INVESTIGATION OF THE MOLECULAR MECHANISMS LINKING CELL-FATE SPECIFICATION AND SHAPE CHANGE IN *C. ELEGANS* GASTRULATION

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ABSTRACT

Allyson Marie Roberts: Investigation of the molecular mechanisms linking cell-fate specification and shape change in *C. elegans* gastrulation
(Under the direction of Bob Goldstein)

There is incredible diversity amongst eukaryotic tissues and overall body plans. Changes in individual cell shapes are essential for generating higher levels of complexity through morphogenetic events. Various molecular mechanisms are responsible for driving cell shape changes during development under tight spatial and temporal regulation. Apical constriction is a process by which cells shrink their apical surfaces through actomyosin contractions to orchestrate tissue-bending events including vertebrate neural tube formation and gastrulation. *C. elegans* is a valuable system for studying mechanisms that drive apical constriction *in vivo*, including how one endodermal cell fate-specifying transcription factor, END-3, drives apical constriction of two endodermal cells, thus beginning gastrulation of the embryo. Here, we screened for new gastrulation genes from among genes regulated by END-3 using RNAi and identified 7 new candidate END-3 targets that contribute to gastrulation. We predict that some of these genes may directly or indirectly regulate apical constriction and gastrulation.

To Bob and Joel, my mentors through different tides.
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LIST OF ABBREVIATIONS

C. elegans	*Caenorhabditis elegans*
dsRNA	Double-stranded ribonucleic acid
EPC	Endodermal precursor cell
Gad	Gastrulation defective
MS cell	Mesodermal precursor cell
RNAi	Ribonucleic acid interference
RNA-seq	Ribonucleic acid sequencing
RPKM	Reads Per Kilobase of transcript, per Million mapped reads

CHAPTER 1: INVESTIGATION OF THE MOLECULAR MECHANISMS LINKING CELL-FATE SPECIFICATION AND SHAPE CHANGE IN *C. ELEGANS* GASTRULATION

Introduction

Apical constriction is a process that drives tissue morphogenesis during metazoan development. In apical constriction, epithelial cells alter their shapes by constricting just the apical side of the cell. This simple change can remodel entire tissues and sculpt various organs including the gut and, in vertebrates, the neural tube[1] **(Figure 1)**. During vertebrate embryonic development, neurulation is driven by apical constriction[2]. It is through neurulation that the neural tube—which will subsequently become the brain and spinal cord—is formed. In mammals, improper or incomplete neurulation can lead to birth defects such as spina bifida, a neural tube defect that occurs in approximately 1 per 1,000 births worldwide[3]. While some prenatal therapeutic treatments against neural tube defects have emerged[4], and many efforts have been made to understand the mechanisms of apical constriction[5], there are still several central questions that remain unresolved surrounding the mechanistic details.

Apical constriction is driven by contractions of the actomyosin cortex, an expansive intracellular network of the actin cytoskeleton and the motor protein non-muscle myosin II. These contractions generate tension at the cellular level that is transmitted across the epithelial layer through cell-cell junctions[1]. Focused along the apical surfaces of neighboring cells, the generated tension acts as a pulling force to shrink the apical

surface, often forming wedge-shaped cells that together orchestrate tissue folding and invagination[6] **(Figure 2A)**.

The nematode *Caenorhabditis elegans* has proven to be a valuable system for understanding the mechanistic details of cell shape change during apical constriction *in vivo*. In *C. elegans*, gastrulation begins with the internalization of two endodermal precursor cells (EPCs)[7]. These two cells are born on the surface of the embryo, change shape through apical constriction, and move inwards towards the center of the embryo until their surfaces are fully covered by neighboring cells **(Figure 2B)**. We note that "apical" is defined differently across biological systems; in the *C. elegans* embryo, "apical" refers to the outer (exposed) cell surface facing the vitelline envelope, while "basolateral" refers to surfaces that are in contact with adjacent cells.

This process is developmentally coordinated and extremely consistent; apical constriction occurs in the same cells with predictable timing across embryos. Individual cells can be identified due to the fact that asymmetry in the *C. elegans* embryo is initiated at fertilization, with the anteroposterior axis being specified by the point of sperm entry[8]. Beginning from the one-cell stage, many subsequent divisions of the early embryo are asymmetric and characteristic, such that cell identity and lineage can easily be tracked[9].

Additionally, *C. elegans* is easily amenable to both genetic and cellular manipulations, and its transparent appearance facilitates subcellular live imaging. The genetic tractability of *C. elegans* permits direct mutagenesis and the creation of endogenous fluorescent protein fusions[10,11]. *C. elegans* also has rapid development: the entire embryonic development of these organisms occurs in 12 hours[12]. These

characteristics make *C. elegans* an extremely useful system for studying apical constriction *in vivo* in a rapid and reproducible manner.

In the *C. elegans* embryo, gastrulation begins at the 26- to 28-cell stage with the apical constriction and internalization of the two EPCs. These two cells, termed Ea and Ep (for E-anterior and E-posterior), originate at the surface of the embryo when born from their mother E cell progenitor. During apical constriction, these two cells shrink their exposed apical surfaces and internalize into the center of the embryo until their entire apical surface is covered by neighboring cells[13]. For simplicity, we use "gastrulation" in this work to refer only to the internalization of Ea and Ep; although, to complete gastrulation in its entirety, this is later followed by internalization of precursor cells of muscle, germline, and other lineages[9].

Previous experiments done by our lab and others have characterized in detail the processes of apical constriction and gastrulation in *C. elegans*. In the internalizing EPCs, cell fate specification and apical constriction appear to be tightly coordinated processes. Within *C. elegans*, endodermal cell fate is specified by the combined action of two partially redundant, endoderm-specific GATA-type transcription factors, END-1 and END-3[14,15]. In the absence of these factors, embryos do not specify endoderm and do not form a gut, ultimately resulting in either embryonic or larval lethality[15,16]. Recent studies have also shown that apical constriction in the E cells occurs via the activation of apically-localized myosin through the myosin kinase MRCK-1, another protein required for apical constriction and gastrulation of these cells[17]. In embryos deficient for END-1 and END-3, or deficient for END-3 alone, Ea and Ep do not apically constrict and do not recruit MRCK-1 to the apical surface[17]. Thus, in addition to directly specifying endodermal fate, it

appears that these partially-redundant endodermal GATA factors somehow also contribute to apical constriction, though their role in this mechanism remains incompletely understood. The primary goal of this research has been to understand by what mechanism END-3 orchestrates apical constriction in the endodermal precursors of the developing *C. elegans* embryo, as a model for how cell fate regulates morphogenesis more generally.

Genetic redundancy is a prevalent complication in studying the morphogenetic events that drive development[18]. The roles of partially or entirely redundant genes are often difficult to detect using traditional, single-knockdown screening techniques; the loss of one gene can be rescued by its redundant counterpart, thereby potentially masking any defect[19]. The partial redundancy of *end-1* and *end-3* in *C. elegans* endoderm specification has been shown previously[14,15], so it stands to reason that several redundant genes may have a role in orchestrating apical constriction downstream of these factors.

One method for overcoming redundancy is using sensitized genetic backgrounds to enhance or expose phenotypes of interest. By screening in mutant backgrounds, it is possible to discover phenotypes that do not emerge with single mutagenesis or deletion[15]. This technique has been used previously in *C. elegans* to elucidate redundant genes implicated in vulval and pharyngeal formation[20], as well as gastrulation[15,20].

We hypothesize that END-3 orchestrates apical constriction and gastrulation of the endodermal precursor cells in *C. elegans* by regulating the transcription of one or several genes that control either new or known cellular mechanisms that contribute to cell shape change and cell positioning. We utilized targeted knockdown of top candidate END-3 targets via RNA interference (RNAi) to screen for gastrulation defects in both wild-type

and sensitized backgrounds. We report the identification of 7 new genes encoding proteins with nonredundant or partially redundant functions in E cell gastrulation. Although these proteins are largely uncharacterized to date, they seem to suggest cell signaling as an important process downstream of E cell specification. These data provide promising new candidates for elucidating mechanistic details connecting cell fate specification and shape change in *C. elegans* and perhaps more broadly across metazoans, as well.

Experimental Approach and Methods

C. elegans strains and maintenance

Worms were cultured and handled as previously described[21]. Experiments were performed using the following worm strains: N2, wild type; VC271, *end-1 (ok558)*; FX02295, *sdz-19 (tm2295)*. Worms were maintained at 20°C.

dsRNA synthesis

Double-stranded RNAs (dsRNAs) were prepared and synthesized as previously described[22]. dsRNAs were synthesized for 21 genes, targeting the entire protein-coding region excluding introns, generated from cDNA. Synthesized dsRNA size and concentration were verified using gel electrophoresis and Nanodrop spectrophotometry, respectively. dsRNAs were stored at -20°C in aliquots either at the original or diluted 1 µg/µL concentration. Aliquots were thawed before use. No aliquots were thawed and refrozen more than once, to maintain dsRNA integrity.

RNA interference injections

RNA interference (RNAi) was performed by injecting dsRNA at a concentration of 1 µg/µL* into the bodies of young adult worms, either into the gut or gonad. Injected worms were moved to new plates seeded with OP50 bacteria to recover at 20°C and incubated for 24-48 hours before embryos were imaged. [*For some constructs, a slightly lower concentration was injected due to a smaller dsRNA yield]

Embryonic lethality assays

Following RNAi injections, 3-5 worms were moved to a single recovery plate seeded with OP50 bacteria. Following 24 hours of incubation at 20°C, all living adult worms from one plate were transferred to a new recovery plate and all dead worms were

removed. This was repeated for all replicate plates per dsRNA genotype. Living worms were transferred at 24, 48, and 72 hours post injection, allowing for 24 hours of embryo laying on each new plate.

Plates were scored twice after transferring all living adult worms to a new plate: (1) 24 and (2) 48 hours after adult worms began laying embryos, i.e., at 0 and 24 hours after the last embryos were laid onto the plate. By 48 hours, all embryos should have hatched when incubated at 20°C. For (1), the total number of embryos and any already hatched larvae were counted. For (2), the total number of unhatched embryos (any remaining embryos) were counted. Unfertilized oocytes were excluded from calculations. For each individual plate of 3-5 injected worms, "% Embryonic Lethality" was calculated as a percentage of (2) / (1).

Mounting embryos for imaging

Embryos were mounted laterally, as described previously[17]. Embryos were dissected from gravid adult worms, and embryos at the 1-6 cell stage were transferred into a droplet of Egg Buffer (118 mM NaCl, 40 mM KCl, 3.4 mM $CaCl_2$, 3.4 mM $MgCl_2$, and 5 mM HEPES [pH 7.2]) on a coverslip coated with poly-L lysine. Coverslips were mounted onto glass slides with 2.5% agarose pads and sealed with VALAP wax (equal parts petroleum jelly, lanolin, and paraffin). Imaging was performed at 20-23°C.

Microscopy

Differential interference contrast (DIC) microscopy images were acquired with a Diagnostic Instruments SPOT2 camera mounted on a Nikon Eclipse 800 microscope. Embryos were filmed under DIC illumination using Z-stacks of 1 μm steps through the entire embryo, at 60 second intervals for 90-150 minutes to image through gastrulation.

Results

Determining candidate END-3 targets as regulators of gastrulation

The central goal of this research has been to determine the role of genes that are controlled directly or indirectly by END-3. Because END-3 is a transcription factor, we hypothesized that it transcriptionally regulates a gene or genes to orchestrate apical constriction in endoderm precursor cells. To identify genes regulated directly or indirectly by END-3, previous members of our lab performed RNA sequencing (RNA-seq) and transcriptome profiling of *C. elegans* embryos at the early E2 stage (containing 2 EPCs) in both wild-type N2 and an *end-3* deletion background (SC Tintori, unpublished)[5]. These experiments gave us reads per kilobase of transcript, per million mapped reads (RPKM) values indicating transcript abundance of genes from whole embryos.

We aimed to identify genes that are both (1) regulated directly or indirectly by END-3, and (2) expressed specifically in the early E lineage before these cells internalize. To determine candidates most likely to be regulated by END-3, we ordered the transcript abundance data described above by the ratio of expression in *end-3 (ok558)* over expression in N2 (WT), such that the genes with the lowest ratio indicated those most down-regulated in *end-3* mutants. In other words, END-3 likely directly or indirectly regulates the expression of these genes. These “candidate END-3 targets” are thus defined as being genes whose mRNA levels decrease in an *end-3* deletion line.

Additionally, we determined which genes were highly and specifically expressed in the E cell and/or its daughter cells, Ea and Ep, using an online tool previously developed by our lab[23]. These analyses demonstrated that 24 of the top 26 genes most regulated by END-3 activity had high transcript abundance in Ea/Ep as compared with

the rest of the 16-stage embryo **(Figure 3)**. This returned a list of genes both positively regulated by END-3 and with abundant transcript levels in the EPCs, representing promising candidate regulators of gastrulation to investigate further.

Depleting candidate END-3 targets gives minimal rates of embryonic lethality

Following the selection of the top 24 potential END-3 targets, we sought to determine the role of these genes in apical constriction and gastrulation. Previous studies have shown that 95% of embryos from an *end-3* genetic null strain fail to gastrulate at the correct time, although there is only a 6% rate of embryonic lethality, because the endodermal precursors typically do eventually internalize[24].

We hypothesized that any gene(s) regulated by END-3 during apical constriction and gastrulation would give a phenotype similar to that seen in an *end-3* genetic null background—namely, embryonic lethality rates no higher than 6% but a high level of gastrulation defects (although, if a candidate also functioned in other necessary processes, depletion could result in a much higher level of lethality). To test this hypothesis, we first synthesized double-stranded RNAs (dsRNAs) to target specific genes by RNA interference (RNAi). We generated dsRNAs targeting 17 of the top 24 genes from our prioritized list. We then assayed for embryonic lethality following RNA injections by quantifying laid embryos that failed to hatch.

For wild-type N2 worms, we observed an average of 2.21% (C.I. -1.25 – 5.67%) embryonic lethality after 48 hours of laying. We found that for all 17/17 genes targeted, RNAi injection resulted in low rates of lethality not significantly different from those seen for WT **(Figure 4)**. As a positive control, we injected dsRNA against *gdi-1*, which encodes a Rab GDP dissociation inhibitor (GDI) with characterized embryonic lethality upon

targeting by RNAi[25]. We observed an average lethality of 38.6% (C.I. 14.1 – 63.0%) after 48 hours and 87.9% (C.I. 64.1 – 111.6%) after 72 hours, thereby validating our experimental technique. Only knockdown of one gene, T19B10.2, resulted in an initially apparent increased rate of 12.2% (C.I. 0.33 – 24.0%) embryonic lethality, which was not statistically significant using a 95% confidence interval.

Visualizing gastrulation in vivo following targeted knockdown implicates candidate END-3 targets in gastrulation

Previous studies have shown that *end-3* null embryos have low levels of embryonic lethality but very high levels of gastrulation defects, including delayed but eventual internalization of endodermal precursor cells[24]. To determine whether our targeted genes displayed similar phenotypes, we chose to directly visualize gastrulation following the targeting of genes by RNAi. During wild-type development, division of one cell of the 4-cell stage embryo gives rise to the endodermal and mesodermal precursor cells (E and MS cells). These two cells in turn divide to give rise to two E daughter cells (Ea/Ep) and two MS daughter cells (MSa/MSp, referred to together as MSx here). Ea and Ep then internalize into the center of the embryo before dividing again once fully internalized, such that no part of any of the 4 EPCs is exposed on the surface.

To screen for gastrulation defective (Gad) phenotypes, we performed RNAi in a wild-type (N2) background to knock down 12 candidate END-3 targets and visualized gastrulation using DIC microscopy. We chose the MSx cell division as a consistent beginning time point for comparing timing across embryos, as any EPC gastrulation defects would not be expected to affect MSx division timing, although it may affect division timings of the E or E2 cells (Ea/Ep). In wild-type embryos, the E2 cells internalized in 17.0

(C.I. 14.9 – 19.1) minutes following the MSx cell division. These E2 cells, once fully internalized, then divided an average of 3.7 (C.I. 1.6 – 5.8) minutes later.

For some candidates targeted by RNAi, we observed Gad phenotypes in which either or both E2 cells did not fully internalize before dividing. Of the 12 candidate END-3 targets screened, we found that 5 showed 0% Gad phenotypes (n = 6-24 embryos per target), while 7 displayed Gad phenotypes, ranging from 5.9 – 38.5% penetrance **(Table 1)**. The majority of these candidates encode uncharacterized, worm-specific proteins with predicted signal peptides (F14H12.6, T19B10.2, T22D1.11), 5-pass transmembrane domains (F19C6.5), or possible mitochondrial function (T25G12.2). The remaining 2 candidates have been characterized as encoding proteins involved in RAS signaling (*dve-1*) or sphingolipid metabolism (*asm-1*), with known homologs in *Drosophila* and humans, respectively[26].

To further characterize the Gad phenotypes of these 7 candidates, we also measured gastrulation timing in these embryos. Across RNAi genotypes, all Gad embryos were characterized by late internalization: either one or both E2 cell was still exposed upon division, but all E4 daughters were fully covered by neighboring cells before dividing again. The severity of this delay varied across different RNAi targets, ranging from 3 minutes (F19C6.5*)* to 14 minutes (T25G12.2) following the E2 division. In contrast, the time between the MSx and E2 division remained consistent between embryos exhibiting Gad and non-Gad phenotypes, suggesting that the observed Gad phenotypes are not due to alterations in cell cycle timing, as occurs in some other mutants[25], or likely in cell fate more generally. Together, these data implicate 7 new genes in E cell internalization.

Targeted knockdown in sensitized backgrounds

Because genetic redundancy is prevalent throughout morphogenetic events in *C. elegans*[24], including endoderm specification and gastrulation[15], and for morphogenesis more generally[18], we hypothesized that END-3 regulates apical constriction through genes that are partially or entirely redundant. To test this hypothesis, we chose to continue screening for gastrulation defects in two sensitized backgrounds: (1) *end-1 (ok558)* deletion, and (2) *sdz-19 (tm2295)* deletion. As mentioned previously, *end-1* encodes endoderm-specifying END-1, a transcription factor partially redundant with END-3[14]. We hypothesized that by targeting END-3 candidates and not END-3 itself, we could identify genes involved in gastrulation while bypassing defects in endoderm specification (as seen following loss of both *end-1* and *end-3*)[15]. On the other hand, *sdz-19* encodes an uncharacterized protein that has been previously implicated in gastrulation[24].

We first wanted to determine whether targeting candidate END-3 targets with RNAi in these two sensitized backgrounds increased rates of embryonic lethality for any gene. I mentored a first-year graduate student as she addressed this question using RNAi by feeding against top candidate END-3 targets in the two sensitized background strains, *end-1* and *sdz-19*. RNAi by feeding in *C. elegans* is a quick and easy way to screen through a list of genes, although this technique can be variable or ineffective[27]. With this in mind, we selectively only looked for targets whose knockdown resulted in greatly increased rates of embryonic lethality. This screen gave us 3 candidates with embryonic lethality rates greater than 30%: F19C6.5, F14H12.6, and *ppt-1* (J Robinson, unpublished; data not shown).

We next sought to determine whether the apparent increase in embryonic lethality in targeted knockdown of these 3 candidates was accompanied by gastrulation defects. We performed RNAi by injection against these candidates in both *end-1* and *sdz-19* backgrounds and imaged the embryos of injected worms. We found that in the un-injected *end-1* deletion strain, 100% of embryos showed Gad phenotypes, with Ea/Ep internalizing an average of 47 (C.I. 43.6 – 50.4) minutes following the MSx division **(Figure 5, Table 1)**. Targeted knockdown of all 3 candidates in the *end-1* deletion background also showed 100% Gad phenotypes, as expected. For F19C6.5 and F14H12.6, we also observed a clear increase in the time required for internalization in some embryos, with maximum internalization times of 102 minutes and 75 minutes, respectively.

In embryos from the un-injected *sdz-19* deletion strain, we observed 16.7% Gad phenotypes, with an average internalization time of 23 (C.I. 16.9 – 19.1) minutes following the MSx division **(Figure 5, Table 1)**. All three knockdown targets showed significant increases in Gad phenotype incidence, ranging from 20 – 42.9%. No targeted candidate displayed any significant change in internalization timing.

We observed that RNAi of *ppt-1* did not result in gastrulation defects in a wild-type background and did not enhance the Gad phenotypes observed in both *end-1* and *sdz-19* deletion backgrounds. From this, we conclude that that *ppt-1* likely does not play an important role in E cell gastrulation. However, for both F19C6.5 and F14H12.6, we observed that targeting by RNAi resulted in Gad phenotypes in wild type, as well as an increase in internalization timing in sensitized backgrounds. We conclude that F19C6.5 and F14H12.6 remain strong candidates of interest as potential regulators of gastrulation, along with the other 5 candidates identified through screening in wild type.

Discussion

Apical constriction is a critical morphogenetic event employed throughout development to generate a variety of tissue shapes and structures[6,28]. *C. elegans* offers a chance to link cell fate specification to the intracellular behaviors that drive this process: the transcription factor END-3 is required for both endoderm fate specification and gastrulation[17,24]. Here, we found that loss of 7 new candidate END-3 targets also resulted in gastrulation defects, implicating them as having an important role in gastrulation.

Our first method of screening through top candidate END-3 targets by assaying for embryonic lethality following RNAi by injection gave no significant results. This is not surprising for two main reasons. First, it has previously been shown that even when END-3 has been depleted in a wild-type background, there is only a 6% rate of embryonic lethality[24]. Even if we hypothesize that END-3 interacts with only 1 downstream target that mediates all of END-3's essential functions, the maximum rate we expect to see would be 6%, a rate that may not emerge as significant based on our statistical analysis. Second, we have no reason to believe that END-3 is interacting with only 1 target gene that mediates all of END-3's essential functions. Genetic redundancy is prevalent throughout biological systems and processes, including in *C. elegans* gastrulation[14,15], and particularly so for morphogenesis[18]. Endoderm is specified by the concerted actions of END-1 and END-3[29], and even apical constriction itself relies on redundant cell-cell junction proteins[30]. For these reasons, we decided to directly image embryos to expose non-lethal gastrulation defects, as seen following *end-3* deletion[24].

Direct injection of dsRNAs targeted against top candidate END-3 targets into adult worms followed by *in vivo* imaging yielded 7 E cell-specific genes whose depletion

affected gastrulation. All of these genes are newly identified as potential regulators of gastrulation and had not emerged in previous screening attempts[24]. Of these genes, 5 encode proteins that appear to be both worm-specific and uncharacterized: F19C6.5, T25G12.2, F14H12.6, T19B10.2, and T22D1.11. One gene, *dve-1*, encodes three proteins (through alternative splicing) with a COMPASS (CMP) domain and has been shown to genetically interact with at least two components of the EGF/RAS signaling pathway[31]. The CMP domain of DVE-1 (Defective proVEntriculus-1, from *Drosophila*) also appears to be homologous to the CMP domain of homeodomain proteins, such as human SATB1, and *Drosophila* DVE[32]. Finally, *asm-1* encodes a sphingomyelin phosphodiesterase[33] and appears to be conserved in many organisms, including humans; loss of the human homologs of *asm-1* results in Niemann-Pick disease, a lipid storage disorder cause by a buildup of lipids within cells and tissues[34,35]. *Asm-1* also appears to have a role in apoptosis in various systems[36]. Previous studies have implicated other genes in both apoptosis and gastrulation in *C. elegans*, as well[5]. This could potentially suggest that the EPCs internalize through activating part of the apoptotic pathway and inducing neighboring cells to produce actin-rich extensions required for internalization of the EPCs[37].

We also used sensitized backgrounds in our screen to attempt to overcome genetic redundancy among regulators of gastrulation. Surprisingly, we saw that 100% of embryos from our *end-1* deletion strain expressed Gad phenotypes, in contrast to previous reports[24] **(Figure 5, Table 1)**. This is similar to the reported phenotype for a deletion of *end-3* alone[24] and suggests that END-1 also has a role in regulating expression of genes important for gastrulation. However, these data could also indicate

that the *end-1* (*ok558*) deletion strain used has picked up a confounding background mutation. We suggest that future users outcross this strain to remove any background mutations or recreate it using CRISPR/Cas9 methods[11].

With a baseline level of 100% Gad phenotypes in *end-1*, we could not assay for increased rate of Gad, but we did see delayed internalization timing following knockdown of F19C6.5 and F14H12.6, further implicating these proteins as regulators of gastrulation. Knockdown of F19C6.5 (but not F14H12.6) in an *sdz-19* background also showed increased internalization defects. This could imply that both F19C6.5 and F14H12.6 regulate gastrulation. We note that for all genes here implicated to have a role in gastrulation, we cannot be sure whether these genes contribute to apical constriction specifically or endoderm specification without further testing.

Apical constriction is a process that occurs to drive a variety of morphogenetic movements across biological systems[6]. With such diversity surrounding this process, one fundamental question is how developmental patterning regulates the force-producing machinery required to orchestrate cell shape change in the right cells at the right time. To do this, it seems that all metazoans that employ apical constriction must have mechanisms in place to spatially and temporally define which cells will constrict, polarize constricting cells (such that only one surface constricts), and initiate the force-generating machinery that will mechanically constrict the cell(s).

Although some mechanistic details of apical constriction have been elucidated in various systems, including *Drosophila*[38]*, C. elegans*[6]*,* and *Xenopus*[39], it remains unclear how conserved aspects of apical constriction are across metazoans—does fate specification regulate apical constriction through similar mechanisms and homologous

proteins? The identification of 7 proteins as potentially having important roles in gastrulation offers the possibility to uncover more of the mechanistic details. This could ultimately help us to identify commonalities across biological systems, evolutionary innovation within *C. elegans*, and/or mechanistic details not previously recognized but broadly used in other systems.

Of the 7 candidates we identified, 5 appear to be worm-specific and uncharacterized, with essentially nothing known about their functions. Based on protein domain predictions, 2 of these appear to have transmembrane domains (TMD) and 3 have predicted signal peptide domains **(Table 1)**. Almost half of the genes in the *C. elegans* genome encode proteins with a signal peptide, a transmembrane domain, or both, meaning that a substantial number of proteins within *C. elegans* are localized to membranes or are secreted[40]. Of the genes identified here, one or all of these proteins may play a role in inter- or intracellular signaling to ultimately help orchestrate apical constriction, through myosin activation or modulating the inter- or intracellular force context in which cortical tension can produce changes in cell shape.

Studies of apical constriction in *Drosophila* have elucidated many mechanistic details about the link between cell fate specification and apical constriction[38]. In *Drosophila,* the mesodermal progenitors undergo apical constriction and internalization. Mesodermal fate is determined by a maternal transcription factor Dorsal; Dorsal then activates transcription factors Snail and Twist. Previous studies have shown that loss of Snail and Twist prevents apical constriction in these cells, and loss of the Twist target protein Folded Gastrulation (Fog) results in failed apical constriction, as well[41]. Fog itself is a secreted protein, and loss of downstream signaling proteins—including Gα and

transmembrane proteins—also results in gastrulation defects[42,43]. Although some of our identified candidates appear to be uncharacterized and worm specific, it seems interesting to consider that parallels might exist between these proteins and known regulators of gastrulation in *Drosophila*.

Future Directions

The screens described here have provided us with a short list of promising END-3 targets with roles in gastrulation; the next steps require characterizing these new proteins and assessing whether expression patterns and functions continue to implicate these proteins as regulators of gastrulation. We also aim to understand the mechanism of apical constriction *in vivo* in *C. elegans*, the specific role END-3 plays, and broadly, how conserved this mechanism may be with those employed by other organisms, including *Drosophila* and mammals.

One of the first future experiments will be to continue to verify the results explained here. In both lethality experiments **(Figure 4)** and imaging for gastrulation defects **(Figures 5,6)**, we had small sample numbers for several genes targeted by RNAi. Especially for the genes that gave Gad phenotypes upon targeting, repeating these experiments will give us a better and more reliable idea of the rate of observing Gad phenotypes, which may help to prioritize genes for subsequent experiments.

To characterize these newly-identified proteins, we first want to know where these proteins are localized. To do this, we will observe subcellular protein localization *in vivo* during development. Our lab has previously generated robust methods for doing this, by using the CRISPR/Cas9 system to generate endogenous fluorescent protein fusions[10,11]. By generating worm strains each with one fluorescently-tagged gene from the 7 identified here, we can then use fluorescence microscopy during embryonic development to assess whether these proteins are enriched apically, basolaterally, or at cell junctions. We can hypothesize that proteins with apical enrichment might contribute to increasing cortical tension before constriction[17], whereas proteins being apically secreted may alter the

extracellular force context against which apical shrinking forces may act[44]. Alternatively, proteins with junctional localization may contribute to the molecular clutch hypothesized to coordinate intracellular actomyosin contractions to apical constriction[28].

Upon determining localization patterns and timing, we will then further characterize the candidates' roles in apical constriction. Previous experiments have shown that prior to apical constriction in *C. elegans*, myosin must be activated by the kinase MRCK-1, which localizes to the apical surface[17]. To determine whether these candidates affect apical constriction through localizing MRCK-1, we will use targeted knockdown of candidates in a worm strain with fluorescently-labeled MRCK-1 or myosin. We expect that if a candidate directly regulates apical constriction, knockdown of the candidate will result in a reduction of apically-localized MRCK-1 followed by a lack of apical constriction and late (or failed) internalization of the E cells. These experiments could be performed in sensitized strains, as well, to overcome any masked, redundant phenotypes.

Another interesting question to address in the future is whether END-3 alone is sufficient to orchestrate apical constriction. This can be tested by ectopically expressing END-3 in other cells of the early embryo. This is complicated by the fact that END-3 is an endodermal fate-specifying transcription factor; however, by expressing END-3 in an already-specified lineage, such as the mesodermal lineage (MS cells), we could hope to avert these complications. If END-3 is sufficient, we could expect that ectopic END-3 expression induces apical constriction and upstream events such as MRCK-1 localization with timings similar to those observed in the E cells. If END-3 does appear to be sufficient for apical constriction, this will suggest to us that all of the factors necessary are present

more broadly than in just the E cells, and that direct regulation of even just one gene could trigger the entire process.

Ultimately, we aim to understand broad mechanisms of apical constriction by expanding our studies to other cells and tissues. Within *C. elegans*, after the E cells internalize, mesodermal and germline precursor cells also internalize into the center of the embryo, some by apical constriction[7]. Within sets of internalizing MS cells, some cells internalize more quickly than others; within 4 cells of C lineage (neuron, epidermis, and muscle precursors) cells, progeny of 2 cells internalize while progeny of 2 others do not[9]. We hypothesize that although these lineages are specified through different mechanisms, they all act through similar proteins to orchestrate apical constriction. Previous experiments and preliminary analysis of single-cell transcriptome data have implicated a family of LIM domain-containing junctional proteins as candidates for having a regulatory role in gastrulation: members of this family have increased expression in gastrulating E and MS cells (SC Tintori, unpublished). Other members in our lab are further characterizing the roles of these proteins using RNAi knockdown and targeted mutagenesis to screen for gastrulation defects in one or several cell lineages (MM Slabodnick, unpublished). These experiments will allow us to better understand the kinds of proteins being utilized in apical constriction across lineages, which will shed light on what may be some of the most broadly used, and possibly conserved, factors within this iterative morphogenetic event.

Together, these experiments will further elucidate specific proteins and mechanisms deployed by cells to orchestrate apical constriction in a spatially- and temporally-regulated manner. *C. elegans* is an important system for studying the detailed

mechanisms involved in apical constriction across various cell lineages and can help to reveal key factors in these processes. By comparing these results with known mechanistic details across species such as *Drosophila* and *Xenopus*, we can begin to understand which mechanistic details are conserved, which have redundant functions but through various factors, and which are innovations by a specific organism or tissue. These data will help us better understand the connection between cell specification and the morphogenetic events required for proper animal development across metazoans.

APPENDIX 1: TABLE OF RNAi SCREEN RESULTS

RNAi Target	Proposed Function or Domains[26,45]	Genetic Background	% Embryonic Lethality	MS div. → E intern. (min)	MS div. → E div. (min)	% Gad (n/n)
Negative Control	---	N2 (wild type)	2.2 ± 3.5	17.0 ± 1.6	20.7 ± 1.3	0 (0/16)
F19C6.5	5-pass TMD	N2 (wild type)	1.0 ± 1.2	18.0 ± 1.4	21.0 ± 0.8	14.3 (3/21)
C29F7.2	kinase-like domains	N2 (wild type)	---	18.0 + 1.4	22.7 ± 1.4	0 (0/10)
nlp-31	neuropeptide-like proteins	N2 (wild type)	3.3 ± 6.3	18.8 ± 1.1	23.5 ± 2.2	0 (0/6)
ugt-23	hexosyl group transferase	N2 (wild type)	0.9 ± 0.9	18.6 ± 1.0	21.7 ± 0.7	0 (0/7)
T25G12.2	mitochondrial function	N2 (wild type)	0.6 ± 0.6	21.8 ± 3.4	22.5 ± 1.0	27.3 (3/11)
C26F1.1	extracellular signal peptide domain	N2 (wild type)	0.7 ± 0.7	18.1 ± 0.7	21.8 ± 1.0	0 (0/16)
dve-1	CMP domain, RAS signaling	N2 (wild type)	1.8 ± 1.4	18.8 ± 2.4	23.0 ± 1.3	9.1 (1/11)
asm-1	sphingomyelin phosphodiesterase	N2 (wild type)	1.0 ± 0.5	24.5 ± 3.8	22.1 ± 0.9	38.5 (5/13)
F14H12.6	signal peptide, 1-pass TMD	N2 (wild type)	5.0 ± 4.2	19.9 ± 2.2	22.8 ± 1.2	5.9 (1/17)
ZK185.2	Mg^{2+} transport	N2 (wild type)	0.6 ± 0.6	---	---	---
T19B10.2	extracellular signal peptide domain	N2 (wild type)	12.2 ± 11.8	21.5 ± 3.8	23.8 ± 1.6	12.5 (1/8)
dpy-14	collagen development	N2 (wild type)	1.7 ± 1.2	---	---	---
T25G12.1	(uncharacterized)	N2 (wild type)	0.4 ± 0.4	---	---	---
F22B7.9	methyltransferase	N2 (wild type)	1.9 ± 3.0	---	---	---
ZK742.3	FMN binding activity, oxido-reductase activity	N2 (wild type)	4.6 ± 9.0	---	---	---
hgo-1	homogentisate oxidase	N2 (wild type)	2.6 ± 0.9	---	---	---
T22D1.11	extracellular signal peptide domain 1-pass TMD	N2 (wild type)	0.60 ± 0.5	21.2 ± 3.2	23.4 ± 2.0	16.7 (1/6)
ppt-1	palmitoyl protein degradation	N2 (wild type)	3.33 ± 2.81	18.4 ± 0.6	22.3 ± 0.6	0 (0/24)

RNAi Target	Proposed Function or Domains[15,16]	Genetic Background	% Embryonic Lethality	MS div. → E intern. (min)	MS div. → E div. (min)	% Gad (n/n)
Negative Control	---	*end-1 (ok558)*	---	47 ± 3.4	14.9 ± 1.2	100 (9/9)
F19C6.5	5-pass TMD	*end-1 (ok558)*	---	56.1 ± 8.8	14.1 ± 0.6	100 (15/15)
F14H12.6	signal peptide, 1-pass TMD	*end-1 (ok558)*	---	52.5 ± 7.7	16.3 ± 1.1	100 (11/11)
ppt-1	palmitoyl protein degradation	*end-1 (ok558)*	---	45.7 ± 2.2	14.4 ± 1.3	100 (10/10)
Negative Control	---	*sdz-19 (tm2295)*	---	23 ± 6.1	22.5 ± 1.4	16.7 (1/6)
F19C6.5	5-pass TMD	*sdz-19 (tm2295)*	---	23.2 ± 3.4	20.5 ± 0.8	38.9 (7/18)
F14H12.6	signal peptide, 1-pass TMD	*sdz-19 (tm2295)*	---	25.3 ± 6.3	23.6 ± 1.0	20 (2/10)
ppt-1	palmitoyl protein degradation	*sdz-19 (tm2295)*	---	26.3 ± 7.6	22.6 ± 1.3	42.9 (3/7)

Table 1 – **Results of RNAi screens.** The "RNAi Target" column gives the gene name or Sequence ID for each targeted gene. Three genetic backgrounds were used for these experiments: N2 wild type; *end-1 (ok558)*, a deletion of endoderm-specifying factor END-1; and *sdz-19 (tm2295),* a deletion of *sdz-19,* encoding a protein previously implicated in gastrulation[24]. "% Embryonic Lethality" displays the average lethality seen at 48 hpi (see Figure 4). "MS div. → E intern." displays the average length of time between the MSa/MSp division and when both Ea/Ep are fully internalized. "MS div. → E div." displays the average length of time between MSa/MSp division and Ea/Ep division. "% Gad" refers to how many of the dsRNA-treated embryos displayed gastrulation defective phenotypes. ± represents a 95% confidence interval.

APPENDIX 2: FIGURES

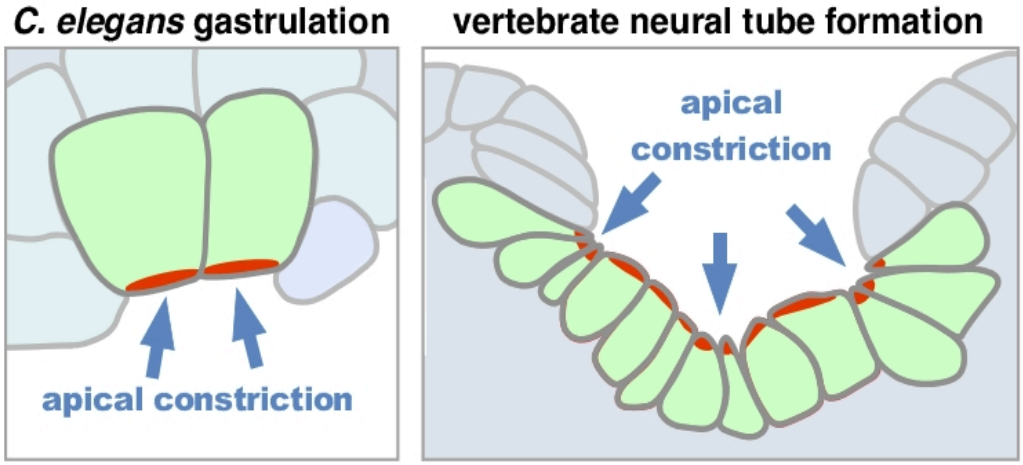

Figure 1. Apical constriction is a conserved developmental process in *C. elegans* gastrulation and vertebrate neural tube formation. The cells highlighted in green are the endodermal precursor cells (EPCs) in *C. elegans* (left) and neural plate cells in vertebrates (right). These cells undergo actomyosin-driven apical constriction (red), to shrink their exposed surfaces. *Adapted from Sullivan-Brown et al. (2010)*[5].

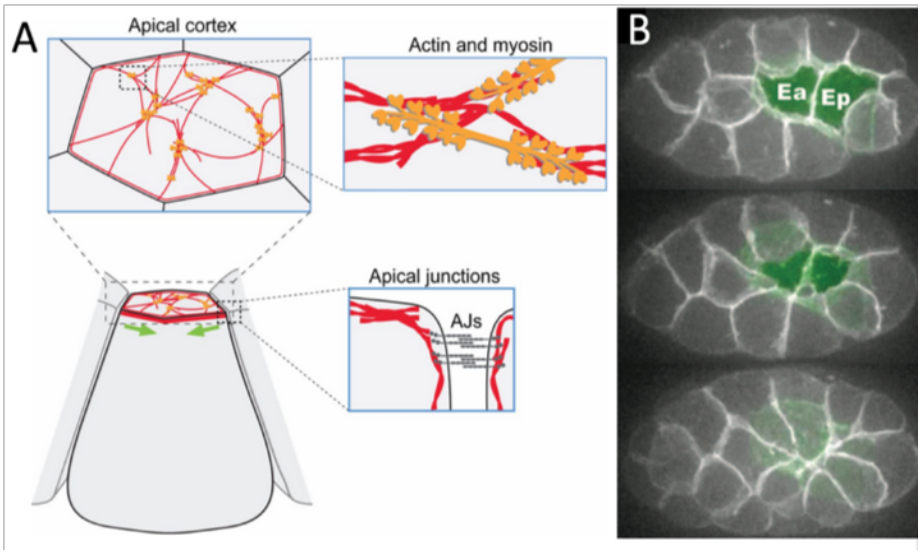

Figure 2. *C. elegans* embryos orchestrate gastrulation of endodermal precursors through apical constriction. (A) Apically-localized networks of actin filaments and myosin motor protein are attached to apical cell-cell junctions (AJs). This network generates tension that pulls on and shrinks the apical surface. *Adapted from Martin and Goldstein, 2014*. (B) A ventral view of a gastrulation-stage *C. elegans* embryo. Endodermal precursors (Ea, anterior; Ep, posterior) are pseudo-colored in green. These cells undergo apical constriction, shrink their exposed surfaces, and fully internalize until covered by neighboring cells (grey). *From Lee et al., 2006*.

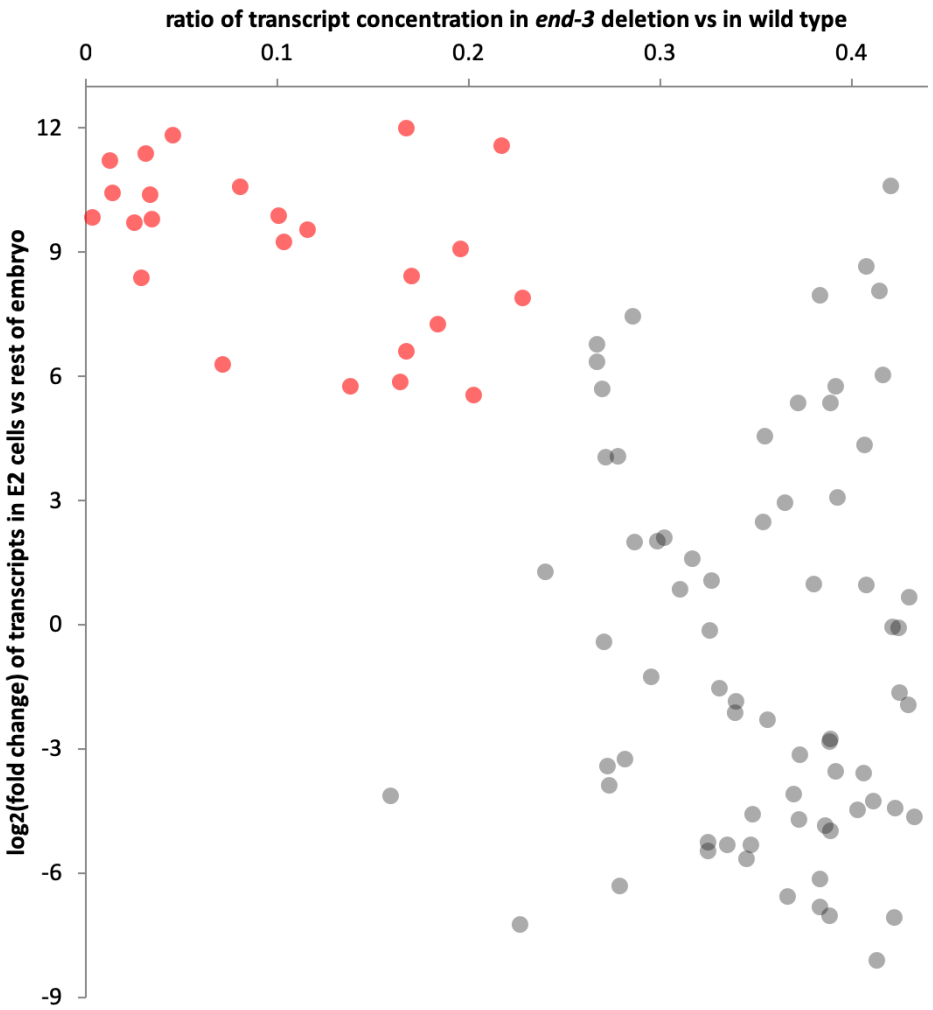

Figure 3. Top candidate genes are regulated by END-3 and display high transcript abundance specifically in E2 cells. Data is shown for 95 of the 100 genes most downregulated in *end-3* deletion vs. in wild type. Log_2(fold change) demonstrates the localized transcript abundance of each transcript, where values greater than 0 indicate abundance enriched in E2 cells. Genes colored red indicate the top 24 candidate targets based on expression pattern and responsiveness to END-3 deletion.

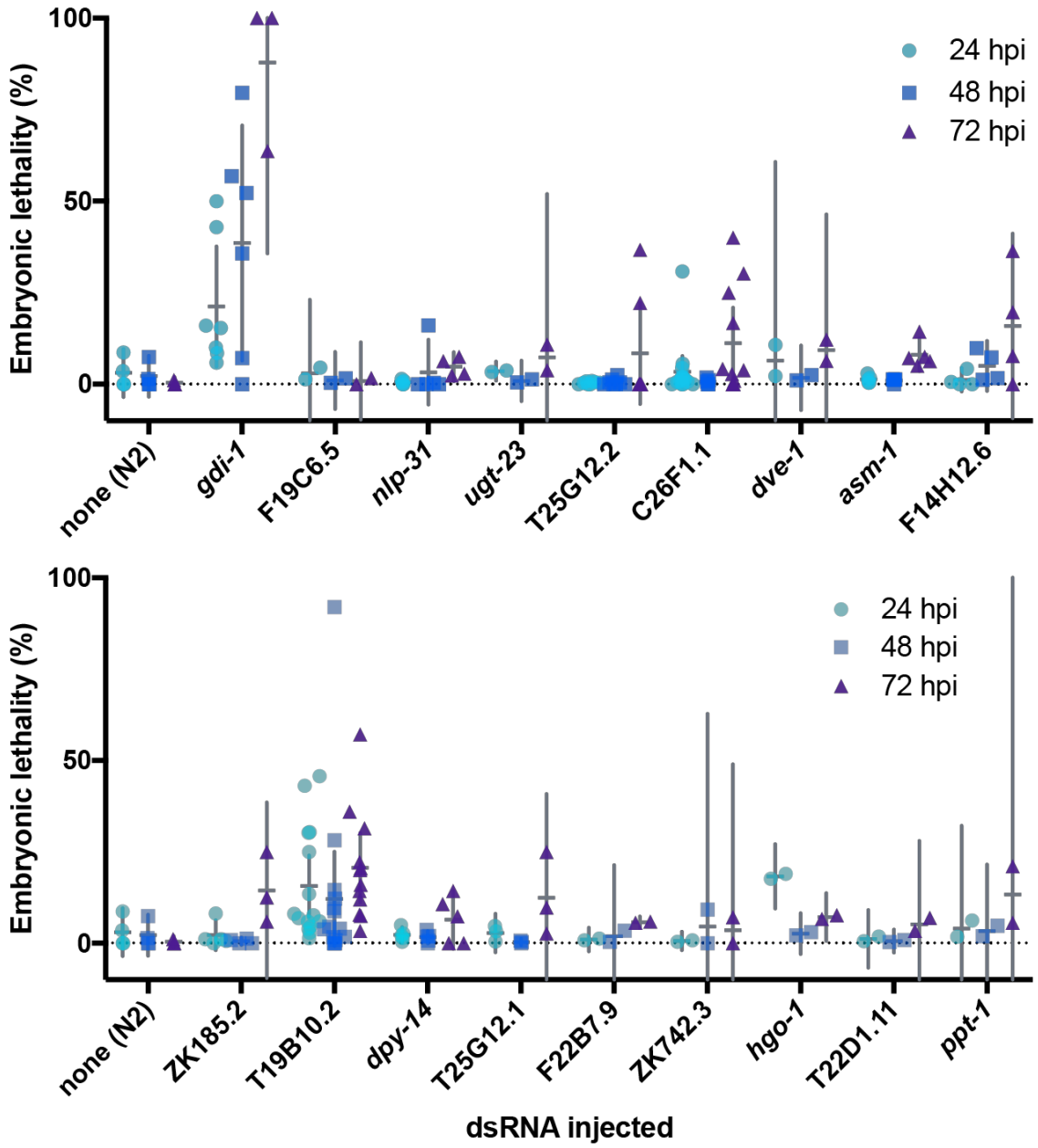

Figure 4. Embryonic lethality rates observed following RNAi by injection. Embryonic lethality was assessed at 24, 48, and 72 hours post injection (hpi) of adult worms to control for RNAi efficacy over time. Points indicate mean lethality, with 95% confidence intervals plotted. The dotted black line designates 0% lethality for ease of comparison. For each dsRNA, 2-12 replicates were performed, with each plate ranging in embryo count from 5 to over 500. The N2 wild-type strain indicates baseline lethality levels. By 72 hpi, adult worms have greatly decreased fecundity, giving smaller numbers of embryos and thus more variable lethality results. For this reason, average embryonic lethality observed at 48hpi was used for comparison across genotypes.

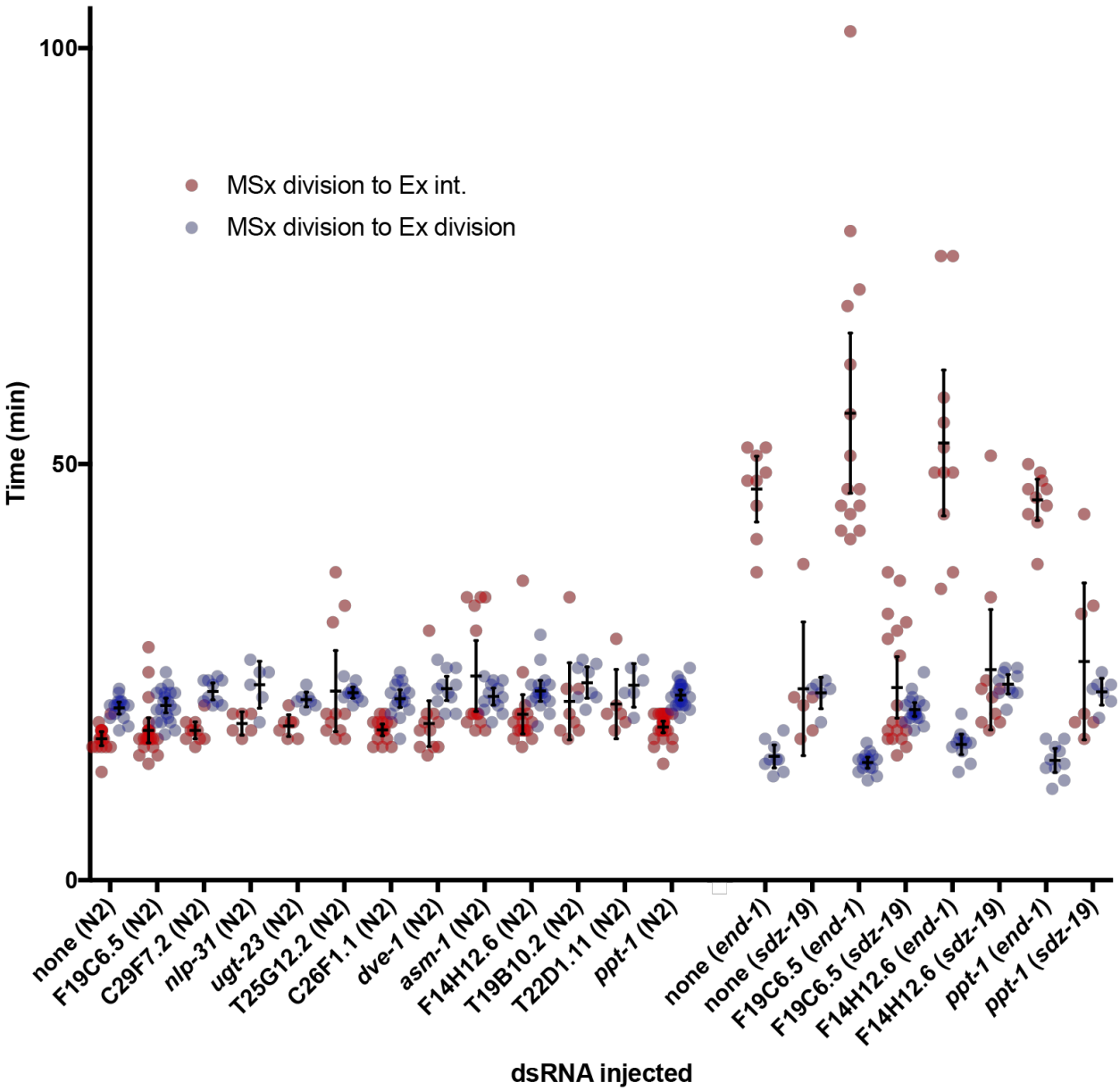

Figure 5. Timing of E cell internalization following RNAi knockdown of candidate END-3 targets in wild type and sensitized backgrounds *end-1 (ok558)* and *sdz-19 (tm2295).* For each background or targeted knockdown, both the timing of Ex internalization (red, left) and Ex division (blue, right) is displayed. The MSx division is used as an easily observable developmental landmark for comparisons across embryos. N2 = WT strain. Both e*nd-1 (ok558)* and *sdz-19 (tm2295)* are deletion strains that act as sensitized backgrounds for screening for Gad phenotypes. Error bars indicate 95% confidence intervals.

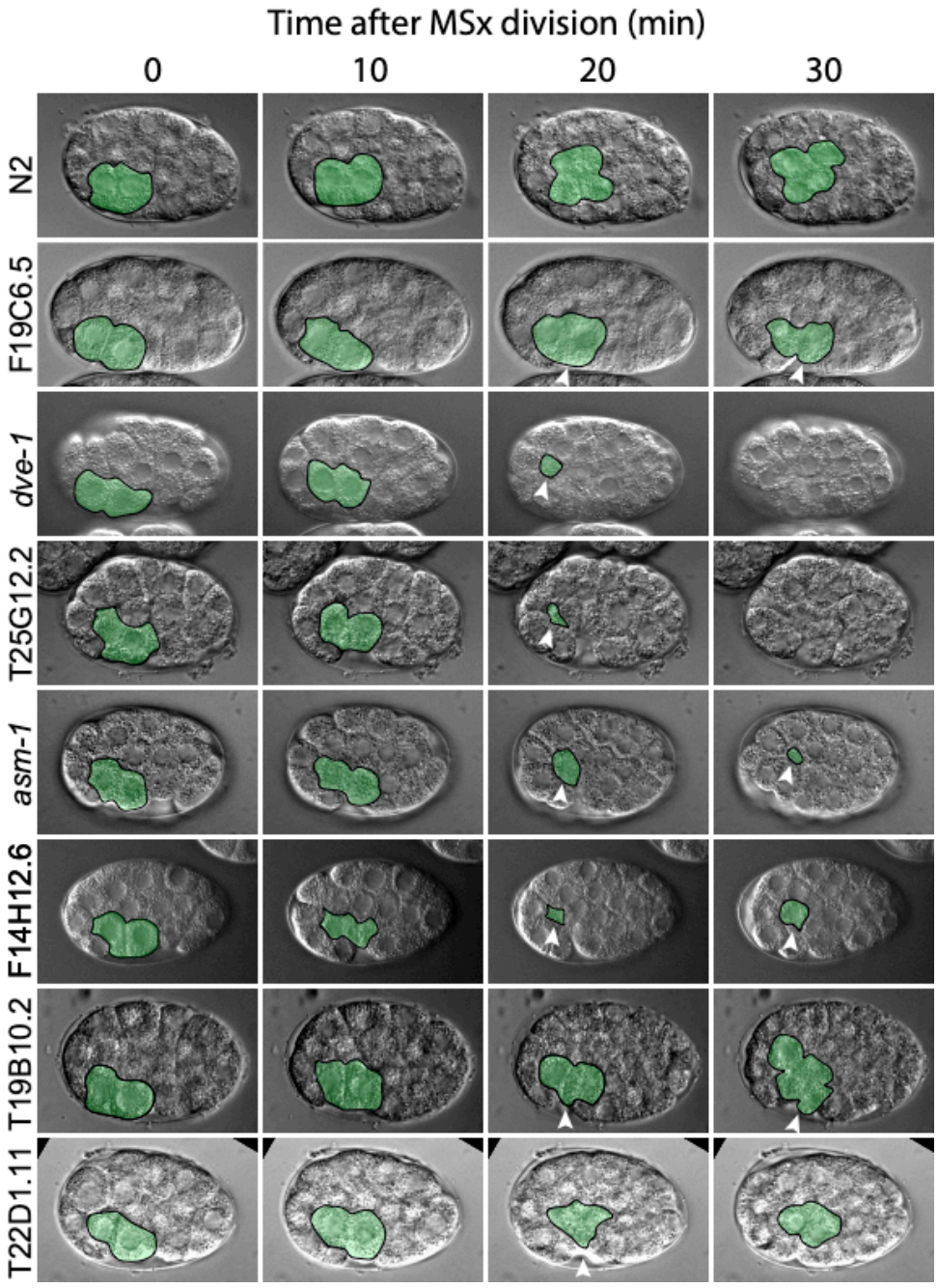

Figure 6. Targeting by RNAi reveals gastrulation defective (Gad) phenotypes, implicating 7 new candidates as regulators of gastrulation. Embryos were imaged using DIC microscopy 24 hours post injection of dsRNA targeted towards one gene (listed to the left). E cells are outlined in black and pseudo-colored green. Images are from single representative embryos displaying Gad phenotypes; individual time points were chosen at MSx division (0 mins), and 10, 20, and 30 minutes following the MSx division. In N2 (wild type), the E cells are fully internalized by 20 minutes and have divided by 30. Embryos were mounted laterally, but some Gad phenotypes (in *dve-1,* T25G12.2, *asm-1*, and F14H12.6) are visible only at the embryonic surface, pictured. White arrowheads indicate exposed surfaces resulting from late internalization.

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