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Report

Endocytosis Is Required for Efficient Apical Constriction during *Xenopus* Gastrulation

Jen-Yi Lee[1,*] and Richard M. Harland[1,*]
[1]Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA 94720, USA

Summary

Coordinated apical constriction (AC) in epithelial sheets drives tissue invagination [1, 2] and is required for diverse morphogenetic movements such as gastrulation [3], neurulation [4, 5], and organogenesis [6]. We showed previously that actomyosin contractility drives AC in *Xenopus laevis* bottle cells [7]; however, it remained unclear whether it does so in concert with other processes. Here we report that endocytosis-driven membrane remodeling is required for efficient AC. We found endosomes exclusively in bottle cells in the early gastrula. Disrupting endocytosis with dominant-negative dynamin or rab5 perturbed AC, with a significant decrease in constriction rate late in the process, suggesting that endocytosis operates downstream of actomyosin contractility to remove excess membrane. Additionally, disrupting endocytosis during neurulation inhibits AC in hingepoint cells, resulting in neural tube closure defects. Thus, membrane remodeling during AC could be a general mechanism to achieve efficient invagination in embryos.

Results and Discussion

Endosomes Are Present Only in Bottle Cells and Traffic along Microtubules

In *Xenopus laevis* embryos, cells in the dorsal marginal zone (DMZ) undergo apical constriction (AC) to form bottle cells, initiating blastopore invagination [8, 9]. Previously, we showed that taxol, which stabilizes microtubules, does not affect bottle cell formation, whereas nocodazole, a microtubule depolymerizing agent, disrupts AC; therefore, microtubules must be intact but need not be dynamic for AC [7]. Because microtubules are necessary for vesicular trafficking [10], we hypothesized that microtubules may facilitate AC by functioning as tracks for endosomes.

To determine whether endocytosis occurs in bottle cells, we employed a cell-impermeable activated biotin that was previously used to surface label *Xenopus* embryos [11, 12]; subsequently, internalized membrane could be visualized with fluorescent streptavidin. We observed biotin-labeled vesicles exclusively in bottle cells (Figure 1A), starting in stage 10 gastrulae (data not shown); therefore, the appearance of vesicles accompanies the onset of constriction.

To confirm that biotinylated vesicles represented endosomes, we used endosome markers EEA1 [13] and rab5-EGFP [14]; labeled vesicles colocalized with both markers, confirming that they are endosomes (Figures 1B and 1C). We therefore exploited the specificity and low background of biotinylation to assay endocytosis.

*Correspondence: jenyilee@berkeley.edu (J.-Y.L.), harland@berkeley.edu (R.M.H.)

To determine where endosomes traffic in bottle cells, we labeled stage 9 embryos with biotin, quenched the reaction, and fixed the embryos at 30 min intervals ("chase"). As cells underwent AC, the number of endosomes also increased, strengthening the correlation between endocytosis and AC. Endosomes trafficked away from the apical membrane, and some vesicles colocalized with the basolateral membrane (Figure 1D).

Previously, we showed that intact but not dynamic microtubules were required for AC [7]. If intact microtubules function as tracks for endosomes, then nocodazole treatment should prevent endocytosis, whereas taxol treatment should not. Indeed, in nocodazole-treated embryos, endosomes remained at the apical membrane (see Figure S1 available online). In contrast, taxol-treated embryos exhibited internalized vesicles indistinguishable from controls (Figure S1). Our data confirm that intact microtubules are required for endocytosis and show that endosomes move toward the basolateral membrane in bottle cells.

Perturbing Endocytosis Disrupts Apical Constriction

Because intact microtubules are required for both AC [7] and endocytosis (Figure S1), we postulated that endocytosis could be required for AC. To test this, we perturbed the initial step of endocytosis with dominant-negative (DN) dynamin, which weakly binds GTP, preventing endosome scission [15, 16]. Targeting DN dynamin to the DMZ perturbed blastopore formation (Figure 2A) but did not affect cell morphology when expressed in non-DMZ cells (Figure S2A). Despite the significant effect on AC (see below) and delay in blastopore formation, DN dynamin-injected embryos (referred to as DN dynamin embryos) developed with no obvious defects (Figure S2F). This is consistent with the finding that development occurs normally even after surgical removal of bottle cells [9].

To confirm that DN dynamin disrupts endocytosis, we counted endosomes in GFP and DN dynamin embryos. DN dynamin embryos had significantly fewer endosomes than control embryos (Figure 2B; Figure S2B), confirming that endocytosis was inhibited.

To determine the effect of inhibiting endocytosis on AC, we measured blastopore depth and bottle cell morphometric parameters, including apical width, apicobasal length, and apical index (length-over-width ratio, AI) [7]. DN dynamin blastopores were 36% shallower than GFP blastopores (Figures 2B and 2C). Additionally, DN dynamin bottle cells were significantly less constricted than control embryos, whereas apicobasal length was indistinguishable between the two groups (Figure 2C). The wider apices in DN dynamin bottle cells accounted for the significant decrease in AI compared to control (Figure 2C). These effects were rescuable with wild-type dynamin, illustrating specificity (Figures 2A–2C). Interestingly, these measurements recapitulate what we previously observed in nocodazole-treated embryos [7], suggesting an overlapping function between endocytosis and microtubules during AC. These results suggest that dynamin-mediated endocytosis is required for efficient AC in bottle cells.

To test whether DN dynamin affects bottle cell AC by perturbing cell fate [16], we examined *chordin*, *xbra*, and *sox17*

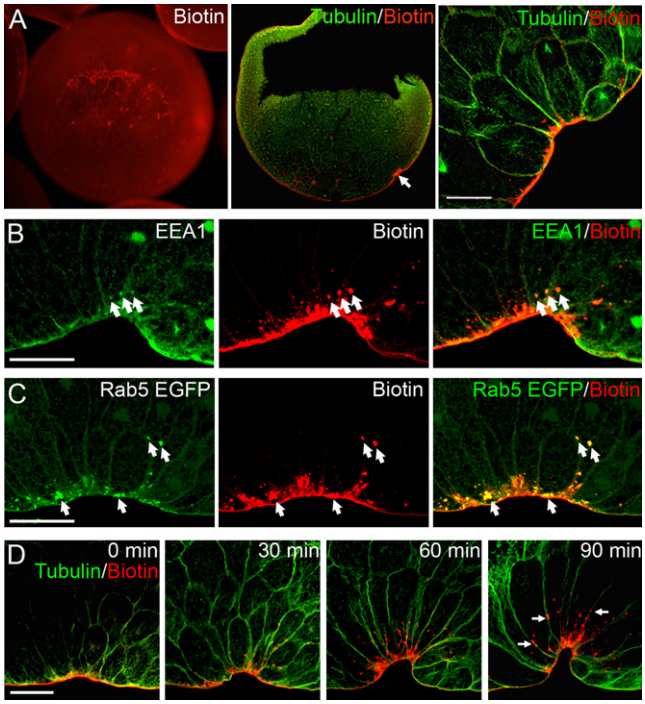

Figure 1. Biotinylated Vesicles Are Present Only in Bottle Cells in *Xenopus* Early Gastrula-Stage Embryos

(A) Vegetal view (left, epifluorescence), midsagittal section (center, confocal), and higher magnification of midsagittal section (right) of stage 10 embryos labeled with NHS-LC-sulfo biotin and anti-DM1α (tubulin). Arrow indicates bottle cells. Scale bar represents 25 μm.

(B) Confocal midsagittal sections of embryos stained with anti-EEA1 and biotin.

(C) Confocal midsagittal sections of embryos injected with rab5 enhanced green fluorescent protein (EGFP) mRNA, then fixed and stained with anti-GFP and biotin. Arrows in (B) and (C) indicate colocalization of biotin with early endosome markers.

(D) Confocal midsagittal sections of embryos following pulse-chase labeling with biotin. Embryos were fixed at the indicated time points and stained with anti-DM1α and streptavidin. Arrows indicate endosomes that appear to be on or near the basolateral membrane. In all midsagittal sections, images are oriented vegetal to the lower left; scale bars for (B)–(D) represent 50 μm. See also Figure S1.

expression as readouts of organizer, mesoderm, and endoderm fate, respectively. Expression was indistinguishable between controls and DN dynamin embryos (Figure S2E). Thus, DN dynamin does not affect AC indirectly through altering cell fate.

To independently assess the role of dynamin during AC, we injected translation-blocking antisense morpholino oligonucleotides (MO) targeting *dnm*-like and *dnm-2*, the two most abundant transcripts during gastrulation [17]. Whereas single MO injections at 160 ng did not affect endocytosis or AC, double injections of 80 ng of each MO significantly perturbed both processes (Figures S3A–S3C).

Because only the double morphants exhibited endocytosis and AC defects, we suggest that both isoforms are involved in bottle cell AC; moreover, the effect on endocytosis and AC is specific and not due to off-target effects. However, both single and double morphants exhibited a range of developmental phenotypes such as failure to close the blastopore, defective convergent extension, and embryonic lethality, presumably caused by MO-related toxicity (data not shown). Because DN dynamin effectively inhibited endocytosis without these deleterious side effects, we chose to use DN dynamin in our experiments as an effective, specific reagent to block dynamin function.

To further elaborate the role of endocytosis during AC, we inhibited rab5, a small GTPase that is required for early endosome sorting, by injection of DN rab5, which binds GTP weakly [18]. Internalized biotin was nearly undetectable in DN rab5 embryos, confirming inhibition of endocytosis (Figure 2D; Figure S2C). DN rab5 embryos had 33% shallower blastopores and less constricted bottle cells relative to uninjected embryos, with no differences in apicobasal length (Figure 2E). As with DN dynamin, cell fates were unaltered by DN rab5 (Figure S2E), consistent with the finding that DN rab5 does not affect activin signaling [19]. Compared to controls, DN rab5 tailbud-stage embryos were short and kinked (Figure S2G), indicative of defective convergent extension [20]. Interestingly, overexpression of constitutively active rab5 had no effect on blastopore formation or bottle-cell morphometrics (Figures S3D and S3E), suggesting that upregulating endocytosis at the level of rab5 cannot increase the rate or extent of AC. Together, the DN dynamin and DN rab5 results strongly argue that endocytosis is required for AC in bottle cells.

Endocytosis Is Required for Apical Constriction Downstream of Actomyosin Contractility

Although endocytosis is required for efficient AC, it did not completely abolish contraction. Thus, we hypothesized that actomyosin contractility folds the apical membrane into microvilli and that endocytosis is then required to remove the excess membrane.

This hypothesis makes three predictions. First, actin and myosin should be apically localized in both GFP and DN dynamin bottle cells. Indeed, both GFP and DN dynamin bottle cells exhibited apical localization of F-actin and activated myosin (Figure 3A), suggesting that endocytosis acts downstream of the establishment of the apical actomyosin machinery. The correct apical localization of F-actin and myosin in *Xenopus* bottle cells also indicates that endocytosis is not required for some aspects of polarity, as it is in *Caenorhabditis elegans* [21] and *Drosophila* [22].

The second prediction is that if endocytosis is required downstream of actomyosin contractility and membrane folding, then microvilli should be present at the apical surface of both GFP and DN dynamin bottle cells. Microvilli have been observed in many apically constricting cells, including bottle cells [23], where they contribute to reduction in apical surface area. Transmission electron microscopy revealed that, whereas animal cells exhibited unfolded apical membrane, both GFP and DN dynamin bottle cells contained numerous microvilli (Figure 3B). Apart from the difference in apical width, there were no apparent disparities between GFP and DN dynamin bottle cells, suggesting that membrane folding is not significantly disrupted in DN dynamin bottle cells.

The final prediction is that both GFP and DN dynamin bottle cells should initially constrict at similar rates but, as membrane accumulates, the DN dynamin bottle cells should slow down. To test this prediction, we monitored the apical surface area at the blastopores of embryos injected with membrane-enhanced GFP (EGFP) alone or GFP plus DN dynamin (Movies S1 and S2; Figure 3C). Measurement of apical surface areas at 3 min intervals over the course of 15 min revealed that both GFP and DN dynamin bottle cells with apices greater than 250 μm^2 exhibited nearly identical rates of AC, but, as expected, the constriction rate decreased as cells became