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## ATP4a is required for development and function of the *Xenopus* mucociliary epidermis - a potential model to study proton pump inhibitor-associated pneumonia

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### Abstract

Proton pump inhibitors (PPIs), which target gastric H<sup>+</sup>/K<sup>+</sup>ATPase (ATP4), are among the most commonly prescribed drugs. PPIs are used to treat ulcers and as a preventative measure against gastroesophageal reflux disease in hospitalized patients. PPI treatment correlates with an increased risk for airway infections, i.e. community- and hospital-acquired pneumonia. The cause for this correlation, however, remains elusive. The *Xenopus* embryonic epidermis is increasingly being used as a model to study airway-like mucociliary epithelia. Here we use this model to address how ATP4 inhibition may affect epithelial function in human airways. We demonstrate that atp4a knockdown interfered with the generation of cilia-driven extracellular fluid flow. ATP4a and canonical Wnt signaling were required in the epidermis for expression of foxj1, a transcriptional regulator of motile ciliogenesis. The ATP4/Wnt module activated foxj1 downstream of ciliated cell fate specification. In multiciliated cells (MCCs) of the epidermis, ATP4a was also necessary for normal myb expression, apical actin formation, basal body docking and alignment of basal bodies. Furthermore, ATP4-dependent Wnt/β-catenin signaling in the epidermis was a prerequisite for foxa1-mediated specification of small secretory cells (SSCs). SSCs release serotonin and other substances into the medium, and thereby regulate ciliary beating in MCCs and protect the epithelium against infection. Pharmacological inhibition of ATP4 in the mature mucociliary epithelium also caused a loss of MCCs and led to impaired mucociliary clearance. These data strongly suggest that PPI-associated pneumonia in human patients might, at least in part, be linked to dysfunction of mucociliary epithelia of the airways.

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#### Authors contribution

PW initiated, conducted and analyzed experiments. TB performed electron microscopy. CH and KF helped significantly with ependymal flow analysis. CM contributed to ATP4 inhibitor experiments. PW, AS and MB planned experiments and interpreted the results. RMH helped with interpretation of data and writing of the manuscript. PW and MB wrote the manuscript with input from all authors.

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## Keywords

ATP4; proton pump inhibitor; cilia; small secretory cells; Wnt; *Xenopus laevis*

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## Introduction

Proton pump inhibitors (PPIs) are a class of therapeutic drugs used during treatment of gastric and peptic ulcers (Shin and Sachs, 2006). In addition, PPIs are prescribed to hospitalized patients to prevent gastroesophageal reflux disease (GERD) (Fohl and Regal, 2011). PPIs, e.g. Omeprazole, belong to the most widely prescribed drugs, with a worldwide volume of >26 billion US \$ and >53 million annual prescriptions in the United States alone (IMS Institute for healthcare informatics, 2011; Reimer, 2013). PPIs inhibit gastric H<sup>+</sup>/K<sup>+</sup> +ATPase (ATP4) function (Shin et al., 2009). ATP4 is a transmembrane proton pump composed of two catalytic  $\alpha$  (ATP4a) and two accessory  $\beta$  (ATP4b) subunits (Shin and Sachs, 1996). ATP4 is highly expressed in the vertebrate stomach and required for acidification of the gastric lumen (Sawaguchi et al., 2004; Shin et al., 2009). In addition to gastric expression of ATP4, it is proposed that ATP4 is expressed in the airways, though these findings are still under debate (Altman et al., 2007; Fischer and Widdicombe, 2006; Herrmann et al., 2007). PPI administration correlates with an increased risk for hospital- and community-acquired pneumonia (Fohl and Regal, 2011). Depending on study design, daily dose, and duration of PPI treatment, the increased risk of developing pneumonia is substantial, but is highly variable (0.91–6.53 fold; confidence interval 95%; de Jager et al., 2012; Fohl and Regal, 2011; Herzig et al., 2014; Jena et al., 2013; Ramsay et al., 2013; Sheen and Triadafilopoulos, 2011). Proposed molecular mechanisms include effects on the immune system or bacterial overgrowth in the stomach, due to increase in gastric pH, enrichment of potential pathogenic microorganisms, and subsequent microaspiration causing airway infections (Fohl and Regal, 2011; Herzig et al., 2014; Reimer, 2013).

The mammalian epithelial lining of the upper respiratory tract constitutes a mucociliary epithelium, which functions as a first line defense against pathogens (Crystal et al., 2008; Mall, 2008; Proud and Leigh, 2011). Dysfunction of the airway mucociliary epithelium causes an increased susceptibility to pulmonary infections (Fliege et al., 2007). The *Xenopus* embryonic epidermis resembles mammalian airway epithelia in many ways, and over the past years it has emerged as an important model for the study of development and function of vertebrate mucociliary epithelia (Hayes et al., 2007; Werner and Mitchell, 2012). It is composed of four cell types: multiciliated cells (MCCs), small secretory cells (SSCs), ion secreting cells (ISCs), and outer/goblet cells (Dubaiissi and Papalopulu, 2011; Dubaiissi et al., 2014; Quigley et al., 2011; Stubbs et al., 2006; Walentek et al., 2014). The apical surface of MCCs is typically decorated with hundreds of motile cilia, whose coordinated beating causes extracellular fluid flow (Mall, 2008; Marshall and Kintner, 2008); the other cell types of mucociliary epithelia secrete substances like mucus and contribute to the airway surface liquid, thereby protecting the epithelium from inhaled particles and pathogens (Crystal et al., 2008; Jeffery and Li, 1997).

Our previous work has revealed that ATP4 is required for cilia-dependent development of the left-right (LR) body asymmetry in *Xenopus*: During gastrulation, ATP4-function is a prerequisite for activation of canonical Wnt/ $\beta$ -catenin signaling and expression of *foxj1* (Walentek et al., 2012), a master transcription factor for motile ciliogenesis (Stubbs et al., 2008; Yu et al., 2008). LRP6 also cooperates in specification of MCCs in the neural epidermis (Huang and Niehrs, 2014). Polarization of cilia at the gastrocoel roof plate is mediated by non-canonical Wnt/planar cell polarity (PCP), and this requires ATP4a function as well (Walentek et al., 2012).

Based on the role of ATP4 in motile ciliogenesis and the rationale that the *Xenopus* epidermis might provide insights into the underlying mechanisms of PPI-associated pneumonia, we tested the role of ATP4 and Wnt signaling in development and function of the mucociliary epithelium. Our results argue for a general role of ATP4 and Wnt signaling in motile ciliogenesis during *Xenopus* development and in the specification of epidermal SSCs. Pharmacological inhibition of ATP4 after development of the mucociliary epidermis also affected extracellular fluid flow. Therefore, we hypothesize that PPI-associated pneumonia might be in part caused by dysfunction of the mucociliary airway epithelium.

## Results

### ATP4 is required for normal development of the *Xenopus* embryonic epidermis

To investigate the potential function of ATP4 in formation of the ciliated *Xenopus* embryonic epidermis, we analyzed the expression of ATP4a protein in the epidermal ectoderm (Fig. S1). ATP4a was found throughout the animal hemisphere during gastrulation (Fig. S1A, A') and neurulation (Fig. S1B, C), within the outer and deep layer of the skin ectoderm (Fig. S1B). The deep layer forms multiciliated cells (MCCs), ion secreting cells (ISCs) and small secretory cells (SSCs) which later intercalate into the outer epithelium (Deblandre et al., 1999; Dubaissi et al., 2014, Stubbs et al., 2006). Furthermore, ATP4a protein was enriched in the epidermal ectoderm during neurulation (Fig. S1C), and continued expression in the epidermis throughout tailbud (Fig. S1D) and early tadpole stages (Fig. S1F, G).

The functional relevance of ATP4 for epidermal development and ciliation was tested by morpholino oligonucleotide (MO) mediated knockdown of *atp4a* (Walentek et al., 2012). Unilateral injections of *atp4a*MO were targeted to the skin ectoderm (confirmed in all experiments by analysis of co-injected lineage tracer). Morphological analysis by scanning electron microscopy revealed fewer and shorter cilia projecting from the apical surface of individual MCCs and reduced numbers of SSCs (Fig. 1A, B). Ciliation defects in *atp4a* morphants, together with our previous finding of ATP4a-dependent *foxj1* expression during gastrulation (Walentek et al., 2012), suggested that *foxj1* expression might also be affected in ATP4a-deficient MCCs; indeed in situ hybridization showed that knockdown of *atp4a* on the injected side resulted in a downregulation of *foxj1* expression, as compared to the contralateral uninjected side (Fig. 1C) or to control morpholino (CoMO) injected specimens ( $p < 0.001$ ; Fig. S2A). Reduced numbers of SSCs in the epidermis further indicated possible defects in SSC specification. Expression of *foxa1* determines SSC specification (Dubaissi et al., 2014), and in agreement with the morphological data, on average only 50% of *foxa1*

expressing cells were detected on the *atp4a*MO injected side as compared to the control side (Fig. 1C; Fig. S2B). In contrast to MCCs and SSCs, ion secreting cells (ISCs) did not show obvious defects, as judged by morphology (Fig. 1A, B) and expression of both the ISC regulator *foxi1* (Fig. 1C; Fig. S2C; Quigley et al., 2011) and the ISC marker  $vH^+$ ATPase (*atp6* subunit expression, Quigley et al., 2011) (Fig. S2D).

These findings were validated by quantitative reverse-transcription PCR (qRT-PCR) (Fig. S3). We used control and injected animal cap explants, which develop into mucociliary epithelium in culture (Sive et al., 2000; Werner and Mitchell, 2013). Four time points were chosen for the analysis: stage 10 (early cell type specification), stage 19 (late specification/early intercalation), stage 25 (late intercalation, when basal bodies are docked to the apical membrane and ciliogenesis starts), and stage 32 (fully ciliated and functional mucociliary epithelium) (Avasthi and Marshall, 2012; Song et al., 2014; Stubbs et al., 2006). Expression levels were calculated relative to stage 10 uninjected control samples in order to reflect the expression dynamics.

Expression of three major transcriptional regulators in MCCs was analyzed: *multicilin* (*mci*; (Stubbs et al., 2012), *myb* (Tan et al., 2013) and *foxj1* (Fig. S2C). *mci* was only weakly expressed at stage 10, peaked at stage 19 and was barely detectable at later stages (25 and 32) of mucociliary development. The *atp4a* morphant explants did not display significant changes in *mci* expression at stages 10 and 19, but there was a slight increase at stages 25 and 32 relative to controls (Fig. S3A). Morphant explants strongly downregulated *myb* and *foxj1* during cell type specification and intercalation stages, but these recovered to normal or above-normal levels by stage 32 (Fig. S3A). These results indicated that not *Mci*-dependent MCC specification was affected in *atp4a* morphants, but downstream gene expression of *foxj1* and *myb*, which are required for MCC ciliation. SSCs are induced later during development, compared to MCCs or ISCs (Dubaisi et al., 2014; Walentek et al., 2014). Accordingly, *foxa1* expression was highest at stage 25 (Fig. S3B). *foxa1* expression was markedly reduced by *atp4a*MO injections at stages 19 and 25. Furthermore, expression of *tph1* (Tryptophan hydroxylase 1; a rate-limiting enzyme for serotonin synthesis and SSC marker; Walentek et al., 2014) was strongly reduced in explants derived from *atp4a* morphants. In contrast, *atp4a* knockdown did not negatively affect *foxi1* expression (Fig. S3C).

We therefore conclude that although ATP4a is not required for *mci* expression, it is required for MCC ciliation and SSC specification. In contrast it is not needed for specification of ISCs and ISC-specific gene expression.

### **ATP4-dependent Wnt/ $\beta$ -catenin signaling regulates *foxj1* expression in epidermal MCCs**

Confocal imaging of immunostained control and *atp4a* morphant specimens confirmed a defect in ciliation following *atp4a* knockdown (Fig. 2A, B), rather than defective specification or intercalation of these cells. In contrast to MCCs of control specimens, which show the characteristic ciliary tuft projecting from the apical membrane of the cell, immunostaining for acetylated- $\alpha$ -tubulin (tubulin) in *atp4a* morphant MCCs revealed intracellular accumulation of tubulin, but few or no cilia projecting from the apical surface (Fig. 2A). ATP4a-deficient MCCs also showed impaired formation of the apical actin

meshwork (Fig. 2A). This phenotype was reminiscent of the airway MCC phenotype in *foxj1* knock-out mice (Gomperts et al., 2004). Loss of *foxj1* in mouse airway MCCs causes ciliation defects due to impaired basal body docking to the apical membrane, a prerequisite for motile cilia formation (Gomperts et al., 2004; Marshall, 2008). We therefore investigated whether ciliation defects correlated with defects in basal body localization in *atp4a* morphants. Embryos were co-injected with a *sas6-gfp* construct in order to label basal bodies (Klos Dehring et al., 2013), and ciliation was analyzed in parallel by tubulin staining (Fig. 2B). *Sas6-GFP* positive basal bodies localized to the apical membrane in ciliated control MCCs, while defects in both ciliation and basal body docking were evident in *atp4a* morphants (Fig. 2B). The MCC phenotype of *atp4a* morphants thus phenocopied the cellular phenotype of *foxj1*-deficient mammalian airway MCCs.

ATP4a is also required for Wnt/PCP-dependent polarization of cilia at the gastrocoel roof plate (Walentek et al., 2012). Dysregulation of Wnt/PCP signaling in MCCs leads to defects in the formation of the apical actin meshwork, basal body docking and ciliation, but in addition it is also required for the uniform alignment of individual basal bodies in MCCs (Mitchell et al., 2009; Park et al., 2008). We therefore analyzed basal body directionality in *atp4aMO* injected MCCs using *centrin4-RFP* and *clamp-GFP* constructs (Park et al., 2008; Fig. 2C). Uniformly aligned basal bodies were found in control specimens, while the alignment was randomized in ATP4a-deficient MCCs (Fig 2C; Fig. S4), suggesting that ATP4a regulates Wnt/PCP-dependent aspects of MCC morphogenesis, in addition to *Foxj1*-dependent processes.

Next, we asked whether ATP4's effects on *foxj1* expression and ciliation in MCCs were caused by interference with Wnt/ $\beta$ -catenin signaling (Fig. 3), as it was shown in the context of LR-axis formation (Walentek et al., 2012). To this end, *atp4aMO* was co-injected with DNA constructs encoding ATP4a (Fig. 3C, C'),  $\beta$ -catenin (Fig. 3D, D') or *Foxj1* (Fig. 3E, E'). DNA injections were chosen to prevent early Wnt-dependent effects such as secondary axis induction, and have previously been effective in such rescues as well as for overexpression (Song et al., 2014; Walentek et al., 2012, 2013, 2014). While full ciliation failed in *atp4a* morphants, the overall morphology of the skin epidermis was remarkably normal; large goblet cells and smaller intercalating cells were clearly visible (Fig. 3A, B) and the total number of tubulin-positive cells (containing cilia or apically enriched tubulin) was not significantly different between *atp4a* morphants and uninjected control embryos (Fig. 3F, right panel). Either of the DNA constructs was sufficient to partially rescue skin ciliation ( $p < 0.001$ ; Fig. 3F) and to restore ciliary motility in *atp4a* morphants (cf. supplementary material Movie1).

In addition to rescue experiments using DNA co-injection, the specificity of the *atp4aMO* was confirmed by a) *atp4a* knockdown using a second non-overlapping translation blocking MO (*atp4aMO-2*; Fig. S5G–H, K), b) a splice-site MO (*atp4a-Sp1MO*; Fig. S5I–K) targeting the second exon/intron boundary of *atp4a* pre-mRNA, which lead to *atp4a* intron 2 retention as confirmed by PCR (see Fig. 4 in Walentek et al. 2015), and c) specific pharmacological inhibition of ATP4 using SCH28080 (Fig. S5A–B, K; Walentek et al. 2012). Each of these treatments caused MCC ciliation defects comparable to *atp4aMO* injections, without significant decrease in tubulin enriched epidermal cells (Fig. S5K).

In addition to partial or full loss of MCC ciliation, we observed increased numbers of MCCs with a relatively small apical surface in SCH28080, *atp4a*MO-2 and *atp4a*SplMO treated specimens, which likely contributes to loss of mucociliary clearance. In contrast to MO-mediated knockdown, pharmacological inhibition of ATP4 by SCH28080 also caused a frequent enlargement of epidermal cells, indicating additional defects in epithelial morphogenesis or homeostasis.

Together these data indicated that ATP4-mediated Wnt/ $\beta$ -catenin signaling was required for *foxj1* expression and maturation of MCCs, without compromising cell type specification or intercalation of MCCs.

### **Wnt/ $\beta$ -catenin signaling acts downstream of Notch/Delta-mediated cell type specification on *foxj1* expression**

Activation of Notch signaling prevents MCC and ISC specification, while inhibition of Notch signaling increases the number of MCCs and ISCs, (Deblandre et al., 1999). To integrate canonical Wnt signaling into the pathway of MCC development, we investigated the interaction of Wnt and Notch signaling. Inhibition of Notch signaling by injection of a dominant-negative mutant of suppressor of hairless (Su(H)-DBM; Deblandre et al., 1999) increased *foxj1* expression in the skin ectoderm in comparison to the uninjected contralateral side ( $p < 0.001$ ; Fig. 4A–B, E), as previously described for other markers of ciliated cells (Deblandre et al., 1999; Ossipova et al., 2007; Stubbs et al., 2012; Tan et al., 2013). In contrast, injection of both *atp4a*MO and Su(H)-DBM led to a marked decrease of *foxj1* expression ( $p < 0.001$ ; Fig. 4C, E). Restoring Wnt signaling by injection of  $\beta$ -catenin DNA reversed the effect on *foxj1* expression, i.e. triple injected specimens revealed an upregulation of *foxj1* ( $p < 0.001$ ; Fig. 4D, E), similar to inhibition of Notch signaling alone (cf. Fig. 4B, E). Injection of  $\beta$ -catenin DNA alone did not significantly alter MCC ciliation or number of MCCs (Fig. S6A–C), in agreement with previously published work, in which gain of canonical Wnt signaling did not increase the number of  $\alpha$ -tubulin expressing cells in the *Xenopus* embryonic epidermis (Ossipova et al. 2012).

In summary, these experiments demonstrated that Wnt/ $\beta$ -catenin signaling acts permissively downstream of Notch-mediated cell type specification and was required for MCC *foxj1* expression.

### **ATP4-dependent Wnt/ $\beta$ -catenin regulates serotonin signaling in the ciliated epidermis**

We have recently shown that small secretory cells (SSCs) synthesize and secrete serotonin, which regulates beating of MCC cilia via serotonin receptor type 3 (5-Htr3; Walentek et al 2014). Furthermore, SSCs are required to protect the embryo from bacterial infection (Dubaissi et al., 2014). In *atp4a* morphants the number of SSCs and *foxa1* expression were greatly reduced (Fig. 1A–C; Fig. S2B; Fig. S3B). To further test how SSCs were affected by ATP4a loss-of-function, we examined serotonin deposition (Walentek et al. 2014). Serotonin was found in vesicle-like structures in control embryos, and these were lost in *atp4a* morphants ( $p < 0.001$ ; Fig. 5A–B, E). Co-injection of *atp4a* or  $\beta$ -catenin DNA restored serotonin in SSCs ( $p < 0.001$ ; Fig. 5C–E), indicating that Wnt signaling regulates SSCs in the epidermis. *tph1*, which is expressed in SSCs (Fig. 5F, F'; Walentek et al. 2014), was also

downregulated in *atp4a* morphants (Fig. 5G, G'). Co-injection of *atp4a* DNA rescued expression of *tph1* to about control levels (Fig. 5H, H').

These data confirmed our findings on *foxa1/tph1* downregulation and showed that ATP4a-dependent Wnt signaling was also required for SSC function in the embryonic epidermis.

### Endogenous Wnt/ $\beta$ -catenin signaling is required for MCC and SSC development in the embryonic epidermis

In addition to canonical Wnt signaling, ATP4a is also required for Wnt/PCP signaling during *Xenopus* LR development (Walentek et al., 2012). Certain aspects of the MCC phenotype in *atp4a* morphants, i.e. apical actin formation, basal body docking and polarized alignment of basal bodies (Fig. 2), could be in part caused by defects in Wnt/PCP signaling as well. In order to dissect the impact of the two Wnt pathway branches on the ciliated epidermis, we inhibited canonical Wnt/ $\beta$ -catenin signaling by overexpression of *dickkopf* (*dkk*) mRNA (Niehrs, 2006). This inhibition caused defects in MCC ciliation ( $p < 0.001$ ; Fig. 6A–C) and SSC serotonin deposition ( $p < 0.001$ ; Fig. 6A–C), similar to ATP4a loss-of-function (cf. Fig. 3,5). MCCs presented fewer and shorter cilia at their apical surfaces and the number of cells positive for serotonin staining was strongly decreased (Fig. 6A', A'', B', B'' and C). Importantly, defects in MCC ciliation in *dkk* injected specimens were less severe compared to *atp4a* morphants and apical actin formation was less affected. We therefore suggest that the MCC phenotype in *atp4a* morphants was the result of combined deficiencies in Wnt/ $\beta$ -catenin and Wnt/PCP signaling.

We also observed a consistent increase in the number of MCCs in *dkk* injected embryos (Fig. 6C). Although the magnitude of this effect was not statistically significant ( $p = 0.07$ ), it might indicate additional roles of Wnt/ $\beta$ -catenin signaling in MCC cell fate specification. To reveal subtle differences between the *atp4a*MO and *dkk* phenotypes, we analyzed transcription of mucociliary genes by qPCR (Fig. S3). In *dkk* injected specimens, we found similar changes in gene expression to *atp4a*MO injected animal caps (Fig. S3), although in contrast to *atp4a*MO, *mci* expression was increased ( $p = 0.06$ ) during early and late cell type specification stages (Fig. S3A) and *foxi1* expression was moderately affected in late cell fate specification and intercalation stages (Fig. S3C). *myb* ( $p > 0.05$ ), *foxj1* ( $p < 0.01/p < 0.001$ ), *foxa1* ( $p < 0.05/p < 0.001$ ) and *tph1* ( $p < 0.001$ ) expression was persistently stronger affected in *atp4a* morphants than after *dkk* injection (Fig. S3).

Taken together, these data support a requirement for ATP4a-dependent Wnt/ $\beta$ -catenin signaling in mucociliary gene expression, and strongly indicates that ATP4 acts through Wnt/ $\beta$ -catenin signaling on the regulation of *foxj1* and *foxa1* expression in MCC ciliation and SSC cell fate specification, respectively.

Recent work reported an additional requirement for Wnt/ $\beta$ -catenin signaling during specification of the deep ectodermal cell layer (Huang and Niehrs, 2014), although our  $\beta$ -catenin DNA overexpression data (Fig. S6) and previous reports by another group (Ossipova et al. 2012) did not reveal any influence of  $\beta$ -catenin injections on the number of epidermal MCCs. Our *atp4a* DNA injections may not suffice to induce an early response to canonical Wnt signaling and, thus, not affect the specification process of deep ectodermal cells.



Therefore, we injected  $\beta$ -catenin mRNA targeted to the epidermal ectoderm at concentrations that were sufficient to induce secondary axis formation upon injection into the ventral marginal zone of 4-cell stage embryos (not shown). Overexpression of  $\beta$ -catenin mRNA ( $\beta$ -cat. RNA; Fig. S7) did not consistently lead to increased numbers of MCCs or SSCs in the epidermis at stage 32 (Fig. S7C–D), although it is noteworthy that in two out of twelve specimens a massive increase in MCCs occurred, indicating a potentially more complex relationship between canonical Wnt signaling and MCC specification.

In summary, our data revealed a developmental requirement for ATP4a-dependent Wnt/ $\beta$ -catenin signaling during Foxj1-mediated motile ciliogenesis and Foxa1-mediated specification of SSCs in the mucociliary epidermis of *Xenopus* embryos.

### **Pharmacological inhibition of ATP4 in the mature mucociliary epidermis decreases the number of MCC and results in loss of extracellular fluid flow**

Our experimental results argue strongly for a role of ATP4 during development of the *Xenopus* mucociliary epidermis, thereby suggesting a potential role for ATP4 in mucociliary clearance in the mammalian airway epithelium as well. In contrast to our early interventions in the embryo, PPIs are administered to adult patients after development of the airway epithelium. In order to address how pharmacological inhibition of ATP4 in mature mucociliary epithelia could influence mucociliary clearance, we performed experiments in which SCH28080 was applied at different stages of epidermal development and function. Both MCC ciliation and serotonin deposition were analyzed as readout (Fig. S5A–F, K; Fig. 7). When applied early (stage 5–30), SCH28080 incubations had essentially the same effects on MCCs and SSCs as MO-mediated knockdown of *atp4a* (Fig. S5A–B, G–J, K; Fig. 3), while later application of SCH28080 during epidermal development, starting at stages 12 or 22, had statistically significant but less pronounced effects on both MCC ciliation ( $p < 0.001$ ; Fig. S5C–D, E–F, K) and number of SSCs ( $p < 0.01/p > 0.05$ ; Fig. S5C–D, E–F, K). These data indicated that ATP4 plays mainly a developmental role in MCC ciliation and specification of SSCs in the embryonic epidermis. In line with this assumption, SCH28080-mediated inhibition of ATP4 starting at stage 29, i.e. when functional MCCs and SSCs were already present, did not significantly affect ciliation or serotonin deposition in MCCs and SSCs, respectively (Fig. S5K; Fig. 7A–B). Nevertheless, a quantitative analysis of extracellular fluid flow (Walentek et al., 2014) revealed significantly reduced velocities of automatically tracked fluorescent beads at stage 35 in tadpoles treated with SCH28080 from stage 29 onwards ( $p < 0.05$ ; Fig. 7E). Prolonged incubation of tadpoles in SCH28080-containing media until stage 41 further decreased extracellular fluid flow velocities, resulting in an almost complete loss of mucociliary clearance ( $p < 0.001$ ; Fig. 7E). Interestingly, tadpoles treated with SCH28080 from stage 30–41 (~72h) displayed a massive loss of MCCs (ciliated MCCs as well as tubulin-enriched cells) in the epithelium, while serotonin-containing SSCs were not affected (Fig. 7C–D). Analysis of epidermal morphology using high-resolution confocal microscopy further revealed loss of cells containing a MCC-specific apical actin meshwork, apical enlargement of ISC-like cells as well as SSCs, and accumulations of actin at cell junctions (Fig. 7F–G). This phenotype suggested that MCCs were lost in the epithelium, leading to a decrease in apical surface

tension and, therefore, allowing other intercalating cell types to expand their apical surface area.

Finally, we analyzed how this late and prolonged SCH28080 incubation affected *foxj1* expression in the epidermis. While epidermal *foxj1* expression in control embryos at stage 40 was rather weak, strong expression was found in SCH28080 treated tadpoles, including a moderate increase in *foxj1*-positive cells (Fig. 7H–I; quantification not shown). These findings suggested that *foxj1* expression might be upregulated in SCH28080 treated embryos in an attempt to regenerate and supply the epidermis with newly specified MCCs. Thus, withdrawal of the drug may lead to rapid recovery of mucociliary function.

In conclusion, inhibition of ATP4 during mucociliary development affected Foxj1-mediated ciliation in MCCs as well as Foxa1-dependent SSC specification and serotonin signaling. Inhibition of ATP4 function in established mucociliary epithelia led to a specific loss of MCCs from the epithelium, without reduction of *foxj1* expression or loss of SSCs, consequently affecting mucociliary clearance at the embryonic epidermis, possibly by a Foxj1/Foxa1-independent mechanism.

## Discussion

Understanding the complex process of formation and function of mucociliary epithelia is important in order to gain insight into the pathophysiological changes underlying human airway diseases. Spatiotemporal control of multiciliated and secretory cell specification, morphogenesis and function is a prerequisite for epithelial barrier function and mucus clearance in mucociliary epithelia, including the mammalian airways. A wide range of human syndromes, collectively addressed as ciliopathies, arise in patients when formation and function of motile cilia are disturbed. Syndromes include - among others - primary cilia dyskinesia (PCD), chronic obstructive pulmonary disease (COPD) and Meckel-Gruber syndrome, which are all associated with dysfunction of respiratory epithelia and increased rates of airway infections (Bergmann, 2012; Fliegauf et al., 2007; Mall, 2008; Zariwala et al., 2011). Similarly, defects in secretory cell function affect the ability of the airways to withstand pathogens (Kim and Criner, 2013; Mall, 2008; Wang et al., 2014).

We have used the *Xenopus* epidermis as a proxy to understand the effects of manipulating ATP4a on the spatiotemporal control of multiciliated and secretory cell specification, morphogenesis and function. Our data indicate that loss of ATP4 function leads to defects in Wnt signaling and, subsequently, impaired ciliogenesis of motile cilia throughout *Xenopus* development, i.e. the gastrocoel roof plate (Walentek et al., 2012), the neuroectoderm (cf. Fig. 1,2 in Walentek et al., 2015) and the mucociliary epidermis (Fig. 3,6). A prominent feature of the *atp4a* morphant phenotype was reduced *foxj1* expression (Fig. 1, S2). Defects in multiciliated cells (MCCs) resulting from *atp4a* knockdown largely resembled loss of Foxj1 function, i.e. impaired ciliogenesis, due to basal body docking defects and lack of apical actin meshwork formation (Fig. 2; Gomperts et al., 2004). Additionally, *atp4a* morphants showed decreased levels of *myb* expression, while *mci* was unaffected (Fig. S3). Thus, while *foxj1* and *myb* dependent ciliogenesis was disrupted, the specification of cells, marked by *mci*, was normal.

MCI and Myb can both induce basal body duplication and multiciliogenesis (Stubbs et al., 2012; Tan et al., 2013), while Foxj1 is necessary for ciliary motility in mono- and multiciliated cells (Stubbs et al., 2008; Yu et al., 2008). myb expression was downregulated and basal body numbers appeared slightly reduced in atp4a morphant MCCs (Fig. 2, S3). Although the aggregation of basal bodies (Fig. 2B, C; Fig. S4), which is often associated with basal body docking defects, prevented accurate quantification, it is possible that the effect on myb expression could lead to impaired basal body multiplication in addition to Foxj1-dependent motile ciliogenesis defects. We have previously shown that ATP4a was not only required for Wnt/ $\beta$ -catenin-dependent aspects of Xenopus development, but was equally important for Wnt/PCP-dependent processes (Walentek et al., 2012). We observed misalignment of basal bodies in MCCs of atp4a morphants (Fig. 2, S4), a feature commonly seen after dysregulation of the Wnt/PCP pathway (Mitchell et al., 2009; Park et al., 2008). Thus it is likely that ATP4 acts through Wnt/PCP signaling in MCCs as well, which could reinforce the cellular phenotype at the level of docking and alignment of basal bodies, as well as ciliation and apical actin organization. We conclude from these experiments that foxj1 downregulation comprised the major phenotype in ATP4a-deficient MCCs, likely accompanied by aspects which originate from defective myb expression and inhibition of Wnt/PCP signaling.

In zebrafish, foxj1 is directly regulated by canonical Wnt signaling (Caron et al., 2012). In Xenopus, ATP4a-dependent Wnt/ $\beta$ -catenin was required for foxj1 expression in the gastrocoel roof plate (Walentek et al., 2012), the epidermis and the neuroectoderm (Walentek et al., 2015), suggesting that foxj1 might be regulated by  $\beta$ -catenin throughout the vertebrates. Expression of Wnt pathway components (e.g. wnt4 and axin2) was shown to increase during air liquid interphase (ALI) culture of human airway epithelial cells (Ross et al., 2007) and multiple roles for canonical Wnt signaling have been proposed during mammalian lung development, in airway stem cells and disease (Hashimoto et al., 2012; Pongracz and Stockley, 2006; Sharma et al., 2010). As the roles of Notch, MCI, Myb and Foxj1 are functionally conserved between the Xenopus skin and mammalian airway epithelia, it is likely that a Wnt-dependent gene regulatory network is also present in the human respiratory tract.

Importantly, we found another process during development of the mucociliary epithelium to be under the control of ATP4 and canonical Wnt, namely the specification and function of small secretory cells (SSCs). Loss of ATP4a through pharmacological inhibition, or MO-mediated knockdown, or inhibition of canonical Wnt signaling by dkk injection decreased serotonin synthesis in SSCs (Fig. 5,6). Furthermore, SSC cell fate specification was likely affected by atp4aMO and dkk injection, because foxa1 expression was reduced by both treatments (Fig. 1, S2B, S3B). Serotonin is a known regulator of ciliary beat frequency in MCCs of mucociliary epithelia, including the human respiratory tract (Bayer et al., 2007; Doran, 2004; Katow et al., 2007; König et al., 2009; Walentek et al., 2014). Additionally, SSCs were required in Xenopus to regulate mucus composition and to prevent bacterial infection (Dubaissi et al., 2014), implicating that loss of SSCs might have additional consequences on other secretory cell types in the epidermis.

In addition to its developmental function as a permissive factor for Wnt signaling activation, ATP4 seems to play an additional role in homeostasis and function of mature MCCs. Decrease in MCCs in the epidermis after late treatment with the specific ATP4 inhibitor SCH28080 and expansion of the apical surfaces of neighboring intercalating cells indicate that MCCs were lost from the epithelium (Fig. 7). Loss of MCCs in this context might possibly be due to over-acidification of the cytoplasm and subsequent apoptosis, a mechanism previously described in human gastric cancer cell lines treated with various PPIs (Yeo et al., 2004). It will be interesting to investigate whether inhibition of ATP4 in mature MCCs leads to intracellular acidification, which would activate pH-dependent Caspase function and subsequently lead to cell-type specific apoptosis.

Several *atp4a* knock-out strains were generated over the past years. Analysis of these mice revealed phenotypes related to abnormal gastric function, i.e. gastric ciliated and mucocystic mataplasia (Judd et al., 2005; Spicer et al., 2000), and iron-deficiency (Krieg et al., 2011). Additionally, ATP4a loss of function affects ion homeostasis in the kidney (Greenlee et al., 2011; Lynch et al., 2008). Apart from these reports, we lack further knowledge on extragastric functions of ATP4a in the mouse. It should be mentioned that *atp4a* knock-out mice were not reported to be more susceptible to airway infections, but a specific function of ATP4a in the mouse airways was not investigated to date and housing of transgenic mice in pathogen-free facilities might have prevented such infections. Furthermore, *atp4a* knock-out mice do not show developmental defects associated with ATP4a loss of function in other species, e.g. left-right axis specification defects (Walentek et al., 2012), indicating either redundancy or that the developmental function of ATP4a was lost in mice during evolution. This suggests that the mouse might not be the best model to investigate all extragastric functions of ATP4a related to human development and disease. Taken together, the data presented here supports the presence of a general ATP4/canonical Wnt/Foxj1 module for the regulation of ciliation and mucociliary function in the frog *Xenopus*. Our findings argue for several roles of ATP4-dependent Wnt signaling during mucociliary development and clearance. The experimental results suggest two potential mechanisms how proton pump inhibitor treatment could act on the human airways and result in an increased susceptibility for acquired infections: airway clearance could be either affected by PPI-mediated down-regulation of ATP4-dependent Wnt signaling, or due to negative effects on mature MCCs. This strongly suggests that PPI-associated pneumonia in human patients might, at least in part, be linked to dysfunction of airway epithelia and highlights the *Xenopus* epidermis as an important model for the study of mucociliary epithelia.

## Materials and methods

### Ethics statement

All animals were treated according to the German regulations and laws for care and handling of research animals, and experimental manipulations according to §6, article 1, sentence 2, no. 4 of the animal protection act were approved by the Regional Government Stuttgart, Germany (Vorhaben A 365/10 ZO “Molekulare Embryologie”).

This work was also done with approval of University of California, Berkeley's Animal Care and Use Committee. University of California, Berkeley's assurance number is A3084-01, and is on file at the National Institutes of Health Office of Laboratory Animal Welfare.

### Statistical evaluation of results

Statistical evaluation of experiments represented by bar graphs was performed using chi-square tests (<http://www.physics.csbsju.edu/stats/contingency.html>). Statistics of experiments represented by box plots were calculated by Wilcoxon sum of ranks (Mann-Whitney) tests ([http://www.fon.hum.uva.nl/Service/Statistics/Wilcoxon\\_Test.html](http://www.fon.hum.uva.nl/Service/Statistics/Wilcoxon_Test.html)). Significance of qRT-PCR results was determined using Student's T-test (<http://www.physics.csbsju.edu/stats/t-test.html>). S.E.M. was calculated using <http://www.miniwebtool.com/standard-error-calculator/>.

### Manipulation of embryos

Embryos were injected at the two- to four-cell stage using a Harvard Apparatus or Picospritzer setup in 1x modified Barth's solution (MBSH) with 4% Ficoll (BioChemica) and transferred to 0.1x MBSH 15 min after injection. atp4aMO (5'-GTCATATTGTTTCCTTTTCCC CATC-3') 1pmol, atp4aMO-2 (5'-CTCTCTCCATCTCCACCGAGAACAT-3') 1pmol, atp4aSplMO (5'-CCCCCCCCCATTTCTTACAATGT-3') 3pmol or CoMO (random control morpholino oligonucleotide; Gene Tools) 1pmol were injected. Drop size was calibrated to about 7–8 nl per injection. Rhodamine-B or Cascade-blue dextran (0.5–1.0 mg/ml; Molecular Probes) were co-injected and used as lineage tracer. DNAs were purified using the PureYield Plasmid Midiprep kit (Promega) and diluted to a concentration of 0.5 ng/μl (foxj1-CS2<sup>+</sup>; Stubbs et al., 2008), 1 ng/μl (ATP4a-CS2<sup>+</sup>MT; Walentek et al., 2012) and β-cat-gfp-CS2<sup>+</sup>; (Miller and Moon, 1997). Synthesis of mRNAs used the Ambion message machine kit and mRNAs were diluted to the following concentrations: Su(H)-DBM and Notch-ICD 150ng/μl (Deblandre et al., 1999); dkk 5ng/μl (Glinka et al., 1998); sas6-gfp 10ng/μl (Klos Dehring et al., 2013); centrin-gfp and clamp-gfp 50ng/μl (Park et al., 2008). SCH28080 (Sigma-Adrich; solvent: DMSO; concentration: 100μM) incubations were performed according to standard procedures on embryos treated with Proteinase K (as described; Sive et al., 2000) in order to permeabilize the fertilization membrane (in case of early incubation experiments). Proteinase K-treated embryos incubated with corresponding concentrations of DMSO were used as controls.

### Quantitative RT-PCR

qRT-PCR experiments were performed in three biological experiments and three technical replicates per experiment. cDNAs were synthesized using iScript reverse transcription supermix (BioRad) from total RNA extracts from 15 animal caps per condition and time point. The following qPCR primers were used:

foxj1: For 5'-CCAGTGATAGCAAAGAGGT-3', Rev 5'-GCCATGTTCTCCTAATGGAT-3';

mci: For 5'-AATCAAAGCATCTGTC-3', Rev 5'-TTTCTTAGAGATTTCCCGCA-3';

myb: For 5'-CAGAATGCTTCATACCCTGT-3', Rev: 5'-CAGTAGTTCTTGCTTCTGTG-3';

foxa1: For 5'-GGAGCAACTATTACCAGGAC-3', Rev: 5'-ATGGGATTCATAGTCATGTAGG-3';

foxi1: For 5'-TACCAAGAGATGAAGATGATC-3', Rev: 5'-CTTGTCAGAAGATAACTGTCCA-3';

tph1: For 5'-CTGGTTTCAAGGACAATGTG-3', Rev: 5'-GGCAGGTTCTTTAGGTACTC-3';

ef1a: For 5'-CCCTGCTGGAAGCTCTTGAC-3', Rev 5'-GGACACCAGTCTCCACACGA-3';

odc: For 5'-GGGCTGGATCGTATCGTAGA-3', Rev 5'-TGCCAGTGTGGTCTTGACAT-3'.

Reactions were performed on a BioRad CFX96 Real-Time System C1000 Touch using SsoAdvanced SYBR Green Supermix (Biorad). Average values from ef1a and odc reactions were used for normalization. The expression levels depicted were calculated as fold expression relative to stage 10 uninjected control.

### Whole-mount in situ hybridization

Embryos were fixed in MEMFA for 1–2 hrs and processed following standard protocols. Digoxigenin-labeled (Roche) RNA probes (atp4a and foxj1, Walentek et al., 2012; atp6V1E1 probe was generated according to NM\_001086298; tph1; Walentek et al., 2014; foxi1, (Quigley et al., 2011); foxa1; (Hayes et al., 2007) were prepared from linearized plasmids using SP6, T3, or T7 RNA polymerase (Promega). In situ hybridization was conducted following standard procedures. In some experiments (Fig. 1C) embryos were bleached after in situ hybridization according to standard procedures (Sive et al., 2000).

### Immunohistochemistry and scanning electron microscopy

Immunohistochemistry followed standard protocols (Sive et al., 2000), using antibodies specific for acetylated- $\alpha$ -tubulin (mouse, 1:700; Sigma), ATP4a (1:500; Walentek et al., 2012), anti-mouse Cy3 (sheep, 1:250; Sigma); serotonin (rabbit, 1:500; Merck), anti-rabbit Alexa-555 (1:250; Invitrogen), anti-mouse Alexa-555 (1:250; Invitrogen), anti-mouse Alexa-405 (1:250; Invitrogen). Cell boundaries were visualized by Alexa 488-conjugated phalloidin (Invitrogen), which stained the actin cytoskeleton. Imaging was performed on a Zeiss LSM710. Lateral projections of confocal z-scans were computed using Zeiss Zen software. Maximum intensity projections of confocal z-scans were computed using ImageJ (Schindelin et al., 2012). Scanning electron microscopy was as previously described (Beyer et al., 2012).

### Imaging of fluid flow

For imaging of skin flow in supplementary material Movie1, stage 29 embryos were anesthetized with benzocaine (Sigma) and exposed to fluorescent latex beads (FluoSpheres® carboxylate-modified microspheres, 0.5  $\mu$ m, yellow-green fluorescence [505/515], 2%

solids, Life Technologies; diluted to 0.04% in 0.1 x MBSH) in a sealed flow chamber. Time-lapse movies (20 sec) were recorded using epifluorescent illumination at 10x magnification on a Zeiss Axioskop 2 microscope and processed in ImageJ.

For imaging of skin flow at later stages (supplementary material Movie2), stage 35 or stage 41 embryos treated with either DMSO (1%) or SCH28080 (100 $\mu$ M) were anesthetized and exposed to latex beads (FluoSpheres<sup>®</sup> carboxylate-modified microspheres, 0.5  $\mu$ m, red fluorescence [580/605], 2% solids, Life Technologies; diluted to 0.04% in 0.1 x MBSH) in a sealed flow chamber. Time-lapse movies (10 sec/50 frames per sec) were recorded using epifluorescent illumination at 20x magnification on a Zeiss Axioskop 2 microscope in combination with a high-speed GX-1Memrecam camera (NACImage Technology) and processed in ImageJ. Particle linking and quantification of extracellular fluid flow velocities was performed as previously described cf. Walentek et al. (2012, 2014).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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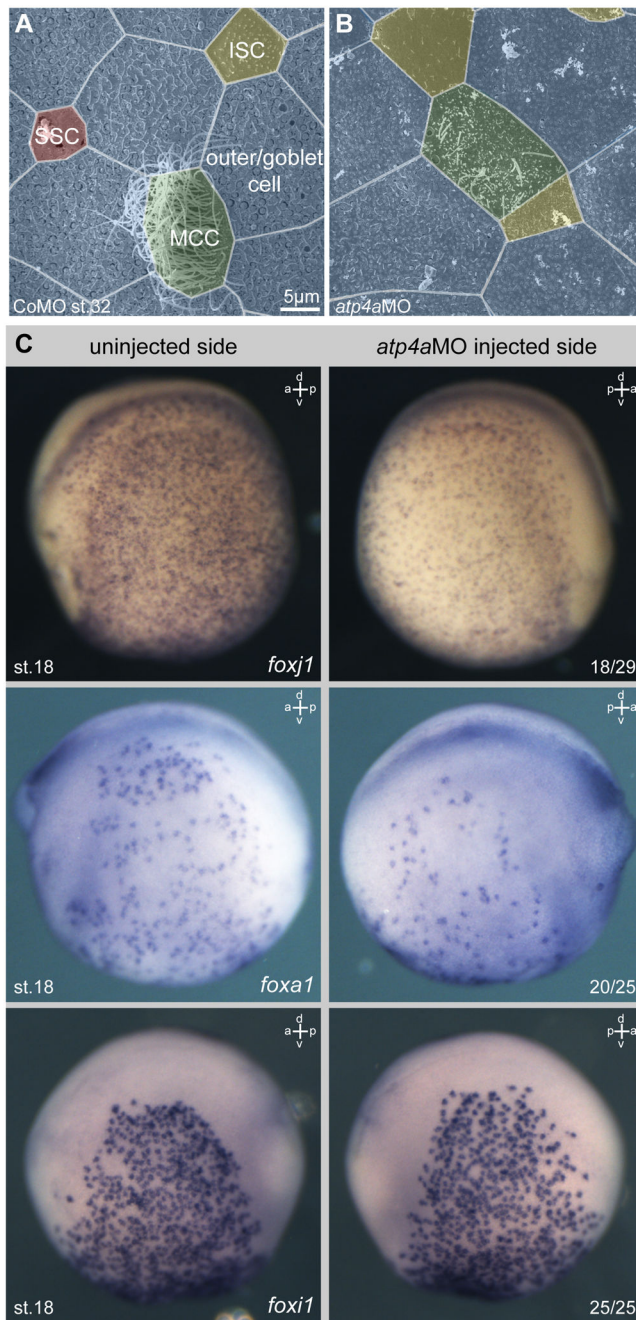
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**Highlights**

- ATP4 is required for development and function of the ciliated epidermis
- ATP4 regulates canonical and non-canonical Wnt signaling during development
- Wnt signaling controls gene expression in multiciliated and small secretory cells
- ATP4-inhibitors cause loss of mucociliary clearance in mature ciliated epithelia



**Fig. 1. ATP4 is required for normal development of the embryonic epidermis**  
 (A–B) Morphological analysis of the embryonic epidermis at stage 32 in control morpholino oligonucleotide (CoMO) (A) and *atp4aMO* (B) injected specimens. Knockdown of *atp4a* lead to defects in ciliation of multiciliated cells (MCCs, green) and reduced numbers of small secretory cells (SSCs, red), but without obvious effects on ion secreting cells (ISCs, yellow) or outer/goblet cells (blue). (C) Knockdown of *atp4a* attenuated *foxj1* and *foxa1* expression, but not *foxi1* expression in the skin epidermis. Embryos were unilaterally injected with *atp4aMO* at the four-cell stage and assayed for *foxj1/foxa1/foxi1* expression by WMISH at stage 15. Correct targeting was confirmed by co-injection of lineage tracer.

Depicted embryos are derived from the same injected batch. Numbers in the right lower corner indicate frequency of phenotype.

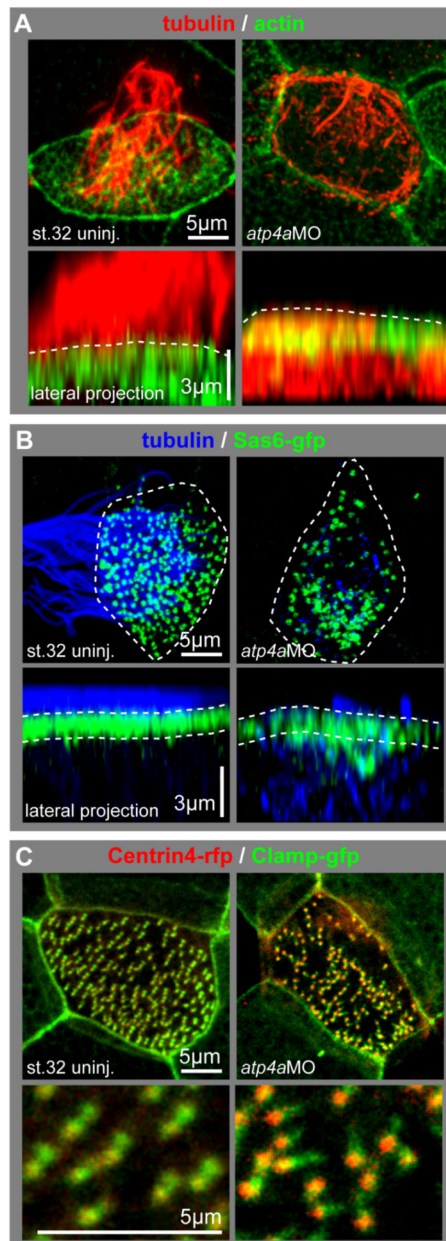
a, anterior; d, dorsal; p, posterior; st., stage; v, ventral.

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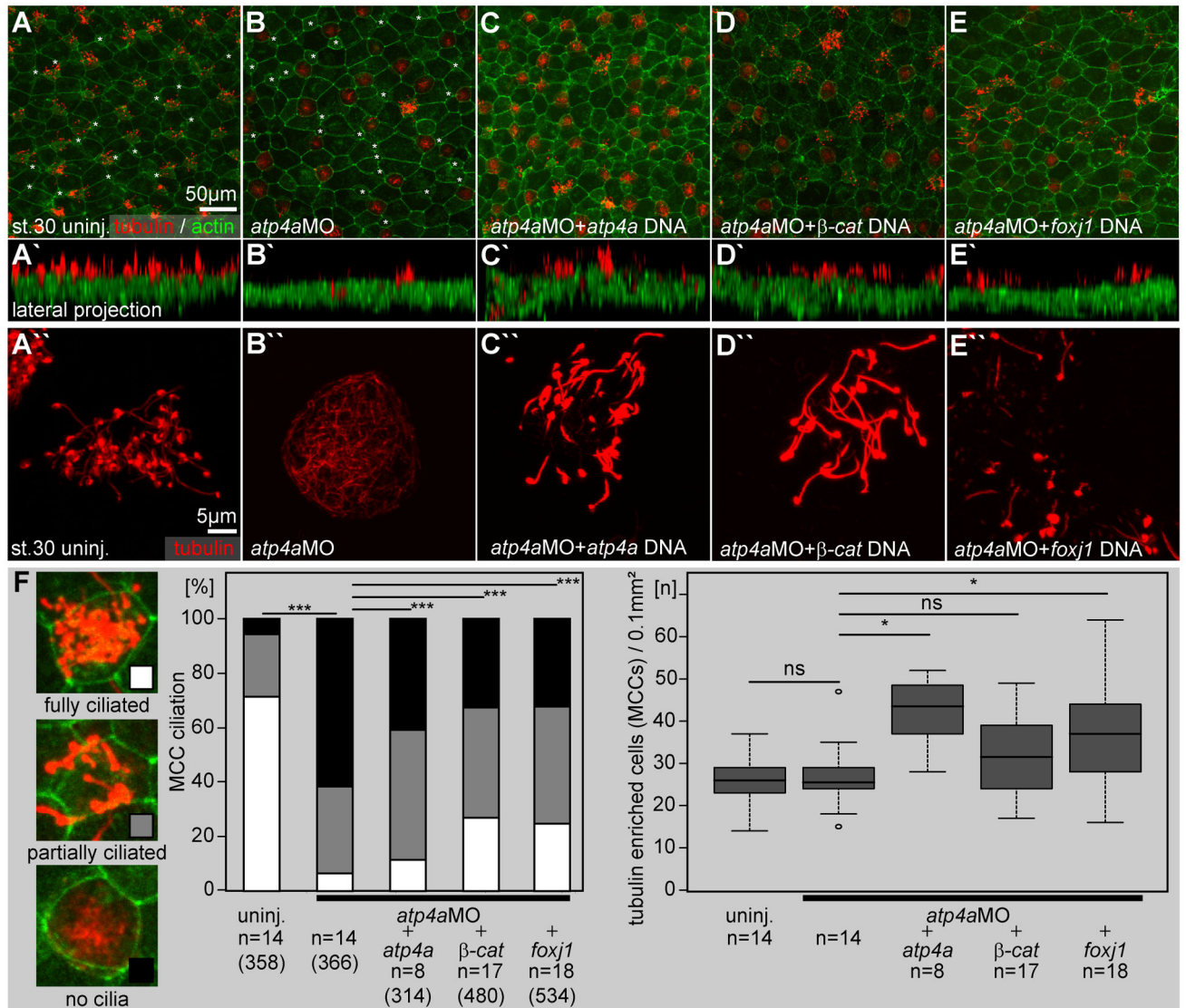
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**Fig. 2. Loss of ATP4 causes defects in MCCs reminiscent of *foxj1* and Wnt/PCP phenotypes** (A–C) Analysis of the cellular phenotype in MCCs using high magnification confocal single cell imaging. (A) Cells were stained with an antibody against acetylated- $\alpha$ -tubulin (tubulin, red) and phalloidin-Alexa488 for actin staining (actin, green). Control (uninj.) MCCs were characterized by the presence of a dense ciliary tuft projecting from the apical surface (dashed lines in lateral projections) and the presence of an apical actin meshwork. In contrast, MCCs in *atp4a* morphants showed reduced ciliation, intracellular accumulation of tubulin and defects in the apical actin meshwork formation. (B) The localization of basal bodies to the apical membrane was analyzed using overexpression of *sas6-gfp* (green) in combination with tubulin (blue) staining, the outlines of the cells are depicted by the dashed lines in the apical views. Control MCCs were fully ciliated and basal bodies aligned close to

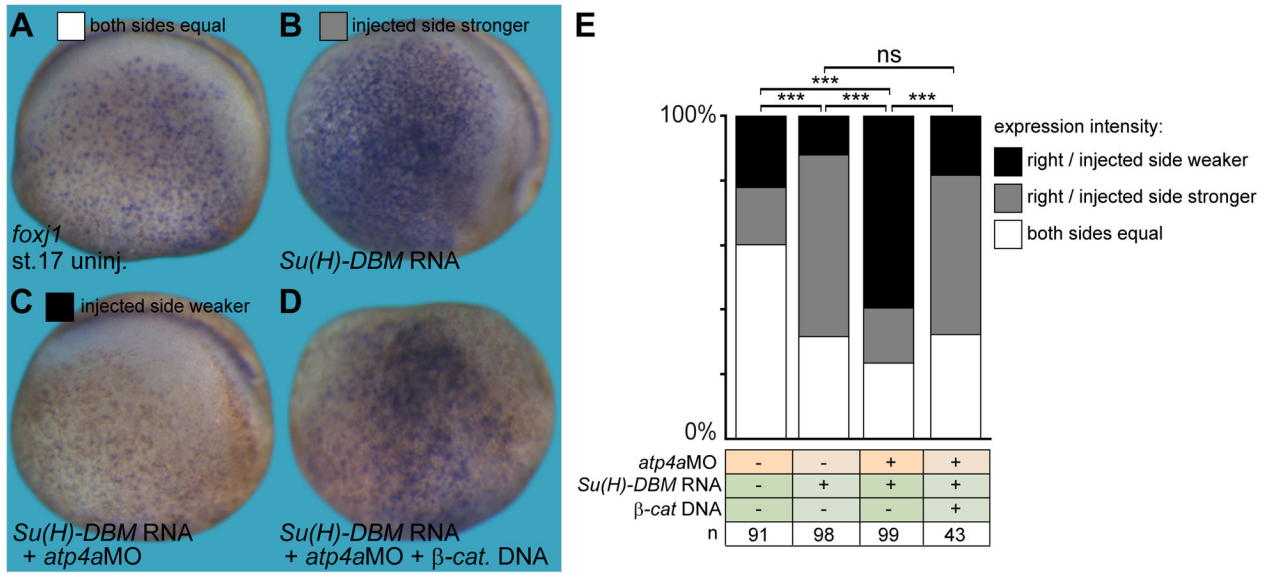
the apical membrane (dashed lines in lateral projections), while *atp4a* morphant MCCs had severe defects in ciliation, which correlated with aberrant basal body distribution within the cell. Basal bodies in *atp4a* morphants were mislocalized to the deep cytoplasm and not uniformly distributed along the apical membrane. (C) Basal body orientation was analyzed using *centrin4-rfp* (red) and *clamp-gfp* (green) overexpression. In control MCCs, basal bodies were mostly uniformly aligned along the anterior-posterior axis, while this alignment was randomized in *atp4a* morphant embryos. Lower panels show a magnified field of basal bodies.



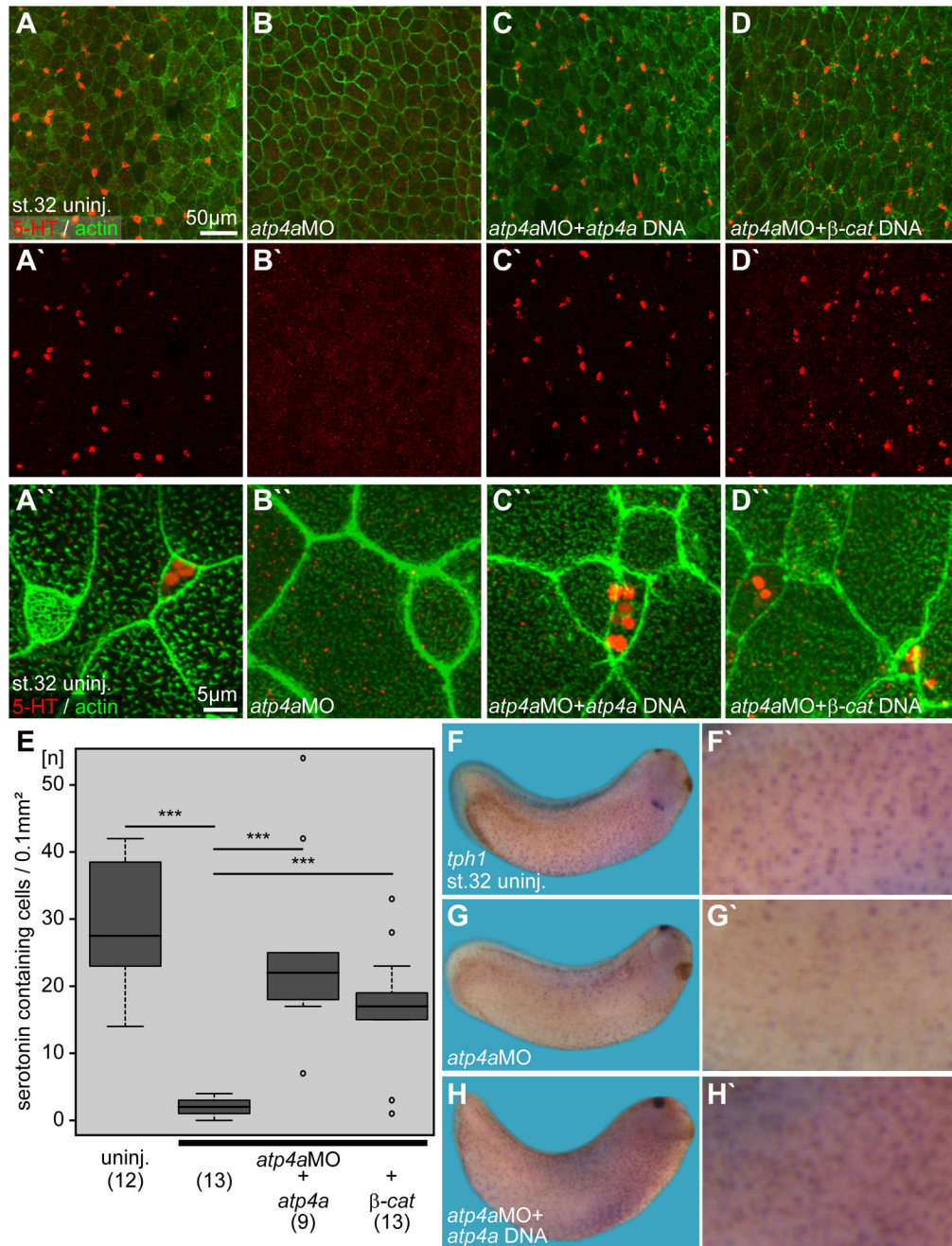


**Fig. 3. ATP4-dependent Wnt signaling is required for *foxj1* expression and ciliation in the *Xenopus* embryonic skin**

(A–E) Immunofluorescent analysis of skin ciliation at stage 30. Embryos were stained for tubulin (acetylated- $\alpha$ -tubulin, red) and actin (phalloidin-Alexa488, green) and analyzed by confocal microscopy. (B) In *atp4a* morphants fully ciliated, partially ciliated and non-ciliated MCCs were found. Smaller intercalating cells with ISC morphology, which are also negative for acetylated- $\alpha$ -tubulin staining are marked with asterisks in A and B. Note that *atp4a* (C),  $\beta$ -catenin ( $\beta$ -cat.; D) and *foxj1* (E) DNAs partially rescued ciliation in *atp4a* morphants. (A'–E') Lateral projections of confocal Z-scans are shown in (A–E). (A''–E'') Higher magnification of representative MCCs. (F) Quantification of results. n, number of embryos; (n), number of cells.



**Fig. 4. ATP4a-dependent Wnt signaling acts downstream of Notch in skin *foxj1* induction**  
 (A–D) *Foxj1* was stained in uninjected control (uninj.) embryos (A), as well as manipulated embryos (injected side shown in B–D). (B) Inhibition of Notch by injection of *Su(H)-DBM* mRNA increased *foxj1* expression, which remained dependent on ATP4a (C) and Wnt/ $\beta$ -catenin ( $\beta$ -cat.; D). (E) Quantification of results. Staining intensity on the injected (right) side was compared to the uninjected (left) control side and quantified as right/injected side stronger, weaker or equal to the control side (G).  
 a, anterior; d, dorsal; n, number of embryos; ns, not significant; p, posterior; st., stage; v, ventral.



**Fig. 5. Serotonin secretion and *tph1* expression in the embryonic epidermis are regulated by ATP4a-mediated Wnt/ $\beta$ -catenin signaling**

(A–D) Immunofluorescent analysis and quantification (E) of serotonin (5-HT; red) deposition in small secretory cells (SSCs) of the skin at stage 32. Actin (phalloidin-Alexa488) staining in green. (A) Uninjected (uninj.) control. (B) Loss of serotonin staining in *atp4a* morphants. (C, D) Rescue of serotonin staining upon co-injection of *atp4a* (C) or  $\beta$ -catenin ( $\beta$ -cat.; D) DNA constructs. (A'–D') Serotonin channel. (A''–D'') Higher magnification of (A–D). (E) Quantification of results. (F–H) WMISH for *tph1*. (F, F')

Uninjected control. (G, G') Loss of *tph1* expression in *atp4a* morphants was rescued by co-injection of *atp4a* DNA (H).  
n, number of embryos; st., stage.

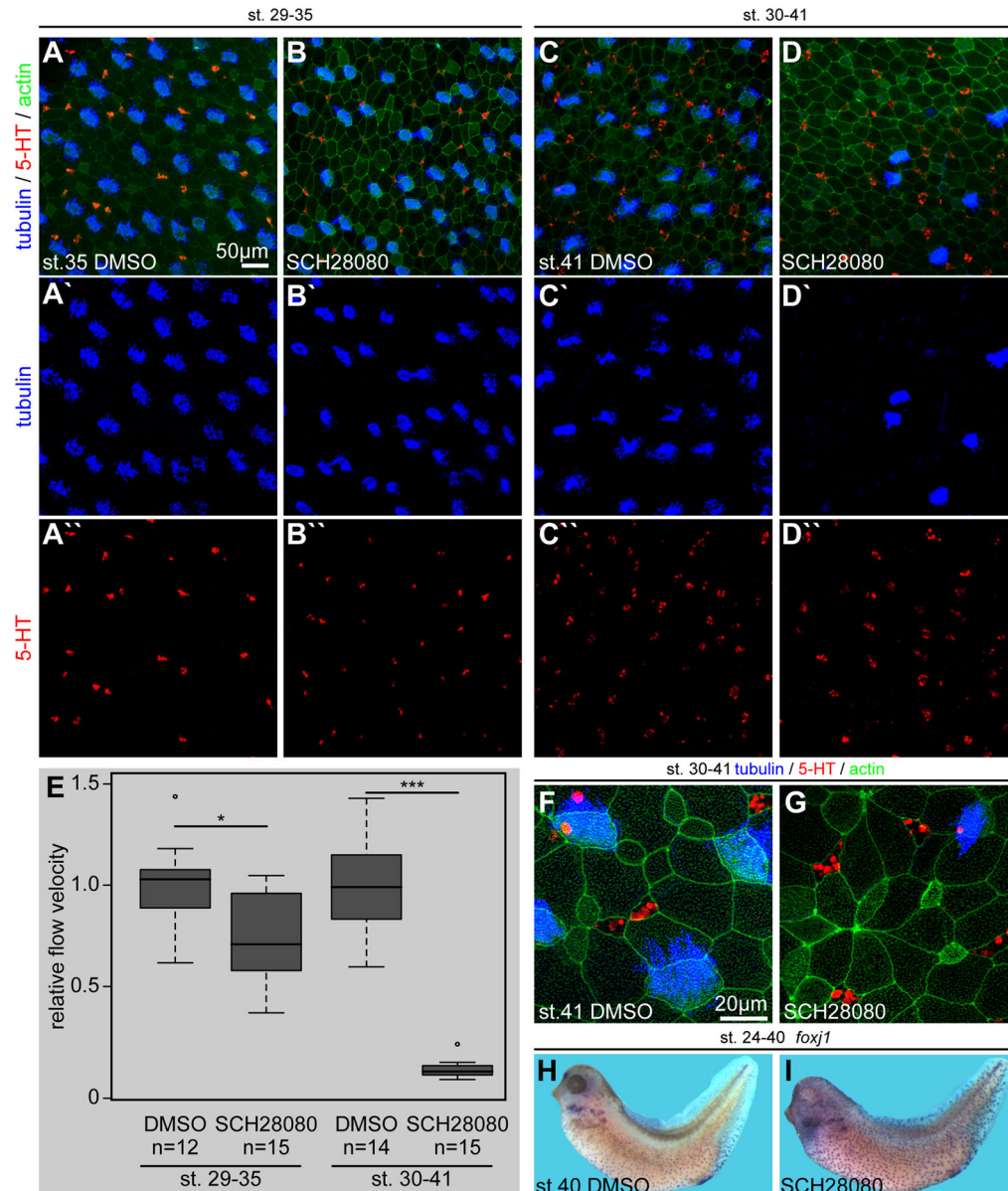
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**Fig. 7. Late pharmacological inhibition of ATP4 causes loss of MCCs and decreased mucociliary clearance**

Immunofluorescent analysis (A–D) of MCC ciliogenesis (acetylated- $\alpha$ -tubulin, blue) and serotonin (5-HT; red) deposition in small secretory cells (SSCs) of the skin at stages 35 (AB) and 41 (C–D). Actin (phalloidin-Alexa488) staining in green. Embryos were treated with DMSO as control or SCH28080 during the indicated stages. Stage 35 (A) and stage 41 (C) DMSO treated controls displayed fully ciliated MCCs (A', C') and the presence of large numbers of serotonin positive SSCs (A'', C''). At stage 35 no apparent defects in MCC ciliation (B') or SSC serotonin deposition (B'') were observed in SCH28080 treated embryos (cf. Quantification in Fig. S3K), although 50% of specimens contained MCCs with a relatively small apical surface area and 60% of specimens were abnormal in their epithelial morphology, i.e. enlarged cells were present (cf. Quantification in Fig. S3K). In contrast,

SCH28080 treatment until stage 41 lead to a massive loss of MCCs (D'), but no effect on SSCs (D''). (E) Quantification of cilia-driven fluid flow velocities revealed a significantly reduced extracellular fluid flow at the epidermis at stages 35 and 41, as compared to DMSO treated controls of the same stage. (F–G) High-magnification confocal imaging on the same set of specimens depicted in (C and D) confirmed the lack of MCCs, but intact SSCs in SCH28080 treated embryos and further indicated apical expansion in ISC-like cells and SSCs as well as enriched actin staining at some cell junctions (G). MCCs and epithelial morphology appeared normal in DMSO treated controls (F). (H–I) Embryos were treated with DMSO or SCH28080 from stage 24–40 and *foxj1* expression was analyzed by in situ hybridization. In comparison to controls (H), *foxj1* staining was increased in SCH28080 treated tadpoles (I).

n, number of embryos; st., stage.