

REVIEW

Switching on cilia: transcriptional networks regulating ciliogenesis

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ABSTRACT

Cilia play many essential roles in fluid transport and cellular locomotion, and as sensory hubs for a variety of signal transduction pathways. Despite having a conserved basic morphology, cilia vary extensively in their shapes and sizes, ultrastructural details, numbers per cell, motility patterns and sensory capabilities. Emerging evidence indicates that this diversity, which is intimately linked to the different functions that cilia perform, is in large part programmed at the transcriptional level. Here, we review our understanding of the transcriptional control of ciliary biogenesis, highlighting the activities of FOXJ1 and the RFX family of transcriptional regulators. In addition, we examine how a number of signaling pathways, and lineage and cell fate determinants can induce and modulate ciliogenic programs to bring about the differentiation of distinct cilia types.

KEY WORDS: Cilia, Ciliogenesis, FOXJ1, Motile cilia, RFX, Transcriptional regulation

Introduction

Cilia and flagella are hair-like cellular projections that have a unique place in the history of cell biology. Identified by Antonie van Leeuwenhoek in 1676, they were the first organelles to be discovered. We now know that these ‘incredibly thin feet, or little legs’, as Leeuwenhoek originally described them, are widely distributed throughout the eukaryotic kingdom (Satir, 1995). The filamentous plasma membrane-bound microtubule core of the cilium, or the axoneme, is an extension of the basal body, a derivative of the mother centriole that anchors the cilium to the apical surface of the cell. Typically, the axoneme is made up of nine radially arranged microtubule doublets with or without a central pair of singlet microtubules – the 9+2 or the 9+0 configurations. The axoneme is built from the basal body by a dedicated kinesin and dynein motor-based transport process called intraflagellar transport (IFT). Although the fundamental design of the cilium and the IFT-dependent assembly process are quite highly conserved (reviewed by Garcia-Gonzalo and Reiter, 2012; Ishikawa and Marshall, 2011), many distinct types of cilia exist in metazoans. Each class of cilia is inextricably linked and highly adapted to a biological function, which can range from fluid movement during left-right patterning of the vertebrate body axis and signal transduction in vision and olfaction, to pathogen clearance from airways, and fertility and reproduction (Fig. 1).

The importance of producing and maintaining properly differentiated cilia during embryonic development and in adult

physiology is best underscored by the large number of human diseases, the ciliopathies (see Box 1), that arise from ciliary dysfunction (reviewed by Hildebrandt et al., 2011). A key step in understanding ciliary biology, and thus the etiology of ciliopathies, is to identify the various components that participate in the generation and function of these organelles. Over the years, a variety of strategies have been used to determine the genes and proteins required in different kinds of cilia (Arnaiz et al., 2009; Gherman et al., 2006; Inglis et al., 2006). These screens have revealed that cilia are complex organelles, with hundreds (if not thousands) of components involved in their assembly, structure and function, the expression of which must be precisely coordinated during cilia formation. In this Review, we focus on how this coordination is achieved and analyze what is presently known about the mechanism by which ciliogenesis is programmed at the transcriptional level. First, we provide an overview of the different types of cilia that can be found, with examples from the vertebrate perspective. We then discuss the major transcriptional regulators that have been linked to ciliogenesis, and the cohorts of genes that are regulated by these proteins.

Diverse cilia types perform various roles in development and physiology

Traditionally, cilia have been classified as either motile or immotile. However, within this simplistic categorization, we need to accommodate the numerous subtypes of cilia that have now been recognized in different organisms (Fig. 2) (Silverman and Leroux, 2009; Takeda and Narita, 2012).

The first category of cilia are the motile cilia. These cilia are usually long, have the classical 9+2 organization of microtubules, and possess dynein arms that use energy from ATP hydrolysis to drive rhythmic movement of the axonemes. Motile cilia can also contain additional protein complexes that are essential for motility, such as the nexin-dynein regulatory complex (N-DRC), which regulates the activity of the dynein arms (reviewed by Lindemann and Lesich, 2010). There are several different types of motile cilia, including motile monocilia (i.e. those existing as a single cilium per cell), such as the prototypical flagella on protozoans and sperm cells, or cilia on the proximal and distal regions of the developing pronephric kidney tubules in the zebrafish embryo. These cilia generally beat in a wavelike or corkscrew fashion in order to generate cellular locomotion or fluid movement (reviewed by Inaba, 2011; Kramer-Zucker et al., 2005). Another type of motile monocilia is found in cells of the organ of laterality in various vertebrate species – the ventral node in mammals, the gastrocoel roof plate (GRP) in frogs and Kupffer’s vesicle (KV) in teleost fishes. In the mouse and the medaka fish, these cilia mostly display the 9+0 configuration, whereas in other organisms, such as the zebrafish, they display the 9+2 structure. Irrespective of their configuration, these cilia move in a rotational manner, and establish a leftward-directed fluid flow within the cavity of the node, GRP or KV (reviewed by Babu and Roy, 2013). The final type of motile cilia is the multiple motile cilia (i.e. those present as more than one cilium per cell) that are designed to move fluid of high viscosity. For

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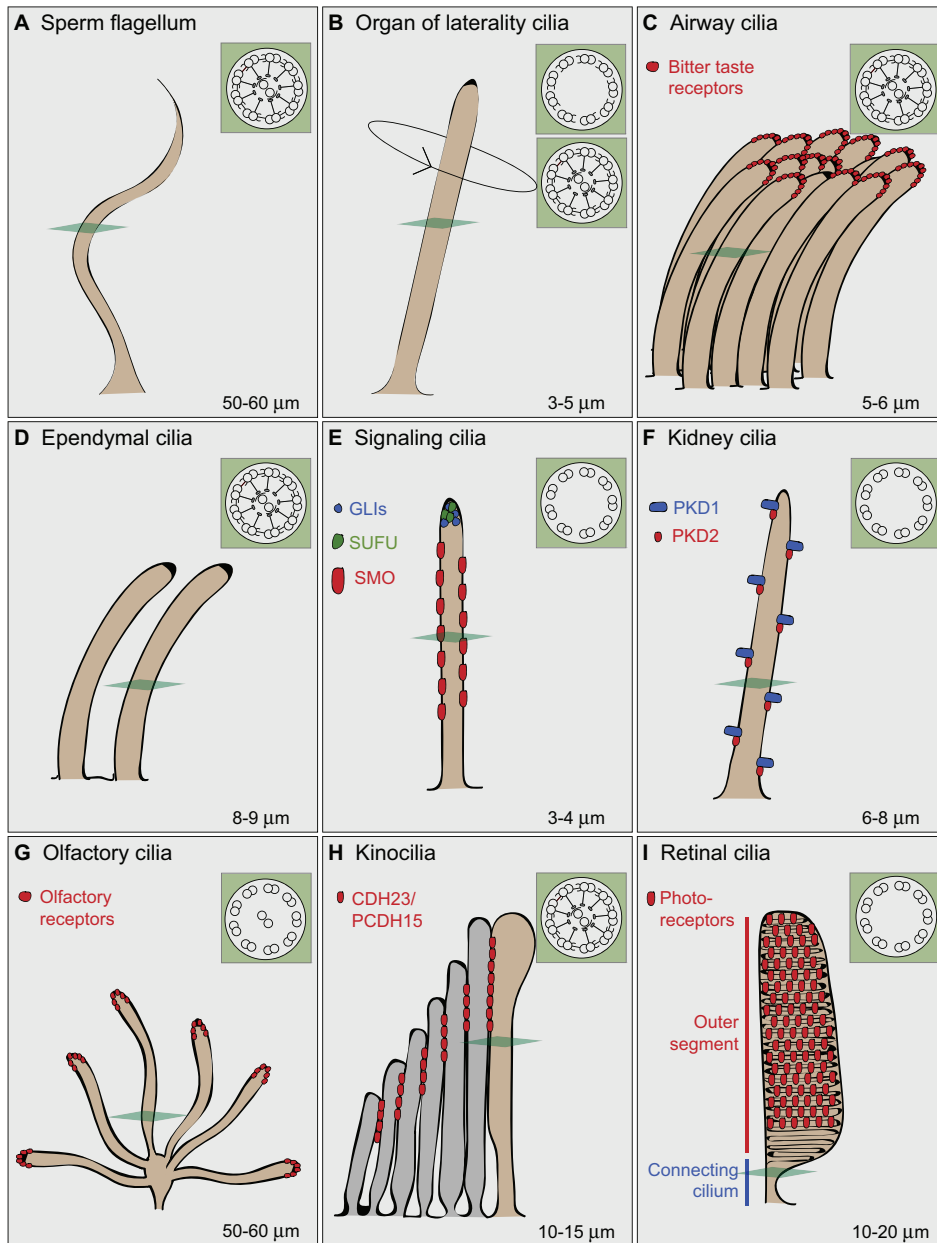


Fig. 1. Diversity of cilia types in vertebrates. Examples of different mammalian cilia are given. The numbers of cilia drawn indicate how many cilia are present per cell (one or many), whereas the average lengths of the cilia found in humans, mice or rats is given in the bottom right of each panel. The insets depict the ultrastructure of a transverse section of the cilium (position of sections indicated in green). Key proteins or receptors that localize to cilia are illustrated. References are given in the text. (A) The sperm flagellum moves with a whip-like motion. (B) Motile nodal cilia, by contrast, move in a vortical manner to establish left-right asymmetry. (C) Bitter taste receptors localize to human airway cilia. (D) Biciliated ependymal cells function to circulate CSF in the spinal canal. (E) Components of the hedgehog signaling pathway, including GLI proteins, SUFU (suppressor of fused homolog) and SMO (smoothed), localize to solitary signaling cilia. (F) By contrast, mechanosensory proteins, such as PKD (polycystic kidney disease) 1 and PKD2, localize to renal cilia to sense urine flow. (G) Olfactory neurons localize olfactory receptors to the distal ends of their cilia in order to sense odorant molecules. (H) The kinocilium serves to polarize the actin-based stereocilia (gray) during development of auditory hair cells. CDH23, cadherin 23; PCDH15, protocadherin 15. (I) Retinal cells have a specialized connecting cilium that gives way to the outer segment – a membrane-dense protrusion packed with photoreceptor molecules. The average length of both the connecting cilium and the outer segment is given in each panel.

example, epithelial cells of the respiratory tract and ependymal cells of the central nervous system of mammals possess anywhere between two and hundreds of motile cilia on their surface. These cilia have a 9+2 microtubule configuration and beat with a metachronal planar stroke to clear mucus in the airways or circulate cerebrospinal fluid within the brain and spinal cord (reviewed by Del Bigio, 2010; Satir and Sleight, 1990). Although the function of motile cilia is principally mechanical, i.e. fluid movement or cellular locomotion, they can also exhibit an array of sensory functions (reviewed by Bloodgood, 2010).

In contrast to the motile cilia, immotile cilia (also called sensory or primary cilia) are generally short and lack motility components, but are specialized morphologically and molecularly in order to sense fluid flow, light, odorants or signaling molecules. Perhaps the most rudimentary and yet the most intensely studied immotile cilia are the solitary signaling cilia found on most quiescent or post-mitotic cells within the vertebrate body. These cilia have a 9+0 microtubule configuration and are used for signal transduction

by a number of important developmental morphogens, notably by those of the hedgehog (HH) family (reviewed by Goetz and Anderson, 2010). Another type of cilia, which fall under the immotile cilia classification and possess a 9+0 microtubule configuration, are the monocilia which extend from epithelial cells lining the mammalian kidney tubules. These cilia project into the tubular lumen, and have a mechanosensory role in perceiving urine flow (reviewed by Praetorius and Leipziger, 2013). Similar flow-sensing cilia decorate the periphery of the mammalian node and are thought to sense the leftward fluid flow generated by motile cilia within the node cavity (reviewed by Babu and Roy, 2013). Immotile cilia are also an essential part of the sensory apparatus of the nose, eyes and ears. Olfactory sensory neurons extend processes called dendritic knobs from the olfactory epithelium, with 10-30 sensory cilia from each of these knobs reaching into the mucosal layer. Localized onto these cilia are odorant receptors, together with all of the downstream signaling machinery necessary for odor detection. Although olfactory cilia have a 9+2

Box 1. Ciliopathies

Ciliopathies are a collection of human disorders that are directly caused by defects in cilia formation or function. Defective immotile cilia cause pleiotropic and highly variable abnormalities, consistent with the extensive distribution of immotile cilia and their wide-ranging functions. Individuals suffering from immotile ciliopathies exhibit combinations of kidney and liver defects (including cysts), obesity, central nervous system defects that can lead to mental retardation, as well as a variety of patterning defects, including abnormalities in limb length, digit number (polydactyly), left-right axis organization (*situs inversus*) and craniofacial patterning. Abnormalities specific to the photoreceptor-connecting cilium can also lead to retinal degeneration and blindness. Examples of immotile ciliopathies include nephronophthisis (NPHP), Senior-Loken syndrome (SLS), Joubert syndrome (JBTS), Bardet-Biedl syndrome (BBS), Meckel-Gruber syndrome (MKS) and orofacialdigital syndrome (OFD) (reviewed by Hildebrandt et al., 2011; Waters and Beales, 2011).

Dysfunction of the motile cilia causes a distinct set of phenotypes that, in humans, is referred to as primary ciliary dyskinesia (PCD). Symptoms of the disease are apparent in cells and tissues that differentiate motile cilia. Poor mucociliary clearance caused by dysfunctional airway cilia leads to chronic infections, sinusitis and rhinitis, which can result in widening of the airways and lung collapse (bronchiectasis and atelectasis, respectively). Lack of motility of sperm flagella and motile cilia in the oviducts can lead to infertility, whereas dysmotility of cilia in the node leads to left-right patterning defects (*situs inversus*, also known as Kartagener's syndrome). In some rare cases, defects in ependymal motile cilia of the CNS can lead to swelling of the brain ventricles or to hydrocephalus (Afzelius, 1976) (reviewed by Boon et al., 2013).

microtubule configuration, in mammals they lack the dynein arms that are necessary for motility (reviewed by Jenkins et al., 2009). Sensory neurons of the retinal photoreceptors also extend short dendrites possessing immotile cilia, which have a very distinct morphology. The connecting region of these cilia generally has a 9+0 microtubule configuration, whereas the distal region, termed the outer segment, contains stacks of ciliary membrane densely packed with light- or color-sensitive opsins (reviewed by Insinna and Besharse, 2008). Within the inner ear, mechanosensory hair cells also possess a single 9+2 cilium. Although this cilium has historically been called a kinocilium ('kino' meaning moving picture), this seems to be a misnomer, because this cilium is immotile. The kinocilium is a transient organelle that plays a crucial role in generating the accurate plane polarized arrangement of the stereocilia – bundles of actin-based microvilli that sense sound vibrations and linear acceleration for hearing and balance (reviewed by Schwander et al., 2010).

RFX transcription factors and their links to ciliogenesis

In recent years, several members of the regulatory factor X (RFX) family of transcription factors have been shown to be required for directing the expression of core components of all types of cilia. All RFX factors share a peculiar winged-helix DNA-binding domain (DBD, see Fig. 3), which achieves DNA sequence recognition by contacting the minor groove with the wing subdomain (Gajiwala et al., 2000). The RFX factors can bind either as monomers or dimers (homo- or hetero-) to a target site known as the X-box, which is found in the promoters of many genes. Based on the high degree of sequence conservation within the DBD, seven mammalian RFX factors have been identified (Aftab et al., 2008; Emery et al., 1996; Reith et al., 1990; Reith et al., 1994b), with an additional member, RFX8, now recognized (ENSG00000196460). The presence of these eight RFX factors has been predicted in all vertebrates analyzed so far, with the exception of fishes, where nine RFX factors can be found, in accordance with an additional genome

duplication event at the base of the actinopterygian lineage (Chu et al., 2010). RFX family members have also been identified in invertebrates such as *Drosophila* and *C. elegans*, and in unicellular organisms such as the yeasts *S. pombe* and *S. cerevisiae* (Fig. 3), demonstrating the evolutionary antiquity (see Box 2) of this transcription factor type (Chu et al., 2010; Durand et al., 2000; Emery et al., 1996; Huang et al., 1998; Otsuki et al., 2004; Piasecki et al., 2010; Swoboda et al., 2000; Wu and McLeod, 1995).

The RFX family can be subdivided into three major groups based on phylogenetic analysis of the DBD (Chu et al., 2010) and on shared protein domains (Fig. 3). One of these groups comprises RFX factors that show only sequence conservation within the DBD. This includes vertebrate RFX5, RFX7 and RFX9, *Drosophila* RFX1 and RFX2, SAK1 from *S. pombe*, and CRT1 from *S. cerevisiae* (Chu et al., 2010; Thomas et al., 2010). These RFX proteins generally control transcriptional cascades not connected with cilia. Members of the other two major groups share several additional conserved protein domains outside the DBD, and are highly similar to the *C. elegans* RFX protein DAF-19 (Fig. 3). These two groups comprise worm DAF-19, *Drosophila* RFX, and vertebrate RFX1-RFX4 and RFX6 (and the recently predicted RFX8). As we discuss below, a growing body of evidence supports an evolutionarily conserved role for members of these two RFX subgroups in programming ciliary differentiation.

C. elegans DAF-19: establishing a link with ciliogenesis

The first experimental evidence that RFX factors are intrinsically tied to the transcriptional regulation of ciliary genes stemmed from work performed in *C. elegans* (Swoboda et al., 2000). The worm genome contains a single RFX factor gene, *daf-19*, that is expressed in all 60 ciliated sensory neurons (CSNs) in the nervous system (Swoboda et al., 2000). These CSNs extend ciliated endings from the tips of their dendrites dedicated to 'smell and taste' functions. Although these 60 sensory neurons are clearly present in *daf-19* mutant animals, they entirely lack sensory cilia, indicating that DAF-19 is necessary for cilia formation. Furthermore, the transcriptional activation of genes encoding IFT subunit genes, such as *che-2*, *osm-1* and *osm-6*, and of many other ciliary genes requires DAF-19 function mediated via functional X-box elements in the promoters of these genes (Burghoorn et al., 2012). Therefore, in *C. elegans*, DAF-19 is the central regulator of ciliogenesis and is specifically required during late differentiation (Senti and Swoboda, 2008; Swoboda et al., 2000). Furthermore, in certain cellular contexts, *daf-19* has been shown to be sufficient for the formation of fully functional cilia (Senti et al., 2009).

Expression and function of vertebrate Rfx genes

Soon after the establishment of a ciliogenic role for *C. elegans* DAF-19, sensory cilia in *Drosophila* were shown to be dependent on RFX (Dubruille et al., 2002). Through later studies in several vertebrate species, a general picture has emerged that Rfx genes are expressed in many ciliated cells and tissues, with some genes exhibiting a more-restricted expression pattern than others (summarized in Fig. 3). Importantly, the disruption of these genes in vertebrates has shown that they play essential roles in the generation of both motile and sensory cilia (see Table 1), and it is likely that the RFX proteins do so by activating core components necessary for both types of cilia.

Rfx1

Rfx1 appears to be an outsider in the group of ciliogenic RFX factors: the ciliary function of this protein is less obvious, and it is

	Mammals	Frogs and fish	<i>D. melanogaster</i>	<i>C. elegans</i>
Lung/airway	A 9+2 (M)	B 9+2 (M)		
Epidermis		C 9+2 (M)		
Reproductive system	D 9+2 (M)	E 9+2 (M)	F 9+0 (I) 9+2 (M)	
Ependyma	G 9+2 (M)	H 9+2 (M)		
	I 9+2 (M)	J 9+0 (M) 9+2 (M)		
Organ of laterality	K 9+0 (M)	L 9+2 (M)		
Kidney	M 9+0 (I)	N 9+2 (M)		
Signaling cilia	O 9+0 (I)	P 9+0 (I)		
Sensory neurons	Q 9+0 (I)	R 9+0 (I)		
	S 9+0 (I)	T 9+2 (I or M)	U 9+0 (M)	V 9+N (I)
	W 9+2 (I)	X 9+2 (I)		

Fig. 2. Cilia types in selected organisms. Microtubule configurations (X+Y: X equals the number of outer microtubule doublets; Y equals the number of central singlet microtubules) and the motility (M, motile; I, immotile; circular arrow, rotational) of each cilia type are indicated. Gray boxes represent tissues/cell types that are not present or that lack cilia. Ultrastructures are shown for: (A) rat airway multicilia (Rhodin and Dalhamn, 1956); (B) *X. laevis* tracheal (Steinman, 1968) and *R. pipiens* pharyngeal (Fawcett and Porter, 1954) multicilia; (C) *X. laevis* epidermal multicilia (Steinman, 1968; Stubbs et al., 2008); (D) Human sperm flagellum and mouse oviduct multicilia (Fawcett, 1954); (E) Zebrafish (Wolenski and Hart, 1987) and *Rana* (Poirier and Spink, 1971) sperm flagella and *R. pipiens* oviduct multicilia (Fawcett and Porter, 1954); (F) *Drosophila* spermatoocyte multiple cilia (Carvalho-Santos et al., 2012; Riparbelli et al., 2012) and sperm flagellum (Acton, 1966); (G) rat brain ependymal multicilia (Brightman and Palay, 1963) [immotile multicilia with a 9+0 configuration also exist in the choroid plexus (Narita et al., 2010)]; (H) *X. laevis* ependymal monocilia and multicilia (Hagenlocher et al., 2013) [these have a 9+2 configuration in *R. temporaria* (De Waele and Dierickx, 1979)]; (I) cilia on mouse spinal canal ependymal cells, which are normally biciliated (Luse, 1956); (J) zebrafish spinal canal ependymal cilia, which can have 9+0 or 9+2 configurations (Kramer-Zucker et al., 2005; Sarmah et al., 2007); (K) mouse nodal monocilia [most have a 9+0 configuration (Jurand, 1974; Sulik et al., 1994) but 9+2 cilia have been described (Caspary et al., 2007) with 9+4 cilia occasionally present in rabbit embryos (Feistel and Blum, 2006)]; (L) zebrafish KV monocilia (Kramer-Zucker et al., 2005); (M) rat kidney monocilia (Latta et al., 1961); (N) zebrafish pronephric multicilia and monocilia (Kramer-Zucker et al., 2005), and *X. laevis* pronephric multicilia (Fox and Hamilton, 1971); (O) rat signaling cilia (Sorokin, 1962); (P) zebrafish signaling cilia (S. Roy, unpublished observations); (Q) mouse retinal photoreceptor connecting cilium (De Robertis, 1956); (R) *R. pipiens* retinal photoreceptor connecting cilium (Peters et al., 1983); (S) mouse ear kinocilia, which lack dynein arms (Sobkowicz et al., 1995); (T) zebrafish otic vesicle kinocilia (Yu et al., 2011) [*L. vulgaris* (another teleost fish) lateral line hair cell cilia have a 9+2 configuration without dynein arms (Flock and Wersall, 1962), whereas analogous cilia from *X. laevis* have a 9+2 configuration with dynein arms (Toyoshima and Shimamura, 1982)]; (U) *Drosophila* chordotonal organ type I sensory cilia (Cachero et al., 2011; Newton et al., 2012) [*Drosophila* also possess external type I sensory neurons, which have a short, immotile connecting cilium]; (V) *C. elegans* sensory cilia (Ward et al., 1975), which have a 9+N configuration where N equals the number of central singlet microtubules and ranges from three to six; (W) rat olfactory neuron multicilia (Lidow and Menco, 1984); (X) adult zebrafish olfactory neuron multicilia. Nonsensory motile multicilia are also found in the olfactory epithelium (Hansen and Zeiske, 1998).

also involved in the regulation of a number of non-ciliary target genes (Iwama et al., 1999; Steimle et al., 1995). *Rfx1* is expressed in several regions of the mouse and rat brain (e.g. the olfactory bulbs, hippocampus and cortex) (Benadiba et al., 2012; Feng et al., 2011; Ma et al., 2006). However, *Rfx1*-null mice are early embryonic lethal, suggesting an important role for *Rfx1* in regulating gene expression that is essential for the initial stages of development (Feng et al., 2009). With respect to the cilium, RFX1, along with RFX2, has recently been found to regulate the transcription of *ALMS1*, a gene that encodes a basal body-associated protein and that is mutated in the ciliopathy Alström syndrome (Purvis et al., 2010).

Rfx2

Rfx2 is preferentially expressed in ciliated tissues such as the brain, organs of laterality, kidneys and testis from early development (Bisgrove et al., 2012; Chung et al., 2012; Horvath et al., 2004; Liu et al., 2007; Ma and Jiang, 2007; Thisse et al., 2004; Wolfe et al., 2004). In addition, *rfx2* expression is enriched in motile multiciliated cells that differentiate in the epidermis of *Xenopus* larvae and within the pronephric kidney tubules of the zebrafish embryo – these cells are similar to motile multiciliated cells of the mammalian airways (Chung et al., 2012; Liu et al., 2007; Ma and Jiang, 2007). The effect of the loss of RFX2 function on ciliary differentiation was first reported for the zebrafish embryo, where a marked reduction in the numbers of immotile primary cilia in the developing neural tube was observed (Yu et al., 2008). In keeping with this, RFX2-deficient *Xenopus* embryos also exhibit reduced and truncated primary cilia in neural tissues, leading to a disruption of HH signaling (Chung et al., 2012). Motile cilia are also dependent on RFX2 for proper differentiation; in multiciliated epidermal cells and the GRP of *Xenopus* embryos, as well as in the zebrafish KV, knockdown of *Rfx2* leads to the truncation and aberrant motility of the motile cilia (Bisgrove et al., 2012; Chung et al., 2012). A handful of putative *Rfx2* target genes have been identified based on their reduced levels of expression in RFX2-deficient *Xenopus* embryos (see Figs 3 and 4).

Rfx3

In the mouse, *Rfx3* is expressed in tissues with ciliated cell types, such as the node and the brain, reminiscent of *Rfx2* expression (Baas et al., 2006; Benadiba et al., 2012; Bonnafe et al., 2004; El Zein et al., 2009). During early stages of brain development, *Rfx3* is transcribed in ciliated ependymal cells of the ventricular lining. During later stages, expression becomes progressively restricted to the cortex and to midline structures, such as the choroid plexus (CP), subcommissural organ (SCO) and the cortical septal boundary (Baas et al., 2006; Benadiba et al., 2012). In addition, *Rfx3* is expressed in the mouse pancreas (Ait-Lounis et al., 2007) and in differentiating multiciliated cells of the *Xenopus* epidermis (Chung et al., 2012).

In line with these expression patterns, mice deficient in *Rfx3* exhibit frequent left-right asymmetry defects (Bonnafe et al., 2004) and the disruption of the differentiation of ciliated cells of the CP and SCO, which leads to the disorganization of these structures and the development of severe hydrocephalus (Baas et al., 2006). Loss of *Rfx3* is also associated with the malformation of the corpus callosum (CC), which normally connects the two brain hemispheres (Benadiba et al., 2012). Finally, in the pancreas, *Rfx3* deficiency causes a significant alteration in the composition of hormone-secreting cells of the islet of Langerhans (Ait-Lounis et al., 2007).

The cilia themselves are affected in multiple ways by the absence of *Rfx3* function: they are shortened (in the node), strongly reduced in number and length (in the pancreas); or overproduced (in the

SCO) (Ait-Lounis et al., 2007; Baas et al., 2006; Bonnafe et al., 2004). Dysregulation of the HH signaling pathway, which manifests as misprocessing of the Gli effector proteins, is the causative trigger for the abnormal development of the CC, and likely also accounts for the alteration of the endocrine lineage of the pancreas (Ait-Lounis et al., 2007; Benadiba et al., 2012). Furthermore, *in vitro* cultures of the multiciliated ependymal cells from *Rfx3* mutant mouse brains have further clarified that RFX3 controls the growth, number and motility of motile cilia by directly regulating the transcription of genes encoding proteins involved in cilia assembly and motility (El Zein et al., 2009) (see Figs 3 and 4).

Rfx4

In mammals, *Rfx4* is expressed in the testis and the brain (Ait-Lounis et al., 2007; Ashique et al., 2009; Blackshear et al., 2003; Morotomi-Yano et al., 2002). In the mouse brain, *Rfx4* is strongly expressed in the SCO and throughout the ependyma from late embryonic stages onwards (Ashique et al., 2009; Blackshear et al., 2003). Haploinsufficiency of *Rfx4* in mice is associated with severe hydrocephalus and reduction or absence of the SCO, whereas homozygous mutant embryos die perinatally, displaying severe dorsal midline defects of the brain and a single central ventricle. Changes in the expression of regional markers, including components of the WNT, bone morphogenetic protein (BMP) and retinoic acid pathways, suggest that RFX4 is required for the establishment of dorsal signaling centers in the developing brain (Blackshear et al., 2003; Zhang et al., 2006). Some of the observed patterning defects are likely to be caused by a loss of cilia integrity and the consequent dysregulation of HH activity (Ashique et al., 2009) due directly to alterations in the expression of genes for ciliary proteins, such as IFT172 (see Figs 3 and 4).

Genes implicated in the ciliopathy Joubert syndrome (see Box 1) provide an interesting example of the regulation of ciliary components via RFX4 (Lee et al., 2012). The transmembrane proteins TMEM138 and TMEM216 are required for ciliogenesis, and mark distinct pools of vesicles around the base of the cilium. These two transmembrane proteins show no obvious sequence homology or shared functional domains, but, when mutated, cause indistinguishable phenotypes in individuals with Joubert syndrome. It has recently been shown that their genes are organized in a head-to-tail fashion on the same chromosome in mammalian genomes, and that their expression responds coordinately to changes in the abundance of RFX4. RFX4 binds to a conserved X-box within the intergenic region, establishing that functional linkage of non-paralogous genes can occur via shared promoter elements (Lee et al., 2012).

RFX factors directly regulate genes for core ciliary components

In summary, there is strong experimental evidence for obligatory but partially redundant roles for vertebrate *Rfx1-Rfx4* in cilia formation and maintenance. These genes share overlapping expression patterns, and the consequences of their loss of function, notably for *Rfx2* and *Rfx3*, are rather similar. The observed phenotypes can be largely explained through changes in ciliary gene expression, which result in structural defects of cilia. Moreover, the fact that inactivation of any single RFX factor translates to a rather ‘mild’ ciliary phenotype further supports a model of functional redundancy and cooperativity among the different RFX factors. This is in line with the highly similar DNA-binding specificity of these proteins (Morotomi-Yano et al., 2002; Reith et al., 1994a).

The target site for RFX factors, the X-box, is a symmetrical promoter motif consisting of an imperfect inverted repeat with two half sites joined by a variable linker of 1-3 nucleotides (e.g.







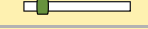
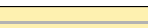







Organisms	RFX TFs	Protein domains	Expression patterns	Ciliary phenotypes		Key ciliary target genes
				Organism wide	Cilia specific	
Vertebrates <i>H. sapiens</i> <i>M. musculus</i> <i>X. laevis</i> <i>D. rerio</i>	RFX1		Brain	Homozygous lethal	Not known	<i>ALMS1</i>
	RFX2		Organs of laterality Brain Kidney Testis Epidermis	Left-right asymmetry defects Defective neural tube closure Perturbed HH signaling	Truncated, dysfunctional motile cilia Fewer and truncated immotile cilia	<i>IFT122</i> <i>IFT172</i> <i>WDPCP</i> <i>TTC25</i>
	RFX3		Organs of laterality Brain Pancreas Epidermis	Left-right asymmetry defects Hydrocephalus Malformation of the corpus callosum Perturbed hormone secretion	Truncated, dysfunctional motile cilia Aberrant number of immotile cilia Truncated immotile cilia	<i>Dync2li1</i> <i>Dnah9</i> <i>Dnah11</i>
	RFX4		Brain Testis	Homozygous lethal Reduction/absence of SCO Patterning defects Hydrocephalus	Truncated cilia	<i>IFT172</i>
	RFX6		Pancreas	n/a	n/a	n/a
	RFX8		Not known	Not known	Not known	Not known
	RFX5		Immune system	n/a	n/a	n/a
	RFX7		Not known	Not known	Not known	Not known
	RFX9		Not known	Not known	Not known	Not known
	Flies <i>D. melanogaster</i>	RFX		Brain Chordotonal and external sensory neurons	Sensory behavioral defects	Structurally abnormal cilia
RFX1			Not known	Not known	Not known	Not known
RFX2			Not known	Not known	Not known	Not known
Nematodes <i>C. elegans</i>	DAF-19		Ciliated sensory neurons	Dauer formation Dye filling and sensory behavioral defects	Absence of all cilia	Numerous
Fungi <i>S. cerevisiae</i> <i>S. pombe</i>	CRT1		Single-celled budding yeast	n/a	n/a	n/a
	SAK1		Single-celled fission yeast	n/a	n/a	n/a

Fig. 3. The expression and function of RFX family transcription factors in various organisms. The members of the RFX family of proteins from selected vertebrates, *Drosophila*, *C. elegans* and fungi are listed. Schematics of each protein are given, with the conserved RFX protein domains highlighted: activation domain (blue); DNA-binding domain (green); domain B (red); domain C (purple); the dimerization domain (yellow). The RFX proteins are divided into subgroups based on functional connections to ciliogenesis: factors directly connected to ciliogenesis (highlighted in blue); those that have not been connected to ciliogenesis (highlighted in yellow); factors that have been loosely associated with ciliogenesis (highlighted in green). Vertebrate RFX factors are grouped according to phylogenetic studies of the DBD domain and the presence/absence of additional protein domains. *ALMS1*, Alstrom syndrome 1; *Dnah*, dynein, axonemal, heavy chain genes; *Dync2li1*, dynein cytoplasmic 2 light intermediate chain 1; *iav*, inactive; *IFT*, intraflagellar transport genes; *n/a*, not applicable; *nan*, *nanchung*; *SCO*, subcommissural organ; *TFs*, transcription factors; *TTC25*, tetratricopeptide repeat domain 25; *WDPCP*, WD repeat-containing planar cell polarity effector.

GTYNKY-AT-RGNAAC) to which RFX dimers make contacts on opposing sides of the DNA (Burghoorn et al., 2012; Efimenko et al., 2005; Gajiwala et al., 2000; Laurençon et al., 2007; Swoboda et al., 2000). The dimer combinations identified for Rfx1-Rfx4 include all homodimers and various heterodimers, supporting the notion that DNA binding and the subsequent transactivation of target genes occurs in a coordinated and closely interdependent fashion (Iwama et al., 1999; Morotomi-Yano et al., 2002; Reith et al., 1994a). Together, these findings have nurtured the view that the RFX factors

regulate overlapping sets of target genes, with functional redundancy for some but not all of these genes (Bonnafe et al., 2004).

A combination of computational searches and experimental approaches, pioneered in *C. elegans* and *Drosophila*, has helped to identify a large number of direct (and candidate) RFX target genes in different species (Ashique et al., 2009; Blacque et al., 2005; Chen et al., 2006; Efimenko et al., 2005; Laurençon et al., 2007; Phirke et al., 2011; Swoboda et al., 2000) (Fig. 4). These genes generally fall into two classes. The first includes X-box-containing target genes

Box 2. Evolutionary conservation of ciliary gene regulation by RFX factors

Cilia are evolutionarily ancient structures found in representatives from all five major eukaryotic branches: Unikonta, Archaeplastida, Excavata, Chromalveolata and Rhizaria. This suggests that the last eukaryotic common ancestor (LECA) was a ciliated, unicellular organism. Accumulating evidence from various organisms for the tight regulation of the expression of ciliary components, such as intraflagellar transport (IFT) genes, by RFX factors leads to questions regarding when and how this co-regulation of ciliary genes has evolved.

Sampling genomes from many different eukaryotic organisms for the presence of RFX factor genes revealed that RFX factors are restricted to only the Unikonta (comprising animals, fungi and amoebzoa), whether ciliated or not (Chu et al., 2010; Piasecki et al., 2010). A comparison of the evolutionary distribution of RFX factor genes and core ciliary genes (e.g. IFT genes) revealed that both existed independently from each other in various fungi and amoebzoa and, thus, must have evolved independently. For example, the yeasts *S. cerevisiae* and *S. pombe* both possess a single RFX factor but no cilia. Conversely, there are multiple examples of Unikonta (e.g. *Physarum polycephalum*) that have cilia but harbor no RFX factor genes in their genomes. In addition, DNA sequence footprints of the X-box promoter motif, the binding site for RFX factors, are found exclusively in ciliary genes within the animal kingdom in co-existence with RFX factors. Therefore, the tight transcriptional control of ciliary genes and cilia formation was most likely 'taken over' by RFX factors early in the animal lineage (Chu et al., 2010; Piasecki et al., 2010).

that encode the so-called core ciliary components involved in basic aspects of cilia formation and function (Fig. 4). Structural components of the basal body (e.g. DYF-17, B9 and BBS proteins), the transition zone (NPH-1 and NPH-4) and the axoneme (DYF-1) fall into this category, as do components of the IFT machinery (e.g. IFT88/OSM-5, IFT172/OSM-1 and XBX-1) (Ansley et al., 2003; Ashique et al., 2009; Burghoorn et al., 2012; Efimenko et al., 2005; Haycraft et al., 2001; Ou et al., 2005; Phirke et al., 2011; Schafer et al., 2003; Signor et al., 1999; Williams et al., 2008; Winkelbauer et

al., 2005). The second class includes cilia subtype-specific X-box-containing genes, many of which have been identified in *C. elegans* and *Drosophila*, that are required for specialized ciliary functions in only certain cell types. Notably, representatives of different candidate receptor families and receptor-associated factors are found within this group (e.g. *C. elegans* ODR-4, ASIC-2, XBX-5, STR-1, STR-13, STR-44, STR-144, SRG-2, SRH-74, SRU-12 and SRX-54; and *Drosophila* Nan and Iav) (Burghoorn et al., 2012; Dwyer et al., 1998; Efimenko et al., 2005; Newton et al., 2012).

RFX factors are thought to orchestrate ciliary differentiation programs after a cell has become committed towards a particular fate. Thus, in *C. elegans*, sensory neurons are clearly present in *daf-19* mutant animals, but they fail to ciliate (Swoboda et al., 2000). Strikingly, however, in both invertebrates and the vertebrates, several transcription factors are also regulated via conserved X-box motifs (e.g. *Rax*, *Zic1*, *Zic3*, *Msx3* and nuclear hormone receptors such as *nhr-44*, *nhr-45* and *nhr-120*), suggesting that besides playing an essential role in promoting ciliogenesis, the RFX factors could also be directly involved in the specification of the ciliated cell types (Burghoorn et al., 2012; Efimenko et al., 2005; Zhang et al., 2006), a hypothesis that clearly requires further exploration. Given this possibility, the cell and tissue-patterning defects that occur in the absence of proper Rfx gene function may not solely be the outcome of perturbed signaling pathways triggered by ciliary abnormalities.

FOX family transcription factors and the discovery of FOXJ1

In recent years, the forkhead box protein J1 (FOXJ1) has emerged as an additional factor important for ciliogenesis, specifically for the biogenesis of motile cilia. FOXJ1 (also known as forkhead-like 13/hepatocyte nuclear factor 3 forkhead homolog 4) is a divergent member of the forkhead box (FOX) family of transcription factors (see Box 3), which play crucial roles in a diverse array of biological processes (Hannenhalli and Kaestner, 2009). *Foxj1* was first cloned by degenerate PCR against the forkhead domain from a rat lung cDNA library (Clevidence et al., 1993). *In situ* hybridization

Table 1. Ciliary transcription factor(s) needed to produce different cilia types in selected organisms

Cilia type	Organism	Transcription factor(s) required	References
Airway motile multicilia	Mouse	FOXJ1	(Brody et al., 2000; Chen et al., 1998)
Epidermal motile multicilia	<i>Xenopus</i>	FOXJ1 RFX2	(Stubbs et al., 2008) (Chung et al., 2012)
Sperm flagellum	Mouse	FOXJ1	(Chen et al., 1998)
Oviduct motile multicilia	Mouse	FOXJ1	(Brody et al., 2000; Chen et al., 1998)
Brain ependymal multiple motile cilia	Mouse	RFX3 FOXJ1	(El Zein et al., 2009) (Brody et al., 2000; Chen et al., 1998)
Brain ependymal monocilia/multicilia	<i>Xenopus</i>	FOXJ1	(Hagenlocher et al., 2013)
Spinal canal ependymal motile cilia	Zebrafish	FOXJ1A	(Yu et al., 2008)
Nodal motile monocilia	Mouse	RFX3 FOXJ1	(Bonnafe et al., 2004) (Alten et al., 2012)
Kupffer's vesicle motile monocilia	Zebrafish	RFX2 FOXJ1A	(Bisgrove et al., 2012) (Stubbs et al., 2008; Yu et al., 2008)
Gastrocoel roof-plate motile monocilia	<i>Xenopus</i>	RFX2 FOXJ1	(Chung et al., 2012) (Stubbs et al., 2008)
Pronephric motile multicilia and monocilia	Zebrafish	RFX2 FOXJ1A FOXJ1B	(Liu et al., 2007) (Yu et al., 2008) (Hellman et al., 2010)
Immotile signaling cilia	Mouse Zebrafish <i>Xenopus</i>	RFX4 RFX2 RFX2	(Ashique et al., 2009) (Yu et al., 2008) (Chung et al., 2012)
Otic vesicle kinocilia	Zebrafish	FOXJ1B	(Yu et al., 2011)
Chordotonal organ sensory motile cilia	<i>Drosophila</i>	FD3F	(Cachero et al., 2011; Newton et al., 2012)
Sensory neurons	<i>Drosophila</i> <i>C. elegans</i>	RFX DAF-19	(Dubruielle et al., 2002) (Swoboda et al., 2000)
Olfactory motile cilia	Zebrafish	FOXJ1A	(Hellman et al., 2010)

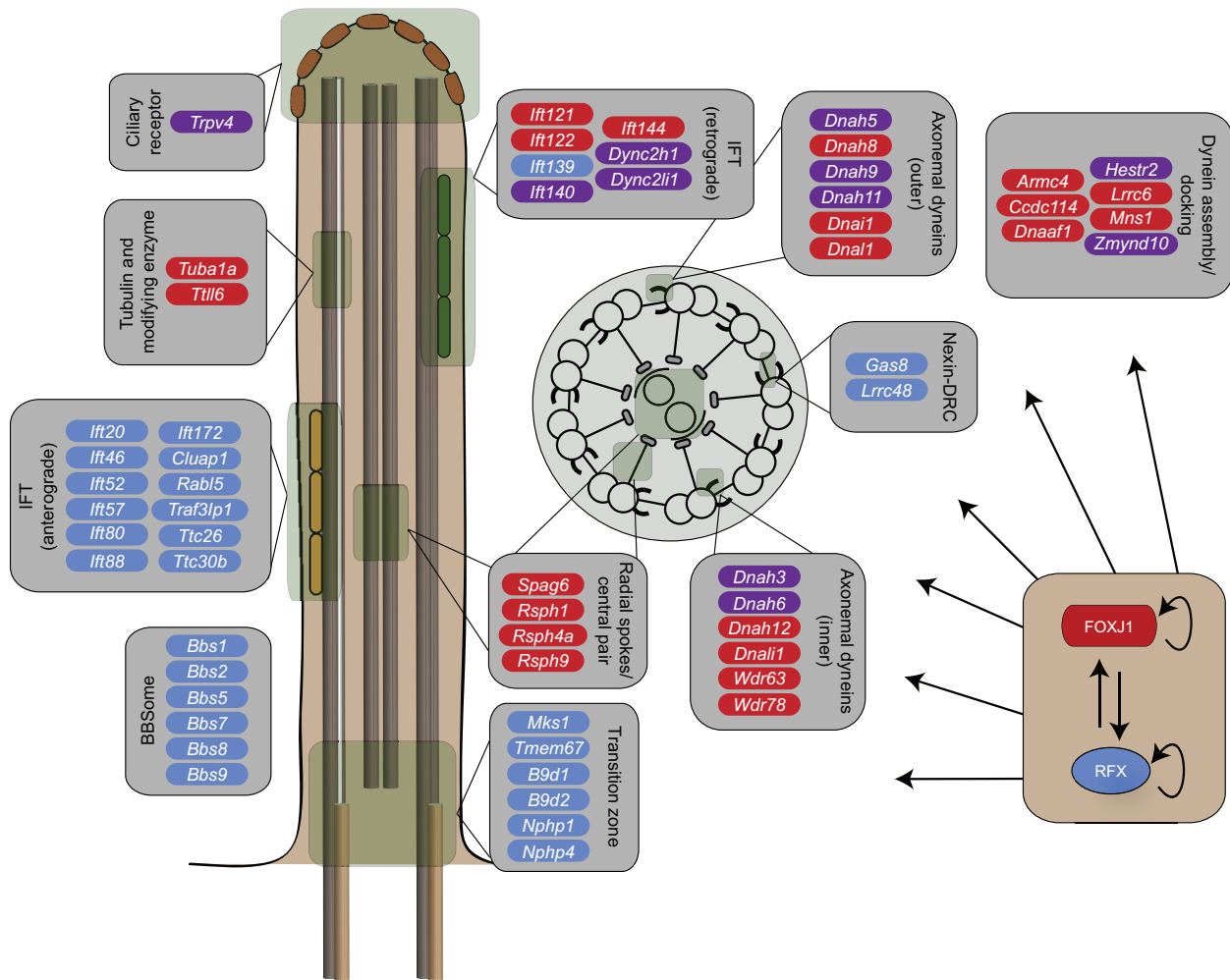


Fig. 4. Direct and indirect targets of ciliary transcription factors. Target genes were collected from previously assembled FOXJ1, FD3F and RFX target sets in *Drosophila* (Newton et al., 2012), *C. elegans* (Burghoorn et al., 2012) or vertebrates (Ashique et al., 2009; Didon et al., 2013; El Zein et al., 2009; Jacquet et al., 2009; Stubbs et al., 2008). Genes are organized by their associations/functions with respect to ciliary structures. Genes in blue are targets of RFX transcription factors, genes in red are targets of FOXJ1 or FD3F (not shown), whereas targets of both transcriptional modules are depicted in purple. The regulation of the target genes listed has been shown in at least one model organism. *Armc4*, armadillo repeat containing 4; *B9d*, B9 protein domain genes; *Bbs*, Bardet-Biedl syndrome genes; *Ccdc114*, coiled-coil domain containing 114; *Cluap1*, clusterin associated protein 1; *Dnaaf1*, dynein, axonemal assembly factor 1; *Dnah*, dynein, axonemal, heavy chain genes; *Dnal1*, dynein, axonemal, light intermediate polypeptide 1; *Dync2h1*, dynein cytoplasmic 2 heavy chain 1; *Dync2li1*, dynein cytoplasmic 2 light intermediate chain 1; *Gas8*, growth arrest specific 8; *Heatr2*, HEAT repeat containing 2; *Ift*, intraflagellar transport genes; *Lrrc*, leucine rich repeat containing genes; *Nphp*, nephronophthisis genes; *Mks1*, Meckel syndrome, type 1; *Mns1*, meiosis-specific nuclear structural protein 1; *Rabl5*, RAB, member of RAS oncogene family-like 5; *Rsph*, radial spoke head homolog genes; *Spag6*, sperm associated antigen 6; *Tmem67*, transmembrane protein 67; *Traf3ip1*, TRAF3 interacting protein 1; *Trpv4*, transient receptor potential cation channel, subfamily V, member 4; *Ttc*, tetratricopeptide repeat domain genes; *Tll6*, tubulin tyrosine ligase-like family, member 6; *Tuba1a*, tubulin α 1a; *Wdr*, WD repeat domain genes; *Zmynd10*, zinc finger, MYND domain containing 10.

revealed that expression of the gene is spatially restricted to a number of mammalian tissues that differentiate motile cilia, including the choroid plexus, lung epithelium, oviduct and testis (Clevidence et al., 1994; Hackett et al., 1995; Murphy et al., 1997). Based on this expression pattern, Murphy and colleagues presciently suggested that FOXJ1 might play a role in ciliogenesis (Murphy et al., 1997). Consistent with a predicted role as a transcriptional regulator, FOXJ1 is a nuclear protein and is detected in a pattern similar to that of *Foxj1* mRNA, with high levels accumulating just prior to ciliogenesis in cells of the mouse lung and trachea, in oviducts and in ependymal cells lining the spinal column and the brain ventricles (Blatt et al., 1999; Tichelaar et al., 1999b). FOXJ1 is also expressed just prior to the appearance of flagella in the spermatids (Blatt et al., 1999), further corroborating the suggestion that FOXJ1 is a transcriptional regulator of motile ciliogenesis.

FOXJ1 is a conserved regulator of motile ciliogenesis

Two independent studies confirmed the hypothesized link between FOXJ1 and motile ciliogenesis, with FOXJ1 knockout mice showing a complete loss of the axonemes of motile multicilia from the airways, choroid plexus and the oviducts, as well as left-right asymmetry defects (Table 1) (Brody et al., 2000; Chen et al., 1998). As a result, most mutant embryos die at birth, with survivors developing hydrocephalus and perishing shortly thereafter. Transmission electron microscopy (TEM) of airway cells showed that loss of *Foxj1* specifically disrupts the 9+2 motile cilia, leaving the 9+0 immotile primary cilia intact. TEM also revealed that the basal body docking to the apical cell membrane was impaired, leading to the observed defects in ciliogenesis (Brody et al., 2000). *In vitro* cultures of airway cells isolated from *Foxj1* mutant embryos further showed that, while the generation of multiple basal bodies

Box 3. The forkhead box transcription factor family

The *fork head* gene was identified in *Drosophila* as a regulator of head and gut development, mutations in which yield ectopic forked structures on the head of the fly (Weigel et al., 1989). In a separate study, the liver-specific transcription factor, HNF3 α (later renamed FOXA1), was isolated from rats (Lai et al., 1990). Weigel and Jäckle astutely recognized the similarity in the DNA-binding domain of both transcription factors, and named this seemingly conserved domain the forkhead domain (Weigel and Jäckle, 1990). The forkhead domain canonically consists of three α -helices and three β -sheets connected to a pair of loops or wings, reminiscent of a helix-turn-helix domain, which directly binds to DNA (Clark et al., 1993). Exploiting this highly conserved 80-100 amino acid DNA-binding domain in searches for homologs, additional family members were identified in organisms ranging from yeast to human. Eventually, 50 human forkhead transcription factors were found, which can be classified into 19 different groups (FOXA-FOXS) (Jackson et al., 2010; Kaestner et al., 2000). These transcription factors play important roles in a wide range of biological processes, including organ development (FOXA transcription factors), insulin signaling and longevity (FOXO transcription factors), and speech acquisition (FOXP2) (Hannenhalli and Kaestner, 2009).

proceeded normally, they failed to dock at the apical membrane (Gomperts et al., 2004; You et al., 2004). Thus, in multiciliated cells, FOXJ1 is essential for basal body docking and for all of the subsequent steps involved in ciliary differentiation.

Besides regulating the formation of the motile multicilia, FOXJ1 is also required to make motile monocilia – such as the flagella of sperm cells (Chen et al., 1998). Though initial reports differed (Brody et al., 2000), it has been recently shown that FOXJ1 function is also necessary for making the 9+0 motile cilia in the node (Alten et al., 2012), which explains the highly penetrant randomization of left-right axis displayed by *Foxj1* mutant mouse embryos (Brody et al., 2000; Chen et al., 1998).

The role of FOXJ1 in controlling motile cilia biogenesis has now been shown to be conserved across the vertebrates (see Table 1), with the knockdown of FOXJ1 in both *Xenopus* and zebrafish causing a loss of all motile cilia (Stubbs et al., 2008; Yu et al., 2008). Furthermore, an in-depth study of the evolutionary history of *foxj1* has clarified that *foxj1* orthologs, like those of the RFX factors, are present throughout the unikonts, but the gene has been secondarily lost from certain lineages (Vij et al., 2012). The authors confirmed this bioinformatics-based analysis by demonstrating a functional association between FOXJ1 and motile ciliogenesis in the flatworm *Schmidtea mediterranea* (Vij et al., 2012). Concurrently, work in *Drosophila* revealed that a forkhead box transcription factor, FD3F, is expressed in a set of proprioceptive and auditory neurons, the chordotonal neurons, which make long mechanosensory cilia that are partially motile (9+0, with dynein arms) (Cachero et al., 2011; Newton et al., 2012). Although FD3F is not a direct ortholog of vertebrate FOXJ1, phylogenetic analysis indicates that it may be a highly derived member of the FOXJ family (Hansen et al., 2007). Consistent with this idea, the chordotonal cilia of *fd3F* mutant flies are devoid of the dynein arms that are necessary for motility (Newton et al., 2012).

FOXJ1 programs motile cilia by activating a network of motile cilia genes

Perhaps the most remarkable aspect of FOXJ1 is its ability to induce, when ectopically expressed, the differentiation of functional motile monocilia in many different tissues in both zebrafish and *Xenopus* embryos (Stubbs et al., 2008; Yu et al., 2008). Though this ciliogenic potential of FOXJ1 has not been as

clearly established in higher vertebrates, there are indications that this ability is conserved. For example, transgenic mice that misexpress FOXJ1 under the control of the surfactant protein C promoter express additional tubulin, suggestive of ectopic cilia, in cells that line the alveoli of the lungs (Tichelaar et al., 1999a). Furthermore, overexpression of FOXJ1 in the chick neural tube and a mouse embryonic fibroblastic cell line (NIH3T3) can induce the formation of long cilia (Cruz et al., 2010). However, overexpression of FOXJ1 in a canine kidney epithelial cell line (MDCK), or in nonciliated human airway epithelial cells (BEAS2B), did not result in the production of motile cilia, and overexpression of FOXJ1 in mouse tracheal epithelial cells (MTECs) did not increase the percentage of ciliated cells (You et al., 2004). This variability could be due to differences in misexpression strategies (such as timing and levels of expression), the dependence of FOXJ1 on specific co-factors or limitations of *in vitro* culture systems, or it could reflect species-specific differences in the ability of FOXJ1 to induce ectopic motile cilia. Nevertheless, taken together, it appears that FOXJ1 plays a master regulatory role in the biogenesis of the motile cilia.

How does FOXJ1 function to program the differentiation of motile cilia? Studies in the mouse, *Xenopus*, zebrafish and *Drosophila* have led to the identification of a cohort of ciliary genes that are regulated by FOXJ1. This includes genes that are generally required for all types of cilia, such as those encoding IFT proteins, tubulins and tubulin-modifying enzymes, as well as genes that are specifically required for different structural and functional aspects of the motile cilia, such as those encoding components to make, assemble, transport and dock the inner and outer dynein arms, radial spokes and the central pair (Didon et al., 2013; Jacquet et al., 2009; Newton et al., 2012; Stubbs et al., 2008; Yu et al., 2008) (Fig. 4). These target genes are consistent with the master regulatory role of FOXJ1 in programming motile cilia differentiation. Although genome-wide chromatin immunoprecipitation (ChIP) will be necessary to estimate the number of direct target genes and to define properly the FOXJ1-binding site, several studies have begun to identify direct targets and preliminary consensus sequences to which FOXJ1 binds. For example, early *in vitro* analyses by protein selection on degenerate oligos, PCR and sequencing, revealed a binding consensus of HWDTGTTTGTGTTA (Lim et al., 1997). This was recently confirmed by *in vitro* binding-site assays, which revealed the consensus sequences TGTGTTA or TGTTGT (Nakagawa et al., 2013). Furthermore, the promoters of two zebrafish cilia genes, *ccdc114* (ENSDARG00000015010) and *wdr78*, are responsive to FOXJ1, bound by the FOXJ1 protein and contain the predicted FOXJ1-binding sites, which are required for their activity in motile ciliated cells (Yu et al., 2008).

Hierarchy, cooperation and redundancy between ciliary transcriptional networks

Based on their loss-of-function phenotypes in multiple model organisms, the RFX factors appear to be required to make both motile and immotile cilia, whereas FOXJ1 is required specifically to make the motile cilia (see Table 1). As these transcription factors function together in cells that make motile cilia, it is important to consider how their two transcriptional programs interface. The first aspect of this interface is the cross-regulation of expression. Data from zebrafish and mouse embryos, and from cultured human airway cells, indicate that FOXJ1 can induce the expression of *Rfx2* and *Rfx3* during motile cilia biogenesis (Alten et al., 2012; Didon et al., 2013; Yu et al., 2008). Conversely, RFX3 has been shown to bind to the *Foxj1* promoter and, in keeping with this, *Foxj1*

expression in cultures of mouse ependymal cells is partially dependent on RFX3 (El Zein et al., 2009).

Besides cross-regulation, several additional scenarios of cooperativity between the RFX and FOXJ1 transcription modules can be envisioned based on current evidence. For example, RFX proteins can enhance the transcriptional activation by FOXJ1 through regulation of target genes downstream of FOXJ1, or by independently binding to DNA of the same target genes to amplify expression. An example of this cooperation occurs in *Drosophila*, which has two types of ciliated neurons: the external sensory neurons have a short connecting immotile cilium, whereas the auditory chordotonal neurons have a long cilium that is mechanosensitive and can be motile. In this system, RFX is required to make cilia in all neuronal types, whereas FD3F is specifically required for proper ciliogenesis in the chordotonal neurons. Three cilia genes specifically expressed in the chordotonal neurons, *nan* (*nanchung*), *iav* (*inactive*) and *Dhc93AB* (*Dynein heavy chain at 93AB*), possess both RFX- and FD3F-binding sites in their upstream regulatory sequences. Mutation of the binding sites for either transcription factor causes a reduction or elimination of target gene expression in these chordotonal neurons. Additionally, overexpression of FD3F leads to misexpression of the target genes, but only in the domain where RFX is expressed (Newton et al., 2012), implying that RFX and FD3F must cooperate to properly regulate chordotonal cilia gene expression in *Drosophila*.

A similar cooperation occurs between RFX3 and FOXJ1 in the human airway cells. In this system, FOXJ1 overexpression alone can induce motile ciliary gene expression. RFX3, however, cannot induce ciliary gene expression on its own, but it can significantly augment FOXJ1-dependent transcription, suggesting that RFX3 functions as a co-factor for FOXJ1 (Didon et al., 2013). Further support for the idea that RFX transcription factors can act as co-factors for FOXJ1 comes from the finding that the two proteins can interact with one another; mouse RFX2 and FOXJ1 have been shown to interact in a high-throughput two-hybrid screen conducted in mammalian cells (Ravasi et al., 2010), and human FOXJ1 and RFX3 can be co-immunoprecipitated when overexpressed in cultured cells (Didon et al., 2013). This raises the interesting possibility that FOXJ1 and RFX factors can form a transcriptional complex, in which FOXJ1 modifies the activity of RFX, in order to provide specificity for motile cilia genes.

In line with the cooperative model, FD3F target genes in *Drosophila* have a modified X-box-binding site for RFX (Newton et al., 2012). This is further corroborated by studies in *C. elegans*, in which core cilia genes have a consensus RFX-binding site, whereas specialized ciliary target genes typically have more degenerate RFX-binding sites (Efimenko et al., 2005). Yet another line of evidence that supports the concept of RFX proteins working together with co-factors comes from studies on the regulation of the major histocompatibility complex class II (MHC-II) genes, where the founding member of the RFX family, RFX5, was originally identified. The promoters of the MHC-II genes contain somewhat 'degenerate' X boxes (one half of the site fits the consensus well, whereas the other half of the site does not), reflecting the fact the RFX factors have partners called RFX-AP (RFX-associated protein) and RFX-ANK (RFX-ankyrin) that drive efficient expression of the MHC-II genes (Reith and Mach, 2001). Such a scenario could also operate in the context of ciliary gene regulation.

The final model of cooperativity between RFX and FOXJ1 is the redundancy model, whereby both transcription factors act in a parallel manner to regulate the formation of a particular cilia type. *Foxj1* and *Rfx3* are both expressed in the floor plate of the mouse

embryo, which makes long 9+0 cilia that are presumed to be motile. In *Foxj1* mutant mice, these cilia are unaffected and the expression of *Rfx3* in the floor plate is not altered (Cruz et al., 2010), suggesting that there may be some redundancy between these two transcription factors in generating the floor-plate cilia. Closer examination, including live imaging to assess motility of the floor plate cilia of *Rfx3* and *Foxj1* single and double mutant mice, will be necessary to determine the extent of redundancy between the two genes in programming the differentiation of these cilia.

To fully uncover the intimate details of the crosstalk between the RFX and FOXJ1 transcriptional networks, it will also be crucial to understand the ciliary gene promoter occupancies in each tissue type to determine whether they are bound by FOXJ1 and an RFX member, or either transcription factor alone. Based on the target genes and phenotypes of mutant animals that have been identified thus far, it appears that RFX factors regulate core cilia genes on their own, and cooperate with FOXJ1 to regulate motility genes in specific cell types. It will also be necessary to determine whether RFX and FOXJ1 can exist as part of the same transcriptional complex when bound to target DNA, as the existing data show only their direct interaction out of context.

Ciliogenic 'selector' genes are deployed to make specialized cilia

How does a cell decide to make a retinal sensory cilium with elaborate membrane stacks versus the multiple motile cilia that beat in the airway epithelium to move mucus? Similar to the action of homeotic transcription factors, we propose that, during development, the ciliogenic programs discussed above are deployed and modified by morphogenetic signaling pathways and cell-type specific transcription factors as selector cassettes in order to make the appropriate variety of cilia (Fig. 5). This is particularly relevant for the motile cilia that are produced only by specific kinds of cells and tissues.

Signaling pathways regulating ciliary diversity

Numerous signaling pathways have been shown to deploy the RFX/FOXJ1 ciliogenic network in order to make motile cilia (see Fig. 5). For example, studies of zebrafish, chick and mouse embryos have demonstrated that HH signaling from the midline induces the expression of *Foxj1* in ciliated floor-plate cells of the spinal cord (Cruz et al., 2010; Yu et al., 2008). However, in the zebrafish, fibroblast growth factor (FGF) signaling induces both *foxj1* and *rfx2* in KV (Neugebauer et al., 2009). Moreover, in multiple tissues in the zebrafish, WNT signaling seems to act downstream of the FGF pathway to directly control *foxj1* expression through TCF/LEF transcription factor-binding sites within the *foxj1* promoter (Caron et al., 2012). This relationship between WNT signaling and *Foxj1* expression is conserved in the *Xenopus* GRP (Walentek et al., 2012). Besides the HH, FGF and WNT pathways, NOTCH signaling has also been heavily linked to motile cilia differentiation. In the zebrafish KV, NOTCH signaling is required for proper *foxj1* expression and ciliogenesis (Lopes et al., 2010), although the relationship between the NOTCH pathway and FGF and WNT in this context is presently unclear. In line with this, a role for the NOTCH pathway has recently been discovered in specifying the correct ratio of the flow-generating motile cilia to the flow-sensing immotile cilia in the *Xenopus* GRP (Boskovski et al., 2013). NOTCH signaling also plays a crucial role in singling out precursors of the motile multiciliated cells in the zebrafish, *Xenopus* and mouse, in this case repressing the multiciliated cell fate (Liu et al., 2007; Ma and Jiang, 2007; Stubbs et al., 2012; Tan et al., 2013). Further experiments will be necessary to understand the

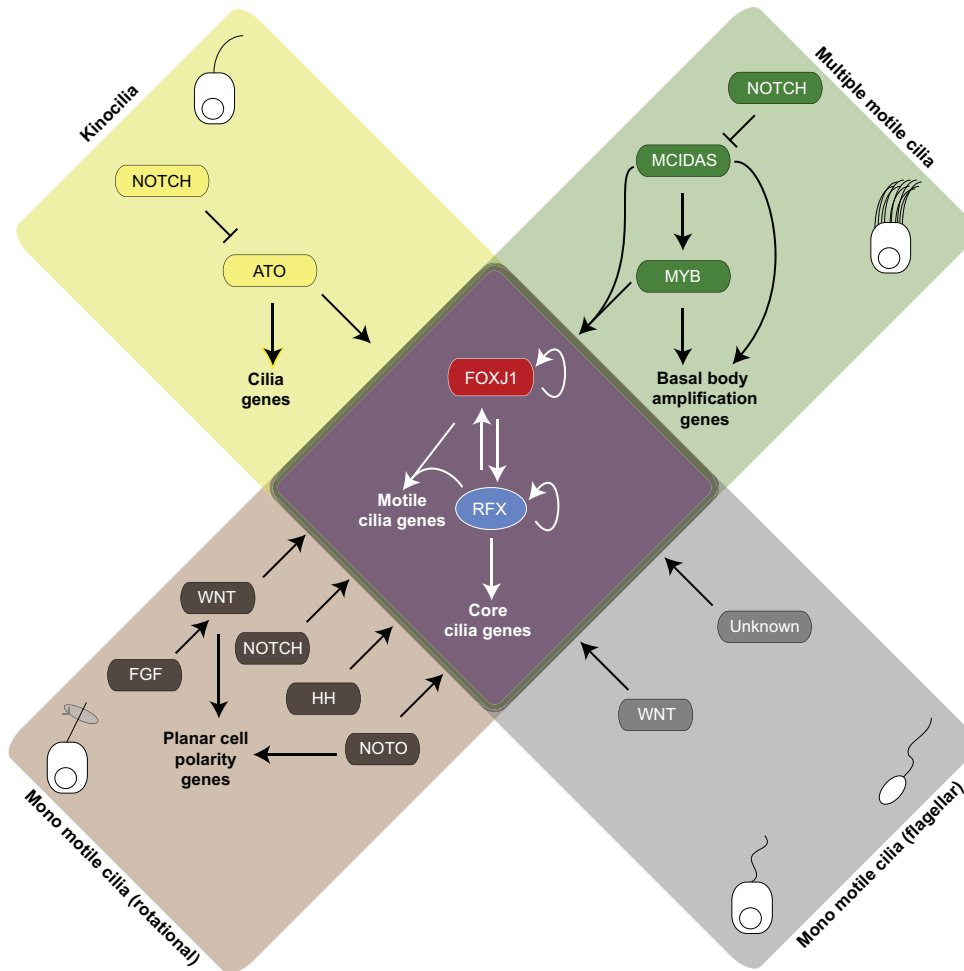


Fig. 5. The regulatory logic of making cilia types that require the RFX/FOXJ1 module. The RFX/FOXJ1 transcriptional cassette is deployed by different signaling pathways and transcriptional modulators to generate ciliary diversity. NOTCH and WNT (the latter acting downstream of FGF) signaling induce the formation of rotational mono motile cilia in organs of laterality in zebrafish and *Xenopus*. The NOTO transcriptional regulator activates FOXJ1 and an RFX factor in the ciliated cells of the mouse node. NOTO and WNT independently regulate cilia polarity in the node, likely by regulating planar cell polarity pathway genes. HH induces the production of rotational motile cilia in the floor plate of both zebrafish and mouse. Kinocilia of the developing ear are produced through the activation of FOXJ1 through ATO. ATO can also independently activate ciliary genes in *Drosophila*. ATO is repressed by NOTCH signaling in the developing zebrafish ear. Multiple motile cilia of the mouse airways, *Xenopus* epidermis or mid-segment of the zebrafish pronephric duct are also inhibited by NOTCH signaling, acting through the transcriptional cascade of MCIDAS/MYB, which activates RFX factors and FOXJ1. MCIDAS/MYB, acting independently from the RFX/FOXJ1 cassette, also regulates genes required for basal body synthesis and docking. Pathways generating monomotile ciliated cells, which move in a flagellar manner, are largely unknown. However, in the zebrafish pronephric duct, FOXJ1 expression is controlled by WNT signaling in the monociliated cells.

effect of each signaling pathway on the expression of RFX/FOXJ1, and to understand how these signaling pathways independently regulate additional genes in order to generate the ciliary diversity present in animal embryos.

Cell type-specific transcription factors that regulate ciliary diversity

Cell type-specific transcription factors can also act through RFX and FOXJ1 to initiate the formation of cilia, while independently regulating specific aspects of ciliogenesis to generate ciliary diversity (see Fig. 5). For example, in order to make the hundreds of motile cilia that exist on the multiciliated cells in the mammalian airways or in the *Xenopus* epidermis, it is first necessary to generate hundreds of basal bodies from which the axonemes will extend. These basal bodies arise *de novo* from procentrioles formed on the surface of deuterosomes – globular structures that serve as organizing centers (Anderson and Brenner, 1971; Sorokin, 1968) that have only recently begun to be molecularly defined (Klos Dehring et al., 2013; Zhao et al., 2013). As discussed in the preceding section, FOXJ1 is not required for the generation of the multiple basal bodies, but functions downstream, in the docking of the basal bodies with the apical cell membrane and subsequent axonemal extension (Brody et al., 2000; Gomperts et al., 2004; You et al., 2004). Recently, a coiled-coil domain-containing protein, multicilin (MCIDAS), was found to be required for the formation of multiciliated cells in the *Xenopus* epidermis and the mouse airways (Stubbs et al., 2012). In both contexts, the transcription factor MYB

acts downstream of MCIDAS to generate multiciliated cells. Another factor appears to act redundantly with MYB, however, as MYB-deficient airway cells show a delay, but not a total loss, of multiciliogenesis (Tan et al., 2013). MYB has also been shown to be required for the formation of multiciliated cells in the zebrafish kidney (Wang et al., 2013). Based on loss-of-function and overexpression experiments, MCIDAS and MYB appear to act in a single pathway, with MCIDAS acting downstream of NOTCH signaling but upstream of MYB, to activate genes that drive multiple basal body formation [such as *Plk4* (polo-like kinase 4) and *Stil* (*Sc1/Tal1* interrupting locus)], on the one hand, and to switch on FOXJ1 to activate genes required for basal body docking, ciliary outgrowth and motility, on the other (Stubbs et al., 2012; Tan et al., 2013; Wang et al., 2013). The molecular details of this process, such as the direct targets of MCIDAS and MYB in the progenitors of the multiciliated cells are presently unknown. This information will be particularly revealing in the case of MCIDAS, which lacks an obvious DBD in its structure, but is able to localize to the nucleus and seems to be capable of activating transcription when overexpressed (Stubbs et al., 2012).

The formation of motile monocilia in the ventral node of the mouse embryo is programmed by a homeobox transcription factor called NOTO (notochord homeobox). Mice lacking NOTO display shortened and malformed nodal cilia and a subsequent disruption of left-right asymmetry (Beckers et al., 2007). In order to generate the monocilia of the node, NOTO deploys FOXJ1, which in turn

activates *Rfx3*. In an elegant experiment, Alten and colleagues replaced the coding region of mouse *Noto* with *Foxj1* (*Noto::Foxj1*), and found that, in contrast to the *Noto* mutants, the expression of ciliary genes (including *Rfx3*) was rescued, and cilia length and motility were restored to normal (Alten et al., 2012). However, these embryos continued to exhibit left-right asymmetry defects because the polarized orientation of nodal cilia, which is dependent on the planar cell polarity (PCP) pathway (Hashimoto et al., 2010; Song et al., 2010), remained disrupted in the *Noto::Foxj1* mice. Thus, NOTO appears to activate *Foxj1* and *Rfx3* to generate the axonemes of motile nodal cilia, while independently establishing their correct posterior positioning on the nodal cells, perhaps by activating the PCP pathway (Alten et al., 2012).

In *Drosophila*, the proneural transcription factor Atonal (ATO) directs the differentiation of chordotonal neurons. As part of this program, ATO activates the expression of both *fd3F* and *Rfx* in order to generate the motile mechanosensory cilia that extend from these neurons (Cachero et al., 2011). Interestingly, ATO has also been shown to activate directly the expression of a ciliary component, Dilatory/CEP131, indicating that ATO can contribute to ciliogenesis independent of the RFX/FD3F cassette (Cachero et al., 2011; Ma and Jarman, 2011). Indeed, this function of ATO may represent a fundamental mechanism for generating mechanosensory cilia because, in zebrafish, an ATO ortholog (ATO1B) activates a *foxj1* paralog in the hair cells of the inner ear, leading to the formation of the immotile kinocilia (Yu et al., 2011).

Target genes of core ciliary transcription factors can generate ciliary diversity

In *C. elegans* and *Drosophila*, the transcriptional programs that function to generate a diversity of immotile cilia types are also beginning to be deciphered. These studies have shown that RFX factors, in addition to regulating the building blocks of all cilia, can also play a role in selecting different primary cilia subtypes through one of several mechanisms. For example, the *C. elegans* RFX, DAF-19, is capable of directly activating genes encoding specific factors that are necessary only in certain specialized sensory cilia subtypes. Examples of these specific ciliary targets include *dyf-2/ift144* and the nuclear hormone receptor *nhr-44*, which are expressed in only a subset of ciliated neurons in the worm (Burghoorn et al., 2012). To accomplish this cell type-specific gene expression induction, DAF-19 probably acts with transcriptional co-factors that are yet to be discovered. Supporting this notion is the presence of an additional DNA motif in close proximity to the X-box, termed the C-box enhancer, in *cis*-regulatory regions of a subset of DAF-19 direct targets that are broadly expressed in all ciliated neurons (Burghoorn et al., 2012; Efimenko et al., 2005).

In flies, differential levels of Rfx gene expression illustrate another mechanism for programming ciliary specialization. Low levels of RFX ensure the expression of core ciliary genes in all sensory neurons, such as many of the genes encoding IFT components. By contrast, high levels of RFX can drive the expression of genes required for ciliary specialization, such as *CG6129/Rootletin*, which is required to make the specialized motile cilia on the chordotonal neurons (Cachero et al., 2011; Newton et al., 2012).

A third RFX-based mechanism for generating ciliary diversity is seen in *C. elegans*, where different isoforms of DAF-19 regulate the expression of distinct cilia genes in various cell types, perhaps through an X-box-independent mechanism. The canonical DAF-19C isoform regulates core ciliary genes, including many of the IFT components. An alternative isoform, DAF-19M, is induced by the

transcription factor EGL-46, in order to activate the expression of the mechanosensory receptor genes *lov-1* and *pkd-2*, and the kinesin-like protein *klp-6* in male-specific ciliated HOB neurons (Wang et al., 2010; Yu et al., 2003).

DAF-19 can also induce the expression of cell type-specific regulatory factors, such as the forkhead factor FKH-2, which in turn activates genes required for the elaboration of the distinctive morphological attributes of cilia. In the AWB odorant-sensing neurons of *C. elegans*, FKH-2 activates the kinesin II subunit *kap-1*, which contributes to the specific branching pattern of the cilia on these neurons (Mukhopadhyay et al., 2007).

Finally, ciliary diversity can also be generated by cell type-specific transcription factors, acting parallel to, or independently of, the ciliary transcription modules. For example, the mammalian transcriptional regulator HNF1 β has been shown to regulate the expression of genes encoding the mechanoreceptors PKHD1 and PKD2 in the kidneys, which allow the renal cilia to sense urine flow (Gresh et al., 2004). Another transcription factor, SOX5, directly regulates the expression of the axonemal central pair component-encoding gene, *SPAG6*, in ciliated human bronchiolar cells. In this instance, SOX5 and FOXJ1 appear to act independently to activate the expression of this ciliary gene (Kiselak et al., 2010), demonstrating yet another transcriptional path that cells can take to generate ciliary diversity.

Conclusions

Ciliated cells have the fundamental problem of precisely coordinating the expression of a complex suite of genes in order to produce functional cilia. Even though the importance of transcriptional regulation in ciliary differentiation was first appreciated through studies of flagellar regeneration in *Chlamydomonas* (Stolc et al., 2005), it is in the metazoans where we find that a set of dedicated transcriptional regulators have been specially delegated for this purpose. Drawing on the cumulative data discussed above, we propose that the expression of genes to create a basic, immotile ciliary template is directed by the RFX transcription factor family. Layering of FOXJ1 control onto this basic program allows a cell to differentiate motile cilia. It will be apparent from this Review that significant gaps remain in our understanding of many aspects of these two major ciliary transcriptional modules. We speculate that much of the future attention will be centered on how the ‘bells and whistles’ unique to the different cilia subtypes are derived from the combinatorial action of the RFX factors and FOXJ1. In addition, there is a need to better understand how these two transcriptional programs are modified by signaling pathways and cell type-specific transcription factors in order to activate specific target genes and generate different kinds of cilia. The findings from these transcriptional studies will have to be integrated with other established mechanisms for generating ciliary diversity, such as variations in IFT (reviewed by Silverman and Leroux, 2009), translational control [e.g. by microRNAs (Marcet et al., 2011; Wang et al., 2013)], membrane trafficking (Olivier-Mason et al., 2013) and post-translational modifications of ciliary components, such as acetylation and glutamylation of tubulin (reviewed by Konno et al., 2012). Importantly, all of this information will have a profound impact on our understanding of how defects in the proper differentiation and function of cilia can cause such a wide and rapidly expanding spectrum of diseases in humans.

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Competing interests

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