Serotonin and MucXS release by small secretory cells depend on Xpod, a SSC specific marker gene

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Summary
Mucus secretion and ciliary motility are hallmarks for muco-ciliary epithelia (MCE). Both, mammalian airways as well as the less complex epidermis of Xenopus embryos show cilia-driven mucus flow to protect the organism against harmful effects by exogenous pathogens or pollutants. Four cell types set up the epidermal MCE in Xenopus. Multi-ciliated cells (MCCs) generate an anterior to posterior flow of mucus. Ion secreting cells (ISCs) are characterized by the expression of ion transporters, presumably to maintain a favorable homeostasis. The largest cell type is represented by goblet cells, which cover most of the epidermis and exhibit secretory properties. Additionally, small secretory cells (SSCs) release mucus, antibiotic compounds, and the monoamine serotonin (5-hydroxytryptamine; 5-HT). We have recently shown that serotonin regulates flow velocity by acting on ciliary beat frequency. Here, we describe the identification and functional characterization of Xenopus polka-dots (Xpod). No homologous genes or proteins were found in other vertebrates, including Xenopus tropicalis. We demonstrate that Xpod serves as an SSC-specific marker, starting to be expressed shortly after SSC specification at neurula stages. Overexpression of a tagged Xpod protein resulted in the localization of secretory granules. Notch signaling induced SSC cell fate, in contrast to its repressing effect on MCC and ISC specification. Xpod loss-of-function revealed that mucus and 5-HT release by SSCs was severely diminished, which impaired the ciliary beating of MCCs. In summary, Xpod specifically marked SSCs and was required for muco-ciliary secretion in Xenopus laevis.

KEYWORDS
Cilia, mucus, notch, serotonin, SSC, Xenopus

1 | INTRODUCTION

Muco-ciliary epithelia (MCE) are characterized by mucus flow driven by motile cilia and are found throughout the animal kingdom. MCEs are multifunctional and, depending on organism, required for locomotion of the animal or embryo (i.e., Lymnea—Aono et al., 2008; planaria—Romanpolas, Patel-King, & King, 2010), feeding (i.e., Amphioxus—Holland, 2006) or protection from environmental hazards (i.e., mammalian airways—Tilley, Walters, Shaykhiev, & Crystal, 2014). In each case, MCEs secrete large amounts of sticky and jellylike mucus, major components of which are high-molecular proteins of the mucin family. In addition to their large size (5–50 MDa), mucin proteins are heavily glycosylated and densely packaged in secretory vesicles. This task is executed by specialized secretory MCE cells, often referred to as...
goblet cells. Upon secretion, changes in pH and ionic composition in the extracellular space unfolds and hydrates the secreted mucins to establish the viscous, gel-like mucus (Ridley & Thornton, 2018). A second highly specialized MCE cell type is multi-ciliated cells (MCCs), each projecting up to 300 motile cilia from the apical surface. Ciliary beating direction of MCCs aligns, resulting in a constant and directional mucus movement (Meunier & Azimzadeh, 2016).

The lining of the airways represents the most relevant MCE for human health. Under normal conditions, the mucus-cilia interplay removes inhaled pathogens and hazardous substances (i.e., tobacco smoke) from the airways. Particles get trapped by mucus, which is then cleared from the airways through cilia motion. If this process is impaired, microbes are able to accumulate and cause severe airway infections. In human primary ciliary dyskinesia (PCD), mutations specifically affect motile cilia function (Afzelius, 2004; Bustamante-Marín & Ostrowski, 2017). The clinical presentation of PCD comprises chronic infections. Mutations impairing mucus functions are known as well. The case best studied is cystic fibrosis (CF), where luminal mucus becomes hyperviscous and thus unable to be moved by cilia. CF patients suffer from clogged airways and chronic infections. Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, encoding a chloride-channel, underlie CF (Livraghi & Randell, 2007). CFTR and other ion-channels and -pumps are required to maintain and regulate ion homeostasis, which is required for maintenance of a normal airway surface liquid layer. If this system is perturbed, luminal water concentrations drop and subsequently affect the dissolution of mucus (Boucher, 2007; Rubin, 2007). Airway clearance by cilia-driven mucus flow thus depicts a rather simple first line of defense mechanism for the organism, before major immune responses are active. However, this simplicity is embedded into a highly complex regulatory and cellular network of interactions to ensure airway functionality, which has to integrate environmental parameters such as humidity as well. Therefore, it is rather difficult to unravel basic mechanisms, which is apparently true for mammalian airways with 50 or even more cell types (Bérubé, Prytherch, Job, & Hughes, 2010).

The epidermis of Xenopus embryos represents a bona fide MCE of low complexity, which protects against bacterial infections in much the same way as the mammalian airways. The embryonic Xenopus skin is composed of only four cell types. Ion secreting cells (ISC) are characterized by the expression of ion channels, which are thought to ensure homeostasis and probably mucus deflation. Both, goblet cells and small secretory cells (SSCs) produce and release mucus. The main cell type of mucus secretion, however, is the SSCs. A Xenopus specific mucin, called MucXS, constitutes the major mucus component. In addition, antibacterial factors and the monoamine 5-hydroxytryptamine/serotonin (5-HT) have been identified to be secreted by SSCs as well (Dubaisi et al., 2018; Dubaisi et al., 2014; Walentek et al., 2014). We have previously demonstrated that the forkhead transcription factor foxA1 is required and sufficient for SSC formation. Loss of foxA1 function impaired SSC specification and thus resulted in the absence of SSCs. Embryos lacking SSCs and consequently major secretion properties disintegrated due to bacterial infection. The protective nature of SSCs was further demonstrated by MucXS gene knockdown through antisense morpholino oligomers (MO), where loss of MucXS facilitated bacterial infections (Dubaisi et al., 2014, 2018). Besides their protective capacity, SSCs are able to regulate ciliary beat frequency of neighboring MCCs via serotonin release. MCCs express the 5-HT receptor Type 3, a ligand-gated ion channel, which impacts on ciliary beating. Therefore, 5-HT adjusts liquid flow velocity along the anterior to posterior axis of the embryo (Walentek et al., 2014). Epidermal MCCs have been successfully used to investigate the multiple stages of ciliogenesis. This is due to the accessibility and paramount embryological manipulation techniques of the Xenopus system (Schweickert & Feistel, 2015). So far, a comparable in-depth analysis of SSC development and function has not been conducted. Yet, the question of transferability to human MCEs has not been solved either. Although SSC secretory products can be visualized by IF (serotonin and MucXS; Dubaisi et al., 2014; Walentek et al., 2014), an SSC specific marker gene with strong mRNA expression is still missing. Genes found to be expressed in SSCs during embryonic development like foxA1, MucXS, enzymes of 5-HT synthesis (tryptophan hydroxylase/tph1; dopamine decarboxylase/ddc) were not SSC-specific and are transcribed in other cell types as well. In addition, most showed rather low expression levels in SSCs when used in situ hybridization (ISH) experiments. Identification of a bona fide marker gene, therefore, would allow us to follow SSC development and function more precisely.

Here, we show that Xenopus polka-dots (Xpod) serves as a reliable, strong and specific SSC marker. Xpod represents a secreted protein, which so far has no homologous counterparts in other vertebrates. Xpod mRNA was used to visualize SSC development starting at neurula stages and to demonstrate SSC fate induction by Notch signaling. Functionally, Xpod knockdown inhibited MucXS and 5-HT release, suggesting a role in the secretory pathway of SSCs.

2 | RESULTS

To identify robust and—importantly—SSC-specific marker genes, a database and literature search was performed, focusing on a regular punctate expression pattern in the larval epidermis. A publication by Yoshii et al. (2011) about an epidermally expressed gene termed Xenopus polka-dots (Xpod) drew our attention, because subcellular Xpod localization showed a high degree of similarity to SSC secretory granules (cf. Figure 3h and i; Yoshii et al., 2011; Dubaisi et al., 2014; Walentek et al., 2014). Unexpectedly, Xpod was not annotated in the Xenopus database Xenbase (Karimí et al., 2018). By homology search we obtained multiple expressed sequence tags (ESTs) but no relevant genomic hits (data not shown). Although EST sequences corresponded to the published Xpod cDNA, we detected a 5' extended reading frame, which added 32 amino-acids to the N-terminus of the published protein, giving rise to a final size of 107 amino-acids (Figure 1a). In silico domain searches revealed the presence of a signal peptide, suggesting that Xpod was a secreted protein. No additional functional
domains could be identified. Surprisingly, homologous gene products were not present in genomes of other vertebrates, including its sister species *Xenopus tropicalis* (data not shown). To identify the nature of Xpod expressing epidermal cells, ISH followed by peanut agglutinin (PNA) staining was performed. The lectin PNA detects sugar residues on MucXS, the mucin secreted by SSCs (Dubaissi et al., 2014, 2018). At tadpole Stage 32 strong Xpod staining in a punctate pattern was apparent in the epidermis (Figure 1b). Magnification of Xpod positive cells showed co-localization with fluorescent PNA signals (Figure 1b’ and data not shown), in contrast to the report by Yoshii et al. (2011), who reported Xpod in a subset of ionocytes as well. In our hands, Xpod correlated with PNA-staining in all cases and at all stages (Figure 1b’ and data not shown), demonstrating that Xpod was specifically expressed in SSCs. The double ISH protocol applied by Yoshii et al. (2011) may have been not sensitive enough to differentiate between neighboring and/or intercalating SSCs and ISCs.

To investigate whether related sequences were present in *Xenopus tropicalis*, an ISH using the *X. laevis* Xpod probe was performed on *Xenopus tropicalis* embryos. Based on the close evolutionary distance of both species, many probes can be used interchangeably. However, no Xpod positive staining was found in Stage 32 *Xenopus tropicalis* embryos (data not shown). To verify that Xpod was a secreted protein, a myc-tag was inserted C-terminally of *Xenopus tropicalis* Xpod. A mycXpod probe was used to localize Xpod in cells, and data not shown, demonstrating that Xpod was specifically expressed in SSCs. The double ISH protocol applied by Yoshii et al. (2011) may have been not sensitive enough to differentiate between neighboring and/or intercalating SSCs and ISCs.

Next, we followed Xpod mRNA expression during SSC development using histological sections. As published, Xpod transcription started at mid-neurula stages in the deep layer of the epidermis and thus marked SSC precursor cells (Figure S1A; Yoshii et al., 2011). During later neurula stages, Xpod signals and the number of SSC precursor cells increased. By early tadpole (Stage 22), SSC intercalation into the superficial cell layer was first observed. At Stage 24, most SSCs had entered the superficial layer, as reported recently (Figure S1B–D; Walentek et al., 2014). Up to Stage 38, Xpod expression in non-swimming tadpoles was restricted to SSCs (data not shown). From these data, we conclude that—beginning with SSC specification—Xpod mRNA expression reliably follows all steps of SSC differentiation.

SSC cell fate specification has been shown to be executed by the FoxA1 transcription factor (Dubaissi et al., 2014). Upstream signaling pathways, however, have not been investigated. Inhibition of Notch signaling has been described as key to MCC and ISC fate determination (Deblandre, Wettstein, Koyano-Nakagawa, & Kintner, 1999; Quigley, Stubbs, & Kintner, 2011). We, therefore, wondered whether SSC fate was equally responsive to Notch signaling. Consequences of Notch gain- and loss-of-function manipulations on cell fate specification were assessed by marker gene expression (Xpod, SSC; *foxj1*, MCCs; *opt6v1e1*, ISC) and by IF (ac. tubulin, MCCs, and PNA, SSCs). As published, injections of Nicd mRNA, encoding a dominant active version of Notch, resulted in the loss of ISCs and MCCs fates, as compared to untreated controls (Figure 2; Figure S2; Table S1; Deblandre et al., 1999; Quigley et al., 2011). Surprisingly, both Xpod expression, as well as PNA positive SSC granules, revealed that numbers of SSCs were increased when Notch signaling was ectopically induced. This result was not anticipated, as Yoshii et al. reported that Xpod positive cells were unresponsive to notch activation. To verify our observation Notch inhibition by dominant-negative suppressor of hairless (Su(H)-DBM) was performed. Su(H)-DBM mRNA injections boosted ISC and MCC formation (Deblandre et al., 1999; Quigley et al., 2011) while

**FIGURE 1** Xpod is expressed in and secreted from SSCs. (a) Xpod amino acid sequence. Translation start sites identified by Yoshii et al. (2011) and in this work marked in red. Signal peptide and cleavage site are indicated in green. (b) Xpod mRNA expression at Stage (st.) 32. (b’) Immunofluorescence for SSC marker PNA co-localized with Xpod positive cells. (c, d) Myc-tagged Xpod (mycXpod) is secreted via PNA (c) and serotonin (d) loaded granules. Orthogonal views are shown in insets (c’) and (d’, d’’).
suppressing SSC development at the same time (Figure 2; Figure S2; Table S1). This result underscores the central role of Notch signaling in MCE cell type specification, with ISC, MCC, and SSC cell fates depending on Notch activation status.

Because Xpod expression emerged early in SSC precursor cells and was very strong in mature SSCs, we wondered what functional relevance this expression had. To that end, a translation blocking morpholino-oligomer was designed (XpodMO), which targeted sequences downstream of the newly identified start codon. A rescue construct was cloned, in which the MO target site was mutated without altering the amino acid sequence (rescXpod). To analyze if Xpod acted on cell specification, embryos were injected ventral-anally at the 4–8 cell stage with XpodMO alone or together with rescXpod mRNA. Embryos were raised up to Stages 30–32 and subsequently analyzed for marker gene expression by ISH. Un-injected controls, Xpod morphants, and rescue specimens (i.e., Xpod gain-of-function in non-SSCs) showed no differences in marker gene expression of SSCs (tph1; Figure S3A-C), ISCs (atp6v1e1; Figure S3D-F) and MCCs (foxj1; Figure S3G-H). We, therefore, excluded an early Xpod function during the process of cell fate determination. In addition, when epidermal cilia were analyzed by IF, we observed no differences between control and XpodMO-injected embryos (Figure S3J,K and data not shown), indicating that ciliogenesis was not impaired in morphants.

Next, we investigated a possible function of Xpod knockdown on SSC secretion. To visualize a potential Xpod activity on secretion, the granular localization of serotonin and MucXS (via PNA stain) was assessed by IF. Cortical actin was stained by phalloidin, as well, to control for overall cellular integrity. XpodMO or rescue mix composed of XpodMO and rescXpod mRNA were injected into the animal-ventral region of 4–8 cell embryos to target the epidermis. Specimens were cultured until Stage 32/33. Abundance of PNA- and 5-HT positive granules per SSCs were assessed and statistically evaluated. Controls showed an average of 13 MucXS and 9 5-HT positive secretory granules per SSC (Figure 3a,d,e). In Xpod morphants, however, the number of PNA (6) and 5-HT (5) granules per SSC was significantly reduced, with an occasional reduction in granule size (Figure 3b,d,e). This effect was specific, as an unrelated MO (gscMO; Sander, Reversade, & De Robertis, 2007; Ulmer et al., 2017) had no impact (Figure S3L-O; Table S1) and because co-injection of rescXpod restored the frequency of loaded granules per SSC to almost wild type levels (10 MucXS and 8 5-HT; Figure 3c,d,e; Table S1). In agreement with our marker gene analysis, we did not detect a significant change in SSC numbers in

![FIGURE 2](image-url) Notch-dependent SSC fate determination. Notch activation (middle) repressed SSC fates, while Notch repression (bottom) induced ectopic SSCs, as compared to wild type controls (top). mRNAs of dominant active intracellular domain of Notch (Nicd) or dominant-negative suppressor of hairless (Su(H)-DBM) were injected into the epidermal lineage at the 4–8 cell stage. At Stages 32–33, expression of marker genes for SSCs (Xpod), MCCs (foxj1), and ISCs (atp6v1e1) were analyzed. In addition, MCCs and SSCs were identified by IF using anti-acetylated tubulin (blue) and anti-serotonin (red) antibodies, respectively. Actin staining by phalloidin (green) marks cell border.
Based on these experiments, we concluded that Xpod played a role during the secretion of MucXS and 5-HT by SSCs.

We, therefore, wondered what consequences Xpod depletion had for MCE function. Loss of SSCs by foxA1 knockdown resulted in the disintegration of tadpoles due to bacterial infections. This lethal effect was potentially due to impaired muco-ciliary clearance, as mucus and antibacterial active substances, which are secreted by SSCs, were missing (Dubaisi et al., 2014). In Xpod morphants, however, we did not observe any impact on embryonic survival (data not shown). Because 5-HT levels were substantially reduced upon Xpod loss, the ciliary beat frequency of MCCs should be reduced, in accordance with our recent work (Walentek et al., 2014). We, therefore, analyzed ciliary beating patterns of control, Xpod morphant and rescued embryos by time-lapse life imaging. Kymographs visualized the ciliary movement of representative MCCs (Figure 4a–c). Ciliary beating of control MCCs showed normal patterns of coordinated power- and recovery strokes (Figure 4a,d; Supporting Information Movie). Most XpodMO injected embryos, however, exhibited impaired ciliary beating, ranging from disorganized and slower cilia sliding to a lack of ciliary movement (Figure 4b,d; Supporting Information Movie and data not shown; Table S1). This effect was specific, because of (a) a dose-dependent MO effect (Figure 4d), (b) rescue of beating patterns upon co-injection of untargeted full-length mRNA (Figure 4d; Supporting Information Movie and data not shown; Table S1), and (c) rescue of ciliary beating by exogenous administration of 5-HT (Figure 4d; Supporting Information Movie and data not shown). From these experiments, we conclude that the loss of Xpod function interfered with ciliary beating patterns, via impaired 5-HT secretion.

### DISCUSSION

The work presented here establishes Xpod as the sole SSC-specific marker gene to date, which should be a valuable tool to unequivocally identify SSCs in wildtype and manipulated Xenopus laevis embryos.
Besides this more descriptive aspect, additional questions on Xpod function and structure arise.

The apparent reduction in MucXS and serotonin release in Xpod morphants suggests that Xpod either plays a role in their synthesis processes or during secretion. Although our data are insufficient to definitely discern between these two possibilities, we favor a secretory function of Xpod because of two arguments: (a) We show that mycXpod was incorporated into secretory granules, which demonstrates that the sole identified structure, the signal peptide, is functional. In addition, Yoshi et al. (2011) using IF detected Xpod in apical blebs, strongly resembling SSC granules. Unfortunately, this antibody was no longer available (Dr. Kinoshita—personnel communication) to conduct a high-resolution subcellular localization study; (b) MucXS and serotonin synthesis differ substantially, which renders an Xpod activity on both processes highly unlikely. Like any other mucin, MucXS mRNA gets translated at the endoplasmic reticulum (ER), the protein modified at the Golgi and subsequently loaded into secretory vesicles. Serotonin, however, is synthesized in the cytoplasm by enzymatic conversion (ddc and tph) of tryptophan to 5-HT, which is shuttled into secretory vesicles via vesicular monoamine transporters (vmat). We have recently shown that vmat1 is uniformly expressed in the epidermis, starting at early neurula stages (cf. Figure S2A,B in Walentek et al., 2014). Vmat1 serves as a monoamine (i.e., 5-HT) hydrogen-ion exchanger, which actively fills the exocytotic vesicle. Therefore, vesicle loading with 5-HT by this canonical pathway should control of an SSC specific promotor, a functional impact on a specific cellular process is still highly unlikely. To our knowledge, de novo gene emergence in a single species has not been reported before.

Interestingly, MucXS is specific to both Xenopus species, although the commonly used mammalian mucins (i.e., mucin5A) are present in both genomes. What properties of MucXS are favorable, compared to other mucins, is not known. The presence of Xpod and MucXS in the

At first glance, it is surprising that Xpod has no vertebrate homolog, not even in its sister species Xenopus tropicalis. The basic process of mucus release via exocytic vesicles, however, is conserved throughout the vertebrates and beyond (Knoop & Newberry, 2018; Perez-Vilar, 2007; Ridley & Thornton, 2018). This raises the question on the evolutionary origin of Xpod. All amphibian genomes, including both Xenopus species, are far from being completely assembled, suggesting that Xpod-like sequences might still be revealed. The mammalian databases have been completed at high resolution, which might indicate that Xpod could be restricted to lower vertebrates. In evolutionary terms, this scenario has the highest probability. On the other hand, our failure to identify homologs could also indicate that Xpod might be a gene specific to Xenopus laevis. Surely, it is hard to imagine how such a species-specific protein could evolve and obtain a crucial function in secretion just once. Although the precise Xpod genomic loci are currently not available, Xpod cDNA fragments match to Xenopus laevis chromosomes 9_10L and S. In Xenopus tropicalis, two homologous but distinct chromosomes 9 and 10 exist, thus chromosome 9_10 reflects an initial fusion result (Matsuda et al., 2015; Session et al., 2016), which potentially could create a species-specific gene product. However, in most cases genomic shuffling results in loss of gene function or in fusion of previously separated gene products. Even if genomic sequences would accidentally become under the control of an SSC specific promotor, a functional impact on a specific cellular process is still highly unlikely. To our knowledge, de novo gene emergence in a single species has not been reported before.
same cell could reflect the same evolutionary advantage that fixed this trait. In that sense, Xpod could be part of a specific MucXS loading complex that is required for vesicle loading. Xenopus tropicalis may use a functional homolog, which differs in sequence. However, serotonin loading was blocked as well in Xpod morphant embryos. Still, it is hard to envisage how Xpod could interfere with both conserved processes. Yet, SSCs are unique because they are all-rounders fulfilling multiple functions, executed by many more mammalian cell types. As various factors have been identified to be secreted by SSCs, the varying loading processes could be linked with each other. It remains to be seen if a mammalian protein is functionally homologous to Xpod. Heterologous experiments in cell culture or Xenopus tropicalis may give answers to this question.

In addition, we demonstrated that activation of Notch signaling induces SSC formation in the epidermis of frog embryos, confirming a recent report (Walentek, 2018). Balanced (repressed or activated) Notch signaling, therefore, accounts for stem cells positioning in the superficial layer, which is differentiating toward MCC, ISC, or SSC fate. Interestingly the different states of Notch signaling induce cell type-specific transcription factors of the forkhead family in each case, that is, foxI (MCCs), foxIe (ISCs), and foxa1 (SSCs). This regulatory code for cell identities is conserved in mammalian tissues (Besnard, Wert, Kaestner, & Whitsett, 2005; Dubaiissi et al., 2014; Esaki et al., 2009; Montoro et al., 2018; Thomas et al., 2010; Wan et al., 2004; Yu, Ng, Habacher, & Roy, 2008), indicating that an SSCs counterpart must exist. Foxa1 and Foxa2 are known inducers of goblet cell identity in mammalian mucus-producing tissues, that is, the gastrointestinal tract (van der Sluis et al., 2008; Ye & Kaestner, 2009). In Xenopus, however, the term goblet cell describes epithelial cells in the outer layer, which are already specified during gastrula stages and continue to proliferate during further development. Although these cells have some secretory properties (Dubaiissi & Papalopulu, 2011; Hayes et al., 2007; Nagata, 2005; Nagata, Nakanishi, Nanba, & Fujita, 2003; Wangkanont, Wesener, Vidani, Kiessling, & Forest, 2016), which have not been addressed in a functional manner so far, SSCs depict all attributes of a mammalian mucin secreting goblet cell. Investigating SSC genesis and function, therefore, could be a valid goblet cell model, like MCCs have been for cilia research.

4 MATERIAL AND METHODS

4.1 Immunofluorescence

Whole-mount embryos were fixed in 4% PFA for 1–2 hr at room temperature. Further handling of embryos was according to standard procedures (Sive, Grainger, & Harland, 2000). Primary antibodies used in this study: mouse monoclonal antibody against acetylated alphatubulin (1:700; Sigma); rabbit polyclonal anti-serotonin antibody (1:500; Merck); mouse monoclonal anti-c-myc 9E10 (Sigma M4439; 1:500). Secondary antibodies: Cy2- or Cy3-conjugated polyclonal rabbit or sheep anti-mouse antibodies (Jackson Immunoresearch or Sigma; both 1:250); Alexa Fluor 488 or 555 conjugated donkey or goat anti-rabbit antibodies (both 1:250; Invitrogen). Actin staining was performed by 30–120 min incubation with Alexa Fluor 488 Phalloidin (1:40; Invitrogen) or Alexa Fluor 405 Phalloidin (1:1000; Thermo Fisher Scientific). PNA conjugated to Alexa Fluor 568 (Life Technologies) was used at a dilution of 1:1000. ImageJ (Fiji) was used for Z-stack analysis taken by Zeiss LSM-700 (Schneider, Rasband, & Eliceiri, 2012).

4.2 High-speed video microscopy of epidermal cilia

At Stage 32/33 specimens (wild type, Xpod morphant and rescue) epidermal ciliary beating (wildtype or reduced) was analyzed using a slide containing a rectangular chamber constructed from duct tape. Recordings were performed using a high-speed Hamamatsu video camera Orca flash 4.0 at 600 frames per second. Significances were calculated by comparing wild type with reduced ciliary beating using Pearson’s chi-squared test (Bonferroni corrected; Statistica 6.1—StatSoft Inc.). To visualize ciliary motion in still pictures, kymographs were generated. Movies were loaded as Z-stack to ImageJ and orthogonal sections were set parallel to the cell surface. Movement of cilia bundles, reflecting power, and recovery strokes (i.e., contrast thereof) were plotted over time. Serotonin incubations were performed as published (Walentek et al., 2014).

4.3 RNA in situ hybridization

Embryos were fixed in MEMFA for 2 hr and processed following standard protocols (Sive et al., 2000). Digoxigenin-labeled (Roche) RNA probes were prepared from linearized plasmids using SP6 or T7 RNA polymerase (Promega). ISH was according to Belo et al. (1997). Sequences of probes used correspond to Xpod (Yoshii et al., 2011) and XtpH1 (L20679; Green, Cahill, & Besharse, 1995).

4.4 mRNA synthesis and microinjections

For in vitro synthesis of mRNA using the Ambion sp6 message machine kit, the plasmid was linearized with NotI. Embryos were injected at the 4- to 8-cell stage, using a Harvard Apparatus. Drop size was calibrated to 4 nL/injection. Used amount of XpodMO (unless otherwise stated): 0.9 pmol. Used amounts of injected mRNA per embryo: rescXpod: 400 pg; mycXpod: 400 pg; Nicd: 320 pg; SulH-DBM: 240 pg (both Deblandre et al., 1999).

4.5 Xpod sequences and used oligonucleotides

Initial EST sequences (i.e., IMAGE:5536832) were used for designing of PCR primers to amplify the Xpod coding region [EcoRI]Xpodcodfor: ATGAATTCAATGAGGGCATATTATCGTGTGCC; XholXpodcodrev: ATC
TCGAGTTAAGAAACAGACACGTCGCG). GenBank accession number of 5’ extended Xpod coding sequence was registered to MK674161. A translation blocking morpholino was used for Xpod loss of function experiments (XpodMO GAGCACACAGGATAAGCCTCCTC AT). To demonstrate MO specificity, a rescue construct was cloned (rescXpod). XpodMO binding-site was changed according to the degenerative genetic code for amino acids. Bold lowercase letters indicate altered nucleotides (rescXpod-forward: ATG cGt ggg ATc ATt TGA GcT GtG TT AGC). Coding sequences of Xpod and rescXpod were cloned into the Cs2+ expression vector. To detect subcellular localization of Xpod, a single myc tag was inserted by site-directed mutagenesis, c-terminal to the signal peptide. Lowercase letters indicate myc tag sequence (Xpodmyc: ctaatcagaaagacctATCTTCTCCTTTTT TCTTCTCCCT; Xpodmycrev: tctgatatgtttttcGTCTTCCCATGCTG AGTCTCCTG).

### 4.6 Experimental animals

According to German regulations and laws (§6, article 1, sentence 2, nr. 4 of the animal protection act) care, handling, and experimental manipulations of Xenopus laevis were approved by the Regional Government Stuttgart, Germany (Vorhaben "Xenopus Embryonen in der Forschung"A379/12 ZO, V340/17 ZO, and V349/18 ZO).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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