14-3-3α directs the pulsatile transport of basal factors toward the apical domain for lumen growth in tubulogenesis

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The 	extit{Ciona} notochord has emerged as a simple and tractable in vivo model for tubulogenesis. Here, using a chemical genetics approach, we identified UTKO1 as a selective small molecule inhibitor of notochord tubulogenesis. We identified 14-3-3α protein as a direct binding partner of UTKO1 and showed that 14-3-3α knockdown leads to failure of notochord tubulogenesis. We found that UTKO1 prevents 14-3-3α from interacting with ezrin/radixin/moesin (ERM), which is required for notochord tubulogenesis, suggesting that interactions between 14-3-3α and ERM play a key role in regulating the early steps of tubulogenesis. Using live imaging, we found that, as lumen begins to open between neighboring cells, 14-3-3α and ERM are highly colocalized at the basal cortex where they undergo cycles of accumulation and disappearance. Interestingly, the disappearance of 14-3-3α and ERM during each cycle is tightly correlated with a transient flow of 14-3-3α, ERM, myosin II, and other cytoplasmic elements from the basal surface toward the lumen-facing apical domain, which is often accompanied by visible changes in lumen architecture. Both pulsatile flow and lumen formation are abolished in larvae treated with UTKO1, in larvae depleted of either 14-3-3α or ERM, or in larvae expressing a truncated form of 14-3-3α that lacks the ability to interact with ERM. These results suggest that 14-3-3α and ERM interact at the basal cortex to direct pulsatile basal accumulation and basal-apical transport of factors that are essential for lumen formation. We propose that similar mechanisms may underlie or may contribute to lumen formation in tubulogenesis in other systems.

14-3-3 | ERM | tubulogenesis | pulsatile directed flow | myosin II

Biological tubes serve as fundamental structural units in a diverse set of organs, including lung, liver, gut, and kidney, playing roles in both the exchange of biological materials and structural support. Tubulogenesis involves changes in cell shape (1–5), vesicle trafficking (6–11), and luminogenesis (12–15). Understanding how these changes occur and how they are coordinated in space and time is essential for understanding tubulogenesis during normal development and is also becoming increasingly important for regenerative medicine.

The notochord of 	extit{Ciona robusta} provides a simple in vivo model for tubulogenesis. The ascidian notochord initially comprises a single stack of 40 postmitotic cells that form the core of the tadpole tail. As the notochord begins to elongate along its anterior/posterior (AP) axis (16–18), extracellular lumen pockets emerge between adjacent cells, expand, and fuse with each other to form a single central lumen (19–21). Although this process has been extensively characterized, the underlying mechanisms remain poorly understood. Recent studies have revealed molecules that are associated with notochord elongation and tubulogenesis. For example, cell elongation is facilitated by actomyosin networks (complexes of actin and myosin II) localizing in the basal contractile ring, together with other components such as α-actinin and coflin (20, 22). Lumen formation requires the establishment of polarity by Par3/Par6αPKC (23) and the cytoskeletal cross-linker ezrin/radixin/moesin (ERM) (20), both of which play roles in other tubulogenesis processes, including those occurring in Madin–Darby canine kidney (MDCK) cells (6, 24, 25), the 	extit{Drosophila} trachea (4, 11), and the 	extit{Caenorhabditis elegans} intestine (12, 26). However, it remains largely unknown how these molecules work together or how cell elongation and lumen formation combine, suggesting that there are novel mediators that affect these genes.

One of the best approaches for elucidating the molecular mechanisms underlying biological processes is a chemical genetics approach utilizing chemical inhibitors. The compounds identified from phenotypic screening have previously led us to identify unexpected molecules responsible for various phenotypes. Furthermore, such compounds enable us to examine the functional role of the target molecule just by changing their treatment time or concentration. This chemical genetics strategy has been useful not only to in vitro cell biology (27, 28), but also

Significance

Ascidians have become a powerful model system in which to uncover basic mechanisms that govern body plan specification and elaboration. In particular, the ascidian notochord is a highly tractable model for tubulogenesis. Here, we use chemical genetics to identify roles for 14-3-3α, and its binding partner ezrin/radixin/moesin (ERM), in tubulogenesis. Combining genetic and chemical perturbations with live cell imaging, we present evidence that 14-3-3α–ERM interactions are required for tubulogenesis and that they act by promoting a directed cytoplasmic flow, previously uncharacterized, which carries lumen-associated components from the basal domain to the apical domain to feed lumen growth. Because many core components of this system are highly conserved, these results have broad implications for tubulogenesis in many other contexts.

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to in vivo developmental biology (29, 30). Here, we demonstrate an in vivo chemical biological approach to identify molecules responsible for tubulogenesis and to elucidate their functional roles in vivo. We identify a chemical inhibitor of tubulogenesis in C. robusta. We identify its target protein 14-3-3a, and we show that 14-3-3a interacts with ERM during lumen formation to control dynamic cycles of basal accumulation and directed transport of 14-3-3a, ERM, myosin II, and other factors from the basal cortex to the lumen-facing apical domain and to promote lumen formation and growth during tubulogenesis.

Results

UTKO1 Was Found to Inhibit Notochord Tubulogenesis Using Unbiased Phenotypic Screening. We began by conducting a phenotypic screen to identify compounds that modulate the morphogenesis of C. robusta. Embryos of C. robusta reach the tailbud stage at 12 h postfertilization (hpf) and become swimming larvae at 21 hpf, at which point they possess their maximum tail length (31). We treated C. robusta embryos at 12 hpf with compounds from our in-house chemical library and assessed their effects on subsequent development. We found that UTKO1, a tumor cell migration inhibitor we developed previously (32, 33), inhibits tail elongation in a dose-dependent manner (SI Appendix, Figs. 1A and B). Interestingly, UTKO1 did not appear to cause any noticeable morphological defects in other body parts, such as the trunk (SI Appendix, Fig. 1C), or defects in other developmental processes, such as ocellus melanization (SI Appendix, Fig. 1D), although it did cause defects in papillae formation and locomotion (SI Appendix, Fig. 1E and Movie S1).

The tail-selective phenotype resulting from UTKO1 treatment prompted us to look more closely at the dynamics of notochord elongation and tubulogenesis in UTKO1-treated embryos. Notochord elongation and tubulogenesis involves four distinct developmental phases (Fig. 1): (phase I) completion of notochord cell alignment into a single file and elongation of notochord cells along the AP axis; (phase II) opening of small extracellular lumen pockets between adjacent notochord cells; (phase III) expansion of the lumen pockets, accompanied by crawling movements of the notochord cells; and (phase IV) connection of the lumen pockets to form a single lumen at the center of the notochord tube (1). Treatment with UTKO1 caused a concentration-dependent delay in the opening and expansion of the lumen, relative to untreated controls (Fig. 1 and SI Appendix, Fig. S1F). However, this inhibitory effect of UTKO1 was not observed when it was added at 16 hpf (SI Appendix, Fig. S1G), suggesting that UTKO1 selectively inhibits phases I and II, which occur from 12 to 16 hpf. Thus, UTKO1 was found to inhibit lumen formation in Ciona notochord during the early stages of tubulogenesis.

14-3-3a Is a Direct Target of UTKO1 in Ciona. To identify the moity of UTKO1 required for its inhibitory activity, we performed a structure–activity relationship (SAR) analysis using UTKO1 analogs that we had previously synthesized (32). Analogs that lacked the hydroxyl group present at C-11 of UTKO1 (UTK09, UTK010, UTK013, UTK014, and UTK016) or the methylene group at C-4 (UTK07), did not inhibit tubulogenesis, suggesting that these two groups are critical for inhibitory activity (Fig. 2 and SI Appendix, Fig. S2A and C). In contrast, an analog that lacks the aldehyde group (UTKO12) and four stereoisomers of UTKO1 retained inhibitory activity (Fig. 2A and SI Appendix, Fig. S2B and D), indicating that the aldehyde group and a specific 3D shape are not required to inhibit tubulogenesis.

Based on these results, we used a UTKO1 analog in which the aldehyde group is replaced by biotin (B-UTKO1ox) (Fig. 2B), to identify target proteins in Ciona lysates that bind UTKO1. Previously, we showed that UTKO1 targets human 14-3-3ζ protein (33), an adapter protein that binds to diverse client proteins (34, 35). Therefore, we used the human anti–14-3-3 antibody as a probe for the UTKO1 target proteins in C. robusta. Proteins that bind to the human anti–14-3-3 antibody were pulled down from Ciona lysate by B-UTKO1ox (Fig. 2C), suggesting that UTKO1 targets one or more of the four isoforms of Ciona 14-3-3: ε, β, γ1, and/or γ2. To identify the specific target(s), we prepared each 14-3-3 isoform as a recombinant protein fused to GST and tested for direct binding to B-UTKO1ox. As shown in Fig. 2D, among the four Ciona 14-3-3 isoforms, only 14-3-3εa was strongly pulled down by B-UTKO1ox, and biotin-free UTKO1 inhibited the interaction (Fig. 2E). Conversely, a UTKO1 analog that lacked the C-11 hydroxyl group (UTKO10) did not show the same pattern. Unlike B-UTKO1ox, B-UTKO1ph did not strongly pull down GST-14-3-3εa (Fig. 2F). These results suggest that UTKO1 binds directly and selectively to 14-3-3εa via the hydroxyl group at C-11, which is important for its inhibitory activity against tubulogenesis. This selective binding of UTKO1 to 14-3-3εa allowed us to explore the functional role of 14-3-3εa in lumen formation during Ciona tubulogenesis.

14-3-3a Is Required for Notochord Tubulogenesis. The 14-3-3εa mRNA was highly expressed during notochord tubulogenesis and peaked at 15 hpf (SI Appendix, Fig. S3A). To investigate the role of 14-3-3εa, we injected a morpholino antisense oligonucleotide (MO) directed against 14-3-3εa into fertilized eggs, to inhibit the synthesis of 14-3-3εa protein, starting from the fertilized egg stage. Treatment with 14-3-3εa MO caused a tail elongation defect that could be detected as early as 12 hpf and increased in severity as development progressed, up until 21 hpf (SI Appendix, Fig. S1 H and I). At 21 hpf, larvae treated with 14-3-3εa MO exhibited a failure of lumen opening or expansion, as seen with UTKO1 treatment (Fig. 2 G–I and SI Appendix, Fig. S1K). On the other hand, 14-3-3εa MO also caused defects in trunk development (SI Appendix, Fig. S1J), consistent with the 14-3-3εa expression pattern in the trunk (36, 37) (SI Appendix, Fig. S3D). The tail defects, but not trunk defects, were rescued by the notochord-specific expression of MO-resistant 14-3-3εa, driven by a notochord-specific brachury promoter (Fig. 2F and SI Appendix, Fig. S1 H–K).
confirming that UTKO1 inhibits notochord lumen formation by binding to 14-3-3α.

14-3-3α Interacts Directly with Ezrin/Radixin/Moesin. We hypothesized that UTKO1 exerts its inhibitory effects by preventing 14-3-3α from binding to one or more client proteins responsible for notochord lumen formation. Since UTKO1 showed greater selectivity toward notochord cells (SI Appendix, Fig. S1 B and C) relative to 14-3-3α MO treatment (SI Appendix, Fig. S1 I and J), we focused on potential clients that are predominantly expressed in notochord cells during tubulogenesis (38, 39). Of these, ezrin/radixin/moesin (ERM) is the most likely candidate, since it is required for lumen formation during Ciona notochord development (20), as well as in other tubulogenesis processes (6), and was recently reported to interact directly with the 14-3-3 protein in human cell lines to regulate cell migration (40).

Indeed, ERM protein purified from E. coli bound 14-3-3 protein from Ciona lysate, detected by anti-14-3-3 (Fig. 3A), and also bound 14-3-3α purified from E. coli (Fig. 3B), supporting a direct interaction between 14-3-3α and ERM in Ciona. Furthermore, the interaction between 14-3-3α and ERM was inhibited in a dose-dependent manner by UTKO1 (Fig. 3B), but not by an off-targeted derivative, UTKO10 (Fig. 3C). Deletion mutants of 14-3-3α lacking amino acids 156 to 206 did not bind to ERM or B-UTKO1ox (Fig. 3 D–F), indicating that ERM and UTKO1 bind to the same 156- to 206-aa domain. This domain corresponds to the ezrin binding domain in human 14-3-3ζ (40), suggesting the existence of a conserved interaction across species. Together, these results implicate ERM as a 14-3-3α client protein that mediates the effects of 14-3-3α on notochord tubulogenesis.

14-3-3α and ERM Co-localize in the Basal Contractile Ring. Immunostaining using specific antibodies (SI Appendix, Fig. S3 B and C) revealed that 14-3-3α was ubiquitously expressed in various cell types, including notochord, epidermis, muscle, and neuronal cells (Fig. 3 G and SI Appendix, Fig. S3D). On the other hand, ERM was almost exclusively expressed in the notochord, with lower levels in the neural tube and in some neuronal cells in the trunk region. Focusing on notochord cells, 14-3-3α and ERM were strongly colocalized in the basal contractile ring, an actomyosin-based contractile ring that forms on the basal surface of notochord cells during notochord elongation and lumen opening at 15 hpf (Fig. 3G, arrowheads). In larvae treated with UTKO1, ERM was still localized in the basal contractile ring, but coenrichment of 14-3-3α at the same location was greatly reduced (Fig. 3H). Since UTKO1 did not affect the amount of 14-3-3α protein expression (SI Appendix, Fig. S3C), the disappearance of 14-3-3α from the basal contractile ring in notochord cells reflects its delocalization due to loss of binding to ERM. We conclude that UTKO1 inhibits the colocalization of 14-3-3α with ERM in the basal contractile ring, by inhibiting their direct interaction in the notochord.

14-3-3α and ERM Undergo Cycles of Basal Accumulation Followed by Basal-Apical Transport. To investigate how the colocalization of 14-3-3α and ERM in the basal contractile ring is related to lumen formation, we performed live imaging of each protein fused with a fluorescent protein under a brachyury promoter. Just before phase II (the lumen opening) (Fig. 1) at 15 hpf, we observed coaccumulation of 14-3-3α and ERM in the basal contractile ring (Fig. 4 A and Movie S2), consistent with the results of immunostaining (Fig. 3G). During phase II (15 to 17 hpf), a behavior emerged in which 14-3-3α and ERM periodically appeared and then disappeared from the basal contractile ring (Fig. 4 B and D, and Movie S3).

To test whether the disappearance of 14-3-3α and ERM from the basal cortex during each cycle is associated with their accumulation elsewhere, we used spatiotemporal cross-correlation analysis to identify other cellular regions that show increases in 14-3-3α or ERM signal intensity that correlate with the disappearance from the basal cortex. Significantly, we found that increases in 14-3-3α and ERM signal intensities in the apical domain were highly correlated with decreases in the basal domain during each cycle, with a consistent peak time lag (0.8 to 1.2 min) (Fig. 4H). This suggests that 14-3-3α and ERM are translocated from the basal to apical domain during each cycle. Indeed, immunostaining of 14-3-3α and ERM at 18 hpf, 3 h after the initiation of phase II, revealed strong apical enrichment (SI Appendix, Fig. S3E).

Indeed, by live imaging (Movie S3), we could detect rapid movements of vesicles containing 14-3-3α and ERM into the apical domain following their disappearance from the basal cortex, sometimes accompanied by visible changes in lumen architecture and size (Fig. 4 J and K). Interestingly, this translocation
of 14-3-3\(\alpha\) and ERM was accompanied by the basal-to-apical movement of cytoplasmic elements, visualized using differential interference contrast (DIC) optics (Fig. 4L and Movie S3), suggesting that a bulk cytoplasmic flow may carry 14-3-3\(\alpha\) and ERM from the basal contractile ring to the apical domain.

**Interaction Between 14-3-3\(\alpha\) and ERM Is Required for Their Pulsatile Accumulation and Basal–Apical Translocation.** In larvae treated with high doses (10 \(\mu\)M) of UTKO1, the pulsatile accumulations of 14-3-3\(\alpha\) and ERM were abolished (Fig. 4C and F), and we could no longer detect obvious directed basal–apical movements of 14-3-3\(\alpha\), ERM, or other cytoplasmic elements (Movie S4) or significant correlations between the apical and basal intensities of 14-3-3\(\alpha\) and ERM (Fig. 4I). At intermediate doses of UTKO1 (3 \(\mu\)M), pulsatile accumulations of 14-3-3\(\alpha\) and ERM persisted, but the time between pulses was increased relative to untreated controls (Fig. 4E and G). Supporting these live imaging results, treatment with UTKO1 also inhibited the apical accumulation of ERM at 18 hpf in fixed immunostained embryos (SI Appendix, Fig. S3 F and G).

These results suggest that an interaction between 14-3-3\(\alpha\) and ERM is required for their pulsatile accumulation and basal–apical translocation. To test this further, we first examined localization of 14-3-3\(\alpha\) in ERM KD embryos, and ERM in 14-3-3\(\alpha\) KD embryos, focusing initially at 15 hpf, when lumen opening is about to begin in the WT. ERM localized normally to the basal contractile ring in 14-3-3\(\alpha\) knocked-down (KD) embryos (Fig. 5A and Movie S5) while 14-3-3\(\alpha\) failed to target the basal contractile ring in ERM KD embryos (Fig. 5B and Movie S6). A truncated form of 14-3-3\(\alpha\) (14-3-3\(\alpha\)ΔC50-100), that cannot interact with ERM, also failed to localize to the basal contractile ring when expressed in place of endogenous 14-3-3\(\alpha\) (Fig. 5C and Movie S7). Thus, 14-3-3\(\alpha\) is not required for accumulation of ERM in the basal contractile ring while the accumulation of 14-3-3\(\alpha\) in the basal contractile ring depends on its ability to interact with ERM. These results are consistent with the diminished accumulation of 14-3-3\(\alpha\), but not ERM, in the basal contractile ring with UTKO1 treatment (Fig. 3G).

We next examined pulsatile accumulation and translocation of 14-3-3\(\alpha\) or ERM during phase II (15 to 17 hpf) in ERM KD, 14-3-3\(\alpha\) KD, and 14-3-3\(\alpha\)ΔC50-100–expressing embryos. We found that pulsatile accumulation of 14-3-3\(\alpha\) (or 14-3-3\(\alpha\)ΔC50-100) in the basal contractile ring was abolished in ERM KD or 14-3-3\(\alpha\)ΔC50-100–expressing embryos. Conversely, pulsatile accumulation of ERM was abolished in 14-3-3\(\alpha\) KD embryos. Under all three perturbations, basal–apical flows of cytoplasm observed by DIC were severely attenuated, correlations between basal and apical accumulations of either 14-3-3\(\alpha\) or ERM were severely attenuated, and lumens failed to form and grow (Control [Fig. 5D and Movie S8]; 14-3-3\(\alpha\) KD [Fig. 5E and Movie S9]; ERM KD [Fig. 5F and Movie S10]; and 14-3-3\(\alpha\)ΔC50-100 [Fig. 5G and Movie S11]). These results suggest that local interactions between 14-3-3\(\alpha\) and ERM at the basal cortex are required for their pulsatile basal accumulation, directed transport toward the apical/luminal domain, and lumen formation and growth.
Pulsatile Basal Accumulation and Basal–Apical Transport of 14-3-3ε and ERM Are Closely Associated with Those of Myosin II. We next asked whether pulsatile basal accumulation and basal–apical transport of 14-3-3ε and ERM are associated with movements of other cortical elements. We focused on nonmuscle myosin II, which is known to be enriched in the basal contractile ring and interacts with actin filaments to promote actomyosin contractility that is critical for notochord cell elongation along the AP axis, and is also somehow associated with lumen opening in the Ciona notochord (20, 41).

As previously described (20), the myosin regulatory light chain fused to EGFP (MRLC:EGFP) showed accumulation in the basal contractile ring (Movie S12). Extending these previous observations, we found that, during phase II (15 to 17 hpf), myosin II showed dynamic cycles of pulsatile accumulation in the basal contractile ring, followed by directed basal–apical flows through the cytoplasm (Fig. 6). Using two-color live imaging, we found that basal accumulation and basal–apical movements of myosin II were tightly coordinated with those of 14-3-3ε and ERM (Fig. 6 and Movie S12). These observations suggest that local interactions between 14-3-3ε and ERM control pulsatile accumulation and directed transport not just of themselves, but also of other cortically associated factors.

Discussion

Studies of Ciona notochord tubulogenesis have focused mainly on candidate genes that are highly expressed in notochord cells or during tubulogenesis in other organisms. In this study, we took
an in vivo chemical genetics approach to identify genes that are responsible for *Ciona* notochord tubulogenesis. This in vivo chemical genetics approach led us to identify UTKO1 to be a selective inhibitor of notochord tubulogenesis (Fig. 1), to identify its target 14-3-3-εa, and gain functional insights into 14-3-3-εa’s role in notochord tubulogenesis.

Because UTKO1 has been reported to inhibit tumor cell migration by targeting 14-3-3-εa, we examined the possibility that UTKO1 also binds to 14-3-3-εa. Our in vitro binding assay clearly showed that, among four 14-3-3 isoforms in *Ciona*, UTKO1 selectively binds to 14-3-3-εa through its 156- to 206-aa domain (Figs. 2D and 3D and E). Furthermore, 14-3-3-εa KD resulted in failed lumen formation in the notochord (Fig. 2 G-J), a similar phenotype to that observed following UTKO1 treatment. Therefore, our in vivo chemical genetics approach enabled us to identify 14-3-3-εa as a molecule responsible for *Ciona* notochord tubulogenesis.

The 14-3-3 proteins are thought to exert their roles as adapter proteins that interact with various kinds of client proteins. Here, we have identified *Ciona* ERM as a client protein through which 14-3-3-εa exerts its effects on notochord tubulogenesis. *Ciona* ERM is required for lumen formation (20) and is predominantly expressed in the notochord (38, 39). In human cell lines, ezrin interacts with 14-3-3ζ to regulate cell migration (40). Indeed, we found that 14-3-3-εa interacted directly with ERM (Fig. 3B) and colocalized with ERM in the basal contractile ring (Fig. 3G). Although we cannot fully rule out other possibilities, the inhibitory effects of UTKO1 toward direct 14-3-3-εa–ERM interaction (Fig. 3B), their colocalization (Fig. 3 G and H), and their dynamics (Fig. 4 B–J) strongly suggest the involvement of ERM as a 14-3-3-εa client protein responsible for the early stage of *Ciona* notochord tubulogenesis.

Our live imaging analysis of 14-3-3-εa and ERM revealed fundamental insights into their roles in lumen formation. Before lumen opening, 14-3-3-εa and ERM were stably coenriched in a basal actomyosin ring (Fig. 4A). However, during lumen opening and growth, we found that 14-3-3-εa and ERM undergo repeated cycles of accumulation in the basal contractile ring, followed by rapid translocation from the basal surface toward the lumen-facing apical domain (Fig. 4H and Movie S3). These movements were tightly correlated with movements of myosin II (Fig. 6) and other cytoplasmic elements toward the apical surface (Fig. 4L), suggesting that they are carried as part of a bulk flow of material from the basal surface to the apical lumen (Fig. 4L and Movie S3). Importantly, neither pulsatile basal accumulation, nor directed basal–apical transport, nor lumen formation were observed when the 14-3-3-εa–ERM interaction was destroyed by pharmacological or genetic methods

Fig. 5. Loss of the pulsatile basal accumulation and basal–apical translocation by the inhibition of 14-3-3-εa–ERM interaction. (A–C) Kymographs for the live images of mCherry:ERM in a 14-3-3-εa KD larva (A), 14-3-3-εa:EGFP in an ERM KD larva (B), and 14-3-3-εaΔC50-100:EGFP in a 14-3-3-εa KD larva at 15 hpf (C). The intensities in the ROI (shown in red or green rectangles) were quantified, normalized along the AP axis, and displayed as a kymograph. The total intensities of all ROIs and time points were plotted along relative position of the AP axis. The yellow arrowheads indicate the accumulation of mCherry:ERM in the basal contractile ring. The blue arrowhead indicates the peak, the shift of which indicates a high correlation between the basal contractile ring and the apical domain. (D–G) The basal ring intensity over time during phase II (15 to 17 hpf, Upper) and the cross-correlation analysis between the intensities of the basal contractile ring and the apical domain (Lower). Yellow arrowheads indicate their pulsatile accumulation in the basal contractile ring. The blue arrowhead indicates the peak, the shift of which indicates a high correlation between the basal contractile ring and the apical domain. (D) Larva injected with control MO; (E) larva injected with 14-3-3-εa MO; (F) larva injected with ERM MO; and (G) larvae expressing 14-3-3-εaΔC50-100:EGFP under 14-3-3-εa MO.
Proposed models of lumen formation in F and other tubulogenesis processes, cell elongation and ERM interaction plays a key role in lumen formation. The notochord has mainly driven its elongation, and Ciona (for 14-3-3) and basal flows could contribute to this process. ERM. The 14-3-3 proteins can also associate with ERM to promote actomyosin contraction and cortical flow (Movie S10). ERM act by regulating the actomyosin network directly to the plasma membrane and to regulate cortical tension and flow in other contexts (44–48). The 14-3-3 proteins can also associate with ERM to regulate cortical tension and flow (49–52). The observation that pulsatile basal accumulation of 14-3-3α–ERM and their subsequent basal–apical translocation are abolished by removing either protein or preventing their interactions [UTKO1 (Fig. 4 D–I and Movie S4); 14-3-3α KD (Fig. 5E and Movie S9); ERM KD (Fig. 5F and Movie S10); and 14-3-3αΔC50-100 (Fig. 5G and Movie S11)] is consistent with the idea that 14-3-3α–ERM interact to promote actomyosin contractility and raises the intriguing possibility that pulsatile contraction of the basal cortex and transient apical–basal flows could be coupled through local regulation of actomyosin contractility by 14-3-3α–ERM.

Our findings reveal a key role for 14-3-3α–ERM in lumen formation (Fig. 7). The basal contractile ring also plays crucial roles in cell elongation along the AP axis (20, 22, 53), which may explain the short-tail phenotype caused by UTKO1 (SI Appendix, Fig. S1B). In Ciona and other tubulogenesis processes, cell elongation and lumen formation are closely related (20, 54, 55). Thus, by promoting both basal contraction and cortical flow, and apical–cytoplasmic flow, 14-3-3α–ERM in the basal contractile ring would play roles in both cell elongation and lumen formation. Until now, lumen formation in the Ciona notochord has mainly been investigated by focusing on apically localizing molecules, such as Par-3/Par-6/aPKC or Sic2aax, as well as ERM itself. Studies of the mechanisms of basal contractility and directed flow are needed to provide molecular or mechanical insights into tubulogenesis, bridging the gap between these two opposite domains.

Materials and Methods

Biological Materials. C. intestinalis were cultivated in Maizuru (Kyoto), Misaki (Kanagawa), and Shinymasita (Kanagawa). Adults were maintained under constant light to induce oocyte maturation. Eggs and sperm were obtained by dissecting the gonadal and sperm ducts, respectively. Eggs were dechorionated using a dechorionation reagent containing 0.05% Actinase E (Kaken Pharmaceutical) and 1% mercaptoacetic acid sodium salt (Wako Pure Chemical Industries) in sea water, and cross-fertilization was performed in plastic Petri dishes. After fertilization, embryos were cultured at 18 °C. The onset of notochord tubulogenesis was at ~12 hpf.

Chemicals and Antibodies. UTKO1 and its analogs were synthesized as previously described (32, 33) and dissolved in methanol. Biotin was purchased from Sigma-Aldrich. The 14-3-3α and ERM antibodies were purchased from Biologica Co. Ltd using the short peptides “EQMKKIAQLGISLT” (for 14-3-3α) and “KQIRSGNTKRIDEFEC” (for ERM) to immunize rabbits and chickens, respectively. GST and a-pan-14-3-3 antibody

Fig. 6. Translocation of myosin II toward the apical domain together with 14-3-3α. (A) Kymograph analysis of the dynamics of myosin II (MRLC) and 14-3-3α (mCherry:14-3-3αa), and their merging during phase II (15 to 17 hpf). The placement of the cross-sections used to generate the kymograph is indicated by white dashed lines. White triangles indicate the intensity of MRLC and 14-3-3αa KD (Fig. 5E and Movie S9); ERM KD (Fig. 5F and Movie S10); and 14-3-3αΔC50-100 (Fig. 5G and Movie S11) are indicated by white dashed lines. White triangles indicate the pulsed accumulation of basal factors and their subsequent transport from the basal cortex toward the lumen-adjacent apical domain. Gray arrowheads indicate the alteration of the lumen architecture.

Fig. 7. Proposed models of lumen formation in Ciona notochord tubulogenesis via the interaction of 14-3-3α and ERM. Orange coloring indicates basal factors, including 14-3-3α, ERM, myosin II, and other cytoplasmic elements. Blue arrowheads indicate cortical flows at the basal surface, directed toward the basal equator, which would enable pulsatile accumulation in the basal contractile ring. Orange arrowheads indicate a directed flow through the cytoplasm from the basal cortex toward the lumen-adjacent apical domain. Gray arrowheads indicate the alteration of the lumen architecture.
were purchased from Santa Cruz Biotechnology, and His and GFP antibody were obtained from Clontech.

**Imaging.** Confocal images for phalloidin staining were prepared as previously described (31). For the immunostaining analysis, Ciona larvae were fixed with 10% formalin in seawater for 30 min. They were washed several times with 0.1% Triton-X in PBS (PBST) and blocked with 10% donkey serum in PBST for 2 h. The antibody for 14-3-3a and ERM was added, and the samples were incubated for 2 h. After several washes in PBST, larvae were incubated with the second antibody for 2 h and again washed several times in PBST. For 14-3-3a and GFP staining, the signal was amplified using Tyramide SuperBoost Kits with Alexa Fluor Tyramides (Invitrogen), according to the manufacturer's instructions. These specimens were photographed using a confocal laser scanning microscope system PV1000 (Olympus). Live imaging was performed by fixing living Ciona larvae with 2% Agarose-LM sieves (nacalai tesque) in seawater.

**Image Processing and Quantification.** We used the open-source program Fiji to perform all basic image-processing tasks, to quantify the average signal intensities of the region of interest (ROI), to measure the size of the ROI, and to calculate the correlation coefficient for the colocalization analysis. In live imaging, the TurboReg plugin was used to register frames to prevent the apparent blurring of the images. To generate kymographs with a heat map in live imaging, the intensities in the basal domain surrounded by the ROIs were quantified and normalized along the AP axis for every time point. Subsequently, kymographs were generated by Fiji. Finally, colors were annotated using the Python programming language to generate the heat map, showing a gradient color scale from red, indicating relatively high intensity, to blue, indicating relatively low intensity. To conduct a cross-correlation analysis, ROIs for the basal contractile ring and the apical domain were manually set for each period (200 s), and their cross-correlation was calculated with MATLAB (MathWorks).

**Plasmid Construction.** The 14-3-3 proteins (ca, cb, γ1, and γ2) were amplified by PCR from a cdna library, using a pair of primers containing the FLAG tag sequence: 5′-CCGGGATCCCTGATTAACAGGATCAGGACATAGTCTACGAGCGCAGGA-3′ and 5′-CCGGGATCCCTGATTAACAGGATCAGGACATAGTCTACGAGCGCAGGA-3′ for 14-3-3a; 5′-CCGGGATCCCTGATTAACAGGATCAGGACATAGTCTACGAGCGCAGGA-3′ and 5′-CCGGGATCCCTGATTAACAGGATCAGGACATAGTCTACGAGCGCAGGA-3′ for 14-3-3b; 5′-CCGGGATCCCTGATTAACAGGATCAGGACATAGTCTACGAGCGCAGGA-3′ and 5′-CCGGGATCCCTGATTAACAGGATCAGGACATAGTCTACGAGCGCAGGA-3′ for 14-3-3c; and 5′-CCGGGATCCCTGATTAACAGGATCAGGACATAGTCTACGAGCGCAGGA-3′ and 5′-CCGGGATCCCTGATTAACAGGATCAGGACATAGTCTACGAGCGCAGGA-3′ for 14-3-3γ. The resulting plasmids were introduced to Bra-mCherry, and Bra-mCherry:ERM, the sequences for 14-3-3a:EGFP, Bra-mCherry:EGFP, and MRLC:EGFP.

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