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# RFX7 is required for the formation of cilia in the neural tube

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ABSTRACT

Regulatory Factor X (RFX) transcription factors are important for development and are likely involved in the pathogenesis of serious human diseases including ciliopathies. While seven *RFX* genes have been identified in vertebrates and several RFX transcription factors have been reported to be regulators of ciliogenesis, the role of RFX7 in development including ciliogenesis is not known. Here we show that *RFX7* in *Xenopus laevis* is expressed in the neural tube, eye, otic vesicles, and somites. Knockdown of RFX7 in *Xenopus* embryos resulted in a defect of ciliogenesis in the neural tube and failure of neural tube closure. RFX7 controlled the formation of cilia by regulating the expression of *RFX4* gene, which has been reported to be required for ciliogenesis in the neural tube. Moreover, ectopic expression of Foxj1, which is a master regulator of motile cilia formation, suppressed the expression of RFX4 but not RFX7. Taken together, RFX7 plays an important role in the process of neural tube closure at the top of the molecular cascade which controls ciliogenesis in the neural tube.



## 1. Introduction

Cilia are cellular organelles that are present on the surface of most vertebrate cells and play crucial roles in physiological and developmental processes (Eggenschwiler and Anderson, 2007; Ishikawa and Marshall, 2011; Pedersen et al., 2008). There are two major types of cilia. One is motile cilia that generate extracellular fluid flows such as mucus flow, cerebrospinal-fluid flow, and leftward flow in the node (Roy, 2009). The second type of cilia, called primary cilia, sense extracellular signals such as growth factors and hormones (Berbari et al., 2009; Gerdes et al., 2009; Goetz and Anderson, 2010; Singla and Reiter, 2006). Substantial studies have revealed that disorders in the formation or the function of cilia result in a wide range of human diseases such as primary ciliary dyskinesia, polycystic kidney disease, Joubert syndrome, Bardet–Biedl syndrome as well as Meckel–Gruber syndrome (Baker and Beales, 2009; Hildebrandt et al., 2011; Mougou-Zerelli et al., 2009; Sattar and Gleeson, 2011; Zariwala et al., 2007). Particularly, Joubert syndrome and Meckel–Gruber syndrome have been reported to be associated with neural tube defects (NTDs) (Delous et al., 2007; Lee et al., 2012). Mice studies have also shown that mutations in genes required for ciliogenesis, such as *C2cd3*, *Fuz*, *Ift122*, *Intu* as well as *Inpp5e*, result in NTDs (Harris and Juriloff, 2010). NTDs are a set of major congenital malformations that are caused by disrupting closure of the neural tube, where neural progenitors are known to have primary cilia (Bay and Caspary, 2012). NTDs divide into two forms, open and closed forms (Katsanis, 2006; Murdoch and Copp, 2010; Robinson et al., 2012). Open forms are seen in the cranial region with anencephaly and in the spinal region with spinal bifida. The entire craniospinal axis can be

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involved in cases of craniorachischisis. Closed forms can present with more subtle phenotypic changes that are skin covered, including encephalocele, meningocele, and spina bifida occulta. While genetic evidence indicates that the function of cilia in the neural tube is crucial for neural tube closure, the role of cilia during neural tube closure is not completely understood.

Cilia are dynamic structures that can form and resorb throughout development. For example, multiple cilia of the cerebral ventricles grow at precise developmental stages (Spassky et al., 2005). Epithelial cells in the cerebral ventricles first harbor primary cilia and switch to multiple ciliary growth under a specific developmental program. Another example is that mouse tracheal epithelial cells in primary cultures differentiate into multi-ciliated cells by exposure to an air–liquid interface (You et al., 2002), indicating that airway epithelial cells can be differentiated by various tracheal conditions during lung development. This differentiation is induced by the function of Multicilin, the *Xenopus* ortholog of human IDAS, which coordinately promotes cell-cycle exit, deuterosome-mediated centriole assembly and the gene expression required for motile ciliogenesis (Stubbs et al., 2012). Therefore, organisms must have developed regulatory mechanisms to induce the assembly of specific cilia subtypes in a temporal and spatial manner. Transcriptional regulation of ciliary gene expression is likely one of these mechanisms. In fact, genes encoding ciliary proteins in *Patella vulgata* are dynamically expressed during development of ciliated tissues and inhibition of transcription impaired cilia assembly (Damen et al., 1994). Recently, several transcription factors, which control the transcription of ciliary genes and are involved in ciliogenesis in vertebrates, have been identified; the RFX (Ashique et al., 2009; Bonnafe et al., 2004; Chung et al., 2012; Laurencon et al., 2007; Liu et al., 2007; Swoboda et al., 2000) and Foxj1 (forkhead box j1) (Brody et al., 2000; Jacquet et al., 2009; Stubbs et al., 2008; Yu et al., 2008) transcription factors. While Foxj1 is a master regulator of motile ciliogenic program in vertebrates (Stubbs et al., 2008; Yu et al., 2008), RFX transcription factors are involved in both primary and motile cilia formation. In vertebrates, seven *RFX* genes have been identified based on a highly conserved DNA binding domain that belongs to the winged-helix family of transcription factors (Aftab et al., 2008; Emery et al., 1996; Gajiwala et al., 2000). While only one RFX transcription factor, known as Daf19 in *Caenorhabditis elegans*, has been identified and reported to be a central regulator of ciliogenesis in *C. elegans* as well as *Drosophila* (Dubruille et al., 2002; Swoboda et al., 2000), it has been reported that not all RFX transcription factors are crucial for ciliogenesis in vertebrates. RFX1, RFX5 and RFX6 do not appear to be essential for ciliogenesis (Reith and Mach, 2001; Smith et al., 2010; Soyer et al., 2010; Steimle et al., 1995; Zhao et al., 2010), whereas RFX2 and RFX3 are more broadly required for the proper development of cilia (Bonnafe et al., 2004; Chung et al., 2012). Importantly, RFX4 has been reported to be a key regulator required for the development of cilia only in the neural tube that are critical in modulating Sonic Hedgehog (Shh) signaling (Ashique et al., 2009). Although RFX7 is ubiquitously and highly expressed in nearly all human tissues examined, with the highest expression in the brain (Aftab et al., 2008), very little is known about the role of RFX7 in ciliogenesis.

Here we report that RFX7 is highly expressed in the central nervous system of *X. laevis* embryos, including the neural tube and eyes. Knockdown of RFX7 resulted in defects of cilia formation and neural tube closure. In addition, *X. laevis* RFX4 was also essential for ciliogenesis and neural tube closure, as previously shown in a mouse model (Ashique et al., 2009). Importantly, RFX7 controls the expression of *RFX4* in the neural plate, and the failure of neural tube closure induced by knockdown of RFX7 was rescued by co-injection with RFX4 RNA. In addition, ectopic expression of Foxj1 suppressed the expression of *RFX4* but not *RFX7* in the neural plate. These data indicate that the role of RFX7 is upstream of RFX4 in the molecular cascade of cilia formation at the neural tube and is not affected by Foxj1, demonstrating that RFX7 plays a key role in neural tube ciliogenesis.

## 2. Results

### 2.1. *RFX7 is essential for ciliogenesis in the neural tube*

To test the possibility of a role for RFX7 in the formation of cilia, we first examined the spatial and temporal expression profile of the *RFX7* gene in *X. laevis* embryos. The *RFX7* gene was maternally expressed and continued to be expressed throughout embryogenesis (Fig. 1A, B). We observed that the *RFX7* gene was expressed on the dorsal side of the ectoderm at stage 10 (gastrula) and in the neural plate at stage 14 (neurula) (Fig. 1A). In addition, *RFX7* is barely detected in the gastrocoel roof plate (GRP), where motile cilia exist during the neurula stage (Schweickert et al., 2007), at stage 14 (Fig. 1C). Then, the expression of *RFX7* was detected in the brain, spinal cord, eyes, otic vesicles, as well as the somites at the tailbud stage (Fig. 1B). *RFX7* is highly expressed in the nervous system including the neural tube, suggesting RFX7 may be important for neural tube formation in *Xenopus* embryos.

Since some vertebrate RFX transcription factors are necessary for ciliogenesis (Ashique et al., 2009; Bonnafe et al., 2004; Chung et al., 2012), we hypothesized that RFX7 is required for ciliogenesis in the neural tube. Various genes related to ciliogenesis are required for neural tube closure and some ciliopathies such as Joubert and Meckel–Gruber syndromes are associated with NTD (Murdoch and Copp, 2010; Vogel et al., 2012). Interestingly, several Bardet–Biedl syndrome (BBS) proteins that are localized to primary cilia and basal bodies (Ansley et al., 2003) cooperate with non-canonical Wnt signaling in planar cell polarity (PCP) (Ross, 2005) that is essential for convergent extension movements of cells (Elul et al., 1997; Keller et al., 1992; Wallingford et al., 2001). In fact, the BBS proteins are required for proper convergent extension movements (Gerdes et al., 2007). Since the failure of convergent extension movements of midline cells in the neural plate resulted in NTDs (Wallingford and Harland, 2002), primary cilia in the neural tube may be necessary for convergent extension movements to close the neural tube. To determine the role of RFX7 in neural tube ciliogenesis, we tested the effect of RFX7 knockdown in neural tube closure. Since the 5′