilia in fishes (Essner et al., 2002; Schweickert et al., 2007). Using acetylated α-tubulin immunostaining, we observed GRP cilia of an average length of about 8.6 μm in wild type embryos (Figs. 4A, A′, D). However, GRP cilia in Rfx2 morphants were much shorter, displaying a 3.5-fold reduction in length as compared to controls (Figs. 4B, B′, D). This reduction in cilia length could be partially rescued by co-injecting GFP-tagged Rfx2 mRNA (Figs. 4C, C′, D), again demonstrating that the MO targets Rfx2 specifically.

Since GRP cilia are essential for LR asymmetry, we next examined whether LR asymmetry was disrupted in Rfx2 morphants by assessing expression for LR asymmetric marker genes. In control embryos, *Pitx2c* is expressed in the left lateral plate mesoderm (LPM), but not in the right side (Figs. 4E, F). However, right-sided, absent, or bilateral expression of *Pitx2c* in the LPM was frequently observed when Rfx2 was disrupted (Figs. 4G–I). Another marker of left–right asymmetry, *Lefty*, also showed randomized expression in Rfx2 morphants (Supp. Figs. 5A–F). In addition to the asymmetric expression of *Pitx2c* and *Lefty* in the LPM, the asymmetric looping of the gut was examined. In control embryos, the gut loops toward to the left. However, the guts fail to loop in Rfx2 morphants (Supp. Figs. 5G–H). Together, these data suggest that Rfx2 is essential for establishing LR asymmetry by regulating GRP ciliogenesis during vertebrate development.

Rfx2 is essential for ciliogenesis but not specification in multi-ciliated cells

The epidermal multi-ciliated cells of *Xenopus* have been shown recently to share many molecular similarities with mammalian multi-ciliated cells. Given the role for Rfx3 in both specification and ciliogenesis in multi-ciliated cells of the mammalian brain (Baas et al., 2006), we hypothesized that the very early expression of Rfx2 in epidermal multi-ciliated cells may reflect a role in their early specification. However, when we targeted delivery of Rfx2 morpholino into the epidermis, we observed no reduction in the number of α-tubulin-expressing cells in Rfx2 morphants at stage 20 (Figs. 6A–B), indicating that ciliated cell specification is unaffected in Rfx2 morphants. We did, however, consistently observe that most multi-ciliated cells in Rfx2 morphants have only a few short axonemes (Figs. 5B, B′), as

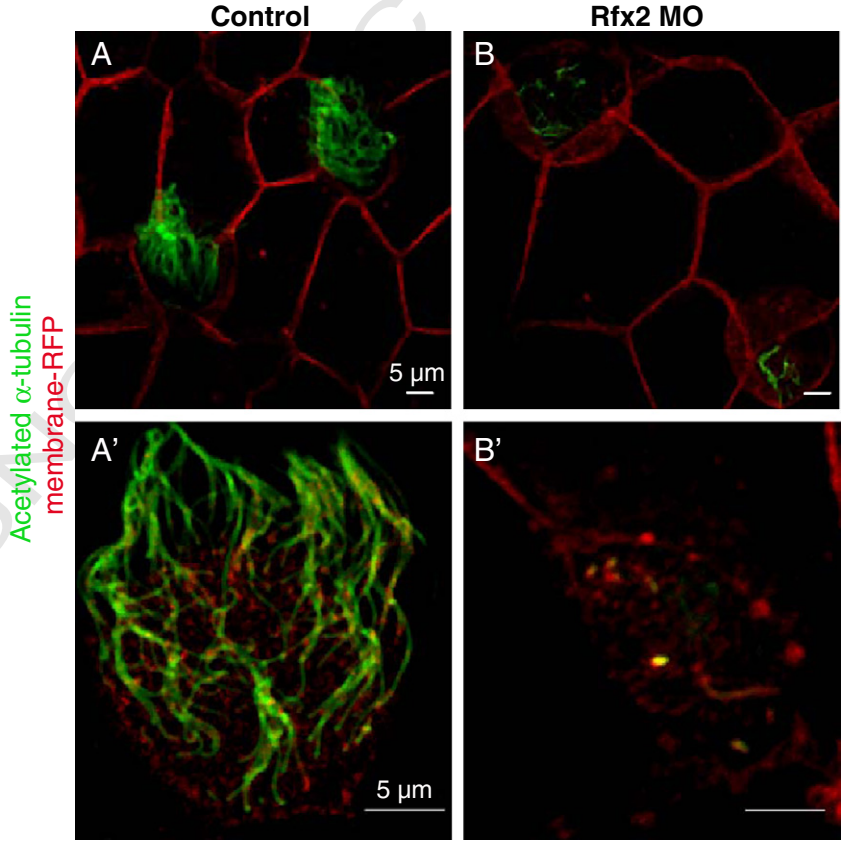

Fig. 5. Rfx2 is essential for epidermal ciliogenesis. (A) A stage 27 control embryo injected with membrane-RFP. Acetylated α-tubulin labels cilia (green) and RFP labels cell boundary (red). (A′) Zoom in view of (A). (B) A stage 27 Rfx2 morphant. (B′) Zoom in view of (B). Note that only a few short axonemes are shown in the Rfx morphant.

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compared to control epidermis, where dozens of long cilia are assembled on multi-ciliated cells (Figs. 5A, A′). Together, these data suggest that Rfx2 is not required for multi-ciliated cell specification, but Rfx2 is essential for motile cilia assembly in these cells.

Rfx2 is required for expression of the ciliogenic gene TTC25

In *C. elegans*, the DAF19 transcription factor is a major regulator of ciliogenesis, controlling the expression of the many essential genes required for making cilia (Swoboda et al., 2000). We therefore hypothesized that Rfx2 would be required for the expression of many, if not all, ciliogenic genes. We previously identified a large set of genes expressed in ciliated cells by a high throughput *in situ* hybridization screen (Hayes et al., 2007). We examined expression of several of these genes by RT-PCR in epidermal animal cap explants upon MO depletion of Rfx2. Expression of some, but not all, of these genes required Rfx2 (Supp. Fig. 6). Based on the expression pattern of *Rfx3*, we propose that it may play partially overlapping roles in epidermal ciliogenesis.

In our animal cap assays, we found that the PCP effector protein Fritz (aka: WDPCP: (Kim et al., 2010)) was very strongly downregulated in Rfx2 morphants, while a multi-ciliated cell-specific α-tubulin was totally unaffected (Supp. Fig. 6). Other RFX factors control expression of genes encoding retrograde IFT proteins, and we observe a similar result. Also downregulated in Rfx2 morphants was *TTC25* (Supp. Fig. 6), which we have previously shown localizes to cilia axonemes and is required for epidermal ciliogenesis and proper neural tube closure (Hayes et al., 2007). Because so little is known about this protein, we selected TTC25 for more in-depth analysis.

First, we further examined TTC25 expression by *in situ* hybridization. Like Rfx2, TTC25 is expressed in the neural tube, epidermal ciliated cells, GRP, otic vesicle, and kidneys (Supp. Fig. 7). In Rfx2

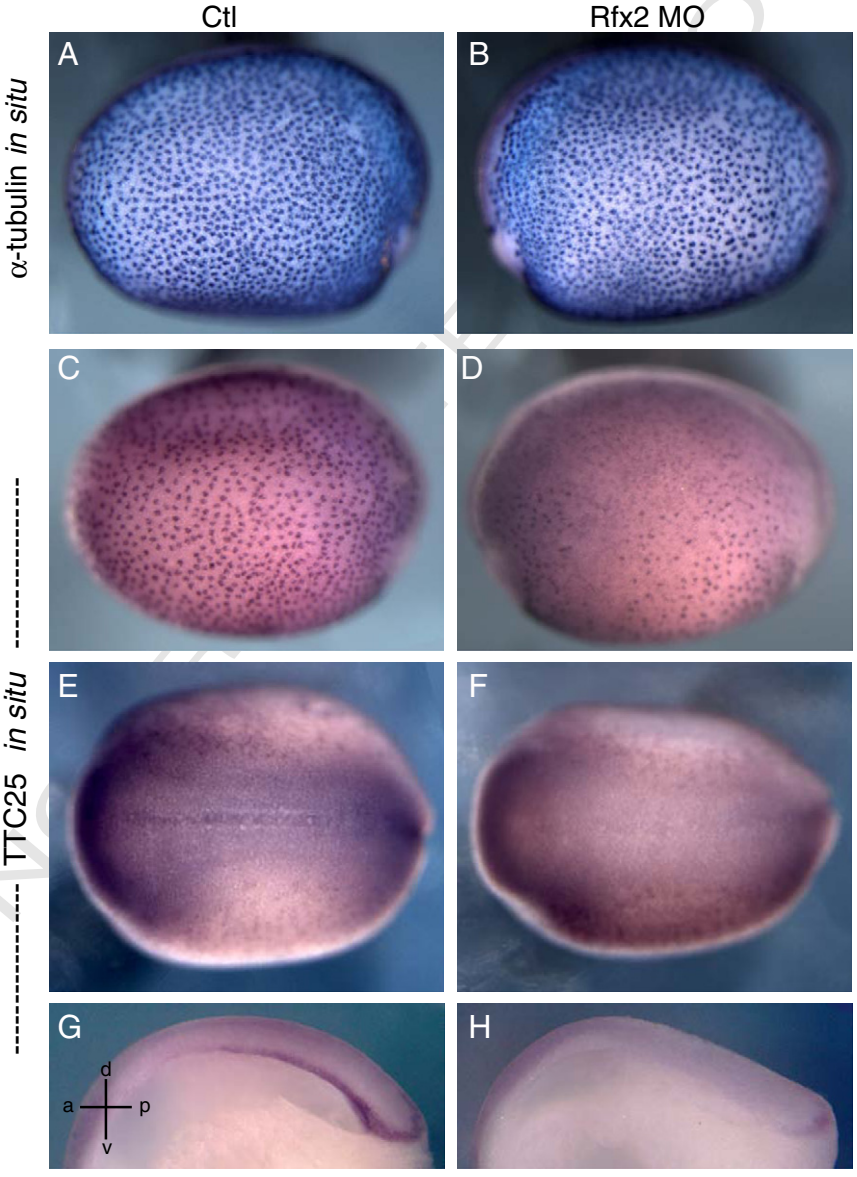

Fig. 6. Rfx2 is required for the expression of a ciliary gene *TTC25*, but not α-*tubulin*. (A)–(D) Rfx2 morpholino is injected into one ventral blastomere at the 4 cell stage. (A) α-*tubulin in situ* hybridization on the control side of epidermis. (B) α-*tubulin in situ* hybridization on the Rfx2 morpholino-injected side. α-*tubulin* expression is not significantly changed (also see suppl. Fig. 4). (C) *TTC25* expression patterns on control epidermis. (D) *TTC25 in situ* hybridization on Rfx2 morpholino-injected side of epidermis. *TTC25* expression is reduced in epidermal multi-ciliated cells. (E)–(H) Rfx2 morpholino is injected into both dorsal blastomeres at the 4 cell stage. (E) Dorsal view of *TTC25* expression on neural plate of a control embryo. (F) Dorsal view of *TTC25* expression on neural plate of an Rfx2 morphant. *TTC25* expression is reduced. (G) Sagittal section view of a control embryo. *TTC25* is expressed in the gastrocoel roof plate (GRP). (H) Sagittal section view of a Rfx2 morphant. *TTC25* expression is reduced in GRP.

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morphants, TTC25 expression is reduced in the neural plate, GRP, and ciliated epidermis (Figs. 6C–H), suggesting that Rfx2 controls expression of TTC25 in these tissues. At the same stage, Rfx2 morphants retain α-tubulin expression in the ciliated epidermis, showing that ciliated cell differentiation is unaffected, and that α-tubulin is not a transcriptional target of Rfx2 (Figs. 6A, B; Supp. Fig. 6).

We showed previously that TTC25 morphants, like Rfx2 morphants, have open rostral neural tubes, a consequence of cilia dysfunction in the neural tube, and that cilia were shorter and less dense in multi-ciliated cells of the epidermis (Hayes et al., 2007). When we examined Hh target genes, we found that *Nkx2.2* in the ventral neural tube was significantly reduced (Figs. 7A–B), while *Vax1b* was not (Figs. 7C–D), consistent with a lack of TTC25 expression in the eye (Supp. Fig. 7E). These data suggest that TTC25 is required for normal assembly of neural tube cilia. However, when we examined neural tube cilia in TTC25 morphants by acetylated α- tubulin staining, we observed a more specific phenotype than the consistent loss of cilia observed in Rfx2 morphants (Figs. 7E–G). At wild type tailbud stages, primary cilia mostly project from the apical surface of cells into the lumen of the neural tube and ventral cilia are much longer compared to dorsal cilia (Fig. 7E). In TTC25 morphants, however, we found that primary cilia are quite short throughout the neural tube (Figs. 7F, G). We could consistently visualize Arl13b protein in these short cilia (Fig. 7F), indicating that at least some proteins are

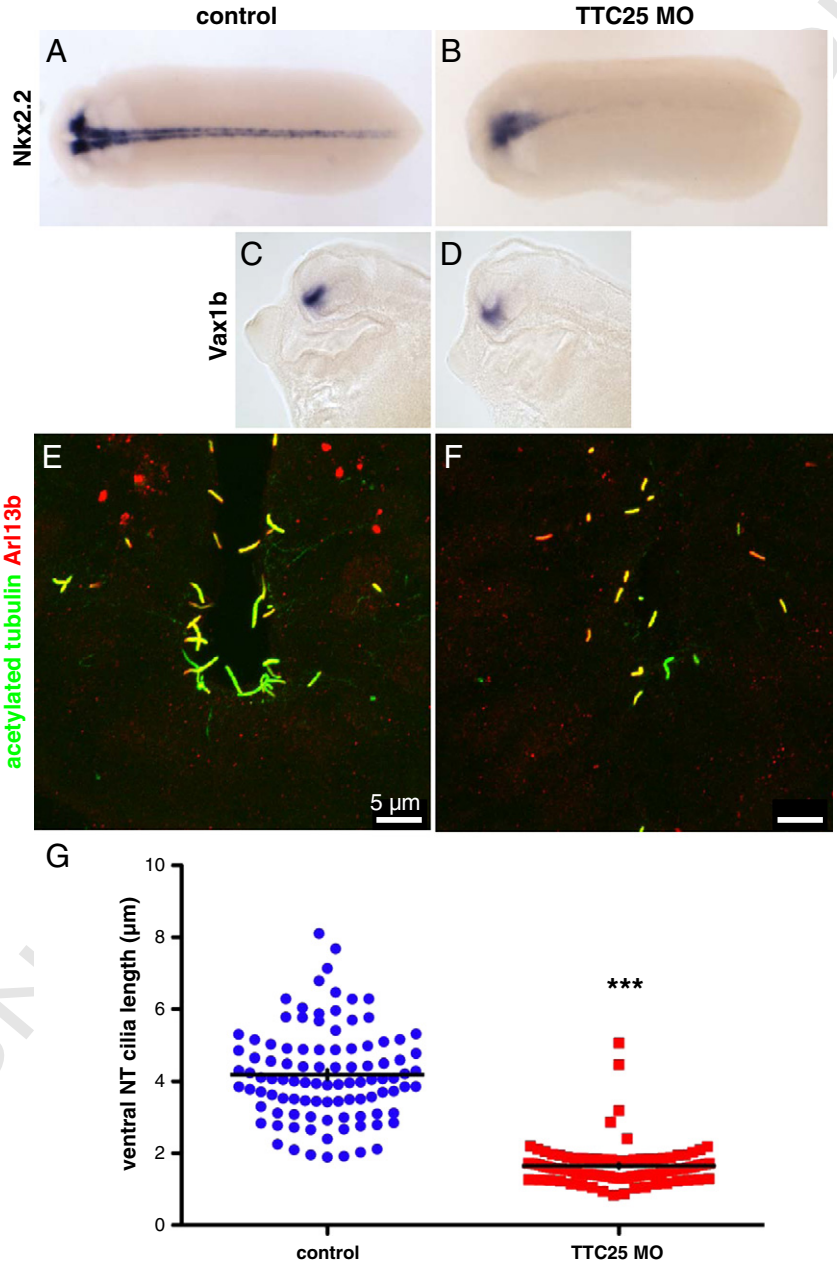

Fig. 7. TTC25 is required for proper neural tube cilia formation and Shh signaling pathway. (A–B) *Nkx2.2* expression at stage 24, dorsal view, anterior left. Expression of *Nkx2.2* in the ventral neural tube (A) is reduced in the spinal cord of TTC25 morphants (B). (C–D) *Vax1b* expression at stage 35, lateral view, anterior left. Expression of *Vax1b* in the ventral forebrain (C) is reduced, but still present, in TTC25 morphants (D). (E–F) Confocal Z-projections of stage 24 neural tube sections stained for cilia markers acetylated α-tubulin (green) and Arl13b (red). (E) In control embryos, most cilia project into the lumen of the neural tube; ventral neural cilia are longer than dorsal ones and express less Arl13b. (F) In TTC25 morphants, the neural tube fails to close and cilia are not found at the luminal boundary (top). Cilia are fewer, shorter, and stain more intensely with Arl13b. (G) Ventral cilia were measured in 3 sections (as in E, F) from 5 embryos each. The 20 longest ventral cilia from each embryo are plotted. Horizontal lines indicate the mean, vertical lines SEM, $p<0.0001$ Mann–Whitney test.

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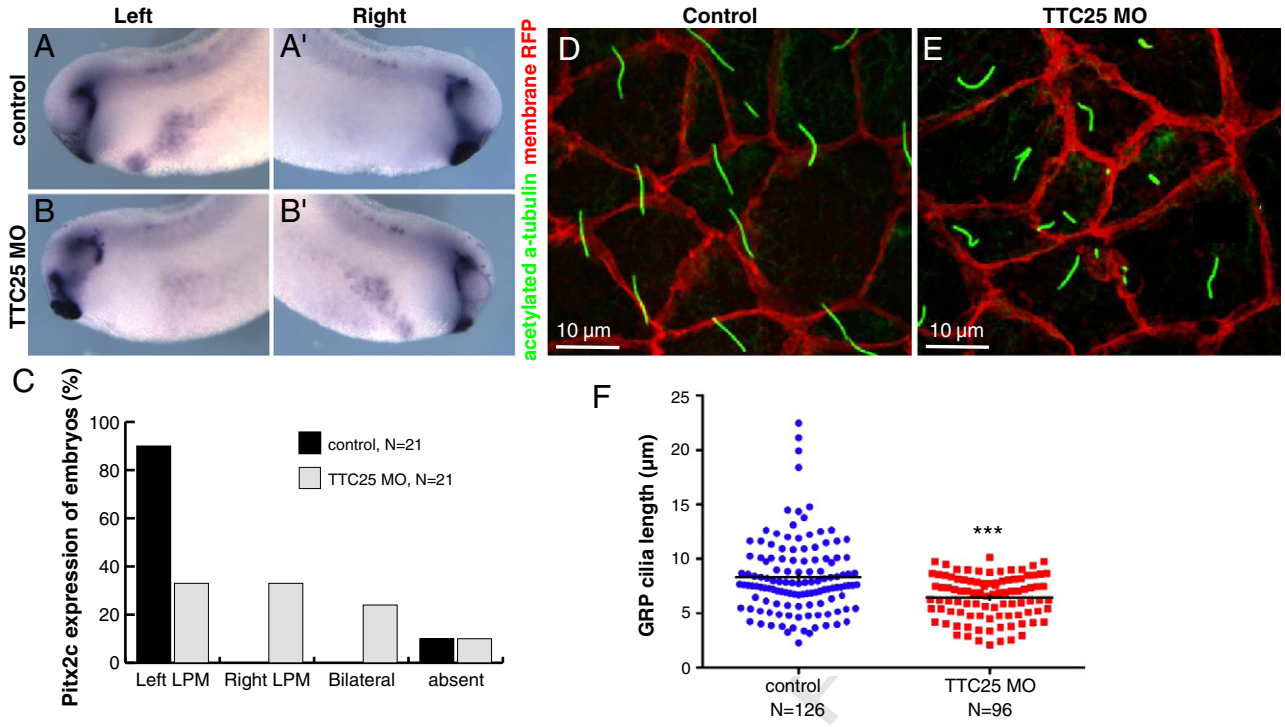

Fig. 8. Rfx2 is required for GRP cilia assembly and the left–right asymmetry. (A–B) *Pitx2c* expression at stage 26, lateral view. In wildtype embryos, Pitx2c is expressed in the left LPM (A, A′, C). In TTC25 morphants, only 33% of embryos show normal left LPM expression (C), and Pitx2c is often expressed in the right LPM (B′, C). (D–E) Confocal images of the posterior GRP (node) stained with acetylated α-tubulin to visualize cilia and injected with membrane-RFP to outline cells. (F) GRP cilia were measured in confocal images as in D, E for 5 embryos each. Horizontal lines indicate the mean, vertical lines SEM, $p<0.0001$ Mann–Whitney test.

able to traffic to cilia normally in TTC25 morphants. Our data suggest that while TTC25 is required for cilia lengthening and Shh signal transduction through cilia, it is not broadly required for the initiation of ciliogenesis in the neural tube.

We next looked at the effect of TTC25 knockdown on left–right asymmetry. Like Rfx2 morphants, TTC25 morphants showed defects in establishment of LR asymmetry, assayed by expression of *Pitx2c* (Figs. 8A–B). We observed a high percentage of TTC25 morphant embryos with situs inversus and symmetric *Pitx2c* expression (Fig. 8C). When we examined GRP cilia structure by acetylated α-tubulin staining, we found a significant shortening of GRP cilia in TTC25 morphants (Figs. 8D–F), though this phenotype was less severe than that of Rfx2 morphants. Again, these data are consistent with TTC25 being required for nodal cilia extension and/or function, but not for initiation of ciliogenesis.

Conclusions

While the protein machinery controlling ciliogenesis has been extensively studied, the transcriptional control of cilia formation remains far more poorly understood (Gherman et al., 2006; Inglis et al., 2006; Thomas et al., 2010). FoxJ1 has been well-characterized as a regulator of motile ciliogenesis (Cruz et al., 2010; Stubbs et al., 2008; Yu et al., 2008); Rfx4 has been shown to govern the growth of primary cilia; and Rfx3 has been shown to regulate the formation of both primary and motile cilia (Ait-Lounis et al., 2007; Ashique et al., 2009; Baas et al., 2006; Bonnafe et al., 2004; El Zein et al., 2009). Here, we report that another Rfx gene, Rfx2, is expressed in ciliated tissues of the body and is required for proper development of primary cilia in the neural tube, motile cilia in the node, and motile cilia on epidermal multi-ciliated cells in *Xenopus*. We find that defects in ciliogenesis following Rfx2 knockdown are associated with developmental defects in neural tube patterning and morphogenesis as well as left–right asymmetry. Rfx2 has also recently been shown to be required for ciliogenesis and left–right patterning in the zebrafish (Bisgrove et al., in press).

These data thus establish Rfx2 as a critical and broadly employed regulator of vertebrate ciliogenesis. We note that Rfx2 and Rfx3 have almost identical expression patterns in most tissues, and moreover, Rfx4 and Rfx5 overlap with specific regions of the Rfx2 and Rfx3 patterns. These overlapping patterns of expression are especially noteworthy because RFX2, RFX3, and RFX4 can heterodimerize (Iwama et al., 1999; Morotomi-Yano et al., 2002; Reith et al., 1994), but the extent to which these proteins cooperate *in vivo* remains entirely unknown. We therefore suggest that further studies of the Rfx transcription factors will be crucial for an understanding of the developmental control of ciliogenesis.

Uncited reference

Smith et al., 2010a

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ydbio.2011.12.029.

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