Published in final edited form as:

Genesis. 2017 January ; 55(1-2): . doi:10.1002/dvg.23001.

What we can learn from a tadpole about ciliopathies and airway diseases – Using systems biology in *Xenopus* to study cilia and mucociliary epithelia

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Abstract

Over the past years, the *Xenopus* embryo has emerged as an incredibly useful model organism for studying the formation and function of cilia and ciliated epithelia *in vivo*. This has led to a variety of findings elucidating the molecular mechanisms of ciliated cell specification, basal body biogenesis, cilia assembly and ciliary motility. These findings also revealed the deep functional conservation of signaling, transcriptional, post-transcriptional and protein networks employed in the formation and function of vertebrate ciliated cells. Therefore, *Xenopus* research can contribute crucial insights not only into developmental and cell biology, but also into the molecular mechanisms underlying cilia related diseases (ciliopathies) as well as diseases affecting the ciliated epithelium of the respiratory tract in humans (e.g. chronic lung diseases). Additionally, systems biology approaches including transcriptomics, genomics and proteomics have been rapidly adapted for use in *Xenopus*, and broaden the applications for current and future translational biomedical research. This review aims to present the advantages of using *Xenopus* for cilia research, highlight some of the evolutionarily conserved key concepts and mechanisms of ciliated cell biology that were elucidated using the *Xenopus* model, and describe the potential for *Xenopus* research to address unresolved questions regarding the molecular mechanisms of ciliopathies and airway diseases.

Cilia and cilia-associated diseases

The cilium (also called flagellum in some cases) is an important organelle present on most mammalian cells (Satir and Christensen, 2007). It is characterized by a membrane-covered core of microtubules, the axoneme, which emerges from a modified centriole, the basal body, and projects from the cell surface into the extracellular space (Avasthi and Marshall, 2012; Satir and Christensen, 2007). Cilia come in various shapes and with several structural modifications, which determine their specific functions, e.g. in cell signaling, perception of environmental stimuli, or generation of extracellular fluid flows (Choksi et al., 2014).

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Cilia types can be broadly subdivided into immotile sensory cilia and motile cilia. The class of sensory cilia includes the primary cilium, which is present in most non-cycling mammalian cells and required for cell signaling perception (e.g. Hedgehog and PDGF signaling) (e.g. Wong and Reiter, 2008), as well as specialized types of immotile cilia, such as the connecting cilium of photoreceptor cells (Falk et al., 2015). Motile cilia can also mediate or alter signaling of the cell (Mitchell et al., 2007; Shah, 2009; Walentek et al., 2014a), but their main function is either to facilitate locomotion or to generate fluid flow along the plane of an epithelium (Ibañez-Tallon et al., 2003): For example, a single motile mono-cilium enables sperm cells to swim towards the oocyte, while a transient epithelium comprised of cells with polarized motile mono-cilia generates an asymmetric fluid flow from right to left during early embryonic development (Blum et al., 2014). This asymmetric fluid flow induces molecular asymmetry along the vertebrate left-right body axis that instructs the correct development and positioning of asymmetric inner organs, such as the heart and the digestive tract. Additionally, many motile cilia decorate the surface of multi-ciliated cells (MCCs) of mucociliary epithelia, which line the brain's ventricles, the fallopian tube and the conducting airways in adult mammals (Ibañez-Tallon et al., 2003). Cilia of MCCs beat in a coordinated fashion to generate directional fluid flows, thereby enabling the generation of cerebrospinal fluid flow, the transport of oocytes, and the removal of inhaled particles and pathogens from the respiratory tract through mucociliary clearance (Freund et al., 2012; Marshall and Kintner, 2008).

Cilia-associated diseases (ciliopathies) are mostly caused by mutations in genes encoding ciliary or basal body components (Barker et al., 2014; Toriyama et al., 2016; Veleri et al., 2014; Waters and Beales, 2011; Zariwala et al., 2011). Because of the omnipresence of cilia in the body, ciliopathies can affect the function of virtually all organs and lead to a broad range of symptoms, including developmental defects (e.g. holoprosencephaly and polydactyly), impaired vision (e.g. retinitis pigmentosa), deafness and anosmia, kidney failure (e.g. polycystic kidney disease and nephronophthisis), obesity, male infertility and tubular pregnancies in women, hydrocephalus, as well as respiratory infections caused by deficient mucociliary clearance (Falk et al., 2015; Ibañez-Tallon et al., 2003; Tilley et al., 2015). The clinical manifestations of ciliopathies are often observed as combinations of the above-mentioned symptoms, leading to complex syndromes affecting multiple organs simultaneously, such as Kartagener's triad (infertility, left-right axis defects, airway infections) (Afzelius, 1976; Zuckerman and Wurtzebach, 2007), Bardet-Biedl syndrome (blindness, obesity, developmental and kidney defects) (Tobin and Beales, 2007) or Joubert syndrome (brain development defects, kidney failure, Polydactyly) (Sattar and Gleeson, 2011). According to the Ciliopathy Alliance (ciliopathyalliance.org), about 20 different ciliopathies have been described to date, and approximately 1 in 1000 people is affected.

Additionally, diseases affecting the respiratory tract are among the most common causes of death worldwide, and chronic lung diseases (e.g. chronic obstructive pulmonary disease (COPD)) and respiratory infections are responsible for over 6 Million deaths per year (WHO report 2012). Impaired lung function and increased susceptibility to airway infections are both caused – at least in part – by loss of cilia function or alterations in the composition and function of the airway mucociliary epithelium (Beers and Morrissey, 2011; Ganesan and Sajjan, 2013; Pain et al., 2014). Currently, we lack crucial knowledge of many aspects of

airway mucociliary development and, thus, we lack tools for early diagnosis as well as therapies for most chronic lung diseases, and lung transplantation is often the last hope for affected patients.

In light of the current unsatisfying state of diagnosis, prevention and treatment of ciliopathies and airway diseases, it is crucial to gain deeper understanding of the normal biology of ciliated cells and ciliated epithelia in order to develop new tools and strategies for these patients. Therefore, new models for the investigation of cilia and ciliated epithelia are necessary for us to take the next steps towards achieving this understanding. The next section will describe why the *Xenopus* embryo provides a powerful and tractable system to address the unresolved questions in the field.

Advantages of the *Xenopus* model for cilia research

Amphibian embryos have been used for developmental biology research since the 19th century, and since the success of the Hogben pregnancy test in the 1930s, *Xenopus* has come to populate laboratories around the world and become one of the favorite model organisms for cell and developmental biology, ion channel physiology and biochemistry (Gurdon and Hopwood, 2000; Harland and Grainger, 2011). With the onset of the 21st century, cilia were “re-discovered” by the broader scientific community and became an attractive subject of modern molecular developmental and cell biological research (Davenport and Yoder, 2005). Subsequently, *Xenopus* has increasingly been used to investigate the formation and function of cilia and ciliated epithelia in the past ten years, and research conducted using *Xenopus* embryos has not only proven that frogs provide a suitable model to study cilia, but has also contributed crucial mechanistic insights to the growing field of cilia biology (some of which are discussed below).

A major advantage of the *Xenopus* system for cell and developmental research is the availability of hundreds of embryos that rapidly develop externally and simultaneously after *in vitro* fertilization (Sive et al., 2000). Furthermore, *Xenopus* oocytes and embryos are large and robust, which allows for a wide range of manipulations, e.g. targeted microinjections of mRNA, DNA, morpholino oligonucleotides (MOs) and CRISPR/Cas9 complexes, tissue grafting, and the generation of organoids from animal cap explants (Asashima et al., 2009; Blitz et al., 2013; Eisen and Smith, 2008; Nakayama et al., 2013; Sive et al., 2000). Targeted microinjections are especially useful to study genes whose inactivation in the entire embryo would lead to early lethality and thus preclude in-depth analysis; they also permit comparison of manipulated and unmanipulated cells within the same organism (co-injection of fluorescent dyes allows tracing of the manipulated cells throughout development). This combination of features facilitates sophisticated experiments, such as parallel testing of many conditions in hundreds of siblings (e.g. for drug screening), manipulation of several genes simultaneously (e.g. to test genetic interaction), knockout analysis in the F0 generation after efficient CRISPR/Cas9 targeting, and rescue of knockdown/-out phenotypes by DNA or mRNA injections. Importantly, MO-mediated knockdown has proven to work specifically and efficiently in *Xenopus*, and defects due to upregulation of *p53* expression in response to MO injection, as reported for zebrafish, was not observed in the frog (Blum et al., 2015). Recent direct comparisons between MO and CRISPR/Cas9 knockout of

transcription factors in *Xenopus* by RNA-sequencing (RNA-seq) further support the specificity of both the transcriptional changes and downstream morphant phenotypes in frog embryos (Campell et al., 2016). The availability of high-quality genome sequences in both *X. laevis* (Session et al., 2016) and *X. tropicalis* (Hellsten et al., 2010) also makes it easy to design specific guide RNAs (gRNAs) for genome editing, and analysis of potential off-target effects by RNA-seq in CRISPR/Cas9-manipulated *Xenopus* embryos revealed extremely efficient effects on the targeted genes (>90% of transcripts expressed from the targeted genes were mutated) and a lack of off-target effects in transcripts harboring potential off-target sequences with as few as 1 to 5 mismatches (Campell et al., 2016). While efficient, targeted indels generated by this manipulation were highly variable, suggesting that Cas9 lesions take place not in single-cell embryos but after a number of cell divisions, as suggested by others working in zebrafish (Campell et al., 2016, Hruscha et al., 2013).

In addition to the advantages for cell and developmental biology research in general, *Xenopus* has features that facilitate easy investigation of cilia and ciliated epithelia. Specifically, *Xenopus* embryos form several ciliated epithelia during development, with conserved functions among vertebrates, which are readily accessible for *in vivo* manipulation, imaging and analysis.

The left-right organizer (LRO)

The LRO is a mono-ciliated epithelium that generates asymmetric fluid flow and induces the asymmetry of the left-right body axis (Blum et al., 2014; Schweickert et al., 2007). This epithelium is the gastrocoel roof-plate (GRP) in amphibians (Fig.1 A) and some basal fish (e.g. sturgeon), and is homologous to the embryonic node in mice and rabbits, or the Kupffer's vesicle in teleost fish (Blum et al., 2007). The GRP forms one day post fertilization (LRO in the mouse is found at E8.0), is relatively large (approximately 3-4 fold larger than the mouse node), and can be easily dissected and used for live cell imaging of fluid flow or cilia movement in culture, or fixed and stained to investigate gene expression, cilia formation and morphology (Blum et al., 2009; Boskovski et al., 2013). Additionally, the ability to target gain- and loss-of-function manipulations on either the left, the right or both sides, which is unique among established vertebrate model organisms, has proved to be a powerful tool to analyze the molecular functions of components and mechanisms of left-right axis specification (Schweickert et al., 2010; Vick et al., 2009; Walentek et al., 2012).

The neural tube and the brain

As in other vertebrates, the developing neural tube in *Xenopus* produces cells that harbor a primary cilium (Fig.1 B). These primary cilia on neural progenitor cells are required to mediate cell signaling (mainly Hedgehog signaling), which patterns the neural tube (Dessaud et al., 2008; Wong and Reiter, 2008). The analysis of Hedgehog-dependent neural gene expression (e.g. *nkx2.2* or *pax6*) by whole mount *in situ* hybridization offers a quick and easy assay to test if primary cilia formation or function is disturbed after manipulation (e.g. Chung et al., 2012; Walentek et al., 2016). In cases where neural signaling defects are recorded, primary cilia formation can be further analyzed using immunofluorescence on tissue sections (Fig.1 B), and again, the ability to target microinjections provides a useful

tool for experimentation, i.e. the possibility to compare one manipulated side of the neural tube to a control injected side within the same individual.

Xenopus is also suitable to examine formation and function of motile cilia of the central nervous system, i.e. motile mono-cilia of the floor plate of the spinal cord, as well as motile mono-ciliated cells and MCCs of the brain (Schweickert and Feistel, 2015; Walentek et al., 2015a). The large size of the tadpole brain and its ventricles makes them amenable to manipulation and analysis of cerebrospinal fluid (CSF) flow, e.g. fluorescent microbeads can be injected directly into the large 4th ventricle and their movement can be imaged and analyzed *in vivo* (Hagenlocher et al., 2013). Through this technique, patterns of CSF were recently visualized in the developing *Xenopus* brain, which revealed a previously underappreciated complexity as well as the contribution of different cilia beating modes in driving dorsal versus ventral CSF flow (Hagenlocher et al., 2013).

The mucociliary embryonic epidermis

Most prominently, mucociliary epithelia line the mammalian conducting airways and the epidermis of many vertebrate and invertebrate embryos (Fig.1 C) (Bartsch et al., 1997; Ganesan et al., 2013; Kemp, 1996; Verdugo, 1982; Webb and Northcutt, 1991). Although these tissues are derived from different germ layers (the ectoderm and endoderm), and hence cannot be considered strictly homologous, the mucociliary epithelia they form are remarkably similar in morphology, cell type composition and function. Mucociliary epithelia are pseudostratified columnar epithelia, characterized by the presence of secretory cell types that release mucus (goblet-like cells) or antimicrobial substances and factors that influence the behavior of the other cell types of the epithelium (e.g. Club and Pulmonary neuroendocrine cells in the airways, and small secretory cells in the *Xenopus* mucociliary epidermis) (Hayes et al., 2007; Quigley et al., 2011; Rock et al., 2010; Walentek et al., 2014a). Additionally, MCCs generate fluid flow and propagate distribution of released substances (Brooks and Wallingford, 2014; Todd, 1836). In the airways, these epithelia clear the respiratory tract of harmful substances and microorganisms to prevent damage and infection, while epidermal mucociliary epithelia prevent colonization and infection of the embryo by bacteria, as demonstrated by the production of factors with anti-microbial properties and functional assays (Dubaiissi et al., 2014; Hayes et al., 2007). The mucociliary epithelium also likely contributes to proper oxygenation of the embryo by transporting fresh water along the epidermis, the major tissue for gas exchange prior to the development of gills and lungs. The mucociliary epidermis evolved much earlier than the lung or trachea, suggesting that it represents the first mucociliary epithelium in the vertebrates, which was likely co-opted and modified in the conducting airways or the reproductive system later on in evolution. Therefore, the *Xenopus* mucociliary epidermis could be regarded as a “prototypic” mucociliary epithelium, and while likely somewhat diverged from more derived organ epithelia, may be used to reveal the principal mechanisms of mucociliary development, regeneration and function (Hayes et al., 2007; Werner and Mitchell, 2012).

The use of the *Xenopus* mucociliary epidermis has several major advantages as compared to the more commonly used models for mucociliary research, i.e. the mouse trachea and human airway epithelial cells (HAECs), which can be differentiated *in vitro* (Fulcher et al., 2005;

Rock et al., 2009): (1) The mucociliary epidermis develops rapidly into a mature mucociliary epithelium within only two days (Fig.3 B), while the airway epithelium in the mouse reaches maturity 1-2 weeks after birth, and the differentiation of HAECs requires a protocol of 3-4 weeks. (2) The exterior localization of the mucociliary epidermis allows *in vivo* manipulations (e.g. drug treatments) and analysis by live-cell imaging. Over the past years, advanced imaging techniques have been developed for use in the *Xenopus* epidermis that take advantage of the large size of *Xenopus* MCCs, as well as the ease of visualizing specific components using mRNA injections of fluorescently tagged constructs, e.g. for cilia, basal body and polarity-complex proteins (Fig.1 B,C) (Brooks and Wallingford, 2015; Werner and Mitchell, 2013). Furthermore, simple imaging methods were developed for the quantitative and qualitative analysis of extracellular fluid flow, which can be used for rapid functional screening and prediction of MCC cilia phenotypes (Walentek et al., 2014a; Walentek et al., 2015b; Walentek et al., 2016). (3) Most importantly, the molecular mechanisms, transcription factors, and expression profiles found in *Xenopus* MCCs share a deep conservation with mammalian airway MCCs (Quigley and Kintner, 2016; Treutlein et al., 2014). Therefore, the *Xenopus* mucociliary epidermis is an emerging model for airway mucociliary research with outstanding potential to facilitate novel approaches and important novel findings in ciliary and mucociliary biology.

Additional sites of ciliation in *Xenopus* embryos and tadpoles

Although the three tissues described above are most frequently employed for cilia research in *Xenopus*, additional tissues form ciliated cells in embryos and tadpoles, and are being actively used or could be used for basic and biomedical research (Schweickert and Feistel, 2015). Among these, the embryonic pronephric kidney is a popular model to study the principles of kidney development and function (Lienkamp et al., 2010; Wessely and Tran, 2011; Zhou and Vize, 2004). In *Xenopus*, the nephrostomes contain MCCs that transport fluid into the proximal tubules of the nephron, which is lined with cells presenting a primary cilium into the lumen, as in the mammalian metanephric kidney, where they sense fluid flow (Berbari et al., 2009; Schweickert and Feistel, 2015). MCCs can be also found at the tadpole nasal pits, where they drive fluid flow towards the embryo, which could help with sampling of the environment. As aquatic vertebrates, frogs also develop a lateral line organ to sense fluid motion around them. This is mediated by neuromasts, which use a primary cilium for mechanosensation of water currents (Elkan and Murray, 1951; Schweickert and Feistel, 2015). Similar hair cells can also be found in the developing inner ear (Chung et al., 2014a; Schweickert and Feistel, 2015). Frog tadpoles also develop a ciliated gut, i.e. the esophagus and the stomach are both covered by MCCs, which mediate fluid transport from anterior to posterior, suggesting that MCCs could be employed in early feeding or transport of bacteria to colonize the intestine (Chalmers and Slack, 1998; Walentek et al., 2015a). Interestingly, the conducting airways of adult *Xenopus* are not densely ciliated like in the mammalian airways (our unpublished observation), further supporting the idea that the mucociliary epidermis represents the more ancestral mucociliary tissue in vertebrates.

Advantages of the *Xenopus* model for systems biology approaches

New next-generation sequencing and proteomics methods allow us now to comprehensively investigate transcriptional changes, genome modifications and architecture, protein composition or transcription factor binding in a systems-wide manner. These systems biology methods were rapidly adapted for the application in *Xenopus*, including RNA-sequencing (RNA-seq) to analyze transcriptional changes, Chromatin immunoprecipitation and DNA sequencing (ChIP-seq) for protein-DNA interactions, Assay for Transposase-Accessible Chromatin (ATAC-seq) to find potential enhancer regions, Chromosome conformation capture (Hi-C) to investigate 3-dimensional chromosomal interactions, as well as proteomics to measure protein dynamics and to detect novel protein-protein interactions (Bogdanovi et al., 2016; Gentzel et al., 2015; Kjolby and Harland, 2016; Ma et al., 2014; Peshkin et al., 2015; Quigley and Kintner, 2016; Walentek et al., 2016; Wühr et al., 2014). The cost for these methods is decreasing while the computational power to analyze the data becomes more and more available. Additionally, commercial services for sequencing and cloud computing make these methods accessible to laboratories that do not have on-campus sequencing and bioinformatics core facilities.

The quality of results obtained from systems biology experiments often relies on the availability of ample biological material. Large numbers of manipulated embryos can be easily generated in *Xenopus*, which makes it an optimal organism for transcriptomics and genomics as well as for proteomics and biochemical studies. Furthermore, large quantities of tissue-specific cells can be obtained through dissection or through the generation of organoids from animal cap explants (Asashima et al., 2009; Sive et al., 2000; Werner and Mitchell, 2013). Mucociliary organoids are especially easy to generate in large numbers, because the default fate of the ectodermal animal cap is to develop into epidermis, while induction of neural fate requires additional inhibition of BMP signaling.

Most researchers use systems biology methods to detect new candidate genes or proteins for further downstream analysis. Since all these methods generate long lists of candidates, even if the most stringent filtering and statistical methods are used, functional testing of results becomes a challenge. Using cost efficient CRISPR/Cas9 for rapid functional screening of dozens or even hundreds of candidate genes is possible in *Xenopus*, especially so in the diploid *X. tropicalis*, where excellent computational tools for gRNA design are available (predicting targeting efficiency and off-target potential) (Bhattacharya et al., 2015; Moreno-Mateos et al., 2015). High-throughput first-pass analysis for cilia defects can be performed using streamlined whole mount *in situ* hybridization protocols, e.g. for asymmetric *pitx2c* expression (motile GRP cilia) (e.g. Blum et al., 2009; Schweickert et al., 2011), or neural *nkx2.2/pax6* expression (primary cilia) (e.g. Chung et al., 2012; Walentek et al., 2016), and fluid flow assays and immunofluorescence can be used to screen for MCC defects in the epidermis or the brain ventricles (e.g. Hagenlocher et al., 2013; Walentek et al., 2015a; Walentek et al., 2015b; Zhou et al., 2016). Additionally, subcellular localization of many candidates can be quickly tested by taking advantage of the *Xenopus* ORFeome collection and gateway cloning technology to generate fluorescently tagged constructs for expression and subsequent imaging (Brooks and Wallingford, 2015; Grant et al., 2015).

Taken together, *Xenopus* embryos are extremely well suited for systems biology approaches, and the possibility to test large sets of candidate genes in a short amount of time will prove to be highly beneficial as datasets and lists of candidates are very likely to grow further in the future.

Elucidating the evolutionarily conserved mechanisms of ciliogenesis and formation of mucociliary epithelia using *Xenopus*

Extensively studied areas in *Xenopus* cilia research include left-right axis specification (reviewed in Blum et al., 2009; Schweickert et al., 2011), polarity signaling pathways (Wnt/planar cell polarity, and Par-mediated apical-basal polarity) in cilia formation and function (e.g. Butler and Wallingford, 2015; König and Hausen, 1993; Mitchell et al., 2007; Ossipova et al., 2007; Wallingford, 2010; Werner et al., 2011), intraflagellar transport (Brooks and Wallingford, 2015), and deuterosome-mediated basal body amplification in MCCs as well as basal body composition/maturation (reviewed in Zhang and Mitchell, 2015; Zhang and Mitchell, 2016). Additionally, *Xenopus* is increasingly being used to elucidate the functions of ciliopathy genes (reviewed in Schweickert and Feistel, 2015).

The major findings in two additional areas are briefly summarized here to highlight the value and potential of systems-wide experiments in *Xenopus* cilia and mucociliary research: (1) Elucidation of the transcriptional cascade that governs MCC specification and motile cilia assembly using *Xenopus* (Fig.2). (2) Studies showing that *Xenopus* could become a very informative model to study signaling control of mucociliary tissue morphogenesis (Fig. 3).

Transcriptional and post-transcriptional regulation of MCCs

The transcriptional control of MCCs relies on the step-wise activation of multiple layers of transcription factors, which collectively regulate cell type specification, massive centriole amplification and cilia formation (Fig.2 A). Efforts over the past years have led to an exceptionally deep understanding of the gene regulatory network that underlies these processes. Data generated in *Xenopus* revealed that upon Notch signaling inhibition in MCC progenitors, a trimeric complex of Multicilin (MCIDAS), E2F4 and Tfdp1 is activated, which initiates the expression of ciliary transcription factors, e.g. *foxj1* and *rfx2* (Ma et al., 2014; Stubbs et al., 2012). The Multicilin complex, together with ciliary transcription factors, regulate a highly conserved set of core MCC genes (~950) required for vertebrate multi-ciliogenesis (Quigley and Kintner, 2016). There is a surprising degree of positive regulation between ciliary transcription factors, leading to multiple layers of amplification and potential redundancy (Fig.2 A), which likely confer robustness to cell fate choice and ciliary gene expression. Ciliary transcription factors also cooperate extensively to activate core MCC genes, which might lead to more precise and robust gene regulation (Chung et al., 2012; Chung et al., 2014b; Ma et al., 2014; Nemajerova et al., 2016; Pan et al., 2014; Quigley and Kintner, 2016; Stubbs et al., 2008; Stubbs et al., 2012; Takahashi et al., 2015; Tan et al., 2013; Zhou et al., 2015). This interpretation is supported by data on Myb and Foxn4, which regulate centriole amplification and activation of motile cilia genes in MCCs, respectively. Inactivation of either Myb (in mouse) or Foxn4 (in mouse and frog) leads to

MCC ciliation defects at stages where MCCs are first formed, but these MCCs recover later during development and are eventually able to form normal cilia (Tan et al., 2013; Campbell et al., 2016). This suggests that Myb and Foxn4 support timely and robust gene expression, which is mainly achieved by the master transcription factors for MCCs (the Multicilin complex) and motile ciliogenesis (Foxj1 and RFX2). Tp73, another transcription factor used in MCC ciliogenesis, is upstream of RFX2/3 and Foxj1, but does not seem to bind to the promoters of the vast majority of MCC genes (Marshall et al., 2016; Nemajerova et al., 2016). By contrast, Foxj1 and RFX2 bind a majority of MCC gene promoters, although Foxj1 in particular may do so indirectly (Quigley and Kintner, 2016). Recent work shows that Foxj1-binding DNA motifs are preferentially found at distal regulatory elements/enhancers, but rarely at core MCC gene promoters, while RFX2 sites or RFX motifs allowing for other RFX protein binding are found at both enhancers and promoters. Interaction of Foxj1 with core MCC gene promoters largely depends on the presence of RFX2, which forms homo- and hetero-dimers with other RFXs. These data hint that RFX2 or other RFX proteins may be required for Foxj1-mediated motile cilia gene expression by facilitating chromatin looping and interaction of enhancer-bound Foxj1 with specific promoters (Quigley and Kintner, 2016). It is tempting to speculate that other RFX factors, implicated in many other developmental and disease contexts, could fulfill essentially the same function of maneuvering distal transcriptional machinery to promoters of target genes (Choksi et al., 2014; Walentek, 2016). Taken together, these data suggest that some upstream combination of E2f4, TfDp1, and Multicilin, along with Tp73, regulates Foxj1 and RFX2. In turn, Foxj1 and RFX proteins perform the heavy transcriptional lifting, with minor assistance from Myb and Foxn4, of the hundreds of genes required to make motile cilia in MCCs.

In addition to transcriptional control, the MCC gene regulatory network also employs post-transcriptional mechanisms for gene regulation (Fig.2 B). The miR-34/449 family of microRNAs (miRs) is highly expressed in MCCs, required for ciliogenesis in human, mouse, zebrafish and *Xenopus* MCCs, and dysregulated in various airway diseases (Graff et al., 2012; Lizé et al., 2010; Marcet et al., 2011; Song et al., 2014; Walentek et al., 2014b; Wang et al., 2013b). Studies in *Xenopus* and mouse have shown that miR-34/449 are directly regulated by ciliary transcription factors (Nemajerova et al., 2016; Walentek et al., 2016), and required for successful ciliogenesis in MCCs through negative regulation of multiple targets, including Notch signaling components, actin regulators and the centriolar protein Cp110 (Cibois et al., 2015; Marcet et al., 2011; Song et al., 2014; Walentek et al., 2016). The case of Cp110 level regulation by miR-34/449 is particularly interesting, as it demonstrates the requirement for precise titration of protein levels during ciliogenesis. Cp110 is able to promote or to inhibit cilia formation in MCCs in a dose-dependent manner, and too little or too much Cp110 equally inhibits cilia formation, though through different mechanisms (Fig.2 B) (Walentek et al., 2016). To achieve robust activation of expression in MCCs, *cp110* is directly activated by ciliary transcription factors (the Multicilin complex, Foxj1 and RFX2). To prevent excess accumulation of Cp110, five of the six miR-34/449 family members are also directly controlled by ciliary transcription factors (the Multicilin complex, Tp73, Foxj1 and RFX2) at two distinct genomic loci, and their high-level expression represses *cp110* to achieve optimal Cp110 protein levels during ciliogenesis

stages (Walentek et al., 2016). These data exemplify how miRNAs are employed within a gene regulatory network, and reveal a versatile mechanism of protein level control in MCCs.

These examples demonstrate how valuable *Xenopus* is for detection and functional testing of ciliary genes, and how systems biology approaches in *Xenopus* can be successfully used to gain insight into the gene regulatory networks that regulate cell fate specification and differentiation. It is likely that mechanisms similar to the ones described for MCCs are employed in other cell types and are dysregulated in diseases, e.g. cancer (Walentek, 2016; Walentek et al., 2014b). Future cilia research in *Xenopus* will therefore not only reveal conserved mechanisms in cilia formation, but also shed light on general principles of gene regulation and cell function in development and disease.

Signaling in mucociliary cell type specification and morphogenesis

The activation of transcriptional networks, such as the one described for MCCs (Fig.2 A), is achieved through signaling control by cell signaling cascades, e.g. Notch, BMP and Wnt. Knowing how cell signaling controls mucociliary development and regeneration is imperative to understand the changes that occur in chronic lung diseases, which are often characterized by tissue remodeling (i.e. changes in cell type composition) or impaired differentiation (e.g. in dysplasia) (Beers and Morrisey, 2011; Ganesan and Sajjan, 2013; Pain et al., 2014). A major challenge in understanding the signaling control of complex tissue formation and morphogenesis is the reiterative and pleiotropic nature of cell signaling pathways, which makes it hard to distinguish between primary and secondary effects on cell types or a given process. In contrast to the airway mucociliary epithelium, which develops late and requires many developmental steps to occur correctly before the epithelium is formed, the *Xenopus* mucociliary epidermis is specified in a relative simple process very early on in development (the epidermal ectoderm is specified by about 12h post fertilization in the animal hemisphere of the embryo; Fig.3 A) (Heasman, 1997; Herriges and Morrisey, 2014). Therefore, the mucociliary epidermis allows investigation of mucociliary development directly, without the prerequisite of successful lung formation. Furthermore, the cell type composition of the *Xenopus* mucociliary epidermis is fully resolved, while it remains uncertain how many rare cell types exist in the mammalian airway epithelium (Brooks and Wallingford, 2014; Crystal et al., 2008; Hayes et al., 2007; Quigley et al., 2011; Walentek et al., 2014a). In addition, the master transcription factors and transcriptional profiles for all epithelial cell types are either published or currently being resolved. These data facilitate analysis of the effects of signaling manipulations on cell type specific gene regulatory networks over developmental time, which will allow us to dissect the different functions of cell signaling in mucociliary epithelia. This understanding will likely provide key insights into mammalian airway epithelial development and disease mechanisms, as roles of major signaling pathways seem to be conserved between the mucociliary epidermis and airway cells.

Notch signaling negatively regulates MCC cell fate, while promoting secretory cell formation (Fig.3 B). This role of Notch signaling was found early on in the *Xenopus* mucociliary epidermis (Deblandre et al., 1999), and holds true for the mouse airways and human airway stem cells, indicating a deeply conserved function (Gomi et al., 2015; Tsao et

al., 2009). Additionally, Notch signaling is also active during a second wave of signaling to subdivide progenitors into more distinct cell lineages, which was shown for *Xenopus* ion secreting cells and airways cell of the mouse (Fig.3 B) (Morimoto et al., 2012; Quigley et al., 2011; Rock et al., 2011).

A recent study has also compared the effects of BMP signaling on *Xenopus* and human mucociliary cells, and revealed a conserved requirement for BMP inhibition in the specification of MCCs and other cells derived from subepithelial progenitor cells (Fig.3 B) (Cibois et al., 2015). In later stages of development, BMP signaling is also required for intercalation of differentiating cells into the epithelium as well as for the expression of goblet cell markers (Fig.3 B), providing another case for reiterative signaling in addition to Notch (Cibois et al., 2015).

Lastly, Wnt/ β -catenin signaling is implicated in several processes in mucociliary development, regeneration and disease, but its precise roles are not completely understood (De Langhe and Reynolds, 2008; Pongracz and Stockley, 2006). Experiments in the *Xenopus* mucociliary epidermis indicate that virtually all cell types require Wnt/ β -catenin signaling at some point during specification and differentiation (Fig.3 B). Specifically, *foxf1* expression in MCCs requires Wnt/ β -catenin signaling in *Xenopus* and zebrafish, where it was shown to be directly regulated by β -catenin (Caron et al., 2012; Walentek et al., 2012; Walentek et al., 2015b). Tp63 is also directly regulated by Wnt/ β -catenin signaling in *Xenopus* and humans, and is an important regulator of stem/progenitor cell proliferation and differentiation (Kjolby and Harland, 2016; Ruptier et al., 2011). Small secretory cells and goblet-like cells also require Wnt/ β -catenin signaling for expression of *foxa1* (which induces small secretory cells; Walentek et al., 2015b) and normal secretion (goblet-like cells; our unpublished results). Similar to the situation in *Xenopus*, Wnt/ β -catenin signaling was implicated in mucus secretion (Mucenski et al., 2005; Okubo and Hogan, 2004), basal stem cell proliferation and differentiation (Brechtbuhl et al., 2011; Ghosh et al., 2013; Hsu et al., 2014), and possibly FOXA1 expression (Lamouille et al., 2014; Wang et al., 2013a) in mice and human cells. Collectively, these findings suggest that a conserved Wnt/ β -catenin dependent gene regulatory network exists in mucociliary epithelia, and contributes to the regulation of cell fate specification and specific differentiation processes in multiple cell types. It is therefore important to resolve the evolutionarily conserved functions of Wnt/ β -catenin signaling in mucociliary epithelia, as well as the functions of other signaling pathways.

These examples all reinforce the argument that the *Xenopus* mucociliary epidermis can serve as a powerful model to study systems-wide effects of mucociliary signaling, which will help to dissect the reiterative and pleiotropic roles of signaling in airway mucociliary regeneration and disease.

In summary, *Xenopus* embryos provide an outstanding model for the investigation of cilia and ciliated epithelia, and the combination of classic advantages of the frog with new technologies for systems biology approaches opens new avenues to address important unresolved questions in the field. These features are further complemented by excellent resources for *Xenopus* research, including genomes, databases, national and international

stock centers, and transgenic lines, which are provided through a highly collaborative and dedicated community of researchers (e.g. Grant et al., 2015; Harland and Grainger, 2011; Hellsten et al., 2010; Karpinka et al., 2014; Session et al., 2016). We look forward to more studies that use *Xenopus* systems biology to address important questions in cilia and mucociliary biology, which will help us to understand how gene regulatory networks function, how complex tissue formation is achieved, and how ciliopathies and airway diseases could be cured in the future.

Acknowledgements

We thank C. Exner and R.M. Harland for critical reading and suggestions on the manuscript. We also thank the cilia and *Xenopus* communities for their contributions to the field, sharing of resources, and fruitful discussions as well as sharing of unpublished data. We thank Xenbase (RRID:SCR_003280) and the National Xenopus Resource (RRID:SCR_013731) as well as the European and Japanese Resource Centers for their invaluable work. PW is funded by the National Heart, Lung and Blood Institute (#K99HL127275).

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Figure 1. Ciliated tissues in *Xenopus laevis* embryos and tadpoles
Immunofluorescent staining of *Xenopus laevis* embryos with anti acetylated- α -tubulin antibody to reveal cilia (green) of (A) the gastrocoel roof plate (GRP) at stage 17, (B) the neural tube at stage 26, and (C) the embryonic mucociliary epidermis at stage 32. The different types of ciliated cells are shown in magnified views. Co-staining: Phalloidin for F-actin (red), anti-serotonin for small secretory cells (5HT; blue). Basal bodies were visualized by expression of *centrin4-rfp* (red) and *gfp-cp110* (blue). Digitized images and developmental data from Nieuwkoop and Faber (1994) Normal Table of *Xenopus laevis* (Daudin) were obtained from Xenbase.org.

Figure 2. Transcriptional and post-transcriptional regulation of multi-ciliated cells
(A) Transcriptional control of core MCC gene expression in multi-ciliated cells. Arrows = activation, T = inhibition, solid line = major contribution, dotted line = supporting contribution. (B) A transcriptional/post-transcriptional regulatory module titrates levels of Cp110 to allow for ciliogenesis in MCCs. Left panel: *cp110*, miR-34b/c and miR-449a-c are directly regulated by ciliary transcription factors (color code is the same as in A). Right panel: optimal (intermediate) Cp110 levels are required for ciliogenesis. Summary of effects of too much or too little Cp110 on MCC cilia formation.

Figure 3. Signaling regulation in the *Xenopus* mucociliary epidermis

Overview of signaling regulation and development of the mucociliary epidermis. **(A)** Developmental processes over the time-course of mucociliary epithelium formation. Corresponding stages of *Xenopus* development are depicted on the left. Digitized images and developmental data from Nieuwkoop and Faber (1994) Normal Table of *Xenopus laevis* (Daudin) were obtained from Xenbase.org. **(B)** Schematic summary of signaling events and key transcription factors regulating mucociliary cell fate specification. Binary decisions based on high versus low level input from signaling events are shown as triangle representing two sides of a signaling gradient (for BMP, Wnt and Notch). Additional contributions from signaling pathways are depicted as, e.g. “+Wnt”. In later stages, BMP signaling (yellow) is also required for intercalation of all cell types derived from subepithelial progenitors.