CORRECTION

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In the supplementary material (mRNA and morpholino injection) the morpholino SB-MO dock1 was incorrectly listed as: 5′-ACACTCTAGTGAGTATAGTGTGCAT-3′. The correct sequence is: 5′-ACCATCCTGAGAAGAGCAAGAAATA-3′ (corresponding to MO4-dock1 in ZFIN).

The authors apologise to readers for this mistake.
The Rac1 regulator ELMO controls basal body migration and docking in multiciliated cells through interaction with Ezrin

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ABSTRACT

Cilia are microtubule-based organelles that are present on most cells and are required for normal tissue development and function. Defective cilia cause complex syndromes with multiple organ manifestations termed ciliopathies. A crucial step during ciliogenesis in multiciliated cells (MCCs) is the association of future basal bodies with the apical plasma membrane, followed by their correct spacing and planar orientation. Here, we report a novel role for ELMO-DOCK1, which is a bipartite guanine nucleotide exchange factor complex for the small GTPase Rac1, and for the membrane-cytoskeletal linker Ezrin, in regulating centriolar/basal body migration, docking and spacing. Downregulation of each component results in ciliopathy-related phenotypes in zebrafish and disrupted ciliogenesis in Xenopus epidermal MCCs. Subcellular analysis revealed a striking impairment of basal body docking and spacing, which is likely to account for the observed phenotypes. These results are substantiated by showing a genetic interaction between elmo1 and ezrin b. Finally, we provide biochemical evidence that the ELMO-DOCK1-Rac1 complex influences Ezrin phosphorylation and thereby probably serves as an important molecular switch. Collectively, we demonstrate that the ELMO-Ezrin complex orchestrates ciliary basal body migration, docking and positioning in vivo.

KEY WORDS: Ciliogenesis, ELMO, DOCK1, Rac1, Ezrin, Multiciliated cells, Xenopus, Zebrafish

INTRODUCTION

Mutations in genes that impair ciliogenesis are implicated in complex human diseases collectively termed ciliopathies (Hildebrandt et al., 2011). Cilia are microtubule-based organelles and commonly divided into primary non-motile and motile cilia (Goetz and Anderson, 2010); the cilia of multiciliated cells (MCCs) are typically motile. Ciliogenesis in MCCs is initiated by the de novo generation of centrioles within the cytoplasm, followed by their migration to the apical cell membrane where they mature into basal bodies (Steinman, 1968; Dawe et al., 2007; Klos Dehring et al., 2013). The transport of the future basal bodies to the apical cell membrane requires an intact actin cytoskeleton and involves actin-myosin-based mechanisms (Lemullois et al., 1988; Boisvieux-Ulrich et al., 1990). The apical cell membrane in MCCs contains a network of actin filaments, and the assembly of this network involves actin regulators such as RhoA and the phosphate loop ATPase Nupb1 (Pan et al., 2007; Ioannou et al., 2013). The docking of the basal bodies modifies the formation of the apical actin network, and defects that impair docking are often associated with a thinner cortical actin network (Park et al., 2006; Ioannou et al., 2013). A complex array of planar polarisation events accompanies the process of basal body docking, including those that determine their distance relative to their neighbours (spacing), their positioning relative to the surface area of the plasma membrane (translational polarity), and their tilting and orientation relative to external cues such as fluid flow (rotational polarity) (Mitchell et al., 2007; Werner and Mitchell, 2012). Basal bodies in mouse multiciliated ependymal cells move, mediated by an actin- and non-muscle myosin II-dependent mechanism, to the anterior side of the apical cell membrane (translational polarity) (Hirot a et al., 2010). Two pools of cortical actin filaments have been described in Xenopus: the apical actin network (at the plane of the basal bodies) and the subapical network (at the plane of the striated rootlets) (Werner et al., 2011). Treatment with a low dose of cytochalasin D, which specifically inhibits the formation of the subapical actin network, leads to defects in basal body spacing and rotational polarity and to subsequent impairment of directional and metachronal ciliary beating and, thereby, to defects in the generation of fluid flow (Werner et al., 2011).

Components of the planar cell polarity (PCP) pathway are important for basal body docking and planar polarisation (Park et al., 2006, 2008; Mitchell et al., 2009). Specifically, Dishevelled interacts with Inturned to control the activation and localisation of the small GTPase Rho at the basal bodies, leading to proper apical docking and polarisation of basal bodies and to Rho-mediated actin assembly (Park et al., 2006, 2008). Basal body migration and docking in MCCs are actin cytoskeleton-dependent steps during ciliogenesis. Rho GTPases are known to be key regulators of the actin cytoskeleton (Hall, 1998). However, the orchestration of the steps and the signalling events leading to changes in actin polymerisation are only incompletely understood.

In ciliogenesis, a role for the Rho family GTPase Rac1 in the positioning of the cilium on the posterior cell surface in mouse ventral node cells has been postulated (Hashimoto et al., 2010), and in Lowe syndrome, a deficiency of Rac1 activity is associated with abnormal primary cilia formation (Madhivanan et al., 2012), but the exact function of Rac1 in these contexts remains unclear. The ELMO-DOCK1 (formerly DOCK180) complex serves as a bipartite guanine nucleotide exchange factor for Rac1 (Brugnera et al., 2002). Several studies have shown the essential function of ELMO1-DOCK1 in remodelling the actin cytoskeleton and controlling cell migration during multiple processes, including engulfment of apoptotic cells, phagocytosis, tumour invasion, myoblast fusion and angiogenesis (Gumienny et al., 2001; Jarzynka et al., 2007; Park et al., 2007; Moore et al., 2007; Elliott et al., 2010;...
Moreover, we analysed the localisation of ELMO1-DOCK1 in non-ciliary axoneme, revealed specific ELMO1 expression at the basal cells (Graser et al., 2007), or acetylated Tubulin, a marker for the either CEP164, a marker for the mother centriole in monociliated epithelial cells from healthy persons. Colabelling of ELMO1 with ciliogenesis remains incompletely understood, we determined the Since the function of Rac1 and its upstream regulators during ELMO directs ciliary basal bodies to the apical surface in spacing, and ultimately to defects in ciliary function. Depletion of any of during the highly coordinated processes of ciliary basal body downstream effector Rac1 cooperate and function as a module with that of ELMO1-DOCK1 and cilia function. Co-injection of elmo1 mRNA partially prevented the pronephric cyst formation of elmo1 morphants, confirming MO specificity (Fig. 1L). Transmission electron microscopy (TEM) studies revealed defective basal body transport to the apical membrane in elmo1 and dock1 morphants (Fig. 1M-O; supplementary material Fig. S4C).

The population of tubular epithelial cells in the zebrafish pronephros consists of monociliated and multiciliated cells (Liu et al., 2007). To confirm the role of ELMO in MCCs, we took advantage of a second animal model, X. laevis, since the epidermis of Xenopus embryos contains isolated MCCs with motile cilia that are easily accessible for high-resolution imaging (Werner and Mitchell, 2012). Xenopus has two ELMO homologues, ELMO1 and ELMO2, which share 70% identity. Both homologues were expressed similarly, including in ciliated tissues (Fig. 2A-F; supplementary material Fig. S5C). The dock1 mRNA expression pattern partially overlapped with that of elmo1 and elmo2 (supplementary material Fig. S5A-C). MOs targeting elmo1 or elmo2 (supplementary material Fig. S6A-C) did not have a significant effect when injected separately. However, combined ELMO1 and ELMO2 knockdown in the medium dose range (each 4 ng) caused defects in the apical migration and membrane attachment of basal bodies, as labelled with Centrin-RFP (Park et al., 2008), and the formation of intracellular polymerised acetylated α-Tubulin-positive structures (Fig. 2G,H; supplementary material Fig. S6D,E). Injection of lower doses of elmo1 and elmo2 MOs (each 2 ng) resulted in partial basal body docking, but the regular spacing of the basal bodies was impaired, causing basal bodies to be attached to each other like ‘beads on a string’ or to form groups (Fig. 2I; supplementary material Fig. S7).

In summary, these results show that ELMO proteins are expressed in ciliated organs in zebrafish and Xenopus. The loss-of-function studies reveal that the ELMO-DOCK1 complex is required for specific aspects of cilia formation, i.e. for coordinated basal body docking and spacing across the apical cell membrane.

**RESULTS**

**ELMO directs ciliary basal bodies to the apical surface in zebrafish and Xenopus**

Since the function of Rac1 and its upstream regulators during ciliogenesis remains incompletely understood, we determined the localisation of the Rac1 regulator ELMO1 in human respiratory epithelial cells from healthy persons. Colabelling of ELMO1 with either CEP164, a marker for the mother centriole in monociliated cells (Grazer et al., 2007), or acetylated Tubulin, a marker for the ciliary axoneme, revealed specific ELMO1 expression at the basal bodies and along the ciliary axonemes in human respiratory epithelial cells (Fig. 1A-E; supplementary material Fig. S1A-E). Moreover, we analysed the localisation of ELMO1-DOCK1 in non-motile cilia of polarised Madin–Darby canine kidney cells (MDCKs) (supplementary material Fig. S1G,H and Fig. S2). Immunostaining revealed a specific staining pattern for ELMO1 as well as for DOCK1 at the base of the cilium in MDCKs. Co-immunostainings with Cep164 confirmed that ELMO1 was localised exclusively at the mother centriole, whereas DOCK1 localised to both mother and daughter centrioles.

Based on the specific ciliary expression of ELMO1 and DOCK1 in primary non-motile cilia of MDCKs and in motile cilia of human respiratory epithelial cells, we investigated the potential role of the Rac1 activator complex ELMO-DOCK1 during ciliogenesis, using the vertebrate model organisms zebrafish and Xenopus laevis. Sections of zebrafish embryos at 36 h post fertilisation (hpf) subject to whole-mount in situ hybridisation (WISH) for elmo1 and cadherin 17 revealed elmo1 expression in the pronephric tubule (Fig. 1F). Since there is no working ELMO1 antibody for immunofluorescence, we immunostained for DOCK1 as a surrogate: in 48 hpf Tg(actb2:Mmu.Arl13b-GFP) zebrafish embryos (Borovina et al., 2010), Dock1 is localised on the base of the cilia, the presumptive basal bodies, in pronephric tubules and otic vesicles (Fig. 1G-I; supplementary material Fig. S3B-B’). Morpholino oligonucleotide (MO)-mediated knockdown of Elmo1 or Dock1 led to pronephric cyst formation and left-right asymmetry defects at 48 hpf (Fig. 1J-L; supplementary material Fig. S4A) and to shortened cilia in the pronephric tube of 24 hpf embryos (supplementary material Fig. S4B). These defects are similar to the phenotypic changes observed in mutant zebrafish lines with defective ciliogenesis (Kramer-Zucker et al., 2005), implicating a link between ELMO1-DOCK1 and cilia function. Co-injection of elmo1 mRNA partially prevented the pronephric cyst formation of elmo1 morphants, confirming MO specificity (Fig. 1L). Transmission electron microscopy (TEM) studies revealed defective basal body transport to the apical membrane in elmo1 and dock1 morphants (Fig. 1M-O; supplementary material Fig. S4C).

Ezrin interacts with ELMO and is required for proper ciliogenesis in zebrafish and Xenopus

Ezrin is localised on ciliary basal bodies in MCCs (Gomperts et al., 2004). Moreover, Ezrin, similar to other ERMs, physically interacts with members of the ELMO family (Grimsley et al., 2006). First, we studied the expression of Ezrin using human respiratory epithelial cells from embryos including in ciliated tissues (Fig. 2A-F; supplementary material Fig. S5C). The dock1 mRNA expression pattern partially overlapped with that of elmo1 and elmo2 (supplementary material Fig. S5A-C). MOs targeting elmo1 or elmo2 (supplementary material Fig. S6A-C) did not have a significant effect when injected separately. However, combined ELMO1 and ELMO2 knockdown in the medium dose range (each 4 ng) caused defects in the apical migration and membrane attachment of basal bodies, as labelled with Centrin-RFP (Park et al., 2008), and the formation of intracellular polymerised acetylated α-Tubulin-positive structures (Fig. 2G,H; supplementary material Fig. S6D,E). Injection of lower doses of elmo1 and elmo2 MOs (each 2 ng) resulted in partial basal body docking, but the regular spacing of the basal bodies was impaired, causing basal bodies to be attached to each other like ‘beads on a string’ or to form groups (Fig. 2I; supplementary material Fig. S7).

In summary, these results show that ELMO proteins are expressed in ciliated organs in zebrafish and Xenopus. The loss-of-function studies reveal that the ELMO-DOCK1 complex is required for specific aspects of cilia formation, i.e. for coordinated basal body docking and spacing across the apical cell membrane.
Immunostaining localised Ezrin to the base of the cilia in pronephric tubular cells and inner ear cells of 48 hpf Tg(actb2:Mmu.Arl13b-GFP) zebrafish embryos (Fig. 3E-G; supplementary material Fig. S3C-C″).

To investigate the role of Ezrin in ciliary function we used an MO that blocks the translation of ezrin (Link et al., 2006). Downregulation of Ezrin led to prominent hydrocephalus and pronephric cyst formation in zebrafish embryos (Fig. 3H-J). Moreover, quantification of cilia length in the pronephric tubule revealed significantly shortened cilia in ezrin morphants compared with the control at 24 hpf (supplementary material Fig. S4B).

Co-injection of ezrin mRNA together with MO partially prevented pronephric cyst formation, confirming MO specificity (Fig. 3J; supplementary material Fig. S8H). Examination of the anterior pronephric tubular segments in 48 hpf ezrin morphants by TEM revealed defective microvillus formation, and showed that multiple basal bodies did not attach to the apical membrane and that ciliary axoneme formation was impaired (Fig. 3K,K″; supplementary material Fig. S4C). The significant reduction of microvilli (Fig. 3K) supports MO specificity, since it is consistent with the reported role of Ezrin during microvillus formation (Berryman et al., 1993; Crepaldi et al., 1997).
To uncover a potential genetic interaction between elmo1 and ezrin during ciliogenesis in zebrafish, we examined whether the cystic phenotype of elmo1 or ezrin morphants could be prevented by ezrin or elmo1 mRNA, respectively. Indeed, Ezrin or Elmo1 overexpression ameliorated the defects caused by elmo1 or ezrin depletion, respectively, suggesting that both proteins act in the same pathway that coordinates basal body migration and docking in zebrafish (Fig. 3L).

In Xenopus, ezrin transcripts were expressed in ciliated epithelial cells, including the otic vesicle, cloaca, pronephros and throughout the epidermis at stage 32 of development (supplementary material Fig. S5C and Fig. S8I). Downregulation of Ezrin using a translation-blocking MO (8 ng) (supplementary material Fig. S8I), led to polymerised acetylated α-Tubulin-positive structures within the cytoplasm that did not project to the outside of the cell like normal ciliary axonemes (Fig. 4A,B; supplementary material Fig. S6D). Strikingly, in ezrin morphants the basal bodies clustered in the cytoplasm close to the cell centre instead of being transported to the apical membrane and distributed uniformly along the cell surface (Fig. 4C-D‴, supplementary material Fig. S6E). Furthermore, the apical actin network of the MO-affected epidermal cells appeared to be thinner than that of controls, with focal points of actin accumulation at the apical membrane directly above the basal body cluster (Fig. 4D-D‴ and Fig. 5A-A‴). TEM confirmed that the basal bodies accumulated at some distance to the apical cell membrane in ezrin morphants (Fig. 5C,F). Some ciliary axonemes formed within the cytoplasm in ezrin morphants, revealing an ultrastructure identical to the properly elongated axonemes of the controls (Fig. 5C‴,C‴,E,G). Injection of lower doses of ezrin MO (2 and 4 ng) led to a phenotype whereby basal body docking and axoneme formation partially occurred, but spacing of the basal bodies was impaired (Fig. 4E-F‴; supplementary material Fig. S7).

Collectively, these results identify a novel role for ELMO and Ezrin in guiding the maturing basal bodies to the cell surface, in their docking, and in their correct spacing underneath the apical membrane in MCCs.

**The ELMO-DOCK1 downstream effector Rac1 is important for basal body docking and spacing**

Rac1 is the major downstream effector of ELMO-DOCK1 (Brugnera et al., 2002). In zebrafish there are two related rac1 genes: rac1 and rac1l. Whereas depletion of rac1 resulted in minor pronephric cyst formation, depletion of rac1l caused prominent pronephric cyst formation (Fig. 6A-C; supplementary material Fig. S9A). Simultaneous knockdown of both proteins resulted in the observed abnormalities in zebrafish, MO depletion of Rac1l partially prevented the pronephric cyst formation of rac1l morphants, confirming specificity of the MO (Fig. 6C). In addition, depletion of rac1 and/or rac1l led to striking defects of cardiac looping in zebrafish embryos at 48 hpf, suggesting an essential role of these gene products during the establishment of normal left-right asymmetry (Fig. 6D). TEM studies of rac1/rac1l double-morphant zebrafish revealed a transport defect and an abnormal accumulation of basal bodies in the cytoplasm at 48 hpf (Fig. 6E-E‴; supplementary material Fig. S4C). Consistent with the observed abnormalities in zebrafish, MO depletion of Rac1 during Xenopus epidermis development resulted in irregular basal body docking and spacing at the apical membrane (Fig. 6F-H‴; supplementary material Fig. S7).

**Rac1 controls ERM phosphorylation during ciliogenesis**

During T-cell activation, Rac1 inactivates ERM proteins in T-lymphocytes, and this results in relaxation of the cortical actin cytoskeleton from the plasma membrane, and leads to the formation of the immunological synapse (Faure et al., 2004; Cemudra-Morollon et al., 2010). To investigate whether Rac1 regulates ERM proteins during ciliogenesis in vertebrates, we isolated pronephric tubules of 24 hpf zebrafish embryos depleted of rac1/rac1l, elmo1 or dock1 and determined the phosphorylation level of ERM proteins by immunoblotting. Increased phospho-ERM levels were observed in all three conditions (Fig. 7A), as well as after Rac1 or Rac1l single knockdown (supplementary material Fig. S9A). Reciprocally, overexpression of...
Elmo1 or Rac1 resulted in significantly decreased phospho-ERM levels in zebrafish (Fig. 7B).

To determine the function of Ezrin in the zebrafish pronephric tubule, wild-type and mutant forms of zebrafish Ezrin, including the phosphorylation-deficient Ezrin(T564A) mutation and the phosphorylation mimetic mutant Ezrin(T564D) corresponding to human Ezrin threonine 567 (Gautreau et al., 2000), were targeted to the zebrafish pronephros using a transient transgenesis approach (Kikuta and Kawakami, 2009). The cadherin 17 promoter-driven expression of both mutant forms caused cyst formation on the left-or right-hand side of the pronephric glomerulus, depending on which side the motile cilia bearing MCCs in the mid-portion of the pronephric tubule were most affected (Fig. 7C-F). The majority of MCCs are located in this mid-tubular segment, creating the fluid flow within the pronephric tubule that is necessary for normal pronephric development (Kramer-Zucker et al., 2005; Liu et al., 2007). Moreover, injection of mRNA of either the phosphorylation-deficient ezrin(T564A) mutation or the phosphorylation mimetic ezrin(T564D) resulted in significantly shortened cilia in the pronephric tubule of 24 hpf zebrafish embryos (supplementary material Fig. S9B-D).

To further confirm a potential role for the ELMO-DOCK1-Rac1 complex in regulating Ezrin phosphorylation levels, we next examined whether overexpression of the phosphorylation-deficient Ezrin(T564A) mutation might prevent the formation of pronephric cysts in Elmo1-, Dock1- or Rac1-deficient zebrafish embryos. Indeed, we were able to show that overexpression of mRNA encoding the phosphorylation-deficient Ezrin(T564A) mutation significantly reduced pronephric cyst formation in all three experimental settings performed at 2 dpf (Fig. 7G).

Taken together, these results indicate that Rac1 plays an essential role during ciliogenesis in vertebrates by acting downstream of ELMO-DOCK1, where it induces Ezrin dephosphorylation.

DISCUSSION

Motile cilia play a central role in embryonic development and human disease. Using two in vivo models, zebrafish and Xenopus, we identified the protein module encompassing Ezrin, ELMO-DOCK1 and Rac1, as a crucial component for cilia biogenesis and function in MCCs.

ELMO1 localises to the basal bodies in both monociliated and multiciliated cells. Cep164, as a marker of the mother basal body in primary cilia, was reported to localise to all basal bodies in MCCs (Lau et al., 2012). Our findings show a colocalisation of ELMO1 with Cep164 in both cell types (Fig. 1A-E; supplementary material Fig. S2C). In addition, in MDCks ELMO1 localises to the mother centriole exclusively, whereas DOCK1 was seen at the mother and daughter centriole. This significant distinction has to be explored in future studies. In non-dividing multiciliated respiratory epithelial cells there is no daughter centriole attached
Depletion of Ezrin, ELMO/DOCK1 or Rac1 by MO injection caused characteristic phenotypes with impaired basal body migration and docking. Rac1 is likely to directly affect filament assembly of the apical actin network, a key structural component of ciliogenesis. Recently, it was proposed that during the process of basal body docking a cytoplasmic actin network, controlled by Nubp1, surrounds the migrating basal bodies and modifies the subapical actin network (Ioannou et al., 2013). Depletion of Nubp1 in Xenopus abolished the internal actin network and led to failure of basal body migration. We showed that knockdown of Ezrin in Xenopus embryos not only impaired centriole/basal body migration, but also decreased overall actin polymerisation at the apical cell membrane (with the exception of the cortical actin ring along the cell-cell junctions) (Fig. 4D and Fig. 5A), similar to the changes reported in Xenopus inturned morphants (Park et al., 2006) or in nubp1 morphants (Ioannou et al., 2013). This supports the concept of actin filaments acting as a guide structure in the apical migration of basal bodies. However, directly above the cluster of centrioles/basal bodies there are actin foci at the prospective site of docking of the basal bodies (Fig. 4D" and Fig. 5A""). This would imply that docking could be the trigger for the proper assembly of the apical/subapical actin network, with its delicate structure (Werner et al., 2011). Since the mechanism of Nubp1 action remains unclear but seems to be independent of RhoA, it is tempting to speculate that the Nubp1-mediated actin modification might involve the ELMO-Ezrin-Rac1 module.

Furthermore, a low dose of nubp1 MO allowed docking of basal bodies, but led to their irregular spacing and to disturbance of the subapical actin pool (Ioannou et al., 2013). Similarly, in Xenopus embryos, treatment with a low dose of cytochalasin D mainly affected the subapical actin network and caused defects in basal body spacing (Werner et al., 2011), virtually identical to our observations using MOs at low dosage: basal bodies were attached to each other like ‘beads on a string’ or formed groups (Fig. 2J-J” and Fig. 4F-F”) instead of being distributed equally throughout the apical cell membrane. In terms of basal body distribution, the changes observed in morphants were significantly different for all MOs compared with the control (supplementary material Fig. S7).

In addition, it has been shown that inhibition of Rac1 in mouse nodal cells leads to disturbed posterior positioning of the basal body (translational polarity) and to left-right asymmetry defects (Hashimoto et al., 2010). These observations support our notion that the ELMO-Ezrin-Rac1 module is necessary for basal body positioning and spacing.

Upon depletion of Ezrin and impaired apical migration of basal bodies, we observed intracellular axoneme formation by TEM (Fig. 5C-C",E,G); similar findings are reported in the literature (Tissir et al., 2010; Werner and Mitchell, 2012; Ioannou et al., 2013). Confocal images showed intracellular polymerised acetylated α-Tubulin-positive structures (Fig. 4B). Whether these acetylated α-Tubulin-positive structures represent intracellular axonomes remains an open question, particularly in consideration of recent data showing that apically formed acetylated microtubules are involved in the radial intercalation of MCCs into the outer epithelial layer of the skin in Xenopus (Werner et al., 2014). Radial intercalation precedes or coincides with basal body migration and docking. The presence of acetylated α-Tubulin-positive structures in morphant embryos could therefore also reflect a failure to remove these acetylated Tubulin structures that were involved in radial intercalation. Even when basal body migration was disturbed in many cells, we still observed shorter cilia, rather than their absence, in many parts of the zebrafish to the basal body, in contrast to differentiating and dividing respiratory epithelial cells exhibiting primary monocilia (Sorokin, 1968; Dawe et al., 2007; Nigg and Raff, 2009). Cep164 has been implicated in the recruitment of Rab8 and docking of vesicles at the motor centriole to initiate the formation of the primary cilium (Schmidt et al., 2012). Cep164 function in MCCs has not been clearly defined as yet, but basal body docking to ciliary vesicles and migration to the apical cell membrane are conceivable (Sorokin, 1968; Park et al., 2008). Ezrin also localises to the putative basal body in MCCs of the human respiratory epithelium (Fig. 3A-D), very likely together with ELMO1; furthermore, ELMO1 resides in part of the ciliary axoneme (supplementary material Fig. S1A-D).

This underlines the role of Ezrin and ELMO1 in ciliogenesis that we have described here.

Fig. 4. Ezrin is required for ciliogenesis in Xenopus. (A,B) In Xenopus embryos (stage 32) injected with Co-MO (8 ng) the cell membrane was highlighted by overexpression of membrane-GFP. Immunostaining for acetylated Tubulin showed the ciliary axonemes protruding to the outside of the cell (A, arrow), whereas in embryos injected with TB-MO ezrin (8 ng) the acetylated α-Tubulin-positive structures mainly formed inside the cell (B, arrows). (C-D”) Basal bodies marked by overexpression of Centrin-RFP aligned properly at the apical cell membrane labelled with phalloidin 488 in Xenopus embryos (stage 32) injected with Co-MO (8 ng) (C-C″), in contrast to the failed migration and docking of basal bodies (arrows) in embryos injected with TB-MO ezrin (8 ng) (D-D″). (E-F″) Injection of a lower dose of TB-MO ezrin (4 ng) together with centrin-RFP and clamp-GFP mRNA (F-F″) revealed partial basal body docking (arrow) and irregular basal body spacing compared with proper apically docked and evenly distributed basal bodies in embryos (stage 32) injected with Co-MO (4 ng) (E-E″). Dashed white line indicates the apical surface. Scale bars: 5 μm.
pronephric tubules (supplementary material Fig. S4B). Since there are monociliated and multiciliated pronephric tubule cells (Liu et al., 2007), one possible explanation is that monociliated cells are less affected by defective ELMO/Ezrin signalling and therefore still form a cilium. Consistent with this possibility, *Xenopus vangl2* morphants are characterised by a reduced number of cilia in skin cells (Mitchell et al., 2009), whereas cilia in the gastrocoel roof plate are reportedly unaffected (Antic et al., 2010; Werner and Mitchell, 2012). In the same way, gastrocoel roof plate cilia are significantly shorter but not missing in *nubp1* morphants (Ioannou et al., 2013).

With respect to the apical actin network, the function of the basal body in the cilium and of the centrosome in the immunological synapse share striking similarities (Griffiths et al., 2010). T-cell receptor signalling initially leads to actin accumulation across the synapse (Ryser et al., 1982). This is followed by actin clearance in the centre of the synapse, forming an outer ring around the synapse, and by migration of the centrosome to the centre of the synapse (Stinchcombe et al., 2006). T-cell receptor signalling also activates Rac1, leading to dephosphorylation of ERMs, which in turn releases the cross-linking between cell membrane and actin filaments and leads to relaxation of the cytoskeleton and to the formation of a stable T-cell–antigen-presenting cell conjugate (Faure et al., 2004; Cernuda-Morollon et al., 2010). In parallel to centriole migration to the centre of the immunological synapse, basal body migration and docking seem to require such regulation of ERM protein activity: we found that Rac1 and its regulators control ERM phosphorylation. Depletion of members of the ELMO-DOCK1-Rac1 module led to an increase in phospho-Ezrin, whereas overexpression led to its decrease (Fig. 7A,B; supplementary material Fig. S9A). The phenotypic effects caused by MO knockdown of ELMO, DOCK1 or Rac1 were reversed by co-injection of the phosphorylation-deficient zebrafish Ezrin(T564A) (corresponding to threonine T567 in human Ezrin). Moreover, the overexpression of either the phosphorylation-deficient Ezrin(T564A) or the phosphorylation-mimetic Ezrin (T564D) caused pronephric cyst formation (Fig. 7C-F). This implies that tight regulation of Ezrin phosphorylation is mandatory to ensure normal embryonic development. Regulated turnover of Ezrin(T567) phosphorylation has been observed in renal epithelial cells; the phosphorylation mimetic Ezrin(T567D) does not allow such a turnover and its overexpression causes aberrant growth of membrane projections in cultured proximal tubule cells (Zhu et al., 2008). Similar findings have been reported in Jeg-3 cells: hyperphosphorylation of Ezrin induced by phosphatase inhibition led to partial mislocalisation of Ezrin; furthermore, the phosphorylation mimetic Ezrin(T567E) mutant mislocalised all over the plasma membrane, whereas the phosphorylation-deficient Ezrin(T567A) mutant was hardly detected at the plasma membrane (Viswanatha et al., 2012). There was also evidence that Ezrin underwent constant C-terminal phosphoryling, i.e. repetitive phosphorylation and dephosphorylation (Viswanatha et al., 2012). Since we were able to show that both mutant forms of Ezrin (T564A and T564D) caused pronephric cyst formation and also reduced ciliary axoneme length in zebrafish pronephros, as in the
knockdown experiments, this suggests that phosphocycling might be necessary during basal body migration and docking, allowing Ezrin to constantly bind to and release actin filaments.

How does Rac1 influence Ezrin phosphorylation? The increase in threonine phosphorylation of ERM proteins observed after knockdown of the ELMO1-DOCK1-Rac1 complex could reflect a Rac1-dependent inactivation of a constitutively active kinase and/or a Rac1-dependent activation of a serine/threonine phosphatase. According toParameswaran and Gupta (2013), the phosphatase that a Rac1-dependent activation of a serine/threonine phosphatase.

Rac1-dependent inactivation of a constitutively active kinase and/or knockdown of the ELMO1-DOCK1-Rac1 complex could reflect a in threonine phosphorylation of ERM proteins observed after Ezrin to constantly bind to and release actin filaments.

Depletion of Ptp4a3 or Ptp4a3l by MO knockdown did not result in pronephric cyst formation (data not shown). So Ptp4a3 and Ptp4a3l do not seem to be the main phosphatases regulating Ezrin phosphorylation downstream of Rac1 in zebrafish. In addition, other potentially relevant phosphorylation sites in different domains of Ezrin have been reported (e.g. Murchie et al., 2014). Thus, the link between Rac1 signalling and the phosphorylation status of ERM proteins remains to be determined and will be the subject of future investigation in our laboratory.

Recently, new gene defects in MCCs of the respiratory epithelium have been identified as causing a distinct mucociliary clearance disorder in humans that is characterised by the reduced generation of multiple motile cilia (RGMC) (Boon et al., 2014; Wallmeier et al., 2014). Individuals show the typical signs of chronic recurrent airway infections, but also hydrocephalus. TEM studies revealed normal microvilli at the apical cell membrane, but a substantial decrease in basal bodies and apical cilia. The responsible genes were identified as MCDJAS and CCNO, which encode multicilin and cyclin O, respectively, and are implicated in the acentriolar, deuterosome-mediated amplification of centrioles in MCCs, which could be confirmed in Xenopus. Since ELMO-DOCK1-Rac1 and Ezrin are potential candidates for RGMC, although with a predominant basal body migration defect, it will be interesting to screen databases of patients with RGMC for defects in these genes.

We present a model for the function of Ezrin and its interactors ELMO and DOCK1 in MCCs (Fig. 8). In conclusion, the Ezrin-ELMO-DOCK1-Rac1 complex represents a newly identified
Fig. 7. The ELMO1-DOCK1-Rac1 complex influences ERM phosphorylation and tight control of phospho-Ezrin is required for proper ciliogenesis. (A) Immunoblot of 24 hpf zebrafish lysates showing elevated phospho-Ezrin (pERM) levels in embryos injected with TB-MO rac1 (4 ng) or rac1l (0.5 ng), SB-MO elmo1 (2 ng) and SB-MO dock1 (2 ng) as compared with Co-MO (4.5 ng). (B) Immunoblot of 24 hpf zebrafish lysates showing reduced pERM levels in zebrasfish embryos injected with elmo1 mRNA (20 pg) or rac1l mRNA (20 pg) as compared with control embryos. Anti-β-Tubulin immunoblots served as a loading control. Immunoblots represent results from one of three independent experiments with similar results. (C-F) Overexpression of Ezrin by transient transgenesis demonstrates the importance of tight regulation of phosphorylation at threonine 564 for the function of zebrafish Ezrin. cadherin 17 (cdh17) promoter-driven expression of either the nonphosphorylatable T564A mutation or the phosphorylation mimetic mutant T564D caused cyst formation on the side of the pronephros where the MCCs in the mid-portion of the pronephric tubule are most affected. Arrowheads indicate the tubular mid-portion, with the cells expressing mutant Ezrin on the side of the formed cyst. The number of individual embryos analysed is indicated above each bar. (G) Quantification of pronephric cyst formation of 48 hpf zebrafish embryos injected with SB-MO elmo1 (2 ng) with or without ezrin(T564A) mRNA (10 pg) (**) p<0.006. SB-MO dock1 (2 ng) with or without ezrin(T564A) mRNA (10 pg) (**) p=0.009 or TB-MO rac1 (4 ng)/rac1l (0.5 ng) with or without ezrin(T564A) mRNA (10 pg) (**) p=0.02. Scale bars: 50 µm.

module that, by influencing the cortical actin web in MCCs, orchestrates ciliary basal body migration, docking and spacing at the apical cell membrane.

MATERIALS AND METHODS

Zebrafish lines and Xenopus, embryo maintenance

All animal work has been conducted according to relevant national and international guidelines [Regional council (Regierungspräsidium) Freiburg, reference number 35-9185.64-1.1] (Westenfeld, 1995). Zebrafish were maintained and the embryos were staged as previously described (Kimmel et al., 1995). The following strains were used: AB/TL wild type (WT), Tg(wt1b:EGFP) (Perné et al., 2007) and Tg(cadherin17-GFP) (Borovina et al., 2010). The Tg(cadherin17-GFP) line was generated by injection of a Tol2 vector (Kawakami et al., 2004) containing a 4 kb fragment from the zebrafish cadherin 17 promoter driving the expression of GFP protein. Methods of X. laevis maintenance and manipulation were as described (Hoff et al., 2013).

Fig. 8. Model illustrating ELMO-DOCK1-Rac1 and their interaction with Ezrin and the effects on basal body migration and spacing in MCCs. (A) The ELMO-DOCK1 complex localises to the ciliary basal body and acts as bipartite guanine nucleotide exchange factor for Rac1. Rac1 in turn dephosphorylates Ezrin via an unknown phosphatase (PPase). Dephosphorylated Ezrin does not bind to actin filaments, whereas phosphorylated Ezrin serves as a linker between the basal body and actin filaments. (B) Constant turnover of phosphorylated Ezrin regulated by ELMO-DOCK1-Rac1 is necessary to promote basal body migration along actin filaments (arrows) and for basal body docking to the membrane and spacing (double arrows) with assembly of the apical and subapical actin network.

Constructs, mRNAs and MOs

Constructs carrying wild-type and mutant forms of zebrafish Ezrin under the cadherin 17 promoter [Cdhl7:Ezrin(WT)-GFP, Cdhl7:Ezrin(T564A)-GFP and Cdhl7:Ezrin(T564D)-GFP] were generated and injected as described in the supplementary methods. Synthesis of mRNAs, MO sequences and their injection are described in the supplementary methods. Translation-blocking and splice-blocking MOs are given the prefix TB and SB, respectively.

Antibodies and reagents

The following antibodies were used for immunofluorescence (IF) and immunoblotting (IB): anti-ELMO1 [EB05297, Everest Biotech; human respiratory epithelial cells, 1:150 (IF), 1:500 (IB); MDCKs 1:200 (IF), 1:500 (IB); Xenopus 1:1000 (IB)], anti-ELMO2 [EB05305, Everest Biotech; Xenopus 1:1000 (IB)], anti-DOK1 (H-4; sc-13163, Santa Cruz Biotechnology; MDCKs 1:200 (IF), 1:500 (IB)]; anti-γ-Tubulin [clone 6-11B-1, Sigma Aldrich; human respiratory epithelial cells 1:10000 (IF); zebrafish and Xenopus 1:500 (IF)]; anti-Tubulin [detyrosinated; AB3201, Upstate; MDCKs 1:200 (IF), 1:500 (IB)]; zebrafish 1:50 (IF); anti-α-Tubulin [clone GTU-88, Sigma Aldrich; zebrafish and Xenopus 1:1000 (IB)] and anti-β-Tubulin [clone 2148, Cell Signaling; human respiratory epithelial cells 1:300 (IF), anti-pERM [63141], Cell Signaling; zebrafish 1:1000 (IB)]; anti-oesin [610401, BD Biosciences; human respiratory epithelial cells, 1:100 (IF), 1:500 (IB); MDCKs 1:200 (IF), 1:500 (IB); Xenopus 1:1000 (IB)]; anti-DOCK1 [H-4; sc-13163, Santa Cruz Biotechnology; MDCKs 1:200 (IF), 1:500 (IB); anti-Cep164 [provided by Erich Nigg, Basel, Switzerland; MDCKs 1:200 (IF), anti-Cep164 [AP23475P-N, Acris Antibodies; human respiratory epithelial cells 1:100 (IF), 1:500 (IB)]; anti-Rac1 [clone 23A8, Upstate; zebrafish 1:1000 (IB)]; anti-GFP [LS-C144578-100, Biozol; zebrafish 1:200 (IF)]; HRP-conjugated antibodies (DAKO, 1:3000). Cy3-, Cy5- or Alexa Fluor 488/546-labelled secondary antibodies were purchased from Jackson ImmunoResearch and Molecular Probes (Invitrogen), 1:1000 dilution. Alexa Fluor 488/568-phalloidin was purchased from Life Technologies (1:200 dilution) and Hoechst 33342 from Sigma Aldrich, 1:1000 dilution. Details of staining and imaging are provided in the supplementary methods.

Immunoblotting

Zebrafish embryos were lysed in 2× SDS protein sample buffer [0.125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% β-mercaptoethanol,
0.004% Bromophenol Blue. For zebrafish pronephric tubule isolation, 60 control or morphant Tg(cadherin17-GFP) embryos were treated with Hank’s solution (Westerfield, 1995) containing 0.1 M DTT, 2 mM Na2VO3 and protease inhibitors (Roche) for 30 min at room temperature, followed by collagenase digestion [5% collagenase (Sigma-Aldrich) in Hank’s solution] for 30 min at room temperature. GFP-fluorescent pronephric tubules were collected and lysed in 2× SDS protein sample buffer. For Xenopus protein analysis, embryos were lysed in buffer containing 50 mM Tris (pH 7.6), 1% Triton X-100, 50 mM NaCl, 1 mM EDTA, 2 mM Na2VO3 and protease inhibitors (Roche). After centrifugation at 13,000 g for 5 min, 2× SDS protein sample buffer was added to the supernatant, and the mixture was heated for 5 min at 95°C. Proteins were separated by SDS-PAGE, transferred to PVDF membrane, and incubated with the indicated antibodies and finally with immunoblot detection reagent (Perbio Science).

**In situ hybridisation analysis and antisense RNA synthesis**
Whole-mount in situ hybridisation (WISH) analysis using Digoxigenin- or Fluorescein-labelled probes was performed as described (Epting et al., 2007) using NBT (blue) or INT (red) (Roche) as substrates. Details of staining and imaging are provided in the supplementary methods. Zebrafish antisense RNA was synthesized from EcoRI-digested ELMO1-pSPORT1 (EST clone MAAP998P118966Q; imaGenes) or NotI-digested Cadherin17-pCR-BlnII-TOPO [amplified from zebrafish cDNA with specific primers (forward: ATTAGTCGCGTAATTCCCGACG; reverse: ATGCGAAGG-CAGGGTGTTCTAAG)] plasmid using SP6 RNA polymerase, and from EcoRV-digested Ezrin-pExpress-1 (EST clone IR82899P10494D; imaGenes) or NotI-digested CMLC2-pBluescript II Sk+ (kindly provided by Wolfgang Driever, Freiburg, Germany) plasmid using T7 RNA polymerase. Xenopus antisense RNA was synthesized from Smul-digested ELMO1-pSII (EST clone 8547184; Open Biosystems), EcoRV-digested ELMO2-pExpress-1 (EST clone 7210931; Open Biosystems), NotI-digested DOCK1-pBluescript II Sk+ (EST clone XL151h21; RIKEN BioResource Center) or SalI-digested Ezrin-pCMV-SPORT6 (EST clone 7011343; Open Biosystems) plasmid using T7 RNA polymerase.

**Electron microscopy**

Xenopus and zebrafish embryos were fixed and subjected to TEM as detailed in the supplementary methods.

**Statistical analysis and quantification**
All data represent results from at least one of three independent experiments with similar results. For the quantification of cilia length in the pronephric tubule, cilia were measured in the anterior and the posterior segment of three zebrafish embryos at 24 hpf for each set of conditions. Numbers of embryos used for analysis are indicated in the respective bar chart unless otherwise stated. Data were analysed by Student’s t-test (two-sided, unequal); error bars represent s.d. Immunoblot signals were quantified using Gel-Pro Analyzer 6.0, INTAS and normalised to respective loading controls. To quantify the regularity of basal body spacing we developed an image analysis pipeline; the individual steps and associated statistical analysis are explained in detail in the supplementary methods.

**Accession numbers**
Corresponding GenBank accession numbers for zebrafish cDNAs: ezrin a (NM_001020490), ezrin b (NM_001030285), elmo1 (NM_213091), dock1 (dock1) (NM_001110011), rac1 (NM_199771), rac1 (KF273902). For X. laevis cDNAs: ezrin (NM_001039239), elmo1 (NM_001090018), elmo2 (NM_001096183), rac1 (BC108884).

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**Competing interests**
The authors declare no competing financial interests.

**Author contributions**

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**Supplementary material**
Supplementary material available online at http://dev.biologists.orglookup/suppl?doi=10.1242/dev.112250/-DC1

**References**


Boehlke, C., Schell, C., Yasunaga, T., Helmsta...
INVENTORY OF SUPPLEMENTAL MATERIAL

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Supplemental Fig. S1. ELMO1 localizes to the ciliary axonemes in MCCs and validation of antibodies used for human respiratory epithelial cells and MDCK studies.
Supplemental Fig. S2. ELMO1 and DOCK1 localize to the basal body of primary cilia
Supplemental Fig. S3. Expression of Dock1 and Ezrin in the ciliary axonemes of the otic vesicle in zebrafish
Supplemental Fig. S4. Knockdown of Elmo1 and Dock1 causes laterality defects in zebrafish and knockdown of Elmo1, Dock1, Ezrin or Rac1/Rac1l leads to impaired ciliogenesis in zebrafish
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SUPPLEMENTAL FIGURE LEGENDS
SUPPLEMENTAL MATERIALS AND METHODS
SUPPLEMENTAL REFERENCES
Fig. S1
Fig. S2

A  ELMO1  Tubulin (detyr.)  Merge

IgG-goat  Tubulin (detyr.)  Merge

B  DOCK1  Tubulin (detyr.)  Merge

IgG-mouse  Tubulin (detyr.)  Merge

C  ELMO1  DOCK1  Cep164  Merge
Fig. S3

![Imagery](image-url)
Fig. S5

A: dock1

B: stage 32

C: acetylated Tubulin

merge

elmo1

elmo2

dock1

ezrin

bar
Fig. S7

A Co-MO

TB-MO elmo1/2

TB-MO ezrin

TB-MO rac1

B

C

D

E

F

p=3.08e−08  p=1.48e−09  p=4.08e−11
Fig. S8

A  B  C  D
512 cells  60%  3 s  20 s

E
22 hpf

F
36 hpf

G
48 hpf

H
Ezrin  y-Tubulin
-  + ezrin mRNA

J
100%  165%

Ezrin  y-Tubulin
Co-MO  TB-MO ezrin

100%  63%

I
stage 32

-9-
Fig. S9

Panel A: Western blot analysis showing the expression of pERM, pERMA, and γ-Tubulin with Co-MO, TB-MO, rac1, rac1l, and rac1/rac1l conditions.

Panel B: Western blot analysis showing the expression of ERM total with Co, ezrin (T564A) and ezrin (T564D) conditions.

Panel C: Immunofluorescence images showing Co, ezrin (T564A), and ezrin (T564D) conditions.

Panel D: Graph showing the cilia length in μm with Co, ezrin (T564A), and ezrin (T564D) conditions, with statistical significance indicated by ** and *.
SUPPLEMENTAL FIGURE LEGENDS

Supplemental Fig. S1. ELMO1 localizes to the ciliary axonemes in MCCs and validation of antibodies used for human respiratory epithelial cells and MDCK studies
(A-D) Human respiratory epithelial cells from controls were double-labelled with antibodies directed against ELMO1 (magenta) and acetylated Tubulin (green). ELMO1 localizes along the ciliary axonemes (white arrow) and the basal bodies (white arrowhead). The nucleus is stained with Hoechst33342 (blue). Immunoblots of human respiratory epithelial cell lysates and MDCK lysates validates the utilized ELMO1 antibody with the appropriate double bands according to the manufacturer (E,G), Ezrin (F) and DOCK1 (H) antibodies. Scale bar, 10 µm (D).

Supplemental Fig. S2. ELMO1 and DOCK1 localize to the basal body of primary cilia
(A) MDCKs were grown to ciliated stage and stained against ELMO1 (green) and the cilia marker Tubulin (detyrosinated) (magenta). The merged image illustrates distinct ELMO1 staining at the base of the cilium. (B) DOCK1 (green) was co-stained with Tubulin (detyrosinated) (magenta). The merged image illustrates distinct DOCK1 staining at the base of the cilium. (C) ELMO1 (green) was co-stained with DOCK1 (magenta) and Cep164 (blue), a marker for the mother centriole. Co-localization of ELMO1 and DOCK1 at the mother centriole is indicated by yellow pixels. Scale bars, 10 µm (A,B), 1 µm (C).

Supplemental Fig. S3. Expression of Dock1 and Ezrin in the ciliary axonemes of the otic vesicle in zebrafish
(A) Immunostaining shows expression of Arl13b-GFP in ciliary axonemes of the otic vesicle of Tg(actb2:Mmu.Arl13b-GFP) embryos at 48 hpf. (B-B``) Dock1 is expressed at the basal bodies (white arrowheads) in the otic vesicle of Tg(actb2:Mmu.Arl13b-GFP) embryos at 48 hpf. (C-C``) Ezrin is expressed at the basal bodies (white arrowheads) and the ciliary axonemes in the otic vesicle of Tg(actb2:Mmu.Arl13b-GFP) embryos at 48 hpf. Scale bars, 5 µm (A,B``,C``).
Supplemental Fig. S4. Knockdown of Elmo1 and Dock1 causes laterality defects in zebrafish and knockdown of Elmo1, Dock1, Ezrin or Rac1/Rac1l leads to impaired ciliogenesis in zebrafish

(A) Quantification of heart looping at 48 hpf by cmfc2 WISH revealed altered heart looping in SB-MO elmo1 and TB-MO elmo1 (each 2 ng), SB-MO dock1 (2 ng) that was slightly enhanced for the double-knockdown by co-injection of SB-MO dock1 (2 ng)/SB-MO elmo1 (2 ng) and SB-MO dock1 (2 ng)/TB-MO elmo1 (2 ng) compared to Co-MO (4 ng) injected zebrafish embryos. Numbers indicate the total number of embryos from three different experiments used for quantification. (B) Quantification of cilia length in either the anterior or the posterior segment of the pronephric tubule of SB-MO elmo1 (2 ng), SB-MO dock1 (2 ng), TB-MO ezrin (2 ng) and TB-MO rac1 (4 ng)/rac1l (0.5 ng) compared to Co-MO (4.5 ng) injected zebrafish embryos at 24 hpf (* p= ≤ 0.05 compared to their respective control). (C) Quantification of electron micrographs for centriole docking defects of SB-MO elmo1 (2 ng), SB-MO dock1 (2 ng), TB-MO ezrin (2 ng) and TB-MO rac1 (4 ng)/rac1l (0.5 ng) compared to Co-MO (4.5 ng) injected zebrafish embryos at 48 hpf. All obtained electron micrographs were analysed for centrioles that were not docked at the plasma membrane. Numbers indicate the total number of centrioles counted from three different embryos per condition used for quantification.

Supplemental Fig. S5. DOCK1, ELMO1/2 and Ezrin show expression in MCCs during Xenopus development

(A,B) Expression of dock1 in the head, branchial arches (white arrow), otic vesicle (white arrowhead), pronephric kidney (black arrow), throughout the epidermis at stage 32, and in the pronephric tubules (white arrow) at stage 42 of Xenopus development. (C) Expression of elmo1, elmo2, dock1 and ezrin co-localized with acetylated Tubulin marking cilia at stage 32. Scale bars, 500 µm (A), 250 µm (B), 2 mm (C).

Supplemental Fig. S6. Knockdown efficiency and defective ciliogenesis upon knockdown of ELMO1/2 and Ezrin in Xenopus

(A) Immunoblot of Xenopus lysates (stage 16 and 22) after injection of TB-MO elmo1 (8 ng) into both blastomeres of two-cell stage Xenopus embryos indicates strong reduction of ELMO1 expression compared to Co-MO (8 ng) injected embryos. Anti-γ-Tubulin immunoblot served as loading control. (B) Immunoblot of Xenopus lysates (stage 22 and 26) after injection of TB-MO elmo2 (8 ng) into both blastomeres of two-cell stage Xenopus
embryos indicates strong reduction of ELMO2 expression compared to Co-MO (8 ng) injected embryos. Anti-γ-Tubulin immunoblot served as loading control. (C) In vitro translated Xenopus ELMO2 protein was detected via immunoblotting by Streptavidin or ELMO2 antibody. ELMO2 protein level decreased in the presence of 0.2 μM and 2 μM of the TB-MO elmo2. Immunofluorescence-based quantification of cells with intracellular acetylated α-Tubulin-positive structures (D) and cells with defective centriole docking (E) of TB-MO elmo1/elmo2 (each 4 ng) and TB-MO ezrin (8 ng) injected Xenopus embryos compared to Co-MO (8 ng) injected Xenopus embryos at stage 32. Numbers indicate epidermal cells of three different embryos used for quantification.

Supplemental Fig. S7. Quantification of the regularity of the centriole spacing
(A) Local basal body density for all recorded multiciliated skin cells in Xenopus embryos. (B) Local basal body density for a cell of a Co-MO (4 ng) injected embryo. (C) Histogram of the local basal body density in the control cell depicted in B. The green lines mark the 25 % and 75 % quantiles. (D,E) Analysis as in B,C for a cell of a TB-MO ezrin (4 ng, low dosage) injected embryo. The larger variation of the local spot density results in a wider distribution with a larger interquartile range. (F) Homogeneity of the basal body distribution measured by the interquartile range of the local spot density. Each point corresponds to one cell. The blue boxes denote the 25 % and 75 % quantile, the red line indicates the median. The p-values (computed with the two-sided Wilcoxon rank sum test) show significant differences to the control. Scale bars: 5μm (B,D).

Supplemental Fig. S8. Expression of Ezrin during zebrafish and Xenopus development
(A) Ubiquitous distribution of maternal ezrin transcripts at 512-cell-stage. (B) At 60 % epiboly stage, ezrin is expressed ubiquitously. (C) At the 3-somite stage, expression of ezrin mRNA is restricted to the polster (black arrowhead). (D) At the 20-somite stage ezrin shows expression in the otic vesicle (black arrowhead) and the developing pronephric tubule (black arrow). (E) At 22 hpf ezrin expression is detected in the forebrain (black arrow), epiphysis (black arrowhead), otic vesicle (white arrowhead) and the pronephric tubule (white arrow). Cross-section of a 36 hpf embryo (F) shows that ezrin (blue) partially co-localizes with cadherin 17 (red) in the pronephric tubule. (G) At 48 hpf, ezrin has a broad expression throughout the brain, epiphysis (black arrowhead) and pronephric tubule (white arrow). (H) Immunoblot of 24 hpf zebrafish lysates after injection of ezrin mRNA indicates a strong upregulation of Ezrin expression in comparison to control embryos. Anti-γ-Tubulin
immunoblot served as loading control. (I) *ezrin* is expressed in the cement gland (white arrow), otic vesicle (white arrowhead), pronephros (black arrow), cloaca (black arrowhead) and throughout the epidermis at stage 32 of *Xenopus* development. (J) Immunoblot of *Xenopus* lysates (stage 32) after injection of TB-Mo *ezrin* (8 ng) into the animal-ventral blastomeres of four-cell stage *Xenopus* embryos indicates a reduction of Ezrin expression compared to Co-MO (8 ng) injected embryos. Anti-γ-Tubulin immunoblot served as loading control. Scale bars, 100 μm (A,B,C,D,E,G), 20 μm (F), 500 μm (I).

Supplemental Fig. S9. Rac1 and Rac1l regulate ERM phosphorylation and overexpression of Ezrin(T564A) or Ezrin(T564D) impairs ciliogenesis in zebrafish (A) Immunoblot of 24 hpf zebrafish lysates showing elevated pERM expression in TB-MO rac1 (4 ng), TB-MO rac1l (0.5 ng) and TB-MO rac1 (4 ng)/rac1l (0.5 ng) injected embryos in comparison to Co-MO (4.5 ng) injected embryos. Anti-Rac1 immunoblot shows efficiency of Rac1 downregulation in Rac1 (27 %), Rac1l (52 %) and Rac1/Rac1l (23 %) depleted morphants compared to control embryos (100 %). Anti-γ-Tubulin immunoblot served as loading control. (B) Immunoblot of 24 hpf zebrafish lysates showing elevated ERM(total) expression in zebrafish embryos upon overexpression by injection of either the *ezrin*(T564A) or *ezrin*(T564D) mRNA (each 20 pg) compared to the control. (C) Representative confocal images showing reduced cilia length (stained with acetylated Tubulin) in the pronephric tubule of zebrafish overexpressing Ezrin(T564A) or Ezrin(T564D) compared to the control at 24 hpf. (D) Quantification of cilia length in the posterior segment of the pronephric tubule of *ezrin*(T564A) or *ezrin*(T564D) mRNA (each 20 pg) injected zebrafish embryos compared to the control at 24 hpf (* p= 0.02; ** p= 0.004). Scale bar, 5 μm (C).

Supplemental Fig. S10. Respective uncropped immunoblots
SUPPLEMENTAL MATERIAL AND METHODS

PCR-based methods
To substitute T564 with A564 or D564 of zebrafish Ezrin, quickchange site-directed mutagenesis PCR was performed with specific primers in which the codon ACG was replaced by GCG (forward: CGAGACAAAGTACAAAGCGCTCCGGGCAGATCCGC, reverse: CCCGGGATCTGGCAGGGTCTTTGTACTTTGTCG) or GAC (forward: CGAGACAAAGTACAAAGACGCTCCGGCAGATCCGC, reverse: CCCGGGATCTGGCAGGGTCTTTGTACTTTGTCG), respectively. This was followed by a PCR using primers to generate full length wildtype and mutant forms of zebrafish Ezrin (forward: GGATCCACCATGCCTAAACCATGAAATGTTCG, reverse: GGATCCTTTAAGGCCTCGAACTCGTCGATTCT). Next, full length PCR products were cloned into the Tol2 vector containing a 4 kb fragment of the zebrafish cadherin 17 promoter to drive the expression of GFP and thus obtain Cdh17:Ezrin(WT)-GFP, Cdh17:Ezrin(T564A)-GFP and Cdh17:Ezrin(T564D)-GFP constructs. All constructs were diluted in 0.1 M KCl to a concentration of 50 ng/μl. One nanoliter of this dilution was injected through the chorion of 1-cell or 2-cell stage embryos.

mRNA and morpholino injection
For synthesis of mRNA, we used full length zebrafish Elmo1-pCS2+ (Epting et al., 2010), Ezrin-pCS2+ (lacks 5' UTR, to which the TB-MO ezrin potentially binds), Ezrin(T564A)-pCS2+, Ezrin(T564D)-pCS2+, Rac11-pCS2+ (with 5bp mismatch, to which the TB-MO rac11 potentially binds), full length *Xenopus* Centrin-RFP-pCS2+, Clamp-GFP-pCS2+ and membrane-GFP-pCS2+ (Ganner et al., 2009). mRNA was prepared from Acc651-linearized Elmo1-pCS2+, KpnI-linearized Ezrin-pCS2+, Ezrin(T564A)-pCS2+, Ezrin(T564D)-pCS2+ and Rac11-pCS2+ and NotI-linearized Centrin-RFP-pCS2+, Clamp-GFP-pCS2+ and membrane-GFP-pCS2+ using SP6 mMessage mMachine Kit (Ambion). Morpholino oligonucleotide (MO) and mRNA injection were performed as described (Ganner et al., 2009). To attenuate possible off target effects, a p53 MO (Robu et al., 2007) was co-injected 1.5-fold to the other MOs used. The following concentrations of Translation/Splicing-Blocking (TB/SB) antisense MOs (Gene Tools) were used for zebrafish: 2 ng of TB-MO elmo1 5’-CAGTCTGCTGGCGTCTCATCCAAG-3’ (Epting et al., 2010), 2 ng of SB-MO elmo1 5’-AGAAAAACAGACACTTACTCTGTGC-3’ (Epting et al., 2010), 2 ng of SB-MO dock1 5’-ACACTCTAGTGATAGTGTGCAT-3’ (Epting et al., 2010), 2 ng of TB-MO ezrin 5’-GATGCGAGCGAGTCGTCCTGCACCAGG-3’ (Link et al., 2006), 4 ng
of TB-MO rac1 5′-CCACACACTTTATGGCCTGCATCTG-3′ (Srinivas et al., 2007), 0.5 ng of TB-MO rac1l 5′-CCACACACTTGATGGCCTGCATGAC-3′, TB-MO p53 5′-GCGCCATTGCTTTGCAAGAATTG-3′ (Robu et al., 2007) and a Standard Control (Co)-MO. For Xenopus: 2 or 4 ng of TB-MO elmo1 5′-GCGGCATTGTCCTTTCTGTGGTTAT-3′, 2 or 4 ng of TB-MO elmo2 5′-GAGGCATTTCCTGCTATGGTTTGCC-3′, 2, 4 or 8 ng of TB-MO ezrin 5′-CCGGTTTGGGCATTTTCACTTCTGC-3′, and 40 ng of TB-MO rac1 5′-GCCTGCATGGCAGCGAATGTCCCG-3′ (Habas et al., 2003). The following quantities of mRNAs were injected: ezrin mRNA (20 pg), elmo1 mRNA (20 pg), rac1l mRNA (20 pg), ezrin(T564A) mRNA (10 or 20 pg) and ezrin(T564D) mRNA (10 or 20 pg). The TNT Quick Coupled Transcription/Translation System (Promega) was used to confirm the morpholino efficiency for TB-MO elmo2 with full length Xenopus elmo2 (ELMO2-pCMV-SPORT6, EST clone: 3399728 (Open Biosystems)).

Microscopy and image acquisition

Immunofluorescence images from human respiratory epithelial cells (Omran and Loges, 2009) were taken with a Zeiss Apotome Axiovert 200 and processed with AxioVision 4.8 and Adobe Creative Suite 4. Confocal imaging for MDCKs, Xenopus embryos and zebrafish embryos was performed using confocal microscopes LSM510 ZEISS (ZEISS objectives: Plan-Neofluar 100x/1.3 oil immersion; LCI Plan-Neofluar 63x/1.3 water-immersion; Achroplan NIR 40x/0.8 water-immersion, respectively). Xenopus embryos were imaged in PBS and zebrafish embryos were embedded in 1 % low-temperature melting agarose (Biozym) in 30 % Danieau’s solution. Vertical projections of recorded stacks were generated using LSM Examiner software (ZEISS), and in special cases Imaris 6.2 (Bitplane) software. MDCK co-localization studies were performed with Imaris Co-localization Tool (Bitplane, Version 7.0). Brightfield images of whole mount in situ embryo stains and of all cross-sections were taken using an Axioplan2 microscope with Axiocam camera and using Axiovision software (ZEISS). Embryos of the Tg(wt1b:EGFP) line were analyzed under a Leica MZ16 stereo-microscope (Leica, Solms, Germany), and non-confocal fluorescent images were taken with a SPOT Insight Fire Wire System (Diagnostic Instruments, Sterling Heights, MI). For electron microscopy, Xenopus embryos and zebrafish embryos were fixed with 4 % PFA/2 % glutaraldehyde in PBS for 1 hour at RT and post-fixed with 1 % osmium tetroxide (Polyscience) for 30 minutes at RT, dehydrated with two 10 minutes ethanol (50 % and 60 %) washings, and incubation in 1 % uranyl acetate (Polyscience) in 70 % ethanol overnight at 4 °C. Embryos were taken through an ethanol dehydration series. After washing
in propylene oxide, embryos were embedded in Durcupan (Fluka). Ultra-thin sectioning was performed using a Leica EM UC6. Zebrafish serial sagittal sections (60 nm) were cut in a rostral to caudal direction at the beginning of the yolk extension and mounted on copper-grids. Sections were viewed and examined in an electron microscope (LEO 906E; Carl Zeiss). All images were exported as TIFF files and imported into Adobe Photoshop software CS2 to arrange figures.

**Histology and histochemistry**

For whole mount antibody staining zebrafish embryos were fixed in 4 % PFA/1 % DMSO overnight at 4 °C, equilibrated in 100 % MeOH at -20 °C for 1 hour, Proteinase K-digested (10 μg/ml) for 20 minutes, treated with ice-cold acetone for 5 minutes at -20 °C, incubated in blocking solution (1 % PBSTT, 1 % DMSO, 2 % sheep serum, 1 % BSA) and then with the indicated primary and secondary antibodies. For cross-section analysis, *in situ*-stained zebrafish embryos were dehydrated in ethanol series, cleared in toluol, embedded in paraffin wax and sectioned (6 μm) using a microtome (Leica). Embryos that were sectioned for Hematoxylin and Eosin staining were fixed in BT-fix (4 % PFA, 0.1 M Na₂HPO₄ buffer (pH 7.3), 3 % sucrose, 0.12 mM CaCl₂) at 4 °C overnight. After being washed in PBS and taken through an ethanol dehydration series, they were embedded in JB-4 resin (Polysciences Inc.) and sectioned at 3–5 μm. Slides were stained for Hematoxylin and Eosin.

Madin-Darby Canine Kidney cells (MDCKs) were grown on glass coverslips for 7 days for cilia stainings. For cilia staining these cells were fixed with 50 % methanol/50 % acetone for 10 minutes at -20 °C, permeabilized with 0.1 % Triton-X100 in PBS and blocked with 2 % gold fish gelatine in PBS as described elsewhere (Boehlke et al., 2010). Human respiratory epithelial cells were obtained by nasal brush biopsy (Engelbrecht Medicine and Laboratory technology) and suspended in cell culture medium. Samples were spread onto glass slides, air dried and stored at -80 °C. Before incubation with the primary antibody (2-3 hours at RT or overnight at 4 °C) and secondary antibody (25 minutes at RT), cells were treated with 4 % PFA, 0.2% Triton-X100 and 1 % skim milk.
Quantification of the regularity of the basal body spacing

To quantify the regularity of the basal body spacing we developed an image analysis pipeline with the following steps: (1) Extraction of the apical membrane as a curved slice from the 3D volumetric image. (2) Segmentation of the cell. (3) Detection of spots and computation of the local spot density. (4) Computation of statistics of the local spot density.

The variation of the local spot density provides a good measure for the regularity of the basal body spacing. For a regular spacing, the spot density is similar everywhere on the membrane. This results in a narrow distribution with a low interquartile range. An irregular spacing leads to very different spot densities on the membrane, which then results in a wide distribution with a large interquartile range. So we can use the interquartile range of the local spot density distribution as a meaningful feature to quantify the regularity of the basal body spacing.

The individual steps are explained in detail in the following sections.

Extraction of the Apical Membrane

The apical membrane has a curved shape (see Fig. 1A), so the cells were recorded as a 3D volumetric image. A simple maximum intensity projection collects not only the signal from the membrane but also spurious signal from deeper layers (see Fig. 1B). Therefore we used the phalloidin 568 channel to find the curved membrane layer (Fig. 2A) and extracted a single curved slice from both channels (Fig. 2B). In this slice the basal bodies have a sharp shape, and spurious signal from deeper layers is not visible.

The z-coordinates of the curved layer $Z : \mathbb{R}^2 \rightarrow \mathbb{R}$ are found as the z-position of the maximal intensity in a Gaussian-smoothed image, i.e.

$$Z(x, y) = \arg \max_{z \in \mathbb{R}} (I_{ph} * K)(x, y, z)$$  \hspace{1cm} (1)

where $I_{ph} : \mathbb{R}^3 \rightarrow \mathbb{R}$ denotes the volumetric image (phalloidin channel), and $K : \mathbb{R}^3 \rightarrow \mathbb{R}$ is a
Figure 2 | Extraction of the apical membrane. (A) Orthogonal slices of the volumetric image. The white lines indicate the automatically found curved slice of the apical membrane. (B) Single curved slice extracted from the apical membrane.

Gaussian smoothing kernel with

\[
K(x, y, z) = \exp \left( -\frac{1}{2} \cdot \left( \frac{x^2 + y^2}{\sigma_{xy}^2} + \frac{z^2}{\sigma_z^2} \right) \right)
\]  

with \( \sigma_{xy} = 1\mu m \) and \( \sigma_z = 0.1\mu m \). The 2D image of the apical membrane, denoted as \( S : \mathbb{R}^2 \rightarrow \mathbb{R} \) for the phalloidin 568 channel and \( T : \mathbb{R}^2 \rightarrow \mathbb{R} \) for the GFP channel, is then extracted from the phalloidin and the Centrin-GFP channel as

\[
S(x, y) = I_{ph}(x, y, Z(x, y))
\]
\[
T(x, y) = I_{GFP}(x, y, Z(x, y))
\]

Segmentation of the cell

Figure 3 | Graphcut segmentation using the phalloidin 568 channel. (A) Initial foreground (red) in the middle of the image and background (blue) at the borders. (B) After graphcut segmentation foreground (red) and background (blue) (C) Overlay of the resulting segmentation borders (white) over raw slice.

The cell is segmented from the slice \( S \) (phalloidin 568 channel) with a graphcut segmentation
(Boykov and Kolmogorov, 2004). As all cells were recorded centered in the image, we selected fixed seeding regions: A small square in the middle of the image as foreground and the image border as background (see Fig. 3A). For each of the seeding regions $M_i : \mathbb{R}^2 \rightarrow \{0, 1\}$, (where $i = 1$ denotes foreground and $i = 0$ denotes background) the mean gray value was computed as

$$g_i = \frac{\int_{\mathbb{R}^2} M_i(x) S(x) \, dx}{\int_{\mathbb{R}^2} M_i(x) \, dx}. \quad (5)$$

From these mean gray values a threshold gray value was computed as $g_t = \frac{g_0 + g_1}{2}$. Using this threshold the foreground and background penalties for each pixel were computed as

$$D_0(x) = \begin{cases} S(x) - g_t & \text{if } S(x) > g_t \\ 0 & \text{else} \end{cases} \quad (6)$$

$$D_1(x) = \begin{cases} g_t - S(x) & \text{if } S(x) < g_t \\ 0 & \text{else} \end{cases} \quad (7)$$

These penalties are used for the unary term in the energy function. The binary term (edge penalties between two neighboring pixels) was computed directly from the Gaussian smoothed intensities in the phalloidin 568 channel, such that high intensities result in a high probability for the cut. I.e. with the Gaussian smoothed image denoted as $S_\sigma = S * G_\sigma$ with $G_\sigma(x) = \frac{1}{\sqrt{2\pi \sigma^2}} \exp\left(-\frac{1}{2} \cdot \|x\|^2 / \sigma^2\right)$ using $\sigma = 0.2\mu m$, the cut penalty for an edge between two pixels at positions $x_1$ and $x_2$ is defined as

$$B(x_1, x_2) = 2 \cdot \left( \max_{t \in \mathbb{R}^2} S_\sigma(t) \right) - \left( S_\sigma(x_1) + S_\sigma(x_2) \right). \quad (8)$$

The energy for a certain segmentation $L : \mathbb{R}^2 \rightarrow \{0, 1\}$ is then

$$E(L) = \sum_{x \in \Omega} L(x) D_1(x) + (1 - L(x)) D_0(x) + \sum_{x_1 \in \Omega} I(L(x_1) \neq L(x_2)) B(x_1, x_2) \quad (9)$$

where $\Omega \subset \mathbb{R}^2$ denotes the set of all discrete pixel coordinates in the image, $\mathcal{N}(x)$ is the set of the 8 neighbors of the pixel at position $x$, and $I$ is the indicator function. The energy is minimized by the min-cut/max-flow algorithm of Boykov and Kolmogorov (2004). The resulting segmentation mask $L$ is illustrated in Fig. 3B and Fig. 3C.

Detection of the Basal Bodies and Computation of the Spot Density

The basal body positions are detected in the extracted slice of the Centrin-GFP channel $T$ (see Fig. 4A). First the image is smoothed with a very small Gaussian kernel ($\sigma = 0.02\mu m$) to introduce a
Figure 4 | Detection of spots in the Centrin-GFP channel. (A) Raw slice with overlaid segmentation borders (white) (B) Detected spots as white circles. (C) Local spot density computed with kernel density estimation

single maximum in flat (oversaturated) regions. The smoothed image is denoted as $T_\sigma$. Then the positions of all local maxima within the cell region are extracted as

$$P_{\text{peaks}} = \{ \mathbf{x} \in \Omega \mid (L(\mathbf{x}) = 1) \land (T_\sigma(\mathbf{x}) > T_\sigma(n) \forall n \in \mathcal{N}(\mathbf{x})) \}.$$  

To eliminate random peaks in the background, an intensity threshold $t$ is computed by applying Otsu’s method (Otsu, 1979) to the set of all peak intensities. Finally noise peaks, that are closer than a basal body diameter ($d = 0.3\mu m$) to a higher peak are eliminated.

$$\mathcal{P} = \{ \mathbf{x} \in P_{\text{peaks}} \mid (T_\sigma(\mathbf{x}) > t) \land (T_\sigma(\mathbf{x}) > T_\sigma(p) \text{ with } p \in P_{\text{peaks}} \setminus \{ \mathbf{x} \} \land \|\mathbf{p} - \mathbf{x}\| < d) \}.$$  

The found basal body positions are illustrated in Fig. 4B.

Based on these spot positions and the segmentation mask $L$ a local spot density is computed using kernel density estimation. The segmentation mask is required to avoid border effects (i.e. an apparent lower spot density at the cell borders, due to missing neighbors outside the cell). Defining the kernel as $K_\sigma(\mathbf{x}) = \frac{1}{2\pi\sigma^2} \exp \left( -\frac{1}{2} \cdot \|\mathbf{x}\|^2 / \sigma^2 \right)$ with $\sigma = 0.75\mu m$ the local spot density is computed as

$$R(\mathbf{x}) = \frac{\sum_{\mathbf{p} \in \mathcal{P}} K_\sigma(\mathbf{x} - \mathbf{p})}{(L * K)(\mathbf{x})}.$$  

The resulting local spot density is depicted in Fig. 4C for a control cell. The full image analysis pipeline was applied to all cells of our experiments. The resulting local spot densities are shown in Supplementary Fig. S7A.
Statistics

To describe a homogeneous or inhomogeneous basal body spacing with a single number, we compute the interquartile range of the local basal body density. This is illustrated in Supplementary Fig. S7B-E. The homogeneous spacing in a control cell (Supp. Fig. S7B) results in a narrow distribution (Supp. Fig. S7C) with a low interquartile range. The inhomogeneous spacing in an Ezrin morpholino cell (Supp. Fig. S7D) results in a wide distribution (Supp. Fig. S7E) with a large interquartile range.

This feature (we denote it as “variation of the local spot density”) was computed for each cell in our experiments. The individual values are plotted in Supp. Fig. S7F. This feature is significantly different in the morpholino cells compared to the control cells. We quantified this difference with a two-sided Wilcoxon rank sum test. The probability of the null-hypothesis was in the range of $10^{-8}$ or below (see Supp. Fig. S7F).
SUPPLEMENTAL REFERENCES


