IN VIVO EXAMINATION OF THE MOLECULAR MECHANICS UNDERLYING APICAL CONSTRICTION'S INITIATION IN *C. ELEGANS* GASTRULATION

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ABSTRACT

Timothy Dennison Cupp: *In vivo* examination of the molecular mechanics underlying apical constriction's initiation in *C. elegans* gastrulation
(Under the direction of Bob Goldstein)

One remarkable finding from research in developmental biology is that surprisingly few cellular behaviors are responsible for the wide variety of morphogenetic events common among all eukaryotes. Molecular mechanisms underlying cell shape changes during tissue restructuring can explain how morphogenesis proceeds *in vivo*. During apical constriction, contractile myosin movements become linked to apical junctions, resulting in junctional pulling that can change cell shape. The process by which this dynamic linkage is achieved remains unknown, though it occurs with strict developmental timing in at least two systems. Since timing and patterning information instruct enrichment of specific mRNAs in the cells that apically constrict in *C. elegans*, we targeted these genes in an RNAi screen, identifying candidates that have an involvement in apical constriction. Our data suggest that zyxin, an important Focal Adhesion protein, may mediate the connections between the actomyosin cortex and adherens junctions during the initiation of apical constriction.

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LIST OF ABBREVIATIONS

C. elegans   *Caenorhabditis elegans*

CCC   Cadherin-Catenin Complex

dsRNAs   Double-stranded ribonucleic acids

E cell   Endodermal Precursor Cell

Focal Adhesions   FAs

Focal Adherens Junctions   FAJs

GFP   Green Fluorescent Protein

Gad   Gastrulation defective

MS cell   Mesodermal Precurosor Cell

TagRFP   Tag Red Fluorescent Protien

RacGEF   Guanine Exchange Factor for Rac (GTPase)

RNAi   Ribonucleic Acid Interference

RPKM   Reads Per Kilobase per Millio

CHAPTER 1: *IN VIVO* EXAMINATION OF APICAL CONSTRICTION.

INTRODUCTION

Apical constriction is a cell shape change that can drive tissue morphogenesis in nearly all metazoans[3]. Vertebrates utilize apical constriction to direct neural tube closure (**Figure 1**) and roughly 300,000 newborns suffer from neural tube closure defects worldwide annually[14]. During this developmentally regulated event, the tension arising from contractions within the actomyosin cortex is transmitted across cell junctions to pull on neighboring cells. The resulting change in cell shape ultimately drives tissue folding and invagination[3,4]. Identifying the key molecules involved and discerning how they behave during apical constriction is crucial to our understanding of this morphogenetic event. Our lab has identified a number of genes involved in apical constriction during *C. elegans* gastrulation, but the precise details of the mechanism are still murky[8,17,21]. The adhesive <u>c</u>adherin-<u>c</u>atenin <u>c</u>omplex (CCC), containing alpha-catenin, beta-catenin, and E-cadherin, becomes apically enriched in E cells upon myosin activation and is required for apical constriction to proceed, somehow dynamically connecting to the tensile cytoskeleton with proper timing. The exact protein-protein interactions that facilitate force transduction between the cytoskeleton and the CCC has been under intense debate in recent years (**Figure 4**)[30,31]. Some camps hold that actin can directly bind to alpha-catenin under certain conditions, though these experiments typically carry caveats stemming from their *in vitro* methodologies[32]. Here, we consider potential interactions that enable protein binding in a developing organism.

In *C. elegans*, two endodermal precursor cells (**E cells**) originate at the surface of the embryo when the E cell progenitor divides. Gastrulation begins at the 26- to 28-cell stage as the E cells apically constrict and internalize **(Figure 1, Figure 2B)**. *C. elegans* is well-suited for identifying and understanding the complex roles of candidate molecular triggers for apical constriction *in vivo*. Several factors make *C. elegans* an attractive candidate for this type of *in vivo* morphogenesis research. The organism's genetic tractability allows for direct edits to the genome, making direct, specific mutagenesis and fluorescent protein fusions a relatively straightforward task[15,16]. *C. elegans* embryos are also transparent, permitting direct observation of fluorescently-tagged protein dynamics throughout development. Because of the animal's short generation time, we can identify, tag, and image any protein of interest all within the span of a month. Since apical constriction progresses in such a spatiotemporally-stereotyped manner and because our model provides many *in vivo* experimental advantages, *C. elegans* is valuable for studying morphogenesis.

Developmental patterning is integrated with spatial information in E cells, instructing their decision to undergo apical constriction at the 26-28 cell stage in the early *C. elegans* embryo with precise timing. Expression of the *end-3* transcription factor confers an endodermal fate to these cells, which promotes the production and apical recruitment of the myosin light-chain kinase, MRCK-1, to apical contact-free cell surfaces[17].

MRCK-1's apical recruitment in E cells results in phosphorylation of myosin's regulatory light chain and its subsequent activation at the apical surface. The apical enrichment and activation of myosin provides the cortical tension that constriction

requires to proceed. Previous hypotheses posited that this sudden increase in tension within cells would alone be sufficient to initiate constriction. While the activation and apical localization of myosin is absolutely required for constriction to proceed[11,12], our lab has shown that these events are not themselves the immediate trigger of apical constriction in either *Drosophila* or *C. elegans*[5]. In fact, myosin's apical enrichment and activation precede the initiation of constriction by several minutes. Myosin activity seems to stabilize the adhesive structures at apical surfaces of cell-cell junctions by preventing the sequestration/internalization of cadherin-catenin complexes, but does not directly initiate tissue rearrangement[19,26].

During this period of increased tension within E cells' apical surfaces before the onset of apical constriction[5,17] myosin puncta flow centripetally along the cell's apical cortex without accompanying movement of associated cell membranes **(Figure 3)**. We refer to this phenomenon as "slippage." With precise timing, these myosin movements "couple" with the cell-cell junctions and apical constriction proceeds, suggesting that tension is transmitted through cell junctions to cytoskeleta of neighboring cells. During coupling, slippage is almost entirely eliminated. The precise mechanism of how myosin and membrane structures achieve this coupling with temporal accuracy remains a mystery.

One hypothesis that can explain this event's nature is the missing link model (**Figure 4**). In the missing link model, there is a disconnect between the actomyosin cortex and the cadherin-catenin complex of apical adherens junctions. According to this hypothesis, to establish a connection and accomplish apical constriction, some crucial protein (or set of proteins) is expressed, recruited, post-translationally modified, or

otherwise made available to interface between the actomyosin cortex and adherens junctions. The formation of this linkage results in force transmission between neighboring cells and constriction can occur.

To test this hypothesis and determine which proteins might be involved in forming this developmentally-regulated linkage, we decided to perform an RNAi screen. A single-cell transcriptomic analysis of the early *C. elegans* embryo provided the basis of our candidate list[9]. In this analysis, expression levels of each transcript were measured in each individual cell through the 16-cell stage of development. With this information, we constructed a candidate list of genes most highly enriched in E cells (16 cell stage) and their progenitor (8 cell stage). We hypothesized that at least one of the candidates in this list will be involved in forming a connection between adherens junctions and the contractile actomyosin network.

To determine which proteins from our candidate list might be involved in apical constriction, we designed the screen to seek defects in *C. elegans* gastrulation (Gastrulation defective = Gad phenotype). We injected dsRNA constructs targeting the candidate gene's mRNA into mature adult worm gonads. Injection of dsRNA leads to highly penetrant knockdown effects (as compared to RNAi feeding strategies)[27]. After knockdown, we established that an embryo is Gad if the E cells fail to fully internalize into the blastocoel before completing their first division. From our candidate list, we isolated a number of genetic targets that result in a Gad phenotype after knockdown in wild-type embryos. In particular, targeting zyxin (*zyx-1*) transcripts with dsRNAs resulted in a high incidence of Gad embryos in both wild-type and sensitized backgrounds. Due

to this phenotype and zyxin's mRNA enrichment in E cells around the time of gastrulation, we sought to consider zyxin's role in apical constriction.

By filming embryos expressing fluorescently-tagged proteins, we were able to track the movements of myosin puncta in relation to cellular borders during apical constriction. We depleted these embryos of zyxin mRNAs to scrutinize these molecular dynamics. In constricting cells, myosin puncta flow centripetally along the apical surface, apparently supplying tension to the cortical network[3,19,20]. As shown in **Figure 3**, wild-type E cells experience several minutes of slippage during which myosin flows without accompanying movement of the cell junctions. These cells then initiate constriction with precise timing and coupling occurs. After targeting zyxin for knockdown, we found that the slippage rate erroneously remained high during the latter stages of gastrulation. We speculate that this sustained slippage explains the Gad phenotype in zyxin-depleted embryos.

Zyxin has long been appreciated for its role in focal adhesion maturation in cells crawling along rigid surfaces, acting as a mechano-sensitive adapter protein between the integrin-signaling and cytoskeletal layers[10-13,18] We show here that zyxin is important even in the absence of a rigid substrate. Our data suggest that zyxin may have an additional role in strengthening the connections between the cytoskeleton and adherens junctions. Future experiments revealing zyxin's expression level, timing, recruitment, and localization will be instrumental in confirming its function during apical constriction.

EXPERIMENTAL APPROACH and METHODS

RNA interference

We injected 1 µg/µL of dsRNA in TE Buffer into the gonads of young adult worms. Each dsRNA construct was designed to target the first kilobase of each candidate gene's exonic code. After 36 hours incubation at 20°C, we dissected out dsRNA-treated embryos and mounted them laterally on glass coverslips no later than the 8-cell stage.

DIC and fluorescence microscopy

We filmed each embryo under DIC illumination at one minute intervals for 1.5 hours to measure its progression through gastrulation. In typical wild-type embryos, E cells don't divide until they have internalized entirely into the blastocoel and are fully covered by their neighbors. We consider an embryo to be Gad if this division occurs before the completion of E cell internalization.

Candidates with an apparent effect on gastrulation were further tested to establish any potential roles in apical constriction. DIC illumination of laterally mounted embryos can only tell us whether gastrulation has been successful **(Figure 6)**, but does not directly reveal the movements of apical components. To reveal these movements, we mounted embryos on their ventral surface so that the apical surfaces of E cells are exposed to the glass coverslip and imaged the ventral surface. Spinning disk confocal microscopy allowed collection of images of the entire apical surface of the E cell **(Figure 7A,B)**. Utilizing worms expressing a myosin-GFP fusion plus a PH domain-mCherry fusion (see Strains and Worm Maintenance, below), we were able to track their movements of myosin and membrane (into which the PH domain embeds itself) with

high temporal resolution (1 image every 3 seconds for 5 minutes), in order to measure the slippage rate during early and late stages of gastrulation. The MTrackJ plugin in ImageJ was used to track myosin and membrane movements and calculate their velocities.

Strains and worm maintenance

Nematodes with cultured and handled as described[28]. The following reporter and mutant strains were used: *MT4417 ced-5(n1812)*; *SU348 sax-7(eq1); LP54 mCherry::PH; NMY-2::GFP.* Imaging was performed at 20°C – 23°C for all strains listed.

RESULTS

Determining Candidate List for RNAi Screen

From the dozens of enriched transcripts in the E cells and the E cell progenitor, we considered three classes of genes: (1) genes whose predicted products are well-characterized and might serve a mechanical purpose; (2) genes with only a few predicted domains; and (3) genes whose products are likely not involved in apical constriction at all. We predict that products in the first class will either interact with the cytoskeleton directly or otherwise sense and transduce force via stretch-induced binding (perhaps revealing cryptic domains as in vinculin, talin) and signaling.

The second class of products only have a few domains listed, merely hinting at prospective functions. Their conceivable involvements in apical constriction range from cell-cell adhesion (e.g. C-type lectin fold protein encoded by F25D7.2) to cell signaling (e.g. tyrosine kinase and phosphatase domains are common features among candidates). Though these proteins aren't well-characterized, we can still offer simple hypotheses positing roles in gastrulation.

The final class of genes encoded lysosomal proteins, glycosylation proteins, Argonaut proteins, etc. and will likely have little direct involvement in the progression of apical constriction since they do not have any proposed mechanical function. We decided to exclude this set of genes was excluded from the screen. The final non-comprehensive list includes 28 candidate genes.

After being targeted for depletion, several candidates display a Gad phenotype

After treating embryos with dsRNAs targeting the candidate genes, we assayed embryos for their ability to progress through gastrulation[3,4]. E cells and the neighboring

MS cells (mesodermal precursor cells) are born within one minute of each other. These MS cells divide into four daughters 25 minutes following their birth, followed shortly thereafter by E cell apical constriction and internalization. In untreated wild-type embryos, E cells fully internalized in 16.2 (+/- 2.0) minutes following MS cell divisions **(Table 1, Figure 6)**. After completing internalization (when no part of either E cell is exposed to the embryo's ventral surface), each E cell divided in 3.7 (+/- 1.7) minutes. We found that targeting certain genes for knockdown often led to delays in internalization. In some cases, E cells failed to completely internalize before undergoing division **(Figure 7)**. This is nearly always due to a delay in internalization rather than a defect in cell division timing **(Table 1, Figure 6)**.

From the candidates tested, we isolated several genes which have an effect on gastrulation in the wild-type background. In addition to severely delaying E cell internalization, treating early embryos with dsRNAs also led to a $\geq$ 25% incidence of the Gad phenotype for 9/23 candidates tested in the wild-type background **(See Table 1, Figure 6)**. To overcome potential genetic redundancies, we also targeted some candidates for knockdown in sensitized genetic backgrounds (See Experimental Approaches and Methods). *Ced*-5, which encodes a RacGEF[38], acts redundantly with *hmr-1* (encoding cadherin) during *C. elegans* gastrulation[21]. Treatment of dsRNAs in the *ced-5* genetic null background led to a very high incidence of Gad phenotypes; 4/6 candidates were Gad in more than half of the embryos. SAX-7 is a transmembrane cell adhesion receptor molecule[29] that functions partially redundantly with cadherin during gastrulation[37]. 8 of the 13 candidates targeted for depletion in the *sax-7* null background displayed Gad phenotype in $\geq$ 25% of embryos. We speculate that these candidates

function in a pathway with cadherin to allow its physical linkage to cytoskeletal elements.

In comparison to earlier screening methods[21], our screen yielded in a high number of promising candidates with highly penetrant effects on gastrulation. Since these genes have an apparent effect on gastrulation, we wanted to uncover any direct roles in apical constriction. One promising candidate has been chosen for further analysis so far.

Determining zyxin's role in promoting completion of gastrulation

Knockdown of zyxin resulted in a noticeable phenotype in both wild-type and sensitized backgrounds. It has also been previously shown to have a role in mechano-sensitive actions. Zyxin is mainly appreciated for its role in biomechanical feedback during focal adhesion maturation[10,12], though recent evidence suggests it can also work at cell-cell contacts[24].

Zyxin is an attractive candidate for additional study for a number of reasons. For one, zyxin's expression pattern is among the most striking of all the candidates in terms of its E cell enrichment. These transcripts are 358 times more enriched in the E cell progenitor than in its neighbors **(Table 1, Figure 5B)**. Additionally, the effect of zyxin targeting on the early embryo is among the strongest that we observed for well-characterized proteins **(Table 1)**. Zyxin's known biomechanical function also offered a clear set of hypotheses detailing how it might function to initiate apical constriction. For these reasons, we predict that zyxin contributes to a developmentally-regulated assemblage that links actomyosin and adherens junctions. Loss of zyxin would weaken

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this connection, thereby preventing membranes from moving in tangent with myosin puncta.

Our gastrulation data raise the possibility that zyxin operates in apical constriction, but the screening method does not allow direct detection of this phenomenon. In our screen, we filmed laterally mounted embryos under DIC illumination. The apical surfaces of E cells face the embryo's ventral surface, so lateral mounts, while quick and technically easy to perform, are good for screens but do not allow for direct observation of constricting apical membranes.

High-speed fluorescent movies of ventrally mounted embryos allow us to measure myosin's rate of flow as well the adjacent cell membrane's rate of centripetal movement simultaneously. We define the difference in speeds as "slippage" between myosin and membrane. As in previous analyses, we define two stages of apical constriction for convenience: The early stage, a period spanning the 10 minutes following MS daughter division; and the subsequent late stage, when E cell apical surfaces actually constrict[5]. During the early stage, apical myosin flows centripetally without accompanying movement of associated membranes. In short, there is a high degree of slippage. As constriction begins, the membrane slowly begins to move at a similar rate as myosin. During this late stage, the slippage rate gradually falls to under 0.5 µm/min in control embryos.

After treating embryos with dsRNAs targeting zyxin, we found that there was no change in myosin's rate of centripetal apical flow in E cells during either the early stage or late stages. During the late stage, however, myosin movements fail to completely couple with membrane movements in each case. As a result, the average slippage rate

remained significantly high **(Figure 8)**. We conclude that depletion of zyxin in the early

C. elegans embryo prevents the tensile actomyosin cortex from linking to adhesive

junctional components, leading to defects in apical constriction and thus in gastrulation.

Zyxin's expression pattern and timing suggest that it may be temporally regulated by

expression timing, and together with these data, hints at a developmentally regulated

role in tissue morphogenesis.

DISCUSSION

C. elegans embryos employ a basic cellular behavior to drive gastrulation. This behavior, apical constriction, drives tissue rearrangement in many different biological systems[4,5]. During apical constriction, myosin contractions elicit tension within the actomyosin cortex. In a constricting cell, this tensile network somehow becomes mechanically connected to cellular junctions, and neighboring cells are pulled over its apical surface, inducing its internalization. The progression of this event is highly stereotypical and appears to be under tight developmental regulation in worms[4,6,17]. However, we can disrupt this process if we deplete early embryos of certain genetic factors. One such gene which appears to be vital to the progression of apical constriction is zyxin.

Direct injection of dsRNAs into adult gonads (in favor of feeding, where knockdown is less effective) and the unique construction of the RNAi candidate list yielded more penetrant effects on gastrulation than previous screening efforts[21]. After targeting zyxin for knockdown with dsRNAs for 36 hours, many embryos demonstrated a Gad phenotype. These defects arose in both sensitized (*sax-7* null, *ced-5* null) and wild-type backgrounds. Knockdown of other cytoskeleton-related transcripts, including alpha-Catulin (*ctn-1*), girdin (*grdn-1*), and formin (*cyk-1*), also led to an increased incidence of Gad embryos **(Table 1, Figure 6)**. Subsequent high temporal resolution imaging has allowed us to directly observe the molecular dynamics at play within constricting cells. These movies reveal that the Gad phenotype detected in zyxin-knocked down embryos is likely due to a defect in membrane-cytoskeleton coupling, as hypothesized. In these cases, slippage rates between myosin and membrane

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movements remain high abnormally late in the process. That is, myosin contractile movements do not efficiently couple with the cellular junctions during the late stages of gastrulation as they should. We hypothesize that zyxin's involvement in apical constriction is at the interface between the contractile cytoskeleton and components within adherens junctions at the apical surface.

The classic literature on zyxin presents this protein as a mechano-sensitive element that is chiefly involved in tension sensing at integrin-based focal adhesions during cellular migration[10,12]. Its stretch-induced recruitment to focal adhesions is required for the subsequent recruitment of other cytoskeleton-associated proteins, such as Ena/VASP and testin[11]. The ultimate consequence of stretch and thus zyxin recruitment appears to be the strengthening actin cables' attachment to adhesive structures on a rigid substrate. Recent evidence suggests that zyxin not only has a role in focal adhesions, but can also be found at the interface between two cells[24].

In a set of *in vitro* experiments[24], Oldenberg et al. pharmacologically induced stretch and used super resolution microscopy to view several members of the focal adhesion complex. They found that stretch induction results in the formation of "Focal Adherens Junctions (FAJs)" between cell neighbors. These FAJs contain zyxin, which is only recruited to cell borders in response to increased cellular tension. Furthermore, Ena/VASP and testin are only recruited to these structures in cells that have not been depleted of zyxin. In control cells, the stretch-induced recruitment of zyxin (and thus Ena/VASP and testin), leads to cytoskeletal strengthening, wherein stress fibers become thicker and more abundant. When zyxin is depleted, FAJs do not form and the actin cytoskeleton fails to develop a robust network of stress fibers. Ena/VASP and

testin are not completely required for cytoskeletal strengthening to occur, but may make the network slightly more robust or mediate an indirect interaction between actin, zyxin, and alpha-catenin. We hypothesize that this *in vitro* zyxin-dependent actin stabilization is also at play *in vivo* during the initiation of apical constriction.

We have previously shown that E cells receive developmental patterning information from cell intrinsic cues as well as signals from their neighbors. E cells integrate this information to prompt an increase in tension via MRCK-1 signaling at the apical surface[17]. Around this time, these cells also specifically expressing high levels of zyxin[9]. We believe that these events must align to properly regulate the timing of apical constriction initiation during *C. elegans* gastrulation.

In our model **(Figure 9)**, zyxin transcripts begin accumulating within E cells while MRCK-1 becomes apically enriched and begins activating myosin. While myosin activation produces tension within the apical cortex before constriction begins, zyxin is being translated and folded. The tensile cortex can then recruit folded zyxin to apical junctions. Once sufficient levels of zyxin have accumulated and integrated themselves into FAJs along with binding partners Ena/VASP and testin, apical constriction will begin. We believe this model can help explain the observed increase in slippage rate after zyxin for depletion in gastrulating embryos.

Future Directions

The following proposed experiments will help address specific questions regarding zyxin's role in apical constriction. One key question is whether zyxin directly physically acts to link the cytoskeleton to adhesive structures at cell junctions and how. What binding partners are necessary to allow zyxin to sense strain, move to cell

contacts, and form connections between components of clutch complex? We also aim to address the crucial question of how developmental regulation guides the progression of this event with temporal precision.

In ascertaining the role of zyxin in apical constriction, we will rely on the CRISPR/Cas system to genetically mutate zyxin, add fluorescent tags, alter cell fate, and change expression pattern to test our model. By attaching a fluorescent tag to zyxin, we will be able to measure its levels while also tracking its movement throughout the embryo. Our expectation is that the timing of zyxin accumulation and junctional recruitment in E cells will be coordinated with the onset of apical constriction. Below we list several experiments that will test the hypotheses stemming from our model **(Figure 9)**.

Genetic truncations and point mutations will reveal which domains are crucial to zyxin's function. Zyxin's N-terminus has been shown to interact with actin, while its C-terminal LIM domains bind with partners like Ena/VASP and testin[25]. An N-terminal truncation should keep zyxin from interacting with tensile actin, thus preventing it from sensing stretch or strengthening the apical cortical meshwork. A C-terminal truncation would prevent binding with other cytoskeletal interactors, but likely has only a marginal effect on cytoskeletal strengthening. If these hypotheses hold true, embryos with zyxin N-terminal truncations will experience abnormally high myosin/membrane slippage during late stages of apical constriction, while embryos with the C-terminal truncation will look relatively normal.

A crucial component of our model is that zyxin's action, under strict developmental regulation, acts with precise timing. We propose that this is due to the

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timing of its expression. To test this hypothesis, we will genetically encode zyxin's

expression to be under control of the *med-1* promoter), resulting in premature zyxin

expression. In this scenario, zyxin will accumulate in E cells several minutes before

MRCK-1 is able to activate myosin at the apical surface. Once myosin activation occurs,

however, we would expect the increase in apical tension to result in near-immediate

recruitment of zyxin to cell junctions. In short, apical constriction would initiate several

minutes early. These experiments will be crucial to explain how developmental

regulation instructs the precise timing of gastrulation in *C. elegans*.

Our model proposes a system in which we are able to comprehensively establish

a connection between developmental patterning and the physical mechanics of early

tissue morphogenesis. Forming this connection requires a set of experiments that will

alter cell fates within the developing embryo. These experiments will utilize the CRISPR

system to make MS cells express the E cell fate, for example. We hypothesize that

these "ectopic E cells" will express zyxin around the time of gastrulation while also

experiencing increased surface tension, ultimately undergoing apical constriction. These

data would provide support to the idea that zyxin and its developmental regulation are

the keystone to the initiation of apical constriction. It is alternatively possible that *end-3*

expression is absolutely required to link spatial information and force generation and

inform cells to constrict. If this is true, early expression of zyxin would not lead to

premature apical constriction.

If the proposed experiments are unable to substantiate zyxin's participation in

apical constriction, we still have many promising candidates from our screen to test.

Alpha-catulin (CTN-1) has domains resembling parts of both alpha-catenin and vinculin.

Since alpha-catenin is an integral part of CCCs[30] and vinculin has been implicated in FAJ formation[33], this is an intriguing candidate for future study. HUM-8 is an unconventional myosin that resembles human Myosin 6. This motor protein is involved in epithelial morphogenesis via its interaction with vinculin at cell borders and allows cells to form cohesive contacts[39]. A formin-related protein, CYK-1, can assemble actin filaments[34] and is directly involved in cytokinesis[35,36]. This direct mechanical action on the cytoskeleton makes it an attractive candidate. For many of our candidates, we only can distinguish a few protein domains. Proteins with kinase or phosphatase domains seem important, though their involvement in apical constriction, if any, is likely to be more indirect.

There is no doubt that a number of experiments are still necessary to fully demonstrate how tensile cortical actin can mechanically effect a cell shape change with precise developmental timing in a living organism. Our data have begun to unravel the linkage between development and biomechanics at the cellular level *in vivo*. With single-cell transcriptomic data informing a genetic screen, and with the ability to watch molecular dynamics *in vivo*, we have provided evidence suggesting an important role for zyxin in apical constriction. We believe the experiments proposed above will further illuminate its role in connecting contractile actomyosin to adhesive structures at cell junctions. Zyxin likely acts with one or more partners throughout this process, and the results from our genetic screen will be a valuable source of information when deconstructing partial redundancies within the system. These data provide us with the beginnings to a course of research that will help us form a thorough understanding of the overlap between mechano- and developmental biology.

APPENDIX 1: TABLE 1 – RESULTS OF RNAi SCREEN RESULTS

KD target	Stage (cells)	Enrichment (Mean RPKM in E over MS)	Proposed Function[29]	Genetic Background	MS div → E intern (min)	MS div → E div (min)	% Gad (n/n)
Negative Control	--	--	--	N2 (wild-type)	16.2± 2.0	19.9± 1.7	0 (20/20)
acp-2	16	878.64/0.2	acid phosphatase	N2 (wild-type)	18.8± 3.4	19.3± 1.6	12.5 (2/16)
add-1	8	8.2/0.0	alpha-adducin: cytoskeleton signaling	N2 (wild-type)	15.7± 2.2	20.5± 3.8	7.7 (1/13)
C46E10.8	8	108.94/0.0	zinc-finger protein	N2 (wild-type)	16.5± 5.7	22.8± 4.8	50.0 (6/12)
C29F7.2	16	955.98/0.45	kinase-like domains	N2 (wild-type)	19.1± 4.8	23.5± 4.8	27.3 (3/11)
C26F1.1	16	699.64/0.57	novel	N2 (wild-type)	17.0± 7.7	18.7± 4.8	0 (0/9)
ctn-1	8	48.48/0.05	alpha-catulin: cytoskeleton, adhesions	N2 (wild-type)	20.0± 8.4	27.6± 7.7	20.0 (2/10)
cyk-1	8	1.08/12.3	formin, actin polymerization	N2 (wild-type)	18.3± 7.9	18.8± 4.5	23.1 (3/13)
dve-1	16	857.9/0.0	CMP domain, RAS signaling	N2 (wild-type)	13.8± 1.7	18.0± 2.6	0 (0/4)
F25D7.5	8	40.93/0.07	C-type lectin fold	N2 (wild-type)	14.6± 2.9	19.2± 0.8	0 (0/5)
F49E10.4	8	134.5/0.42	mitochondria-eating protein	N2 (wild-type)	20.5± 4.5	26.4± 3.6	0 (0/13)
grdn-1	8	9.8/0.0	actin-associated	N2 (wild-type)	11.6± 10.8	19.6± 7.0	33.3 (3/9)
H24G06.1	8	45.86/0.0	mitogen-activated protein kinase binding protein	N2 (wild-type)	15.0± 3.6	20.3± .7	20.0 (2/5)
hum-8	16	26.48/0.0	unconventional myosin	N2 (wild-type)	24.8± 7.9	20.6± 1.7	66.7 (6/9)
let-4	8	25.06/0.0	organizes ECM	N2 (wild-type)	11.9± 2.3	19.6± 4.0	0 (0/17)
pssy-1	8	137.97/1.94	phosphatidylserine synthase	N2 (wild-type)	17.0± 5.2	23.6± 2.4	7.1 (1/14)
R05D3.2	16	32.49/0.2	LMBR1 human ortholog – Shh signaling	N2 (wild-type)	23.4± 7.7	23.5± 2.9	51.3 (7/13)

KD target	Stage (cells)	Enrichment (Mean RPKM E / MS)	Proposed Function[29]	Genetic Background	MS div → E intern (min)	MS div → E div (min)	% Gad (n/n)
R06B10.2	8	6.19/.16	tyrosine phosphatase	N2 (wild-type)	23.7± 1.2	22.6± 5.6	71.4 (5/7)
T14E8.1	8	13.7/0.0	tyrosine kinase	N2 (wild-type)	19.4± 5.3	21.3± 1.6	18.2 (2/12)
tnc-2	8	54.46/0.32	troponoin	N2 (wild-type)	16.3± 2.9	19.3± 1.0	0 (0/9)
Y53C10A. 10	8	19.16/0.0	novel	N2 (wild-type)	17.5± 3.1	22.4± 2.1	8.3 (2/12)
Y57G11C. 6	8	32.48/0.18	tyrosine phosphatase	N2 (wild-type)	18.2± 2.6	23.1± 1.1	30 (3/10)
zig-5	8	27.05/0.0	secreted immunoglobulin	N2 (wild-type)	17.2± 4.3	23.0± 4.1	37.5 (3/8)
zyx-1	8	75.28/0.21	zyxin, focal adhesions	N2 (wild-type)	18.6± 4.0	22.8± 5.4	25 (5/20)
Negative Control – sax-7 (eq1)	--	--	--	sax-7 (eq1)	16.7± 2.3	21.3± 1.8	0 (0/7)
btb-15	16	132.4/1.9	zinc-finger protein	sax-7 (eq1)	16.0± 1.4	19.5± 1.3	0
C29F7.2	16	955.98/0.45	kinase-like domains	sax-7 (eq1)	18.7± 18.8	21.6± 6.7	28.6 (2/7)
C26F1.1	16	699.64/0.57	novel	sax-7 (eq1)	29.7± 12.0	23.8±	66.7 (4/6)
cyk-1	8	1.08/12.3	formin, actin polymerization	sax-7 (eq1)	23.0± 12.8	24.3± 5.6	73.3 (11/15)
acp-6	16	72.27/0.0	acid phosphatase	sax-7 (eq1)	18.0± 4.1	22.2± 1.6	20.0 (1/5)
F49E10.4	8	134.5/0.42	mitochondria-eating protein	sax-7 (eq1)	23±4	31±7	9.1 (1/11)
grdn-1	8	9.8/0.0	actin-associated	sax-7 (eq1)	26.5± 16.6	24.3± 5.6	56.5 (13/23)
hex-5	16	220.17/0.0	hexosaminidase	sax-7 (eq1)	23.9± 3.4	27.0± 6.8	33.3 (3/9)
kin-9	8	45.82/0.12	tyrosine kinase	sax-7 (eq1)	18.7± 1.2	22.3± 3.7	50.0 (2/4)
let-4	8	25.06/0.0	organizes ECM	sax-7 (eq1)	12.4± 1.5	18.0± 1.0	0 (0/4)
tnc-2	8	54.46/0.32	troponin	sax-7 (eq1)	20.5± 3.5	23.7± 1.2	33.3 (1/3)
zyx-1	8	75.28/0.21	zyxin, focal adhesions	sax-7 (eq1)	24.4± 11.2	24.5± 6.2	66.7 (10/15)

KD target	Stage (cells)	Enrichment (Mean RPKM E / MS)	Proposed Function[29]	Genetic Background	MS div → E intern (min)	MS div → E div (min)	% Gad (n/n)
ZK1053.3	8	53.07/0.0	novel	sax-7 (eq1)	23.7± 11.6	24.3± 1.2	33.3 (1/3)
Negative Control – ced-5 (n1812)	--	--	--	ced-5 (n1812)	17.4± 1.9	20.0± 2.2	0 (0/5)
acp-2	8	878.64/0.2	acid phosphatase	ced-5 (n1812)	33.7± 9.8	25.8± 7.2	69.2 (9/13)
btb-15	16	132.4/1.9	zinc-finger protein	ced-5 (n1812)	22.6± 3.3	19.5± 2.1	40.0 (2/5)
F49E10.4	8	134.5/0.42	mitochondria-eating protein	ced-5 (n1812)	23.7± 6.4	19.2± 3.3	83.3 (5/6)
grdn-1	8	9.8/0.0	actin-associated	ced-5 (n1812)	16.8± 5.7	22.0± 5.5	0 (0/4)
tnc-2	8	54.46/0.32	troponin	ced-5 (n1812)	32.4± 8.5	22.9± 5.8	72.7 (8/11)
zyx-1	8	75.28/0.21	zyxin, focal adhesions	ced-5 (n1812)	27.6± 9.2	22.2± 3.0	62.5 (5/8)

Table 1 – **Results of RNAi screen.** "KD target" column gives gene name or Cosmid ID for each targeted gene. "Enrichment" and "Stage" columns show data from Tintori et al[9], comparing Reads Per Kilobase per Million (RPKM) between an E cell precursor/MS cell precursor pair (8 cell stage of embryogenesis) or an E cell/MS cell pair (16 cell stage). Three genetic backgrounds were used to avoid potential redundancies in the system. Sax-7 (eq1) is a null allele of a Cell Adhesion Molecule and Ced-5 (n1812) is a null allele of a cell engulfment protein involved in Ras signaling. The column "MS division → E internalization" shows the time it takes an E cell to fully internalize (see text for definition) following MS division. +/- represents 95% confidence interval. The column "MS division → E division" displays how long after MS division E cells divide with +/- representing 95% confidence. The "% Gad" column shows the proportion of dsRNA-treated embryos that were Gastrulation defective.
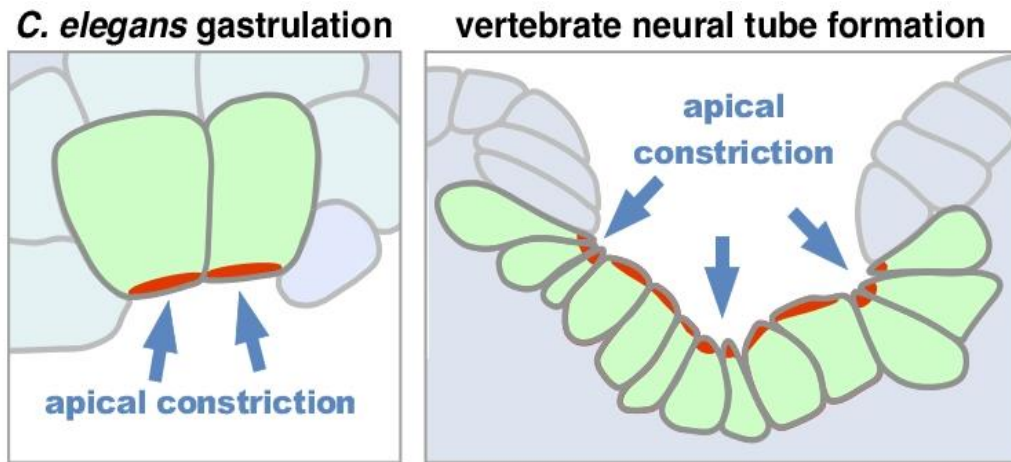
Figure 1. Apical constriction is a conserved process in *C. elegans* gastrulation as well as vertebrate neural tube formation. The cells highlighted in green are E cells in *C. elegans* and neural plate cells in vertebrates and undergo apical constriction. Myosin is enriched and activated (red) in the apical cortex of specific cells, eliciting tension[3,8] *Adapted from Bob Goldstein.*
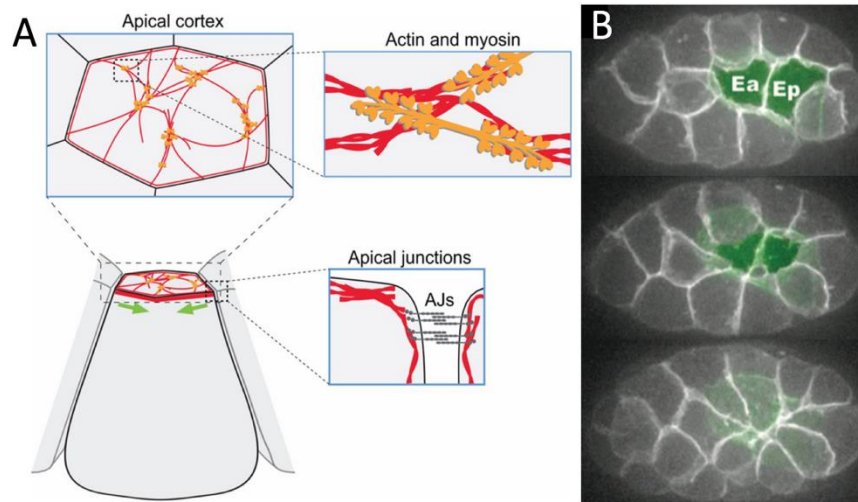
Figure 2. *C. elegans* **embryos achieve gastrulation via apical constriction.**
(A) Apical constriction initiates when the tensile actomyosin network physically connects to apical adherens junctions. This connection appears to form with precise timing in gastrulating *C. elegans* embryos. (B) shows a ventral view of a gastrulation-stage embryo, with E cells (Ea, anterior; Ep, posterior) pseudocolored in green undergoing apical constriction. *Adapted from Martin & Goldstein 2014 and Lee et al. 2006.*
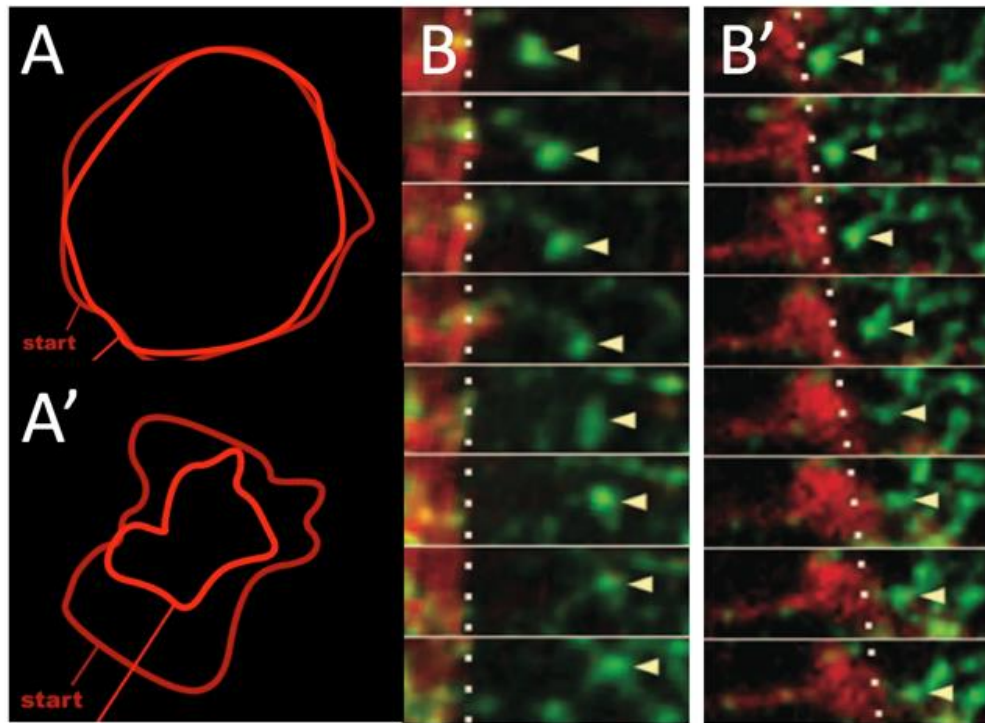
Figure 3. Pseudo-kymograph of apical myosin (green) and membrane (red) dynamics during apical constriction. (A) Apical membrane trace of an E cell in the "Early" Stage at 0 and +3 min. **(A')** Apical membrane trace of an apically constricting E cell in the "Late" stage at 0 and +3 min. **(B), (B')** show the molecular dynamics associated with **(A)** and **(A')** respectively. Early **(A, B)**, there is a large degree of slippage between myosin (green) and membrane (red). Late **(A', B')**, there is a low degree of slippage, and constriction proceeds. *Adapted from Roh-Johnson et al. 2012*
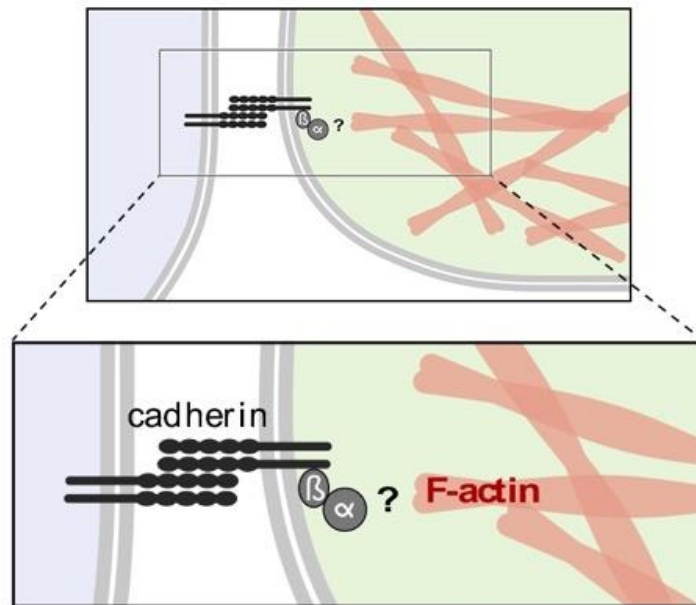
Figure 4: The temporally-regulated link between adherens junctions and F-actin.
The molecular link between the cadherin-catenin complex and F-actin in apically
constricting cells remains a mystery, and continues to be a source of controversy.
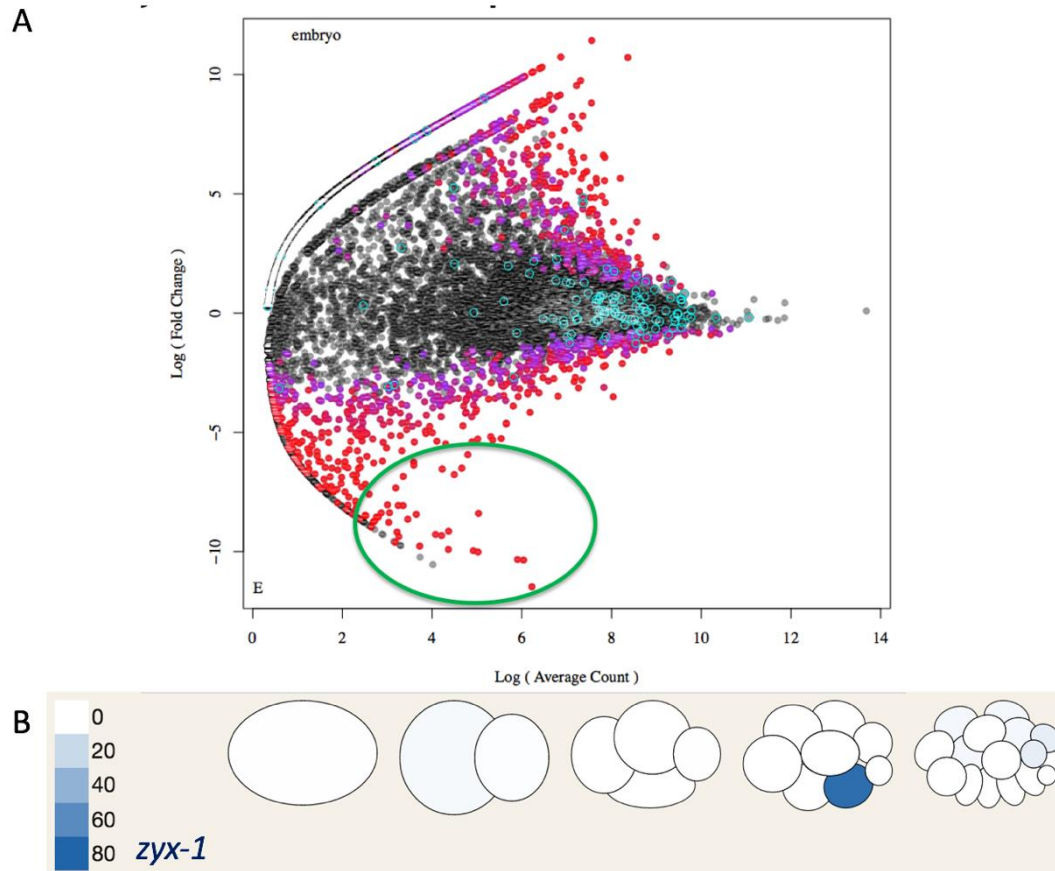*From Bob Goldstein*

Figure 5. Choosing candidates based on expression profile in the early embryo.
(A) Genes with transcriptional enrichment in a given cell (8-cell stage E cell progenitor here) are colored red. Candidates considered were chosen from within the circle. **(B)** Zyxin is highly enriched in the E cell progenitor cell at 80 RPKM at the 8-cell stage, compared to its low levels in all other neighboring cells. *Adapted from Tintori et al. (2016)[9].*
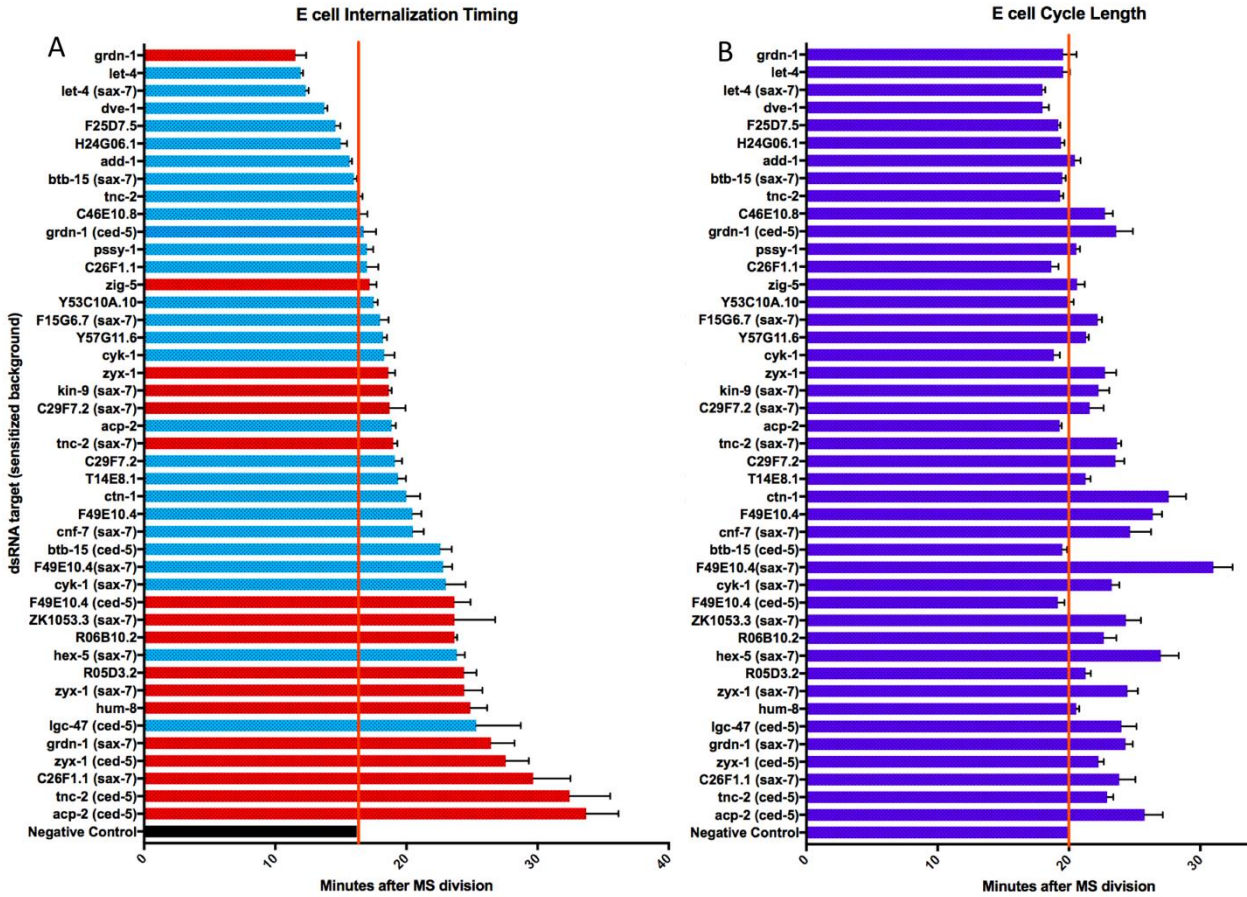
Figure 6. Results of RNAi screen. Graphical Representation of data from **Table 1.** Refer to **Table 1** legend for details. **(A)** Timing of E cell Internalization, corresponding to the "MS div > E intern (min)" Column. Red Bars represent a gastrulation defect incidence in $\geq$25% of tested embryos. **(B)** Timing of E cell division, corresponding to the " MS div > E div (min)" Column.
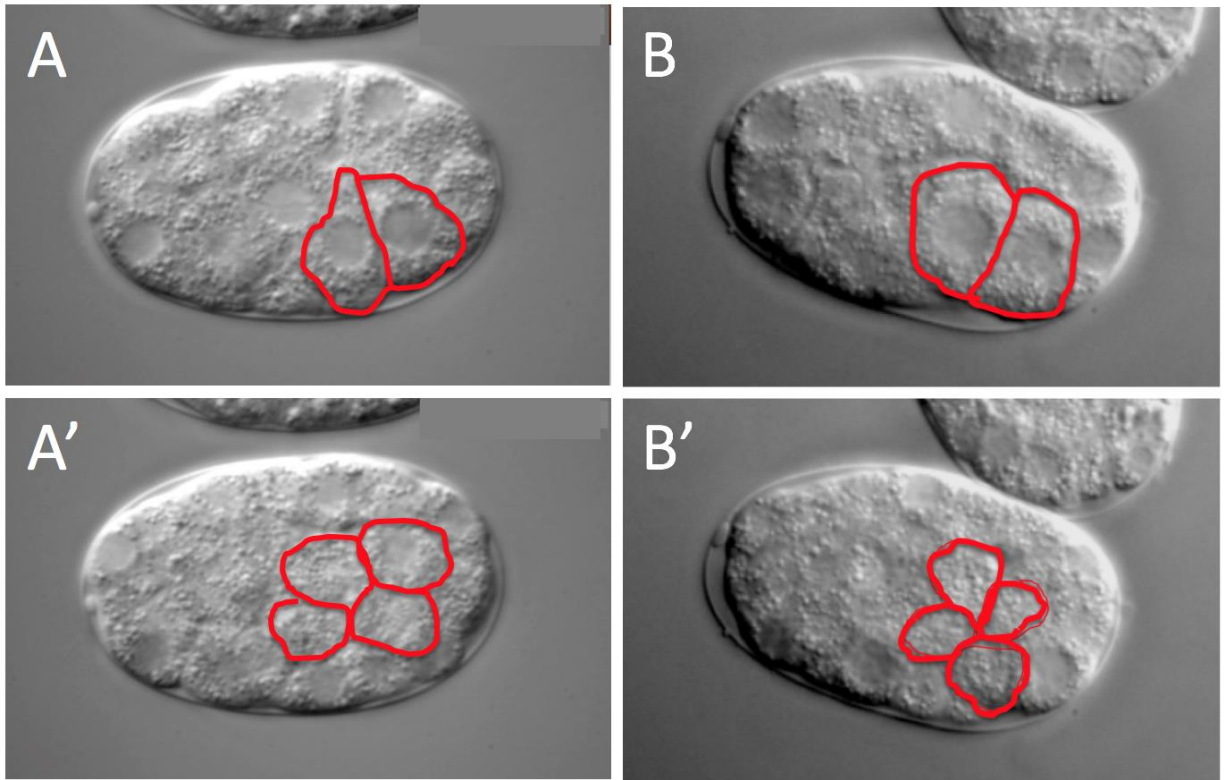
Figure 7. E cells divide at ventral surface in Gad embryos. Lateral mounted embryos at the gastrulation stage. **(A, A')** Wild-type progression of gastrulation where E cells (top, red outline) divide after fully internalizing. **(B, B')** A Gad embryo after targeting zyxin for depletion, where an E cell daughter is born at the ventral surface. **(A, B)** 90 minutes after fertilization. **(A', B')** 120 minutes after fertilization.
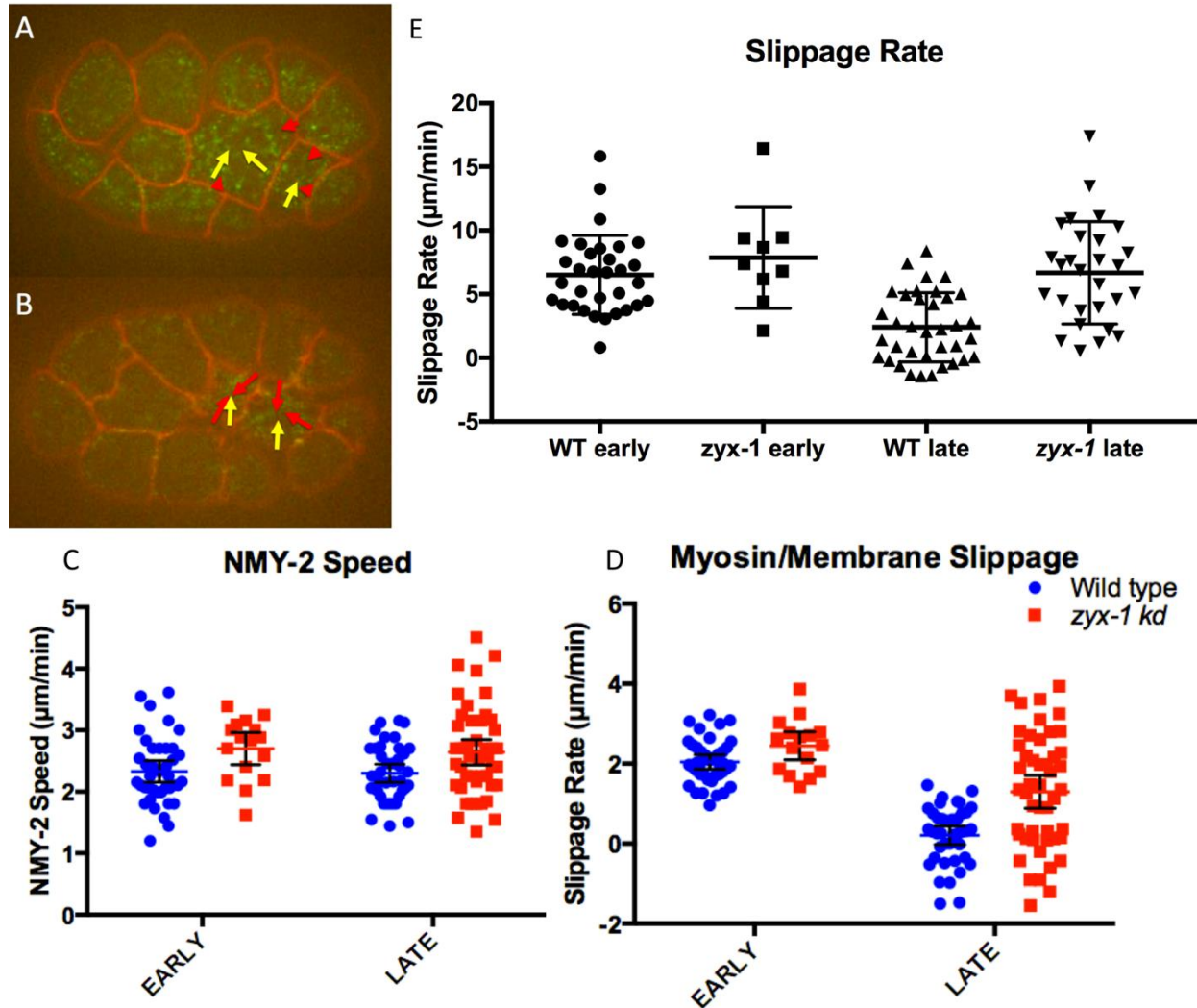
Figure 8. Slippage Rate remains abnormally high after zyxin depletion. (A,B)
Snapshot of ventrally mounted wild-type embryos in Early and Late stage, respectively.
Yellow arrows represent myosin puncta movements in E cells. Red arrows represent
movement of associated membrane. **(C)** Myosin's rate of centripetal flow does not
change from Early to Late stage. The dsRNA treatment targeting zyxin does also not
cause a change in myosin flow rate. **(D)** In both wild-type (blue circiles) and zyxin-
depleted (red squares) embryos, there is a high degree of slippage between myosin
and membrane in Early Stage E cells. The slippage rate in Late Stage E cells zyxin-
depleted embryos remains high abnormally. **(E)** The same embryos analyzed by
kymography in **C** and **D** were independently measured using the ImageJ mTrackJ
plugin in a double-blind study (credit: Terrance Wong). **(C,D,E)** n = 5 wild-type and 6
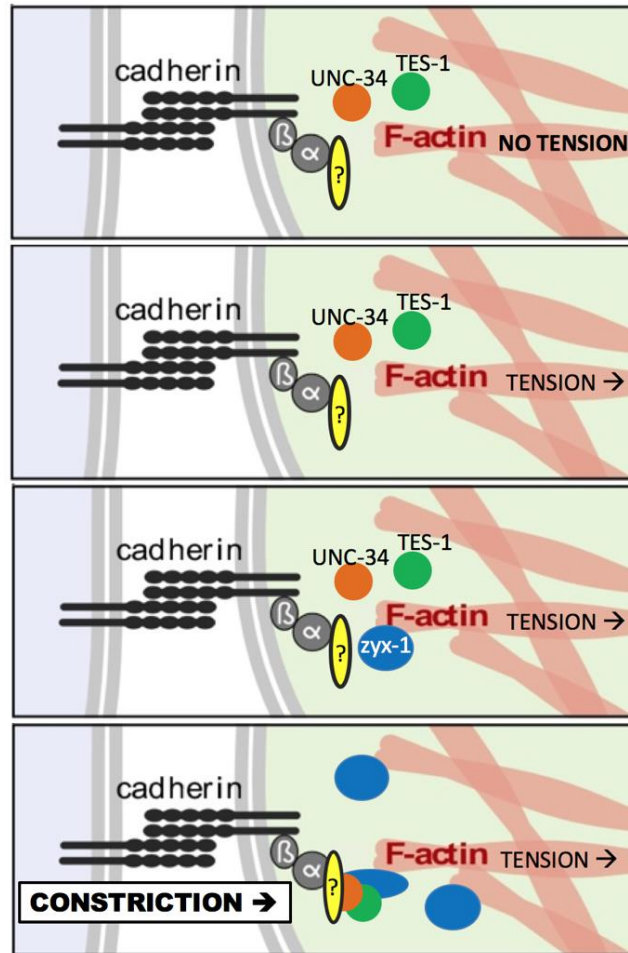*zyx-1*-targeted embryos.

Figure 9. Our model of zyxin's action during apical constriction. In our model, an early increase in tension fails initiate constriction because the actomyosin network is not connected to the cadherin-catenin complex. Once zyxin has accumulated to sufficient levels, it forms a complex between actin, Ena/VASP (UNC-34), Testin (TES-1), and the CCC at apical cell junctions, allowing constriction to proceed.

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