

TOOLS

A Lifeact-EGFP quail for studying actin dynamics in vivo

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Here, we report the generation of a transgenic Lifeact-EGFP quail line for the investigation of actin organization and dynamics during morphogenesis in vivo. This transgenic avian line allows for the high-resolution visualization of actin structures within the living embryo, from the subcellular filaments that guide cell shape to the supracellular assemblies that coordinate movements across tissues. The unique suitability of avian embryos to live imaging facilitates the investigation of previously intractable processes during embryogenesis. Using high-resolution live imaging approaches, we present the dynamic behaviors and morphologies of cellular protrusions in different tissue contexts. Furthermore, through the integration of live imaging with computational segmentation, we visualize cells undergoing apical constriction and large-scale actin structures such as multicellular rosettes within the neuroepithelium. These findings not only enhance our understanding of tissue morphogenesis but also demonstrate the utility of the Lifeact-EGFP transgenic quail as a new model system for live in vivo investigations of the actin cytoskeleton.

Introduction

During morphogenesis, tissue is formed by a complex interplay of changes in cellular geometry and cellular movements. The forces required to shape developing tissues are generated by actomyosin networks that are coupled to cell-cell and cell-matrix junctions (Clarke and Martin, 2021; Perez-Vale and Peifer, 2020). Contractions of the actomyosin network allow cells to pull on their neighbors or the matrix. These active forces at the cellular level are translated into dramatic changes in shape at the tissue level, which underly morphogenesis.

Remodeling of the actin cytoskeleton is the basis of a toolbox of common morphogenetic processes that drive tissue formation. These range from the subcellular scale, such as cellular protrusions, to changes in shape at the cellular scale, up to multicellular structures such as actin cables and cellular rosettes. Live imaging of actin dynamics has emerged as a powerful tool to unravel these processes (Blankenship et al., 2006; Christodoulou and Skourides, 2015; Fierro-Gonzalez et al., 2013; Phng et al., 2013; Samarage et al., 2015; Schimmel et al., 2020; Zenker et al., 2018).

To visualize actin dynamics in real-time during morphogenesis in a highly accessible vertebrate model, we generated the TgT2[UbC:Lifeact-EGFP] transgenic quail. This transgenic line, expressing an EGFP fusion of the actin-binding Lifeact

peptide (Riedl et al., 2008), allows for the high-resolution visualization of actin structures within the living embryo, from the subcellular filaments that guide cell shape to the supracellular assemblies that coordinate movements across tissues. The unique amenability of avian embryos to live imaging facilitates the investigation of previously intractable processes during embryogenesis (Benazeraf et al., 2010; Huss et al., 2015; Li et al., 2019; Nerurkar et al., 2019; Saadaoui et al., 2020; Xiong et al., 2020). We present novel insights into the dynamic behaviors of cellular protrusions in different tissues, apically constricting cells, and the assembly of multicellular structures. These findings not only enhance our understanding of tissue morphogenesis but also demonstrate the utility of the TgT2[UbC:Lifeact-EGFP] transgenic quail as a new model system for live in vivo investigations of the actin cytoskeleton.

Results

Generation of a transgenic Lifeact-EGFP quail line

To avoid potential overexpression artifacts, we used the low-expression ubiquitin promoter (Qin et al., 2010) to drive Lifeact-EGFP expression in our transgenic quail line (TgT2[UbC:Lifeact-EGFP]). The UbC-Lifeact-EGFP cassette was inserted in

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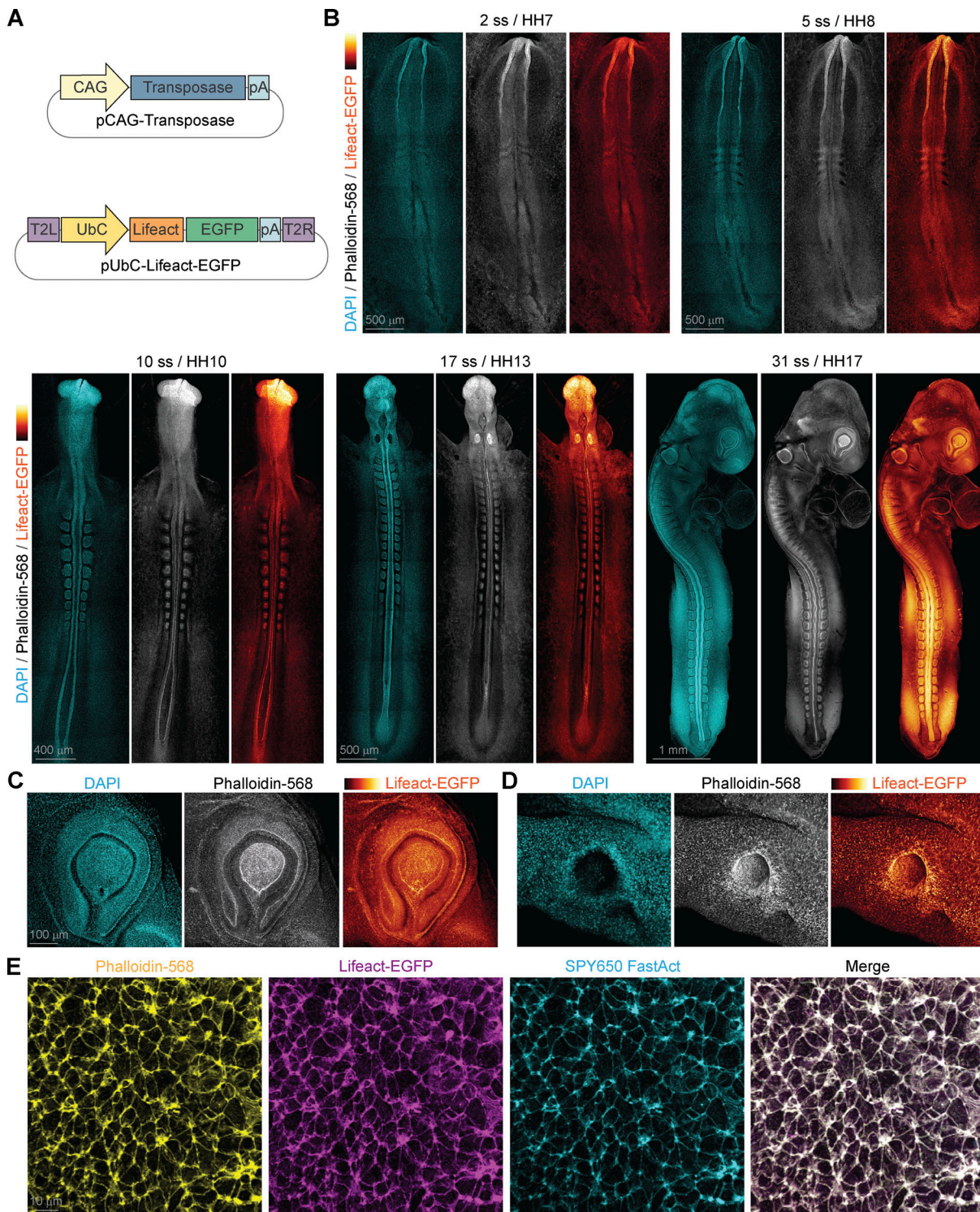


Figure 1. The endogenous actin cytoskeleton is fluorescently labeled in the developing TgT2[Ubc:Lifect-EGFP] transgenic quail embryo. (A) Plasmid vectors were used to generate the transgenic line. **(B)** Tiled confocal images show that the Lifect-EGFP signal matches the labeling of the actin cytoskeleton by Phalloidin-568 throughout early embryonic development. **(C and D)** Lifect-EGFP and Phalloidin-568 labeling in the eye (C) and the otic pit (D). **(E)** Higher magnification images of non-neural ectoderm demonstrate the similarity of the Lifect-EGFP signal to staining with Phalloidin-568 and SPY650FastAct.

between 5' and 3' Tol2 (T2) transposable elements to facilitate stable integration into the quail genome (Fig. 1 A). Using a direct injection technique (Barzilai-Tutsch et al., 2022; Serralbo et al., 2020; Tyack et al., 2013), we transfected the blood-circulating

primordial germ cells in vivo. Fifty wild-type embryos at stage HH16 (E2.5) were injected in the dorsal aorta with a mix of lipofectamine 2000, the UbC-Lifect-EGFP plasmid, and a pCAG-Transposase construct. One male and one female founder

were identified and mated with wild-type quails to establish lines. After further breeding, the lines were indistinguishable and the line from the male founder was selected for long-term maintenance. The TgT2[UbC:Lifect-EGFP] quails are viable, phenotypically normal, and fertile and can be maintained as heterozygotes or homozygotes. The transgene inheritance followed a Mendelian distribution, indicating that the gene was likely integrated at a single location in the genome. We could detect robust Lifect-EGFP expression from Hamilton and Hamburger stage 3 (HH3) onwards (Hamburger and Hamilton, 1951) (Fig. 1 B and Fig. S1). The Lifect-EGFP fluorescence overlapped with Phalloidin-568 and SPY650 FastAct staining in fixed embryos and was enriched in regions of increased Phalloidin-568 intensity (Fig. 1, B–E). The intensity of Phalloidin staining appeared higher than the Lifect-EGFP fluorescence in the developing eye at HH17 (Fig. 1, B and C), likely due to differences in the binding affinities of these actin probes for various actin structures (Belin et al., 2014).

Live imaging of actin dynamics during morphogenesis

We performed live imaging of the TgT2[UbC:Lifect-EGFP] quail line at a range of scales from the subcellular to the tissue level to examine the actin cytoskeleton dynamics during common morphogenetic tissue processes.

Imaging cellular protrusions

Many actively migrating cells reorganize their actin cytoskeleton and generate polarized protrusions (Schaks et al., 2019). Although this has been visualized in multiple tissues (Lamb et al., 2020; Mishra et al., 2019; Olson and Nechiporuk, 2021; Omelchenko, 2022), the actin cytoskeleton has never been imaged live at high resolution during fusion of the early heart tube in vivo. Vertebrate heart formation begins with the migration of bilateral sheets of cardiogenic mesoderm toward the midline where they fuse to form the primitive heart tube (Kirby, 2007; Rosenquist, 1966; Stalsberg and DeHaan, 1969). Here, we performed high spatiotemporal resolution live imaging of mesodermal cardiac progenitors crawling over the underlying endoderm of the anterior intestinal portal (AIP) in the TgT2[UbC:Lifect-EGFP] quail embryo at HH7–HH8 stage (E1). The bilateral collectives of cardiac progenitor cells form many filopodia that are enriched in Lifect-EGFP and protrude outward, making contact with the surrounding tissues (Fig. 2 A and Video 1). Fusion of the bilateral heart fields is initiated when progenitor cells from each side first make contact via their filopodia (Fig. 2 B). Similar to the migration of *Drosophila* myotubes (Bischoff et al., 2021), we did not find any lamellipodia, suggesting the cardiac progenitor cells use a filopodia-dependent migratory mechanism. Cardiac progenitor cell filopodia are on average $9.1 \mu\text{m} \pm 0.5 \mu\text{m}$ long and highly dynamic with an average persistence time of $389.1 \text{ s} \pm 22.9 \text{ s}$ ($n = 86$ filopodia, 4 embryos). Filopodia that contact the surrounding tissues are significantly longer and more persistent than those that do not make contact ($11.2 \mu\text{m} \pm 0.7 \mu\text{m}$, $n = 42$ and $523.6 \text{ s} \pm 34.5 \text{ s}$, $n = 37$, compared to $7.2 \mu\text{m} \pm 0.4 \mu\text{m}$, $n = 44$ and $276.0 \text{ s} \pm 20.5 \text{ s}$, $n = 44$, Fig. 2, C–E). Immunolabeling showed that the tissues surrounding the cardiac progenitor cells are covered in an extracellular matrix

rich in fibronectin, which also extends along some of the filopodia (Fig. S2).

To examine cellular protrusions in a different tissue context, we turned to the developing neural tube. Unlike the stereotypical filopodia of the cardiac progenitor cells, cellular protrusions of varying morphologies are proposed to be required for correct neural tube closure (Rolo et al., 2016). Despite many descriptions of these protrusions in fixed specimens as cytoplasmic threads, ruffles, filopodia, blebs, or lamellipodia over the last 50 years (Bancroft and Bellairs, 1975; Geelen and Langman, 1979; Rolo et al., 2016; Waterman, 1976), there has been very little live imaging of their dynamics (Massarwa and Niswander, 2013; Pyrgaki et al., 2010; Ray and Niswander, 2016). We performed high spatiotemporal resolution live imaging of TgT2[UbC:Lifect-EGFP] quail embryos during spinal neural tube closure at stage HH9–HH10 (E1) (Ainsworth et al., 2010). Clusters of protrusions highly enriched for Lifect-EGFP were clearly visible protruding into the open lumen of the neural tube (Fig. 2 F). Close to the zippering point of the closing neural tube, some protrusions reached across the open neural tube lumen, contacted the opposing neural fold, and appeared to assist in pulling the neural folds together (Fig. 2 G and Video 2). Zippering of the neural tube progressed faster in embryos with more protrusions (Fig. 2, H and I), supporting an active role for the protrusions in neural tube closure. The morphology of these protrusions was variable, consisting of both lamellipodia-like and filopodia-like structures (Fig. 2 J), which constantly changed shape and area for >60 min until the approaching zippering point of the neural tube reached them (Fig. 2 J and Video 3). As filopodia and lamellipodia appeared concurrently and could not be separated, we computationally masked the Lifect-EGFP signal for the entire cluster of protrusions and measured the change in area over time to reflect their highly dynamic nature (Fig. 2 K).

Building on the observed dynamic protrusions in the developing neural tube, we shifted our focus to the migrating surface ectoderm which is also crucial for neural tube formation (Christodoulou and Skourides, 2022; Galea et al., 2017; Maniou et al., 2021; Marshall et al., 2023; Moury and Schoenwolf, 1995). As the neural tube is closing, the surface ectoderm migrates over it toward the embryonic dorsal midline. During this migration, transient apertures in the ectodermal sheet were observed (Fig. 2 L). High-speed spinning disk confocal imaging of the TgT2[UbC:Lifect-EGFP] quail embryo revealed distinctive oscillations of the Lifect-EGFP at cell junctions and the formation of lamellipodia as surrounding cells tried to close the gap (Fig. 2 M; and Video 4). The fluctuating cell junctions indicate pulsed actomyosin contractions and occur at intervals of ~ 2 min (Fig. 2 N and Box 1). Simultaneously, a retrograde actin flow at an approximate rate of $2.6 \mu\text{m min}^{-1}$ was identified, propelling the formation of lamellipodia (Fig. 2 N, Box 2 and O). These lamellipodia, in turn, facilitated the forward movement of cells, contributing to the resolution of the temporary openings and sealing of the ectodermal sheet.

Together, our live imaging confirmed that the TgT2[UbC:Lifect-EGFP] quail line is an excellent model system for studying the rapid remodeling of the actin cytoskeleton during protrusive cell behaviors in vivo.

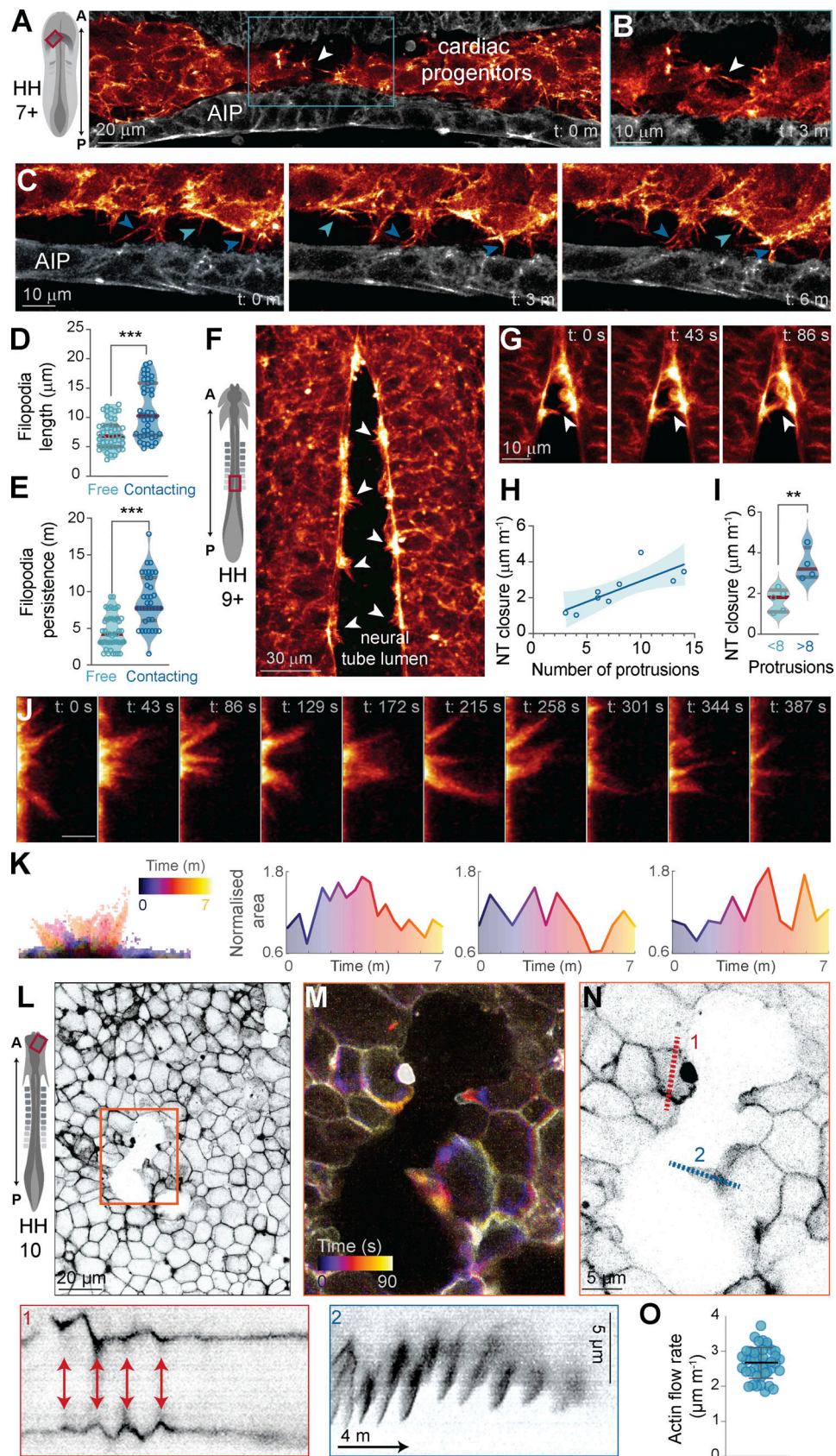


Figure 2. Live imaging reveals the dynamics of cellular protrusions in different tissue contexts. (A) Schematic indicates embryonic stage and the red box shows imaging location in the migrating heart field. A indicates anterior and P, posterior. Mesodermal cardiac progenitor cells extend filopodia toward the surrounding tissues as the bilateral heart fields migrate toward the midline over the Anterior Intestinal Portal (AIP). White arrowhead indicates leading cardiac

progenitor cells extending filopodia towards each other. The tissues surrounding the cardiac progenitor cells are pseudocoloured for clarity. **(B)** Inset of boxed region in A, 3 m later shows the first contact via a filopodium (white arrow) between the leading cardiac progenitor cells from each side. **(C)** Time series of filopodia projecting from cardiac progenitor cells towards the AIP. Filopodia contacting AIP cells (dark blue arrowheads) are longer and have higher persistence than free filopodia (light blue arrowheads). **(D and E)** Quantified in D and E, $n = 86$ filopodia from 4 embryos. *** $P < 0.001$ (Welch's test for D and two-sided Mann-Whitney test for E). Violin plots show mean and first and third quartiles. **(F)** Schematic indicates the embryonic stage, and the red box shows the imaging location in the neural tube. Cellular protrusions of varying morphologies (white arrowheads) extend from the edges of the neural folds into the open lumen of the neural tube. **(G)** Protrusions reach across the neural tube lumen (white arrowhead) and contact the opposite neural fold close to the zippering point. **(H and I)** Neural tube (NT) closure is faster in embryos with more protrusions, $n = 9$ embryos. Shaded area in H indicates 95% C.I. ** $P < 0.01$ (Student's t test) in I. Violin plots show mean and first and third quartiles, $n < 8$: five embryos and $n > 8$: four embryos (Student's unpaired two-sided t test). **(J)** High spatiotemporal resolution imaging reveals the highly dynamic nature of the neural tube protrusions. **(K)** Computationally masking the protrusions (left panel) allowed measurement of the area change over time, three examples shown from two embryos. **(L)** Schematic indicates the embryonic stage, and the red box shows the imaging location in the ectoderm. The ectodermal sheet contains transient apertures indicated by orange box. **(M)** Higher magnification of inset from L, pseudocoloured with a temporal code to reveal dynamic actin-based structures. **(N)** Kymographs made from regions indicated demonstrate contractile pulsations in cell junctions (1) and formation of lamellipodia by actin flow (2). **(O)** Speed of actin flow measured at lamellipodia, $n = 39$ flow waves from four cells. Graph shows mean $\pm$ SD.

Actin dynamics during apical constriction

Another common morphogenetic process during tissue development is apical constriction, in which cells change geometry by shrinking their apical surface (Martin and Goldstein, 2014). Epithelial remodeling requires apical constriction in a wide variety of contexts to attain the correct form and architecture of the tissue. Different dynamic patterns of actomyosin network contraction can drive apical constriction (Martin and Goldstein, 2014). In invertebrates, pulsed contractions of a medioapical meshwork drive shrinkage of the apical cell surface (Martin et al., 2009; Solon et al., 2009). However, a purse-string-like contraction of a circumferential cellular actomyosin cable has long been proposed to drive apical constriction in vertebrates (Baker and Schroeder, 1967; Schroeder, 1970). More recent work imaging the actin label Utrophin-GFP in the *Xenopus* neuroepithelium (Christodoulou and Skourides, 2015) and ZO1-GFP in the mouse epiblast (Francou et al., 2023) suggests that pulsatile contraction of a transient apical actin network also drives stepwise shrinkage of the apical surface in constricting vertebrate cells, but this has not yet been investigated in the avian neural plate.

To visualize the actin cytoskeleton and cell dynamics during apical constriction, we performed live imaging of the neuroepithelium in the open region of the caudal neural plate of TgT2 [UbC:Lifect-EGFP] quail embryos at HH8–HH9 (E1) (Fig. 3 A). Computational image segmentation was performed to segment all cells within the image and identify those undergoing apical constriction. To achieve this, we first used a custom local Z projection strategy to project the curved apical surface of the neuroepithelium onto a 2D plane. Next, we used the Cellpose 2.0 segmentation algorithm (Pachitariu and Stringer, 2022) to identify cell boundaries labeled by Lifect-EGFP. Using a custom MATLAB script, we identified cells undergoing apical constriction—defined as cells with a rate of area decrease $>0.02 \text{ min}^{-1}$ (Fig. 3 B and Video 5). Data from constricting cells were analyzed and averaged within embryos to exclude any effects from slight variations in developmental timing between embryos. Measuring the ratio of Lifect-EGFP signal at the apical cortex relative to the cell junctions revealed an average increase of $71.7\% \pm 2.9\%$ during the first 25% of the reduction in apical cell area (Fig. 3 C and Fig. S3, A and B). The inverse correlation between mean Lifect-EGFP intensity at the apical

cortex and mean apical cell area is highly significant (Fig. S3 B). Furthermore, the decrease in apical cell area was not constant but increased toward the final stages of apical constriction (Fig. 3, C and D). There was a moderate, but highly significant correlation between the rate of change in Lifect-EGFP intensity at the apical cortex and the change in apical cell area for individual cells (Fig. S3 C). The apical constriction events identified here are similar to those recently described during epiblast cell ingression at the mouse primitive streak where isolated cells constrict their apical surface over a 25–90 min period (Francou et al., 2023). Dye-labeling experiments in chick embryos have previously suggested that some cells may ingress in the caudal neural plate at HH8–HH9 (Dady et al., 2014); however, this had not been directly visualized live. Although the apical surface area of all cells we tracked in the quail neuroepithelium fluctuated over time, these area fluctuations were significantly larger in cells undergoing apical constriction (Fig. 3, D and E). Consistent with observations in the mouse primitive streak, the rate of area change in apically constricting quail neuroepithelium cells was faster during the constriction phases than during the expansion phases (Fig. 3 F).

Formation of multicellular rosettes

Multicellular rosette formation is associated with tissue reorganization in a variety of species (Harding et al., 2014; Miao and Blankenship, 2020). During rosette formation, two rows of cells become aligned. Myosin-driven contraction constricts the shared interfaces of the aligned cells, pulling them into a rosette structure with a centrally shared interface. These multicellular rosettes are a common feature during the morphogenesis of various organs (Gompel et al., 2001; Lienkamp et al., 2012; Villasenor et al., 2010).

In the avian embryo, epithelial rosettes are thought to be important for the formation of the neural tube (Nishimura et al., 2012; Nishimura and Takeichi, 2008). However, this has only been studied in fixed tissue where aligned rows of cells or rosettes can be identified but the process of rosette formation cannot be followed. Using live imaging, we visualized the alignment of cells and their formation into rosettes in the neuroepithelium of the TgT2 [UbC:Lifect-EGFP] quail embryo at HH8–HH9 (E1) (Fig. 4 A and Video 6). We focused on the region caudal to the closed neural tube, where the neural plate was still

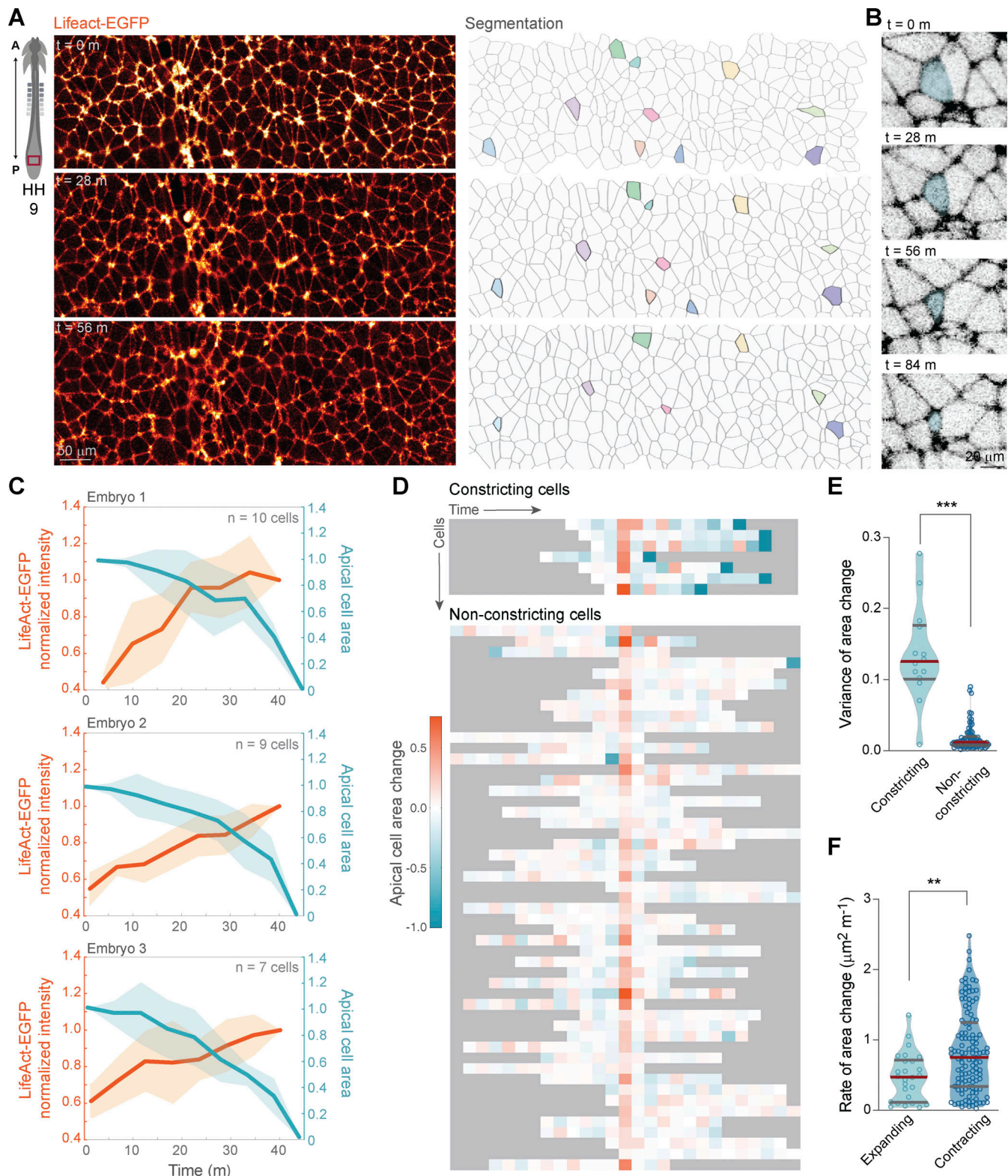


Figure 3. Cells undergo apical constriction in the developing neuroepithelium. (A) Schematic indicates the embryonic stage and the red box shows the imaging location in the caudal neural plate. Computational segmentation (right panel) of the Lifeact-EGFP signal (left panel) in live imaging of the neuroepithelium identifies cells undergoing apical constriction. (B) Time series of an apically constricting cell (blue cell in segmented image in A). (C) The ratio of Lifeact-EGFP intensity at the apical cortex relative to the cell junctions increases as the apical cell area shrinks. Graphs show mean (colored line) $\pm$ SEM (shading) for the normalized Lifeact-EGFP intensity and apical cell area over time in three embryos. (D) Heatmap of apical area change over time in cells undergoing apical constriction and non-constricting cells. Example from 1 embryo, $n = 7$ constricting cells and 47 non-constricting cells. Cells are aligned by the maximal observed area. (E) Constricting cells have a higher variance of apical area change than non-constricting cells, $n = 14$ constricting cells and 62 non-

constricting cells from 2 embryos. *** $P < 0.001$ (two-sided Mann-Whitney test). (F) Rate of area change during expansion and contraction phases of constricting cells, $n = 19$ cells from three embryos. Area change is faster during contraction phases. ** $P < 0.01$ (bilateral Mann-Whitney test). Violin plots show mean and first and third quartiles.

relatively flat. The transverse view of the 3D stack revealed a slight curvature along the midline of the neural plate (Fig. 4 A, $t = 0$ h, x, z view). We identified mediolaterally aligned rows of 10 or more cells. The mediolateral junctions shared between the rows of aligned cells are enriched for diphosphorylated myosin light chain, suggesting they are contractile (Fig. S4 A). Live imaging for 2 h revealed that the mediolateral junctions contracted and the curvature of the neural plate increased along the mediolateral axis (Fig. 4 A, $t = 2$ h, x, z view, B).

As the contraction of shared interfaces between aligned cells can generate multicellular rosettes, we used computational segmentation and a custom MATLAB script to identify rosettes consisting of 5–8 cells (Fig. 4 C). The rosettes were enriched for diphosphorylated myosin light chain (α -ppMLC) at their central vertex (Fig. S4 B). Surprisingly, even when the neural plate was only slightly curved, most cells were already part of a multicellular rosette. 79.8% of these rosettes contained five cells, 17.8% contained six cells, and only 2.9% contained seven cells (Fig. 4, C and D). After 2 h, the total number of rosettes had increased by 45.4%. Furthermore, the distribution had shifted toward higher-order rosettes with only 49.4% containing five cells but 29.2% containing six cells, 8.9% containing seven cells, and 3.7% containing eight cells. As the mediolateral junctions forming the rosettes contracted, the Lifeact-EGFP intensity (normalized to junction area) significantly increased, suggesting continued actin accumulation during junction contraction (Fig. 4 E).

To examine the stability of the actin remaining at the center of the multicellular rosettes following the contraction of the mediolateral junctions, we used fluorescence recovery after photobleaching (FRAP). Comparing five-cell rosettes to eight-cell rosettes revealed a higher immobile fraction and a decreased recovery time in the higher-order rosettes (Fig. 4, F–I). Our results show that a greater proportion of the actin molecules are stationary and not exchanging with the surrounding pool of actin within the center of the eight-cell rosettes. This may indicate a higher degree of actin stabilization or stronger binding interactions that prevent the actin molecules from diffusing away. However, within the mobile pool of actin, there is also a higher rate of actin turnover or a more efficient replenishment in the center of the higher-order rosettes, possibly due to greater accessibility or a higher rate of actin polymerization at the site. These complex dynamics may reflect the tuning of actin remodeling within multicellular rosettes to balance structural integrity with the flexibility required for ongoing morphogenesis.

To perturb the formation of multicellular rosettes, we treated the embryos with the actin polymerization inhibitor Latrunculin A (Coue et al., 1987). During 2 h of live imaging, we observed a decrease in mediolateral curvature of the neural plate and extension of the mediolaterally aligned shared junctions (Fig. 4, J and K). Latrunculin A treatment also decreased the total number of rosettes by 48.0% (Fig. 4 L). Moreover, there was a corresponding shift from higher-order toward lower-order multicellular

rosettes (Fig. 4, L and M). During Latrunculin A treatment, there was a significant decrease in the Lifeact-EGFP intensity along the shared mediolateral junctions, consistent with a reduction in actin polymerization (Fig. 4 N). Together, our results support a role for rosette formation in the anisotropic bending of the neural plate during neural tube formation.

Discussion

Understanding the remodeling of the actin cytoskeleton, which enables cellular movement and facilitates force transmission across tissues, is crucial to comprehending the diversity of forms generated during morphogenesis. Toward this goal, we generated the transgenic quail line, TgT2[Ubc:Lifeact-EGFP], designed to facilitate the high-resolution visualization of actin structures during embryogenesis in a higher vertebrate. The flat morphology, in ovo development, and robustness of the quail embryo enable long-term imaging of previously intractable developmental processes. The TgT2[Ubc:Lifeact-EGFP] line expresses the actin-binding Lifeact peptide fused with EGFP, allowing detailed observation of actin dynamics in vivo. By employing cutting-edge live imaging, we have investigated the dynamics of the actin cytoskeleton in different contexts, and at various scales, from subcellular filaments to tissue-level assemblies in the developing embryo.

Our approach provided the first high-resolution live imaging of cellular protrusions in migrating cardiac progenitor cells in the avian embryo. We demonstrated that these migrating cardiogenic cells extend many filopodia, but not lamellipodia. The filopodia that contact the surrounding tissues are significantly longer and more persistent than those that do not. As integrins are known to be present at filopodial tips (Galbraith et al., 2007; Lagarrigue et al., 2015), the higher persistence of filopodia in contact with surrounding tissues may indicate a force-dependent stabilization of the filopodia (Alieva et al., 2019). This indicates that these filopodia could have signaling roles, as proposed previously (Francou et al., 2014), and/or mechanical roles during cardiac progenitor cell migration.

Cellular protrusions have been described in the closing neural tube for several decades (Bancroft and Bellairs, 1975; Geelen and Langman, 1979; Rolo et al., 2016; Waterman, 1976). More recent work suggests they are required for successful neural tube closure (Rolo et al., 2016); however, their exact role in this process remains unclear. Performing live imaging close to the zipper point of the closing neural tube in the TgT2[Ubc:Lifeact-EGFP] transgenic quail enabled the first high-resolution visualization of the dynamics and behavior of neural tube protrusions in the live embryo. We found they can reach across the open neural tube lumen and contact the opposing neural fold, pulling it closer. By directly measuring the speed of neural tube closure, we showed that embryos with more protrusions zipper their neural folds together faster. Our results suggest that actin-

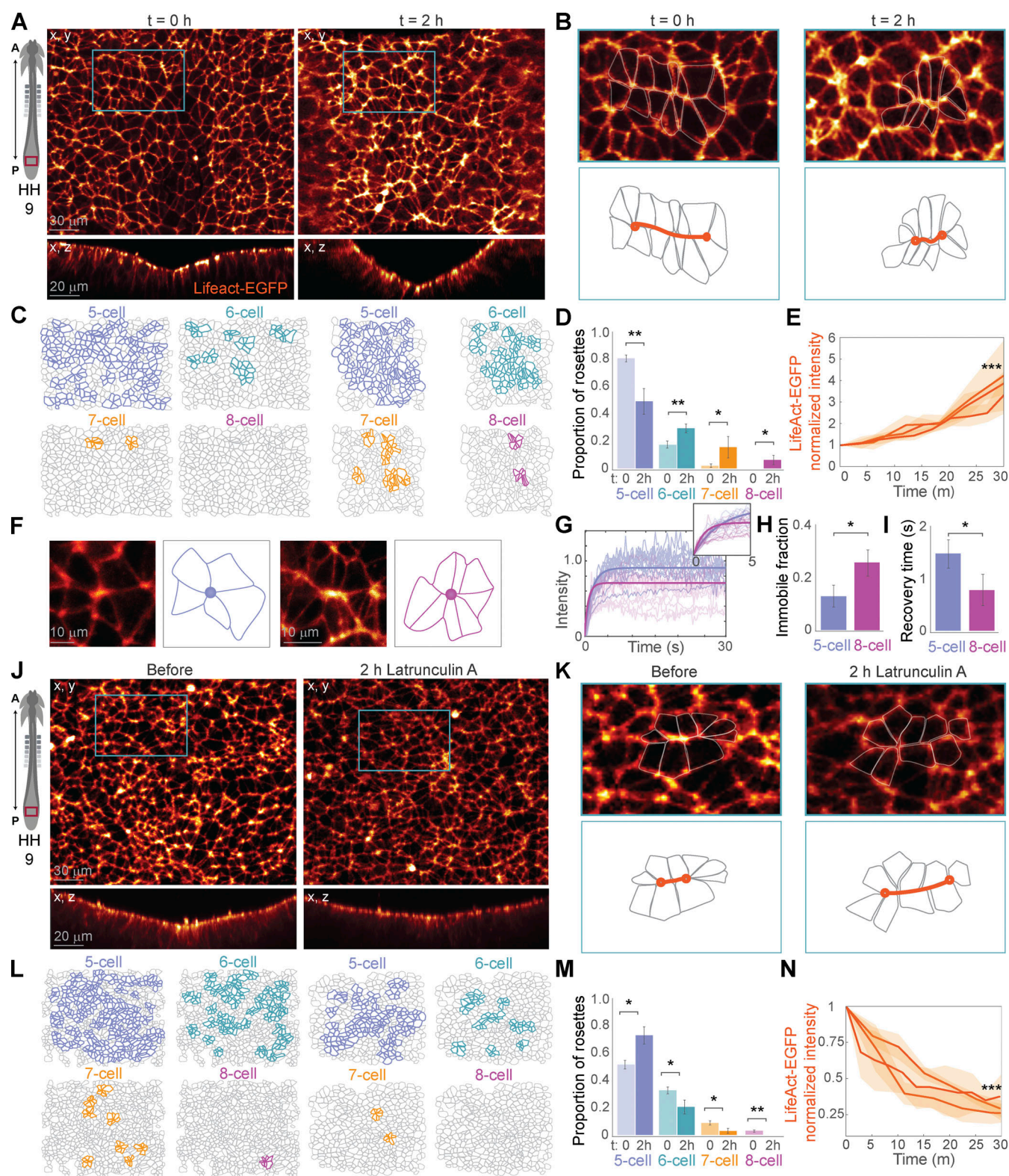


Figure 4. Formation of multicellular rosettes in the developing neuroepithelium. **(A)** Schematic indicates the embryonic stage, and the red box shows imaging location in the neural plate. Aligned rows of cells (blue boxes) form in the relatively flat neural plate and contract their shared mediolateral junctions within 2 h, concomitant with bending along the midline (x,z views). **(B)** Insets show aligned cells indicated in A. **(C and D)** Computational segmentation of areas shown in A reveals an increasing number and complexity of rosettes over time, quantified in D. Bar graphs show mean $\pm$ SEM, $n = 827$ rosettes from 4 embryos. ****** $P < 0.01$, ***** $P < 0.05$, Student's t test. **(E)** The intensity of LifeAct-EGFP increases as the shared mediolateral junctions contract (normalized to junction area). Graph shows mean (orange lines) $\pm$ SEM (shaded areas) from three embryos. ******* $P < 0.001$, Wilcoxon Signed Rank Test, $t: 0$ m versus $t: 30$ m. **(F-I)** FRAP of the actin at the central vertex shows a higher immobile fraction and a faster recovery time in eight-cell rosettes ($n = 17$ rosettes, 6 embryos) compared with five-cell

rosettes ($n = 17$ rosettes, 6 embryos), inset shows first 5 s of fluorescence recovery. Graphs show mean $\pm$ SEM * $P < 0.05$, Student's t test. **(J and K)** Schematic indicates embryonic stage and red box shows imaging location in the neural plate. Treatment with Latrunculin A causes relaxation of shared mediolateral junctions and flattening of the neural plate. **(L and M)** Computational segmentation of areas shown in J reveals that Latrunculin A reduces the number and complexity of rosettes. Bar graphs show mean $\pm$ SEM, $n = 2,044$ rosettes from 4 embryos. ** $P < 0.01$, * $P < 0.05$, two-sided Student's t test. **(N)** The intensity of Lifeact-EGFP localized to shared mediolateral junctions is decreased by Latrunculin A treatment (normalized to junction area). Graph shows mean (orange lines) $\pm$ SEM (shaded areas) from three embryos. *** $P < 0.001$, Wilcoxon Signed Rank Test, t : 0 m versus t : 30 m.

based cellular protrusions play an active mechanical role during neural tube closure.

Imaging the migrating surface ectoderm of the TgT2[Ubc:Lifeact-EGFP] quail revealed pulsed junction contractions and lamellipodia in cells surrounding gaps in the tissue. We measured a retrograde actin flow propelling the lamellipodia formation, which helped the cells migrate to seal the gaps in the ectodermal sheet. Interestingly, as described previously in vitro (Anon et al., 2012), the lamellipodia appeared to form preferentially along the gap edges with the lowest curvature.

In addition to investigating subcellular actin structures, the TgT2[Ubc:Lifeact-EGFP] quail is a valuable model for analyzing cellular actin dynamics and supracellular actin assemblies. By combining live imaging with computational image segmentation, we confirmed that a subpopulation of cells in the caudal avian neural plate apically constrict and ingress. Although this had been suggested by dye-labeling experiments in the chick embryo (Dady et al., 2014), ours is the first live observation of this process. We also used the live imaging and computational image segmentation approach to reveal the formation of multicellular rosettes in the developing neuroepithelium. As the rosettes formed, the mediolateral curvature of the neural plate increased, and this was reversed by inhibition of actin polymerization. The increasing number and complexity of the multicellular rosettes during neural plate bending suggest that the persistence of these structures is higher than the transitory rosettes involved in tissue remodeling in *Drosophila* (Blankenship et al., 2006). Furthermore, the accumulation of higher-order rosettes in the neural plate may contribute to the reported decrease in tissue fluidity during neurulation (Bocanegra-Moreno et al., 2023; Yan and Bi, 2019).

Together, the insights gained from this research offer a deeper understanding of the processes underlying tissue morphogenesis and establish the TgT2[Ubc:Lifeact-EGFP] transgenic quail as an invaluable resource for real-time, in vivo study of the actin cytoskeleton.

Materials and methods

Generation of transgenic Lifeact-EGFP quail line

The direct injection technique was performed as described previously (Serralbo et al., 2020; Tyack et al., 2013). Briefly, plasmids were purified using a Nucleobond Xtra Midi EF kit. 1 μ l of injection mix containing 0.6 μ g of pUbc-Lifeact-EGFP Tol2 plasmid, 1.2 μ g of CAG Transposase plasmid, and 3 μ l of Lipofectamine 2000 CD (Thermo Fisher Scientific) in 90 μ l of OptiPro was injected in the dorsal aorta of 2.5-day-old embryos. Eggs were then sealed and incubated until hatching. Chicks

were grown for 6 wk until they reached sexual maturity. Semen from the male was collected using a female teaser and massage technique as described previously (Serralbo et al., 2020). First, the male cloacal gland was emptied of foam by applying pressure to the gland. The male was then introduced into a cage containing a female. When the male was ready to mate, it was removed and inverted. The cloaca was massaged until the semen was expelled and could be collected. Genomic DNA from semen was extracted and PCR was performed to test for the presence of the transgene. Males showing a positive band were kept and crossed with wild-type females. F1 offspring were selected using GFP goggles and confirmed by genotyping 5 days after hatching by plucking a feather.

Maintenance of quails

Transgenic quails were hosted and bred at the University of Queensland according to local animal ethical policies (Ethics approval: 2023/AE000559). Fertilized quail eggs from transgenic quail lines were collected daily by the animal facility. Eggs were kept at 14°C before use.

Embryo staining

Embryos were fixed at room temperature for 30 min in 4% paraformaldehyde (P6148-500G; Sigma-Aldrich), and extra-embryonic tissues were trimmed before adding Phalloidin-Rhodamine (1:1,000, AB235138; Abcam), Spy650 FastAct (1:1,000, SC505; SpiroChrome), and DAPI (1:1,000, MBD0015-15ML; Sigma-Aldrich) for 3 h at room temperature. After incubation, embryos were washed and mounted with fluoromount aqueous mounting medium (F4680-25ML; Sigma-Aldrich) for imaging on glass slides (Epredia).

Whole-mount immunostaining

Embryos were fixed at room temperature for 30 min in 4% paraformaldehyde (P6148-500G; Sigma-Aldrich), and extra-embryonic tissues were trimmed before immunostaining. Embryos were permeabilized in PBS with 0.5% Triton X-100 (PBTX, T9284-500mL; Sigma-Aldrich) and blocked in 0.5% PBTX, 0.2% bovine serum (BSA, A2153-50G; Sigma-Aldrich), and 0.02% sodium dodecyl sulfate (SDS, L4509-25G; Sigma-Aldrich). Embryos were then incubated in primary antibody: Mouse anti-Fibronectin (1:1,000, B3/D6; DSHB) or Rabbit anti-Di-phospho-MLC (Thr18/Ser19) (1:100, 95777S; Cell Signalling Technology) in blocking buffer overnight at 4°C, followed by washing and incubation in Goat anti-mouse IgG Alexa 647 (1:1,000, A31571; Thermo Fisher Scientific) or Goat anti-rabbit Ig Alexa 647 (1:1,000, A21245; Thermo Fisher Scientific) for 3 h at room temperature. After incubation, embryos were washed and mounted on a six-well glass bottom plate (P06-1.5H-N;

Cellvis) with fluoromount aqueous mounting medium (F4680-25ML; Sigma-Aldrich) for imaging.

Quail embryo culture

Eggs were incubated horizontally at 37.5°C in a humidified atmosphere until the desired stages. After incubation, quail eggs were cooled down for ~1 h at room temperature (RT) before culture. While keeping the egg horizontal by placement onto a customized egg holder, 1–2 ml of thin albumen was aspirated out with a 5 ml syringe (Nipro) and an 18G sterile needle (BD PrecisionGlide) from the blunt end of the egg to create a space between the embryo and the shell. A small window (~1.0 × 0.5 cm) was cut on the top of the eggshell to facilitate visualization and staging. Embryos were staged based on morphological criteria as described previously (Ainsworth et al., 2010). Embryos were cultured according to a protocol described elsewhere (Williams and Sauka-Spengler, 2021). Briefly, the egg yolk and albumin were carefully slid out of the shell into a 35 mm petri dish with the embryo resting at the top of the yolk. The albumin covering the surface of the embryo was carefully wiped away with Kimwipe tissue. A piece of 1.5 × 1.5 cm square filter paper with a 0.2 × 1.0 cm hole in the center was placed on the surface of the embryo, with the embryo centered in the hole. The filter paper was then cut along the edge with a pair of sharp dissection scissors to release the embryo from surrounding tissues while maintaining biological tension. The filter paper, with the embryo attached, was lifted away from the yolk using fine forceps and placed into a 35-mm one-well dish precoated with 1 ml agar-albumin. The yolk was washed away from the embryo using preheated Hanks' Balanced Salt solution (HBSS). After washing, the embryo was transferred into another 35-mm one-well dish precoated with 1 ml agar-albumin mixture consisting of 0.3% wt/vol bacto-agar and 50% vol/vol albumin. Before further use, the one-well dish containing the embryo was kept in a humidified environmental chamber at RT.

Latrunculin A treatment

A 20-μl drop of 20 μM Latrunculin A (L5163; Sigma-Aldrich) was placed on the agar-albumin-coated plate. Cultured embryos at HH8–HH9 (E1) were placed on top of the drop dorsal side down and an additional 20-μl drop of Latrunculin A was added to the ventral side. Embryos were allowed to rest in a humidified environmental chamber at 37.5°C for 1 h before imaging.

Fixed embryo imaging

Mounted embryos were imaged in a Zeiss Axiovert 200 Inverted Microscope Stand with LSM 710 Meta Confocal with a 20×/NA0.8 or 40×/1.3.

Live imaging

Following culture, embryos were allowed to rest for at least 1 h at RT before imaging and then transferred dorsal side facing down to a six-well imaging plate precoated with 250 μl of agar-albumin. All live imaging was done at the IMB microscopy facility (The University of Queensland), which is supported by the Australian Cancer Research Foundation. Live imaging was performed on the following systems: a Zeiss Axiovert 200 Inverted

Microscope Stand with LSM 710 Meta Confocal Scanner fitted with dedicated GaAsP 488 and 561 nm detectors for increased sensitivity, a Zeiss Axiovert 200 Inverted Microscope Stand with LSM 710 Meta Confocal Scanner, and Airyscan super-resolution detector and Mai Tai eHP 760–1040 nm laser with dedicated GaAsP NDD detectors controlled by Zeiss Zen 2012 Black software or an Andor Dragonfly spinning disc confocal equipped with dual Andor Zyla 4.2 sCMOS cameras, controlled by Fusion Software (Andor). Microscope environmental chambers were maintained at 37.5°C while imaging.

Image processing

Images were processed in Imaris 10.0.1 (Bitplane) or FIJI. 3D Images were displayed in Imaris as a volume using Maximum Intensity Projection mode and render quality set at 1 for capture. Pseudocolouring for Fig. 2 was performed in Photoshop 2022 (Adobe) by manually selecting and desaturating surrounding tissue areas. Temporal color coding was performed using the Hyperstack function in FIJI.

Filopodia quantifications

Length and persistence time were measured in FIJI. The maximal length of a filopodium was measured using a straight segmented line. Persistence was calculated as the final time point at which a filopodium is visible minus the first time point at which it is observed.

Protrusion dynamics were quantified by the area of the positive pixels in a masked image of the Lifeact-EGFP channel over time. Masking was performed using Otsu's binarize function in MATLAB.

Speed of neural tube closure

The closure speed of the neural tube was calculated by tracking the displacement of the zippering point of the neural folds divided by the elapsed time.

FRAP

FRAP experiments were performed with a Zeiss 40X (W-Plan Apochromat NA1.10) objective at five times magnification. A 4 × 4 μm region of interest (ROI) was photobleached with a two-photon laser (Mai Tai Laser System) at 840 nm with 30% laser power and imaged every 0.2 s for 30 s.

For FRAP analysis, the mean fluorescence intensity of the photobleached region was corrected by background fluorescence and normalized to a non-photobleached reference and the intensity after bleaching. The average of the prebleach fluorescence intensities was set to 1 and the value after bleaching to 0. The normalized mean fluorescence intensities were then fitted with an exponential function to obtain the decay time and the plateau intensity (Ip). The immobile fraction was calculated as I-Ip. The analysis was performed in MATLAB (MATLAB ver. R2021a).

Lamellipodia dynamics

The ectoderm of the TgT2[Ubc-Lifeact-EGFP] transgenic quail at HH7–8 was imaged using the Andor Dragonfly spinning disc confocal with a 60× objective and a time interval of 5 s. To

quantify the F-actin retrograde flow, a 1- μm wide ROI line was drawn manually from the center of the cell outward across the lamellipodium of a given cell. The kymograph was then generated using the KymoResliceWide FIJI plugin. The fronts of actin flow waves were visually identified on the kymograph and manually traced by drawing lines along the front of the actin flow waves. The actin flow rate was then calculated by measuring the slope of the traced lines.

Signal-to-noise calculation

Signal-to-noise was measured as the ratio between mean intensity and standard deviation on a representative ROI that contained fluorescence and background. The measurements were repeated at different locations (>5) within the image and the mean value was reported.

Statistical analysis

Statistical analysis was performed using Prism Graphpad software (version 10.0.2). For all the statistical analysis: ns, P value >0.05; *, P value <0.05; **, P value <0.01; ***, P value <0.001. Shapiro-Wilk normality test was applied to the data to select between parametric and non-parametric analyses.

Correlation coefficients (r) between the mean apical cell area and the mean medial/junctional Lifeact-EGFP intensity for each embryo were obtained using Spearman's correlation. The same method was used to test the correlation between the change in area and the change in Lifeact-EGFP intensity (medial/junctional) for individual cells over time.

Computational segmentation

Images were segmented using Cellpose human-in-the-loop workflow (Pachitariu and Stringer, 2022). The cyto3 model was trained with five images by manually correcting cell boundaries, and then the refined model was applied to all time points. Obtained masks were exported as png and used for the following analyses.

Cell tracking

Cell tracking algorithm pairs segmented cell at time t with the same cell at time $t + 1$. Cells are positive pairs when the cell position at time $t+1$ is within the distance of 2-cell radius from time t and the area change is not higher than three times. Cells from the image at time t that could not be paired with cells at time $t+1$ are identified and excluded from tracking. The algorithm is repeated for each cell and all the time points of the movie. The cell tracking algorithm is implemented in MATLAB (available in Online supplemental material).

Detection of apical constriction

All cells were tracked for 50–90 min. The cell boundaries were converted to polygons in MATLAB and the apical cell area was measured using the polyshape toolkit. Constriction events were identified by normalized apical area rate decrease $>0.02 \text{ min}^{-1}$. The rate of area change for each time point was calculated as $(\text{area}_{t+1} - \text{area}_t)/\Delta_t$. Cell expansion was defined as positive area rate change and cell constriction was defined as negative area rate change. The rate of apical area change was defined as

follows: $\Delta \text{area}(t) = (\text{area}_{t+1} - \text{area}_t)/\text{area}_t$. The variance of area change was calculated for each cell time series. Custom MATLAB script is available in Online supplemental material.

Lifeact-EGFP measurements during apical constriction

The intensity of apical surface Lifeact-EGFP was measured by isotropically reducing the segmented cell polygon by 20%. The Lifeact-EGFP at the cell boundary was measured by isotropically expanding the segmented cell polygon by 20% and then subtracting the apical surface measurement.

Rosette detection

The segmented cell polygons were smoothed using `reducepoly` function in MATLAB to obtain the positions of cellular vertices. Rosettes were defined as a group of cells with a shared vertex. Shared vertices were identified as those positioned within the sqrt of the mean area of the group of cells. The custom MATLAB script is available in the Online supplemental material.

Mediolateral junction measurements

Lifeact-EGFP quantification during mediolateral junction contraction and after Latrunculin treatment was measured with FIJI. The junctions shared by the aligned cells were enclosed using the freehand selection tool at each time point to measure the mean value of the Lifeact-EGFP channel. The values were normalized to the junction area.

Online supplemental material

Fig. S1 shows that Lifeact-EGFP fluorescence is similar to Phalloidin staining at early embryonic stage HH3 and that there is no difference in the signal-to-noise of the Lifeact-EGFP and Phalloidin in the fixed embryos shown in Fig. 1. In Fig. S2, immunostaining shows that fibronectin is deposited on the surface of the AIP where migrating cardiac progenitor cells make contact, supporting Fig. 2. Fig. S3 shows that the intensity of Lifeact-EGFP at the apical cortex is inversely correlated with apical cell area in three separate embryos, supporting Fig. 3. The enrichment of diphosphorylated myosin light chain along mediolateral junctions and in the vertices of multicellular rosettes is shown by immunostaining in Fig. S4, supporting Fig. 4. Video 1 shows live imaging of filopodia extending from cardiac progenitor cells in the migrating heart field, supporting Fig. 2. Video 2 shows live imaging of protrusions close to the zipper point of the closing neural tube, supporting Fig. 2. A zoomed movie of a single cluster of protrusions in the closing neural tube is shown in Video 3, supporting Fig. 2. Video 4 shows lamellipodia extending from ectodermal cells around a gap in the tissue, supporting Fig. 2. Video 5 shows live imaging of the neuroepithelium with apically constricting cells highlighted in blue, supporting Fig. 3. Video 6 shows live imaging of neuroepithelial cells arranging into a multicellular rosette (highlighted in purple), supporting Fig. 4. Text S1 is the code for cell tracking. Cellpose segmentation masks converted to polygons are tracked at each time point by minimizing distance and cell area change. Text S2 is the code for detecting apically constricting cells. Cell area change for each tracked cell is fitted and cells with a slope lower than a threshold value are defined as apically constricting cells. Text S3 is the code

for the FRAP analysis. Mean photobleached ROI intensity is normalized and fitted with an exponential function to obtain the immobile fraction and the decay time. Text S4 is the code for rosette detection. Rosettes were detected and classified according to the number of cells sharing a vertex.

Data availability

The data are available from the corresponding author upon reasonable request.

Acknowledgments

Microscopy was performed at the Institute for Molecular Bioscience Microscopy Facility which was established with the support of the Australian Cancer Research Foundation and incorporates the Dynamic Imaging, Cancer Biology Imaging, and Cancer Ultrastructure and Function Facilities. We thank the IMB Microscopy facility staff.

M.D. White was supported by a Future Fellowship (FT200100899) and a Discovery Project grant (DP220101878) from the Australian Research Council (ARC), and an Ideas Grant (2013027) from the National Health and Medical Research Council of Australia (NHMRC). I. Noordstra was supported by the European Molecular Biology Organization (EMBO ALTF 251-2018). A.S. Yap was supported by grants (GNT1163462, 201070) and fellowships (GNT1136592) from the NHMRC and the ARC (DP19010287, 190102230).

Author contributions: Y.D. Alvarez: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing - review & editing, M. van der Spuy: Formal analysis, Investigation, Methodology, Writing - original draft, J.X. Wang: Formal analysis, Investigation, Methodology, Writing - review & editing, I. Noordstra: Formal analysis, Investigation, Writing - review & editing, S.Z. Tan: Investigation, Methodology, Writing - review & editing, M. Carroll: Formal analysis, Writing - review & editing, A.S. Yap: Investigation, O. Serralbo: Resources, M.D. White: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

Disclosures: The authors declare no competing interests exist.

Submitted: 12 April 2024

Revised: 15 May 2024

Accepted: 3 June 2024

References

Ainsworth, S.J., R.L. Stanley, and D.J.R. Evans. 2010. Developmental stages of the Japanese quail. *J. Anat.* 216:3–15. <https://doi.org/10.1111/j.1469-7580.2009.01173.x>

Alieva, N.O., A.K. Efremov, S. Hu, D. Oh, Z. Chen, M. Natarajan, H.T. Ong, A. Jégou, G. Romet-Lemonne, J.T. Groves, et al. 2019. Myosin IIA and formin dependent mechanosensitivity of filopodia adhesion. *Nat. Commun.* 10:3593. <https://doi.org/10.1038/s41467-019-10964-w>

Anon, E., X. Serra-Picamal, P. Hersen, N.C. Gauthier, M.P. Sheetz, X. Trepat, and B. Ladoux. 2012. Cell crawling mediates collective cell migration to

close undamaged epithelial gaps. *Proc. Natl. Acad. Sci. USA.* 109: 10891–10896. <https://doi.org/10.1073/pnas.1117814109>

Baker, P.C., and T.E. Schroeder. 1967. Cytoplasmic filaments and morphogenetic movement in the amphibian neural tube. *Dev. Biol.* 15:432–450. [https://doi.org/10.1016/0012-1606\(67\)90036-X](https://doi.org/10.1016/0012-1606(67)90036-X)

Bancroft, M., and R. Bellairs. 1975. Differentiation of the neural plate and neural tube in the young chick embryo. A study by scanning and transmission electron microscopy. *Anat. Embryol.* 147:309–335. <https://doi.org/10.1007/BF00315078>

Barzilai-Tutsch, H., V. Morin, G. Toulouse, O. Chernyavskiy, S. Firth, C. Marcelle, and O. Serralbo. 2022. Transgenic quails reveal dynamic TCF/ β -catenin signaling during avian embryonic development. *Elife.* 11: e72098. <https://doi.org/10.7554/eLife.72098>

Belin, B.J., L.M. Goins, and R.D. Mullins. 2014. Comparative analysis of tools for live cell imaging of actin network architecture. *Bioarchitecture.* 4: 189–202. <https://doi.org/10.1080/19490992.2014.1047714>

Bénazéraf, B., P. Francois, R.E. Baker, N. Denans, C.D. Little, and O. Pourquié. 2010. A random cell motility gradient downstream of FGF controls elongation of an amniote embryo. *Nature.* 466:248–252. <https://doi.org/10.1038/nature09151>

Bischoff, M.C., S. Lieb, R. Renkawitz-Pohl, and S. Bogdan. 2021. Filopodia-based contact stimulation of cell migration drives tissue morphogenesis. *Nat. Commun.* 12:791. <https://doi.org/10.1038/s41467-020-20362-2>

Blankenship, J.T., S.T. Backovic, J.S. Sanny, O. Weitz, and J.A. Zallen. 2006. Multicellular rosette formation links planar cell polarity to tissue morphogenesis. *Dev. Cell.* 11:459–470. <https://doi.org/10.1016/j.devcel.2006.09.007>

Bocanegra-Moreno, L., A. Singh, E. Hannezo, M. Zagorski, and A. Kicheva. 2023. Cell cycle dynamics control fluidity of the developing mouse neuroepithelium. *Nat. Phys.* 19:1050–1058. <https://doi.org/10.1038/s41567-023-01977-w>

Christodoulou, N., and P.A. Skourides. 2015. Cell-Autonomous Ca(2+) flashes elicit pulsed contractions of an apical actin network to drive apical constriction during neural tube closure. *Cell Rep.* 13:2189–2202. <https://doi.org/10.1016/j.celrep.2015.11.017>

Christodoulou, N., and P.A. Skourides. 2022. Distinct spatiotemporal contribution of morphogenetic events and mechanical tissue coupling during *Xenopus* neural tube closure. *Development.* 149:dev200358. <https://doi.org/10.1242/dev.200358>

Clarke, D.N., and A.C. Martin. 2021. Actin-based force generation and cell adhesion in tissue morphogenesis. *Curr. Biol.* 31:R667–R680. <https://doi.org/10.1016/j.cub.2021.03.031>

Coué, M., S.L. Brenner, I. Spector, and E.D. Korn. 1987. Inhibition of actin polymerization by latrunculin A. *FEBS Lett.* 213:316–318. [https://doi.org/10.1016/0014-5793\(87\)81513-2](https://doi.org/10.1016/0014-5793(87)81513-2)

Dady, A., E. Havis, V. Escriou, M. Catala, and J.L. Duband. 2014. Junctional neurulation: A unique developmental program shaping a discrete region of the spinal cord highly susceptible to neural tube defects. *J. Neurosci.* 34:13208–13221. <https://doi.org/10.1523/JNEUROSCI.1850-14.2014>

Fierro-González, J.C., M.D. White, J.C. Silva, and N. Plachta. 2013. Cadherin-dependent filopodia control preimplantation embryo compaction. *Nat. Cell Biol.* 15:1424–1433. <https://doi.org/10.1038/ncb2875>

Francou, A., K.V. Anderson, and A.K. Hadjantonakis. 2023. A ratchet-like apical constriction drives cell ingression during the mouse gastrulation EMT. *Elife.* 12:e84019. <https://doi.org/10.7554/eLife.84019>

Francou, A., E. Saint-Michel, K. Mesbah, and R.G. Kelly. 2014. TBX1 regulates epithelial polarity and dynamic basal filopodia in the second heart field. *Development.* 141:4320–4331. <https://doi.org/10.1242/dev.115022>

Galbraith, C.G., K.M. Yamada, and J.A. Galbraith. 2007. Polymerizing actin fibers position integrins primed to probe for adhesion sites. *Science.* 315: 992–995. <https://doi.org/10.1126/science.1137904>

Galea, G.L., Y.J. Cho, G. Galea, M.A. Molè, A. Rolo, D. Savery, D. Moulding, L.H. Culshaw, E. Nikolopoulou, N.D.E. Greene, and A.J. Copp. 2017. Biomechanical coupling facilitates spinal neural tube closure in mouse embryos. *Proc. Natl. Acad. Sci. USA.* 114:E5177–E5186. <https://doi.org/10.1073/pnas.1700934114>

Geelen, J.A., and J. Langman. 1979. Ultrastructural observations on closure of the neural tube in the mouse. *Anat. Embryol.* 156:73–88. <https://doi.org/10.1007/BF00315716>

Gompel, N., N. Cubedo, C. Thisse, B. Thisse, C. Dambly-Chaudière, and A. Ghysen. 2001. Pattern formation in the lateral line of zebrafish. *Mech. Dev.* 105:69–77. [https://doi.org/10.1016/S0925-4773\(01\)00382-3](https://doi.org/10.1016/S0925-4773(01)00382-3)

Hamburger, V., and H.L. Hamilton. 1951. A series of normal stages in the development of the chick embryo. *J. Morphol.* 88:49–92. <https://doi.org/10.1002/jmor.1050880104>

- Harding, M.J., H.F. McGraw, and A. Nechiporuk. 2014. The roles and regulation of multicellular rosette structures during morphogenesis. *Development*. 141:2549–2558. <https://doi.org/10.1242/dev.101444>
- Huss, D., B. Benazeraf, A. Wallingford, M. Filla, J. Yang, S.E. Fraser, and R. Lansford. 2015. A transgenic quail model that enables dynamic imaging of amniote embryogenesis. *Development*. 142:2850–2859. <https://doi.org/10.1242/dev.121392>
- Kirby, M.L. 2007. Cardiac Development. Oxford University Press, Oxford, UK. <https://doi.org/10.1093/oso/9780195178197.001.0001>
- Lagarigue, F., P. Vikas Anekal, H.-S. Lee, A.I. Bachir, J.N. Ablack, A.F. Horwitz, and M.H. Ginsberg. 2015. A RIAM/lamellipodin-talin-integrin complex forms the tip of sticky fingers that guide cell migration. *Nat. Commun.* 6:8492. <https://doi.org/10.1038/ncomms9492>
- Lamb, M.C., K.K. Anliker, and T.L. Tootle. 2020. Fascin regulates protrusions and delamination to mediate invasive, collective cell migration in vivo. *Dev. Dyn.* 249:961–982. <https://doi.org/10.1002/dvdy.186>
- Li, Y., F.M. Veceli, W.G. Gonzalez, A. Li, W. Tang, C. Lois, and M.E. Bronner. 2019. In vivo quantitative imaging provides insights into trunk neural crest migration. *Cell Rep.* 26:1489–1500.e3. <https://doi.org/10.1016/j.celrep.2019.01.039>
- Lienkamp, S.S., K. Liu, C.M. Kerner, T.J. Carroll, O. Ronneberger, J.B. Wallingford, and G. Walz. 2012. Vertebrate kidney tubules elongate using a planar cell polarity-dependent, rosette-based mechanism of convergent extension. *Nat. Genet.* 44:1382–1387. <https://doi.org/10.1038/ng.2452>
- Maniuk, E., M.F. Staddon, A.R. Marshall, N.D.E. Greene, A.J. Copp, S. Banerjee, and G.L. Galea. 2021. Hindbrain neuropore tissue geometry determines asymmetric cell-mediated closure dynamics in mouse embryos. *Proc. Natl. Acad. Sci. USA*. 118:e2023163118. <https://doi.org/10.1073/pnas.2023163118>
- Marshall, A.R., G.L. Galea, A.J. Copp, and N.D.E. Greene. 2023. The surface ectoderm exhibits spatially heterogeneous tension that correlates with YAP localisation during spinal neural tube closure in mouse embryos. *Cells Dev.* 174:203840. <https://doi.org/10.1016/j.cdev.2023.203840>
- Martin, A.C., and B. Goldstein. 2014. Apical constriction: Themes and variations on a cellular mechanism driving morphogenesis. *Development*. 141:1987–1998. <https://doi.org/10.1242/dev.102228>
- Martin, A.C., M. Kaschube, and E.F. Wieschaus. 2009. Pulsed contractions of an actin-myosin network drive apical constriction. *Nature*. 457:495–499. <https://doi.org/10.1038/nature07522>
- Massarwa, R., and L. Niswander. 2013. In toto live imaging of mouse morphogenesis and new insights into neural tube closure. *Development*. 140:226–236. <https://doi.org/10.1242/dev.085001>
- Miao, H., and J.T. Blankenship. 2020. The pulse of morphogenesis: Actomyosin dynamics and regulation in epithelia. *Development*. 147:dev186502. <https://doi.org/10.1242/dev.186502>
- Mishra, A.K., J.A. Mondo, J.P. Campanale, and D.J. Montell. 2019. Coordination of protrusion dynamics within and between collectively migrating border cells by myosin II. *Mol. Biol. Cell*. 30:2490–2502. <https://doi.org/10.1091/mbc.E19-02-0124>
- Moury, J.D., and G.C. Schoenwolf. 1995. Cooperative model of epithelial shaping and bending during avian neurulation: Autonomous movements of the neural plate, autonomous movements of the epidermis, and interactions in the neural plate/epidermis transition zone. *Dev. Dyn.* 204:323–337. <https://doi.org/10.1002/aja.1002040310>
- Nerurkar, N.L., C. Lee, L. Mahadevan, and C.J. Tabin. 2019. Molecular control of macroscopic forces drives formation of the vertebrate hindgut. *Nature*. 565:480–484. <https://doi.org/10.1038/s41586-018-0865-9>
- Nishimura, T., H. Honda, and M. Takeichi. 2012. Planar cell polarity links axes of spatial dynamics in neural-tube closure. *Cell*. 149:1084–1097. <https://doi.org/10.1016/j.cell.2012.04.021>
- Nishimura, T., and M. Takeichi. 2008. Shroom3-mediated recruitment of Rho kinases to the apical cell junctions regulates epithelial and neuroepithelial planar remodeling. *Development*. 135:1493–1502. <https://doi.org/10.1242/dev.019646>
- Olson, H.M., and A.V. Nechiporuk. 2021. Lamellipodia-like protrusions and focal adhesions contribute to collective cell migration in zebrafish. *Dev. Biol.* 469:125–134. <https://doi.org/10.1016/j.ydbio.2020.10.007>
- Omelchenko, T. 2022. Cellular protrusions in 3D: Orchestrating early mouse embryogenesis. *Semin. Cell Dev. Biol.* 129:63–74. <https://doi.org/10.1016/j.semcdb.2022.05.005>
- Pachitariu, M., and C. Stringer. 2022. Cellpose 2.0: How to train your own model. *Nat. Methods*. 19:1634–1641. <https://doi.org/10.1038/s41592-022-01663-4>
- Perez-Vale, K.Z., and M. Peifer. 2020. Orchestrating morphogenesis: Building the body plan by cell shape changes and movements. *Development*. 147:dev191049. <https://doi.org/10.1242/dev.191049>
- Phng, L.K., F. Stanchi, and H. Gerhardt. 2013. Filopodia are dispensable for endothelial tip cell guidance. *Development*. 140:4031–4040. <https://doi.org/10.1242/dev.097352>
- Pyrgaki, C., P. Trainor, A.K. Hadjantonakis, and L. Niswander. 2010. Dynamic imaging of mammalian neural tube closure. *Dev. Biol.* 344:941–947. <https://doi.org/10.1016/j.ydbio.2010.06.010>
- Qin, J.Y., L. Zhang, K.L. Clift, I. Huhur, A.P. Xiang, B.Z. Ren, and B.T. Lahn. 2010. Systematic comparison of constitutive promoters and the doxycycline-inducible promoter. *PLoS One*. 5:e10611. <https://doi.org/10.1371/journal.pone.0010611>
- Ray, H.J., and L.A. Niswander. 2016. Dynamic behaviors of the non-neural ectoderm during mammalian cranial neural tube closure. *Dev. Biol.* 416:279–285. <https://doi.org/10.1016/j.ydbio.2016.06.030>
- Riedel, J., A.H. Crevenna, K. Kessenbrock, J.H. Yu, D. Neukirchen, M. Bista, F. Bradke, D. Jenne, T.A. Holak, Z. Werb, et al. 2008. Lifeact: A versatile marker to visualize F-actin. *Nat. Methods*. 5:605–607. <https://doi.org/10.1038/nmeth.1220>
- Rolo, A., D. Savery, S. Escuin, S.C. de Castro, H.E. Armer, P.M. Munro, M.A. Molé, N.D. Greene, and A.J. Copp. 2016. Regulation of cell protrusions by small GTPases during fusion of the neural folds. *Elife*. 5:e13273. <https://doi.org/10.7554/eLife.13273>
- Rosenquist, G. 1966. Migration of precardiac cells in the chick embryo: A radioautographic study. *Contrib. Embryol.* 38:111–121.
- Saadaoui, M., D. Rocancourt, J. Roussel, F. Corson, and J. Gros. 2020. A tensile ring drives tissue flows to shape the gastrulating amniote embryo. *Science*. 367:453–458. <https://doi.org/10.1126/science.aaw1965>
- Samarage, C.R., M.D. White, Y.D. Álvarez, J.C. Fierro-González, Y. Henon, E.C. Jesudason, S. Bissiere, A. Fouras, and N. Plachta. 2015. Cortical tension allocates the first inner cells of the mammalian embryo. *Dev. Cell*. 34:435–447. <https://doi.org/10.1016/j.devcel.2015.07.004>
- Schaks, M., G. Giannone, and K. Rottner. 2019. Actin dynamics in cell migration. *Essays Biochem.* 63:483–495. <https://doi.org/10.1042/EBC20190015>
- Schimmel, L., D. Fukuhara, M. Richards, Y. Jin, P. Essebie, E. Frampton, M. Hedlund, E. Dejana, L. Claesson-Welsh, and E. Gordon. 2020. c-Src controls stability of sprouting blood vessels in the developing retina independently of cell-cell adhesion through focal adhesion assembly. *Development*. 147:dev185405. <https://doi.org/10.1242/dev.185405>
- Schroeder, T.E. 1970. Neurulation in *Xenopus laevis*. An analysis and model based upon light and electron microscopy. *J. Embryol. Exp. Morphol.* 23:427–462. <https://doi.org/10.1242/dev.23.2.427>
- Serralbo, O., D. Salgado, N. Véron, C. Cooper, M.J. Dejardin, T. Doran, J. Gros, and C. Marcelle. 2020. Transgenesis and web resources in quail. *Elife*. 9:e56312. <https://doi.org/10.7554/eLife.56312>
- Solon, J., A. Kaya-Copur, J. Colombelli, and D. Brunner. 2009. Pulsed forces timed by a ratchet-like mechanism drive directed tissue movement during dorsal closure. *Cell*. 137:1331–1342. <https://doi.org/10.1016/j.cell.2009.03.050>
- Stalsberg, H., and R.L. DeHaan. 1969. The precardiac areas and formation of the tubular heart in the chick embryo. *Dev. Biol.* 19:128–159. [https://doi.org/10.1016/0012-1606\(69\)90052-9](https://doi.org/10.1016/0012-1606(69)90052-9)
- Tyack, S.G., K.A. Jenkins, T.E. O'Neil, T.G. Wise, K.R. Morris, M.P. Bruce, S. McLeod, A.J. Wade, J. McKay, R.J. Moore, et al. 2013. A new method for producing transgenic birds via direct in vivo transfection of primordial germ cells. *Transgenic Res.* 22:1257–1264. <https://doi.org/10.1007/s11248-013-9727-2>
- Villasenor, A., D.C. Chong, M. Henkemeyer, and O. Cleaver. 2010. Epithelial dynamics of pancreatic branching morphogenesis. *Development*. 137:4295–4305. <https://doi.org/10.1242/dev.052993>
- Waterman, R.E. 1976. Topographical changes along the neural fold associated with neurulation in the hamster and mouse. *Am. J. Anat.* 146:151–171. <https://doi.org/10.1002/aja.1001460204>
- Williams, R.M., and T. Sauka-Spengler. 2021. Ex ovo Electroporation of early chicken embryos. *STAR Protoc.* 2:100424. <https://doi.org/10.1016/j.xpro.2021.100424>
- Xiong, F., W. Ma, B. Bénazeraf, L. Mahadevan, and O. Pourquie. 2020. Mechanical coupling coordinates the Co-elongation of axial and paraxial tissues in avian embryos. *Dev. Cell*. 55:354–366.e5. <https://doi.org/10.1016/j.devcel.2020.08.007>
- Yan, L., and D. Bi. 2019. Multicellular rosettes drive fluid-solid transition in epithelial tissues. *Phys. Rev. X*. 9:011029. <https://doi.org/10.1103/PhysRevX.9.011029>
- Zenker, J., M.D. White, M. Gasnier, Y.D. Alvarez, H.Y.G. Lim, S. Bissiere, M. Biro, and N. Plachta. 2018. Expanding actin rings zipper the mouse embryo for blastocyst formation. *Cell*. 173:776–791.e17. <https://doi.org/10.1016/j.cell.2018.02.035>

Supplemental material

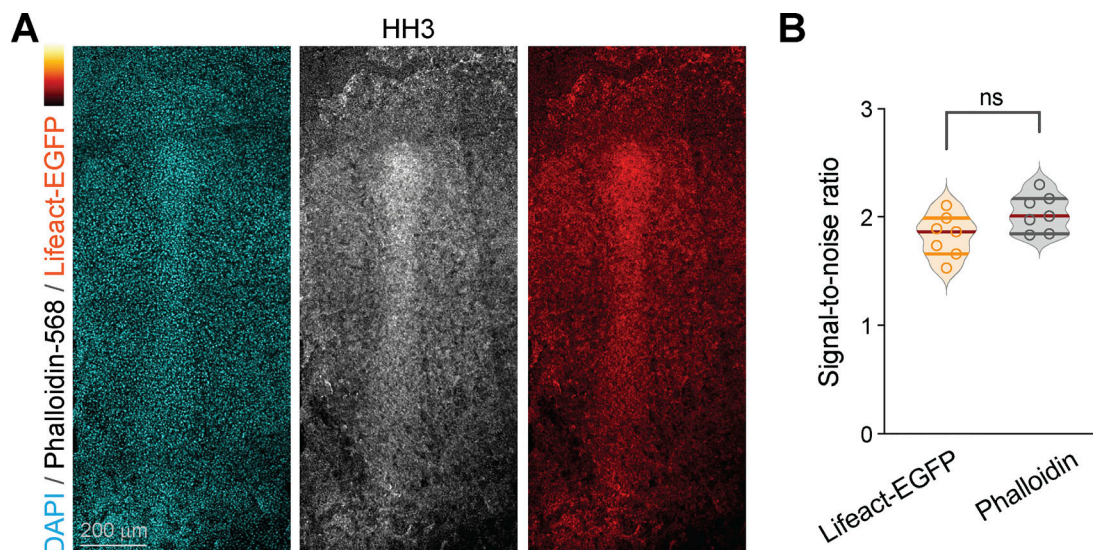


Figure S1. **Lifeact-EGFP is expressed at early embryonic stages and has comparable signal-to-noise to Phalloidin.** (A) The Lifeact-EGFP signal matches Phalloidin labeling of the actin cytoskeleton in the primitive streak at HH3. (B) There is no significant difference in the signal-to-noise of the Lifeact-EGFP and Phalloidin staining of fixed embryos in Fig. 1 ($n = 7$ ROIs from three embryos, Welch's two-sided t test).

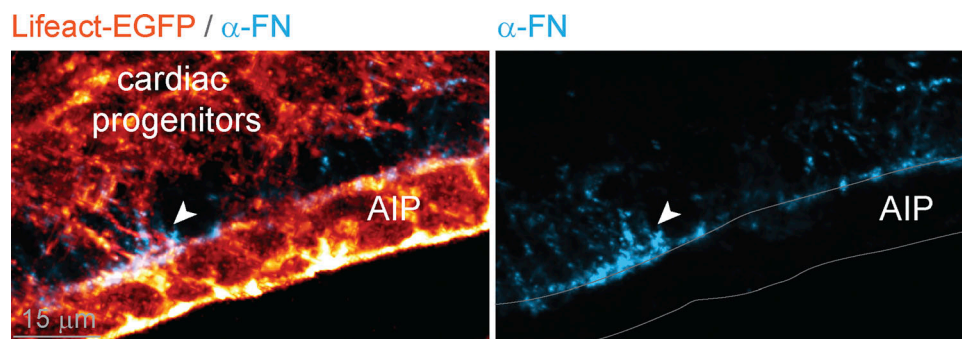


Figure S2. **Fibronectin is deposited on the surface of the Anterior Intestinal Portal.** Immunostaining shows fibronectin (blue) on the surface of the Anterior Intestinal Portal (AIP) where filopodia from migrating cardiac progenitor cells make contact (arrowhead). AIP has been outlined for clarity in the right panel.

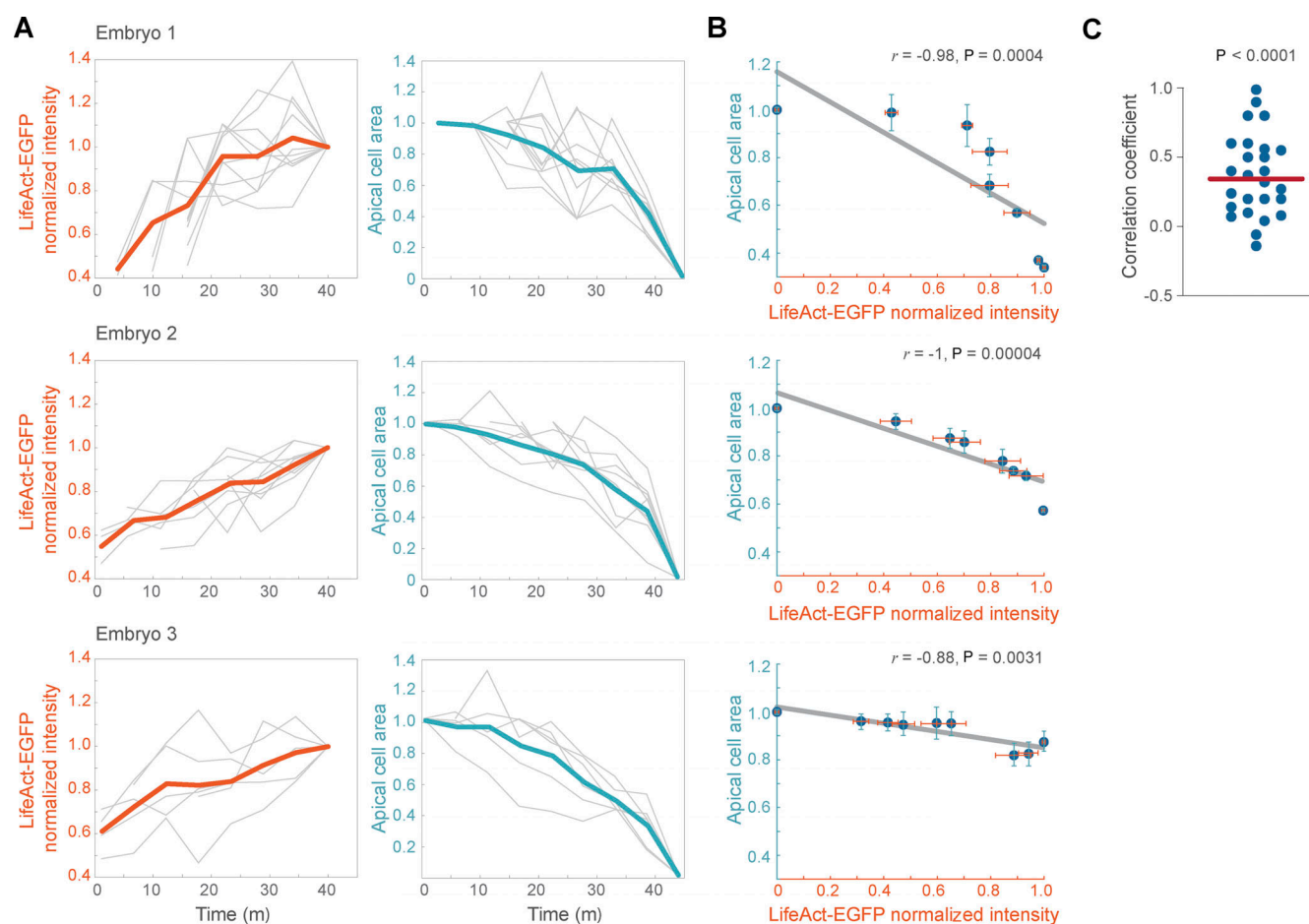


Figure S3. **The intensity of Lifeact-EGFP at the apical cortex is inversely correlated with apical cell area.** (A) The ratio of Lifeact-EGFP intensity at the apical cortex relative to the cell junctions increases as the apical cell area shrinks. Graphs show mean (colored line) and individual cells (grey lines) for the normalized Lifeact-EGFP intensity and apical cell area over time in three embryos. (B) Spearman's rank correlation was computed to assess the relationship between mean Lifeact-EGFP intensity at the apical cortex (medial/junctional) and mean apical cell area for each embryo. (C) Correlation coefficients for the change in Lifeact-EGFP intensity at the apical cortex versus the change in apical cell area for individual cells. There is a moderate, but highly significant correlation ($***P < 0.0001$, $n = 26$ cells from 3 embryos, one sample, two-sided t test).

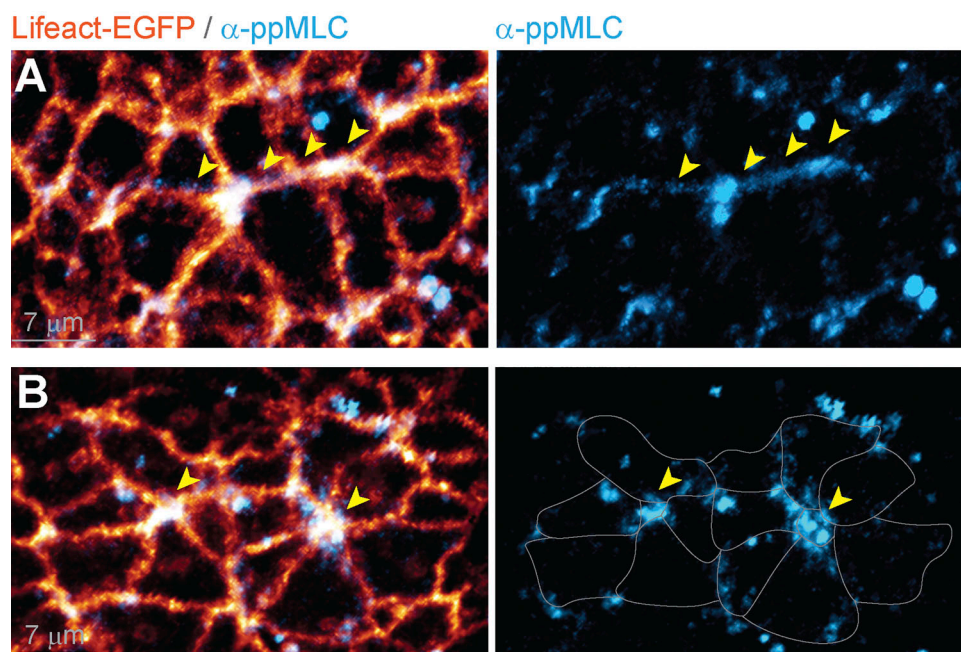


Figure S4. **Multicellular rosettes are contractile structures.** (A and B) Immunostaining reveals diphosphorylated myosin light chain (blue) is enriched along the shared mediolateral cell junctions (arrowheads) and in the central shared vertices of multicellular rosettes. The rosettes have been outlined for clarity in the right panel of B.

Video 1. **Live imaging of mesodermal cardiac progenitors crawling over the Anterior Intestinal Portal in the TgT2[UbC:Lifeact-EGFP] quail embryo at HH7–HH8 stage (E1).** Z-stack images were taken at 40× every 93 s. Mesodermal cardiac progenitor cells extend filopodia toward the surrounding tissues as the bilateral heart fields migrate toward the midline. The tissues surrounding the cardiac progenitor cells are pseudocolored in the last frame for clarity. Playback speed = 5 frames per second.

Video 2. **Live imaging of cellular protrusions during spinal neural tube closure in a TgT2[UbC:Lifeact-EGFP] quail embryo at stage HH9–HH10 (E1).** Protrusions close to the zipper point reach across the lumen and contact the opposing neural fold. Z-stack images were taken at 40× every 43.5 s. Playback speed = 5 frames per second.

Video 3. **Live imaging of cellular protrusions during spinal neural tube closure in a TgT2[UbC:Lifeact-EGFP] quail embryo at stage HH9–HH10 (E1).** Z-stack images were taken at 40× every 43.5 s. Playback speed = 5 frames per second.

Video 4. **High-speed spinning disk confocal imaging of the ectoderm of the TgT2[UbC:Lifeact-EGFP] quail embryo at HH8–HH9.** Images were taken at 60× every 5 s. Playback speed = 30 frames per second.

Video 5. **Live imaging of the neuroepithelium of the TgT2[UbC:Lifeact-EGFP] quail embryo at HH8–HH9.** Z-stack images were taken at 63× every 5.5 min. Apically constricting cells are highlighted by computational segmentation in blue. Playback speed = 3 frames per second.

Video 6. **Live imaging of the neuroepithelium of the TgT2[UbC:Lifeact-EGFP] quail embryo at HH8–HH9.** Z-stack images were taken at 63× every 5.5 min. Computationally segmented cells (purple) are forming a multicellular rosette. Playback speed = 3 frames per second.

Provided online are four supplemental text files. Text S1 is the MATLAB code for cell tracking. Text S2 is the MATLAB code for detecting apically constricting cells. Text S3 is the MATLAB code for the FRAP analysis. Text S4 is the MATLAB code for rosette detection.