A high-content imaging approach to profile *C. elegans* embryonic development

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ABSTRACT

The *C. elegans* embryo is an important model for analyzing mechanisms of cell fate specification and tissue morphogenesis. Sophisticated lineaging approaches for analyzing embryogenesis have been developed but are labor-intensive and do not naturally integrate morphogenetic readouts. To enable the rapid classification of developmental phenotypes, we developed a high-content method that employs two custom strains: a Germ Layer strain expressing nuclear markers in the ectoderm, mesoderm and endoderm/pharynx, and a Morphogenesis strain expressing markers labeling epidermal cell junctions and the neuronal cell surface. We describe a procedure that allows simultaneous live imaging of development in 80-100 embryos and provide a custom program that generates cropped, oriented image stacks of individual embryos to facilitate analysis. We demonstrate the utility of our method by perturbing 40 previously characterized developmental genes in variants of the two strains containing RNAi-sensitizing mutations. The resulting datasets yielded distinct, reproducible signature phenotypes for a broad spectrum of genes involved in cell fate specification and morphogenesis. Our analysis additionally provides new *in vivo* evidence for MBK-2 function in mesoderm fate specification and LET-381 function in elongation.

SUMMARY STATEMENT: This manuscript provides a high-content imaging-based method for phenotypically profiling genes required for cell fate specification and tissue morphogenesis in the *C. elegans* embryo.

INTRODUCTION

The *C. elegans* embryo is an important model for studying cell fate specification and morphogenesis (Armenti and Nance, 2012; Chisholm and Hsiao, 2012; Jackson and Eisenmann, 2012; Lamkin and Heiman, 2017; Loveless and Hardin, 2012; Priess, 2005; Spickard et al., 2018; Vuong-Brender et al., 2016; Wang and Seydoux, 2013). *C. elegans* embryonic development occurs in two phases (**Fig. 1A**). During the six hours following fertilization, 10 rounds of cell division coupled to cell fate specification generate the three germ layers (ectoderm, mesoderm, and endoderm; (Sulston et al., 1983)); the subsequent 7 hours are largely post-mitotic and consist of morphogenetic events that structure the differentiated tissues (Chisholm and Hardin, 2005; Vuong-Brender et al., 2016).

In a pioneering study, the invariant lineage of the embryo was manually determined by DIC microscopy (Sulston et al., 1983). Subsequent work imaging fluorescent histones enabled semi-automated tracking of nuclei through the first 9-10 rounds of cell division (Bao et al., 2006; Giurumescu et al., 2012; Santella et al., 2010). Newer methods have combined histone-based lineaging with monitoring of specific markers to assess the outcome of cell fate decisions (Du et al., 2015; Du et al., 2014; Ho et al., 2015; Murray et al., 2008; Murray et al., 2012). In one recent approach, the fate of cells in the 13-cell stage embryo was uniquely barcoded by monitoring the combinatorial expression of lineage-specific markers among their progeny 5 cell divisions later (Du et al., 2015; Du et al., 2014). While lineaging-based approaches are very powerful, they also have limitations. In particular, they are labor-intensive, require expertise beyond that available in the majority of labs, and are relatively low throughput, which limits their utility for larger-scale screening efforts. Extending lineaging-based analysis to inform on morphogenetic events during the second half of embryogenesis has also been challenging (Christensen et al., 2015).

Here, we describe a technically straightforward method that complements existing approaches to enable rapid profiling of a broad spectrum of developmental processes during embryogenesis. The method is based on a pair of custom-engineered strains: a Germ Layer strain expressing nuclear markers in the ectoderm, mesoderm and endoderm/pharynx, and a

Morphogenesis strain that labels epidermal cell junctions and the neuronal cell surface. We describe a filming procedure that allows the simultaneous monitoring of 80-100 embryos in 3D at 20-minute time resolution and provide user-friendly custom software that locates individual embryos within a larger field, to generate cropped, oriented image stacks; we further provide a FIJI script that compiles the cropped sequences into composite 4D movies. We demonstrate the utility of our approach by characterizing the knockdown phenotypes of 40 previously characterized developmental genes. Manual scoring revealed that our method generates reproducible and informative phenotypic signatures that enable the functional classification of genes involved in a broad spectrum of developmental processes. We also uncovered previously unreported functions for two genes in the 40-gene set. Specifically, our approach provided *in vivo* evidence that MBK-2 acts in anterior fate specification and that LET-381 acts in the late stages of embryo elongation.

RESULTS

Transgenic markers for high-content imaging of germ layer specification and morphogenesis during C. elegans *embryogenesis*

To monitor cell fate specification and morphogenesis during *C. elegans* embryogenesis, we developed a pair of strains expressing different sets of fluorescent markers. The three germ layers—endoderm, ectoderm, and mesoderm-—are a central organizing principle of embryonic development (Hall, 1999; Wolpert et al., 2015). An analysis of the *C. elegans* genes expressed in each germ layer suggested that the endoderm evolved first, followed by the ectoderm and mesoderm (Hashimshony et al., 2015), suggesting that embryogenesis may employ germ layer-specific programs. To monitor effects on cell proliferation and positioning in the three germ layers, we constructed a "Germ Layer" reporter strain by using germ layer specific promoters to express fluorescent nuclear markers. The ectoderm includes the epidermis and nervous system; to represent both components, we constructed a composite transgene on Chromosome II that uses a modified *dlg-1* promoter (P*dlg-1Δ7*; (Sheffield et al., 2007)) and the *cnd-1* promoter (Murray et al., 2008) to drive expression of mCherry-tagged histone (HIS-72) in the epidermis and ~40% of the neurons in the embryo (**Fig. 1B; Fig. S1**). For neuronal expression, we also tested three other promoters; however, these were not optimal due to nonspecific *(*P*unc-33*, P*unc-119)* or lack of (P*rgef-1*) expression during embryogenesis. A GFP-tagged ectoderm transgene was also generated that was not used in the final strain (for a list of all transgenes generated, including ones not in the final strains, see **Fig. S1, Table S1).** The composite mesoderm transgene, constructed on Chromosome IV, includes mCherry- and GFP-tagged histones in tandem under control of the *hlh-1* promoter which drives expression in body wall muscle (P*hlh-1*; (Krause et al., 1994)); **Fig. 1B**). Combining green and red reporters, rather than using a YFP tag, allows 3 color imaging by collecting z-stacks in two, rather than three, fluorescent channels, increasing the number of fields that can be imaged in a given time interval. To mark endoderm nuclei, we initially used the *elt-2* promoter to express mCherry- or GFP-tagged histone (HIS-72) in the intestine. However, for the final strain we instead chose a transgene that expresses a GFP

fusion with the PHA-4 transcription factor (**Fig. 1B**; (Fakhouri et al., 2010; Zhong et al., 2010)), because it labels nuclei in the pharynx as well as the intestine. The pharynx is a complex organ of mixed lineage that is part of the digestive tract (Altun and Hall, 2009; Mango, 2007). In summary, we generated a toolkit of strains with transgenes expressing fluorescently-tagged nuclear markers in the three germ layers (**Fig. 1B, Fig. S1; Table S1**), and combined three of these, which respectively mark the ectoderm, mesoderm, and endoderm/pharynx in red, yellow, and green in a composite Germ Layer reporter strain (**Fig. 1B**). To capture morphogenetic movements and cell shape changes during the second half of embryogenesis, we also generated a Morphogenesis reporter strain that uses the P*dlg-1Δ7* and P*cnd-1* promoters to express the epithelial junction marker DLG-1::GFP in the epidermis and an mCherry-tagged plasma membrane marker in a subset of neurons (**Fig. 1B, Fig. S1, Table S1**).

A high-content imaging method to capture germ layer specification and morphogenetic events during* C. elegans *embryogenesis

C. elegans embryogenesis takes ~13 hours, making it difficult to image enough embryos using one-at-a-time filming methods. To solve this problem, we developed a protocol using a spinning disk confocal with point visiting capacity and a holder for 384-well glass bottom plates (Yokogawa CV1000; **Fig. 1C**). For each condition, 2 to 8-cell stage embryos manually dissected from ~10-15 hermaphrodites are loaded into one well with a mouth pipet. Although only 14 or fewer of the 384 wells are used in each experiment, the advantage of the 384-well plate is that it confines embryos to a small area, enabling rapid identification of multiple fields containing several embryos each. To ensure that embryos develop in a roughly synchronized fashion, the dissection media and imaging plate are kept on ice to stall embryogenesis until all wells have been loaded. We limit time on ice to one hour, which is enough time for one person to dissect worms for ~7-8 conditions; up to 14 conditions can be imaged by having two people dissect in tandem.

The fluorescent reporters in our strains are first expressed about 2.5 hours after first cleavage. Thus, there is enough time to perform a low resolution (10X) scan and select fields for high resolution imaging before the markers are expressed. The close proximity of the wells allows high resolution imaging with a 60X, 1.4NA oil lens. We typically collect green, red, and bright field z-series (15 to 18 z-planes at 2 µm intervals) of each field every 20 minutes for 10 hours in a temperature-controlled room that allows us to maintain embryos between 21-23°C. This procedure allows imaging of 80-100 total embryos from up to 14 conditions in a single overnight run. Following imaging, we perform a low resolution (10X) well scan to assess embryonic lethality and larval abnormalities. Embryos subjected to dissection and imaging exhibit only slightly higher levels of embryonic lethality than embryos subjected to dissection alone (~11-12% for dissected, filmed embryos versus 6% for dissected embryos in both strains; **Fig. 1D**) suggesting that the procedure is well tolerated.

Visualization of developmental events in the Germ Layer and Morphogenesis strains

In this section, we highlight developmental events as they appear in embryos from the Germ Layer and Morphogenesis strains imaged using the above protocol (**Movies S1 and S2**). The comma stage (t=0, corresponding to ~395 minutes post fertilization) is used as a temporal landmark to compare events in the two strains.

Morphogenesis Strain: mCherry-marked neurons appear in two patches positioned on the left and right sides of the embryo anterior about 120 minutes before the comma stage (**Fig. 2A,** top row, -120 and -100 minute panels). Slightly later, two additional patches appear on the left and right sides in the embryo mid-region, so that the neurons appear partitioned into four quadrants (**Fig. 2A,B;** -60 minute panels). During the 60 minutes prior to the comma stage, the neuron patches on the two sides of the embryo move towards each other until they meet at the midline (**Fig. 2A**,**B**; -60 to 0 minute panels). The overlying epidermis, marked with the GFP-tagged apical junction marker, moves with the neurons, and seals at the midline to encase the embryo in skin (Ventral Enclosure; **Fig. 2A-D**). On the dorsal side of the embryo, the intercalation and subsequent fusion of the epidermal cells occurs during the same time

interval (**Figs. 2E, S2A**; Dorsal Intercalation, (Chisholm and Hardin, 2005)). During the 80 minutes that follow the comma stage, the epidermis in the head is organized in concentric rings that appear to constrict and extend towards the anterior to encase the head in skin (**Fig. 2F**, Anterior Enclosure). During anterior enclosure, the bi-lobed neuronal structure formed when the anterior patches met at the midline extends towards the anterior and is structured to form the early head ganglia and nerve ring (**Fig. 2A,B,F**; (Hallam et al., 2000; Shah et al., 2017; Sulston et al., 1983)). Between 60 and 120 minutes after the comma stage, the neurons below the midline intercalate via convergent extension to generate the ventral nerve cord (**Fig. 2A,G**; Ventral Nerve Cord Elongation, (Shah et al., 2017)).

Germ Layer Strain: The ectoderm, mesoderm, and endoderm/pharynx nuclear markers are expressed beginning ~120 minutes prior to the comma stage (**Figs. 2A, S2A**). Since the yellow mesoderm is a superposition of red and green signals, comparing images of the Germ Layer strain with images of strains expressing the three transgenes individually (**Fig. S2B**) can be helpful to understand the composite strain. PHA-4::GFP is in nuclei in the pharynx and endoderm, which are positioned in the central core of the embryo throughout development (**Fig. S2B**). The mesoderm nuclei, which are visible in both the red and green channels, form a cup-like structure that surrounds the green Endoderm/Pharynx nuclei in the posterior 3/4 of the embryo (**Fig. S2B**). Since the mCherry fluorophore takes longer to mature than GFP, the mesoderm nuclei start out more green and then become fully yellow as the mCherry signal intensifies (**Fig. S2B**). When the neurons in the four patches become visible in the red channel in the Morphogenesis strain, the nuclei in the same neurons become visible in the red channel in the Germ Layer strain (-60 minute timepoint in **Fig. 2A** and **Fig S2B**). The nuclei in the epidermis, which are enriched at the lateral edges of the embryo and form a cap that sits over its anterior end, also appear in the red channel in the Germ Layer strain (-60 minute timepoint in **Fig. S2B**). We conclude that the positioning of the germ layers and morphogenetic events during embryogenesis are clearly visualized using our strains and filming method.

Penetrant phenotypes are observed after RNAi in sensitized variants of the Germ Layer and Morphogenesis Strains

A comparison of injection, soaking and feeding as delivery methods for dsRNAs targeting a small set of test genes revealed that injecting L4 hermaphrodites with dsRNA and incubating them at 20°C for 24 hours prior to dissection yielded the most reproducible results (*data not shown*). Next, we determined whether using a previously described RNAi-sensitized background (*nre-1(hd20) lin-15b(hd126)*; Schmitz et al., 2007) would enhance the penetrance and severity of developmental phenotypes. We targeted 40 genes involved in a spectrum of developmental processes (**Fig. 4B**, **Table S2.7**) in four strains: (1) the Germ Layer strain (Germ Layer Control), (2) the Germ Layer strain with RNAi-sensitizing mutations (Germ Layer Sensitized), (3) the Morphogenesis strain (Morphogenesis Control), and (4) the Morphogenesis strain with RNAi-sensitizing mutations (Morphogenesis Sensitized). Time-lapse sequences of embryos (60X) and post scans (10X) were acquired (**Fig. 3A**).

In the absence of RNAi, addition of the sensitizing mutations to the Germ Layer and Morphogenesis strains did not alter hatching rates (**Fig. 3B**), indicating that the sensitizing mutations did not cause embryonic lethality on their own. However, for both strains, embryonic lethality was significantly higher following RNAi targeting the 40 test genes in the strains carrying the RNAi-sensitizing mutations compared to the control background (points above the diagonal in **Fig. 3C;** for a breakdown of which genes were enhanced see **Fig. S3A** and **Table S2.1**). To assess phenotypic severity, we scored the predominant arrest point for each depletion in each strain (**Fig. 3A**; from least to most severe—larval abnormal/lethal, embryonic lethal, 3-fold arrest, 2-fold arrest, 1.5-fold arrest, comma arrest, early arrest). In both strains, arrest points were shifted from less to more severe in the sensitized background compared to the control (**Fig. 3E,F**; note that arrest point phenotypes for the same RNA tended to be slightly more severe in the Germ Layer strain compared to the Morphogenesis strain). As an example of a typical shift in severity between the control and sensitized backgrounds, RNAi of *dsh-2*, one of three *C. elegans* Disheveled homologs that contributes to WNT signaling, led to a late elongation arrest (2-3 fold) in the control background and 1.25-fold arrest with defects in

neuronal organization and head morphology in the sensitized background (**Fig. 3D, Movie S3**).

Manual annotation of developmental defects observed in the Germ Layer and Morphogenesis strains partitions development genes into four broad classes

To assess the utility of our strains for profiling developmental phenotypes, we scored the time-lapse sequences for more detailed features (**Fig. 4A**; for defect examples and descriptions see **Fig. S4** and **Table S2.2**). In the Germ Layer strain, the nuclear pattern was visually assessed at the comma stage (or arrest point, if earlier than comma stage). Embryos with an aberrant nuclear pattern, which can result from defects in either cell fate specification or cell positioning, were scored as having a "cell fate/positioning defect"; nuclear abnormalities were also scored (**Fig. S4B,C**). In the Morphogenesis strain, we scored defects in nervous system and epidermal morphology, including defects in the row of seam cells, intercalation of the epidermal cells on the dorsal side of the embryo and ability of the epidermis to enclose the ventral side or head of the embryo (**Fig. S4D-F**). Note that the penetrance of three defects (ventral rupture, dorsal intercalation and seam cell defects) may be an underestimate, because the ability to score them depends on embryo orientation (for the number of embryos in each orientation see **Table S2.3**). The defects scored in the RNAi-sensitized background are summarized in Figure 4B (for all defects scored in the control and RNAi-sensitized backgrounds for both strains see **Table S2.3**). Overall, this assessment partitioned the 40 genes into four broad classes (**Fig. 4B**): (1) genes whose inhibition led to cell fate and/or positioning defects, which generally arrested at or prior to the comma stage (Cell Fate or Positioning), (2) genes whose inhibition led to arrest at the comma or 1.5 fold stage accompanied by ventral or anterior rupture and other morphogenetic defects (Early Morphogenesis), (3) genes whose inhibition led to defects in elongating past the 2-fold stage without a detectable effect on nuclear specification or positioning (Elongation), and (4) genes whose inhibition led to an arrest at or after the 3-fold stage and/or to larval abnormalities (Late Morphogenesis/Larval Development). Inspection of published expression profiles for the

genes in our broad classes ((Levin et al., 2012); **Table S2.5**) revealed that genes in the groups required for cell fate specification and/or positioning (group 1) and early morphogenesis (group 2) showed expression signatures consistent with either maternal expression (initially high and then falling gradually during embryogenesis) or relatively constant expression (suggesting both maternal and ongoing zygotic expression). In contrast, expression of genes required for elongation (group 3) or late morphogenesis/larval development (group 4) tended to be upregulated later during development, most commonly between the 7th AB-division and ventral enclosure, a time proposed to correspond to a nematode phylotypic stage because of the number of critical developmental genes that turn on (Levin et al., 2012).

Custom programs to crop and orient individual embryos for 4D data visualization and analysis

To isolate embryos for quantitative analysis, we developed custom software that crops out individual embryos from broader fields (**Fig. 4C**) and orients them based on asymmetry in the red channel signal (**Fig. 4D**), while also performing background subtraction and correcting for signal attenuation with imaging depth. This program, which is accompanied by a graphical user interface (GUI), is compatible with image (tif) stacks acquired on most platforms and saves each embryo to a new, tightly cropped, anterior-posterior oriented stack (for details see Materials and Methods). We used the cropping program to generate tif files for individual embryos and assembled them into composite movies for each condition in each strain. The composite movies for each of our 40 RNAi conditions are available at (https://datadryad.org/review?doi=doi:10.5061/dryad.kb79121).

High-content imaging in the Germ Layer and Morphogenesis strains provides an efficient means to functionally profile genes required for cell fate specification and positioning

To evaluate the utility of the methodology described above for functionally profiling, we began by analyzing the 18 genes in our test set that were scored with defects in cell fate

specification or cell positioning and arrested prior to the 2-fold stage. These genes can be partitioned into three classes (**Fig. 5A**): (1) genes whose inhibition leads to incomplete cell fate specification because embryos arrest prior to the completion of cell division, (2) genes whose inhibition leads to normal numbers of each nuclei in each germ layer, despite an abnormal visual pattern, suggesting a role in cell positioning, and (3) *bona fide* genes required for cell fate specification. To partition the 18 genes between these classes, we segmented and counted the number of red, green, and yellow nuclei in cropped Germ Layer image stacks at the comma stage (or arrest point, for embryos that arrested prior to the comma stage; **Fig. 5B**).

Genes whose inhibition results in incomplete cell fate specification show low nuclear counts for all three germ layers. The strongest example in our test set was PLRG-1, a component of the CDC5L complex required for pre-mRNA splicing (Ajuh et al., 2000; Ajuh et al., 2001); *plrg-1* knockdown embryos exhibited an early arrest prior to marker expression in both strains (**Fig. 5A-C**; **Movie S4**). The *plrg-1(RNAi)* phenotype resembled that previously reported to result from blocking zygotic transcription (Nance and Priess, 2002; Powell-Coffman et al., 1996). Consistent with this idea, inhibition of a key RNA polymerase II subunit in our strains resulted in a similar early stage arrest prior to marker expression (*rpb-3(RNAi)*, data not shown). Embryos in which *aps-1* was knocked down exhibited weaker incomplete specification, arresting with ~25 fewer nuclei of each type than controls (**Fig. 5B**), along with severe epidermal and nervous system morphology defects (**Movie S4**). There were five class 2 genes which, despite exhibiting an abnormal nuclear pattern, had normal (*arx-1, arx-3, let-19, gex-2,*) or only slightly reduced (*die-1*) nuclear counts for the three germ layers, suggesting a primary defect in cell positioning and/or early morphogenesis. Three of these encode regulators of actin assembly: ARX-1 and ARX-3 are components of the Apr2/3 complex (Pollard, 2017; Sawa et al., 2003), and GEX-2 is a component of the WAVE complex, which regulates the Arp2/3 complex in response to Rac signaling (Patel et al., 2008; Soto et al., 2002; Sullivan-Brown et al., 2016). Consistent with prior work, knockdown of all three genes led to a ventral rupture phenotype, particularly well captured in the morphogenesis strain, in

which the epidermis compressed into a patch on the dorsal side of the embryo (**Fig. 5C**; **Movie S4**), concurrent with an inside out inversion that ejected the embryo contents out of its ventral side. Knockdown of the two other class 2 genes, *die-1* and *let-19*, gave distinct morphogenesis defects. DIE-1 is a C2H2 zinc finger transcription factor implicated in cell intercalation (Heid et al., 2001; Rasmussen et al., 2013). In *die-1* knockdown embryos, the neuron patches on the right and left sides of the embryo came together as in controls, but dramatic defects in epidermal morphology, particularly on the dorsal and ventral sides of the embryo, prevented elongation and further development (**Fig. 5C**; **Movie S4**). In *let-19* knockdown embryos, defects in epidermal morphology were accompanied by anterior rupture (see **Movie S4** and legend).

Eleven cell fate/positioning genes exhibited altered nuclear counts that suggested a primary defect in cell fate specification (**Fig. 5B**). These included 4 genes required for WNT signaling (*pop-1*, *mom-2*, *wrm-1* and *lit-1*), which controls asymmetric divisions in which the two daughter cells adopt distinct fates. POP-1 is a TCF/LEF family protein that is an essential component of a transcriptional repressor that prevents the expression of genes that specify endoderm fate; thus, *pop-1* inhibited embryos have extra endoderm (Jackson and Eisenmann, 2012; Sawa and Korswagen, 2013). *C. elegans* has several WNT proteins, but the most important of these in the embryo is MOM-2. Binding of the MOM-2 ligand to its receptor converts POP-1 from a transcriptional repressor to an activator that promotes endoderm fate. The divergent β-catenin WRM-1 and the Nemo-like kinase LIT-1 promote POP-1 nuclear export, which is essential for transduction of the WNT signal; thus, *mom-2*, *wrm-1* and *lit-1* knockdown embryos all lack endoderm and are expected to exhibit similar phenotypes (Jackson and Eisenmann, 2012; Sawa and Korswagen, 2013). Consistent with this expectation, RNAi of all three genes led to embryos that arrested with a similar sectored appearance (**Fig. 5D**, **Movie S5, Fig. S5A**). The similarity was particularly striking for *lit-1* and *wrm-1(RNAi)* embryos, where a large patch of green nuclei ran along the length of the embryo on one side opposite a patch of red nuclei on the other side with a cap of yellow nuclei at one end (**Fig. S5A,** regions marked with dashed outlines); both also exhibited similar disorganized

epidermal and neuronal marker expression in the morphogenesis strain (**Fig. 5D**, **Movie S5**). As expected, the *pop-1* phenotype was distinct from the phenotypes of the other three genes (Jackson and Eisenmann, 2012). In *pop-1* inhibited embryos, a stripe of green nuclei (presumably endoderm) appeared in the central core of the embryo. As development proceeded, patches of green nuclei appeared to be encased by an outer layer of red nuclei in one half of the embryo and an outer layer of yellow nuclei in the other half (**Fig. S5A,** regions marked with dashed outlines). Although the phenotypes were clearly distinct, the effects of the two classes of WNT perturbation on nuclear counts (**Fig. 5B**) were similar due to the fact that the green PHA-4 marker is expressed in both pharyngeal and endoderm nuclei, which masks conversions between endoderm and pharyngeal fates characteristic of WNT signaling defects in a counting assay. In addition to genes controlling WNT signaling, the cell fate specification genes also included two genes (*aph-1* and *lag-1*) that encode components of the Notch pathway that mediates cell-cell contact-based signaling required for anterior pharynx specification (Greenwald and Kovall, 2013; Sjoqvist and Andersson, 2017). APH-1 is a component of gamma secretase that cleaves the Notch receptor upon ligand binding to generate a fragment that translocates into the nucleus, where it interacts with LAG-1 to promote target gene transcription (Greenwald and Kovall, 2013). In the Germ Layer strain, *aph-1* and *lag-1* knockdown embryos exhibited an “empty head” phenotype in which green pharyngeal nuclei were absent in the head region (**Fig. 5B,E,F**; **Movie S5**); the epidermis and neurons also exhibited similar abnormal dynamics in the morphogenesis strain (**Fig. 5D**). Thus, high-content filming in the two reporter strains yielded distinct, reproducible signature phenotypes following inhibition of components of the WNT and Notch pathways.

Four of the *bona fide* cell fate specification genes were tissue-specific fate regulators. Inhibition of *pha-4*, a transcription factor that specifies the pharynx (Mango, 2007), resulted in a strong reduction in the number of green endoderm/pharynx nuclei (**Fig. 5B**) and an empty head phenotype similar to loss of Notch pathway components; green endoderm nuclei were also absent (**Fig. 5E,F**). This was expected since our green endoderm/pharynx reporter is PHA-4::GFP and confirms that our RNAi conditions target PHA-4 efficiently. The embryos also

displayed defects in epidermal and neuronal organization and elongation consistent with loss of the pharynx (*pha-4* sequences at https://datadryad.org/review?doi=doi:10.5061/dryad.kb79121). Knockdown of *elt-1*, which encodes a transcription factor required for epidermal specification (Gilleard and McGhee, 2001; Page et al., 1997), led to a marked reduction in red ectoderm nuclei in the Germ Layer strain (**Fig. 5B**) and a striking lack of an outer epidermal layer in the Morphogenesis strain (**Fig. S4E,F**). Two cell fate specification genes were involved in mesoderm specification, *pal-1* and *mex-3*. PAL-1 is a conserved homeodomain protein that specifies the C and D blastomeres, which produce primarily body wall muscle (Hunter and Kenyon, 1996). Knockdown of *pal-1* in the Germ Layer strain led to a reduction in the number of mesoderm nuclei (**Fig. 5B**) and arrest with a visible void in the posterior where the mesoderm normally resides (**Fig. 5E,F**). MEX-3 binds the 3'UTR of the *pal-1* message and represses its expression in the embryo anterior (Hunter and Kenyon, 1996; Pagano et al., 2009). Thus, *mex-3* inhibition leads to ectopic PAL-1 expression and an anterior-to-posterior cell fate transformation that produces extra muscle (Draper et al., 1996). Consistent with this, *mex-3* knockdown in the Germ Layer strain led to a dramatic increase in number of yellow muscle nuclei at the expense of red and green nuclei (**Fig. 5B,E**). In summary, distinct reproducible phenotypes, consistent with their established roles, were observed for each of the four established tissue-specific regulators in our test gene set.

A gene for which our analysis provided new insight is *mbk-2*, which encodes a DYRK (dual-specificity YAK-1-related) family kinase that controls the maternal-to-zygotic transition. Penetrant MBK-2 depletion leads to a complex phenotype that includes cell division defects due to failure to degrade the microtubule severing complex katanin at the meiosis-to-mitosis transition (Robertson and Lin, 2015). Under our conditions in the Germ Layer strain with the RNAi-sensitizing mutations, *mbk-2(RNAi)* led to a bimodal phenotype: half of the embryos exhibited an early arrest with relatively few nuclei, consistent with cell division defects; however, the other half, presumably those in which *mbk-2* was only partially inhibited, exhibited a muscle in excess (Mex) phenotype, similar to *mex-3* inhibition (**Fig. 5B,E**; **Movie**

S6). Consistent with the idea that the Mex phenotype results from partial inhibition of *mbk-2*, 6 out of 6 embryos exhibited the Mex phenotype in the control background (**Fig. S5B**, **Table S2.3**). MEX-3 lies downstream of MEX-5/6, two partially redundant zinc finger proteins that prevent MEX-3 degradation in the embryo anterior (Hunter and Kenyon, 1996; Schubert et al., 2000); as expected, *mex-5*/*6* knockdown resulted in a phenotype essentially identical to *mex-3* knockdown (**Movie S6**). Prior biochemical work suggested that MBK-2 activates MEX-5/6 by phosphorylation at a specific site that enables docking of a second kinase, PLK-1 (**Fig. 5G**; (Nishi et al., 2008)). Despite the elegant *in vitro* biochemistry, direct *in vivo* evidence for MBK-2 function in cell fate specification has been lacking (Robertson and Lin, 2015). Our observation that *mbk-2* inhibited embryos can exhibit a classic Mex phenotype fills this gap, providing *in vivo* support for proposed role of MBK-2 in preventing PAL-1 expression in the embryo anterior (**Fig. 5G**). These results suggest that high-content analysis in our strains has the potential to provide new insights for other well-characterized development genes.

Filming in the Germ Layer and Morphogenesis strains enables functional classification of genes required for elongation

After epidermal enclosure, the embryo elongates 4-fold in length accompanied by a 2-fold decrease in diameter to achieve its final shape ((Vuong-Brender et al., 2016); **Fig. 6A**). Five genes in our test set encoded proteins that first become essential at this stage (**Fig. 4B**, **Fig. 6B**). Elongation can be divided into two phases that begin prior to and after the onset of muscle contraction ((Vuong-Brender et al., 2016); **Fig. 6A**). During the first phase, elongation is driven by changes in epidermal cell shape that require stimulation of the small GTPase RhoA (RHO-1 in *C. elegans*) leading to increased actomyosin contractility in seam cells (green in **Fig. 6A**), concurrent with suppression of the same pathway in the dorsal and ventral epidermis (red in **Fig. 6A**; (Vuong-Brender et al., 2016)). RhoA promotes actomyosin contractility by activating the LET-502/Rho kinase, which controls the activity of myosin II (Gally et al., 2009; Wissmann et al., 1997). The second phase begins when the muscles start to contract (Vuong-Brender et al., 2016). Muscles, which are internal to the epidermis, are

attached to the cuticle outside the epidermis via transepithelial attachment structures consisting of intermediate filaments anchored to fibrous organelles that span the plasma membrane on the apical and basal sides of the epithelium (**Fig. 6A**; (Zhang and Labouesse, 2010). Two of the five genes required for elongation were *let-502*, which is required for actomyosin contractility during phase 1 (Gally et al., 2009; Wissmann et al., 1999), and *vab-19*, which is essential for transepithelial muscle attachment during phase 2 ((Ding et al., 2003); **Fig. 6A,B**). Although both inhibitions led to arrest at the 2- to 3-fold stage, their phenotypes were clearly distinct. Embryos in which *let-502* was knocked down arrested without further movement, whereas *vab-19* knockdown embryos continued to quiver and the epidermis exhibited a crumpled end-stage phenotype (**Figs. 6C; Movie S7**).

Three additional genes exhibited specific elongation defects (*ceh-43*, *pha-1* and *let-381*). We will discuss *ceh-43* and *let-381* here (for a discussion of *pha-1* see **Fig. S6**) CEH-43 is the sole *C. elegans* ortholog of the Distalless/Dlx homeobox genes involved in appendage development in animals (Aspock and Burglin, 2001; Panganiban and Rubenstein, 2002). Prior work has shown that CEH-43 is expressed in the epidermis, neurons, and neuronal support cells in the head, and that cells leak out of the embryo from a hole in the epidermis at the tip of the head in *ceh-43* knockdown embryos (Aspock and Burglin, 2001). It was also reported that the pharynx disconnected from the buccal cavity (mouth) in these embryos and retracted into the embryo ("Pun" or pharynx unattached phenotype). Following *ceh-43* knockdown in the Germ Layer strain, we observed pharyngeal cells leaking out through a hole in the head; however, all of the pharyngeal cells ended up outside the embryo and did not subsequently retract (blue arrows in **Figs. 6C, S5C; Movie S7**). Since expulsion of the pharynx precludes its connection to the buccal cavity, it is not surprising that elongation, which likely requires the mechanical integrity of this connection, fails in *ceh-43* inhibited embryos.

FoxF forkhead transcription factors are generally implicated in mesoderm development (Amin et al., 2010; Mazet et al., 2006). Consistent with this, the single *C. elegans* FoxF-related transcription factor, LET-381, is required to specify the fate of the non-muscle mesoderm descendants of the M mesoblast (coelomocytes) during the larval stages (Amin et al., 2010).

LET-381 is also expressed in a number of unidentified cells in the head during embryogenesis (Amin et al., 2010). Mutants in *let-381* are embryonic lethal and hatched *let-381* larva have been reported to exhibit short or “dumpy” phenotypes (Amin et al., 2010; Simmer et al., 2003); however, embryonic elongation arrest phenotypes have not previously been described. Our analysis indicates that *let-381* knockdown results in phenotypes in the Germ Layer and Morphogenesis strains that are essentially identical to the *vab-19* knockdown phenotypes (**Fig. 6C**). Like *vab-19*, and in contrast to the *ceh-43* and *pha-1* knockdowns, *let-381* knockdown did not lead to head-specific defects, suggesting that LET-381 may have a role in the assembly of transepithelial attachment structures. Further investigation will be needed to address this possibility.

Overall, the analysis of the 40 gene set indicates that high-content filming in the germ layer and morphogenesis strains provides a rapid and effective means to generate precise phenotypic signatures for functional classification of genes involved in morphogenesis as well as cell fate specification during embryonic development.

DISCUSSION

Here, we describe a high-content imaging approach that captures both early and late developmental events during *C. elegans* embryogenesis. We also present a data collection procedure that allows simultaneous monitoring of development in 80-100 embryos and a custom program that generates cropped, oriented image stacks of individual embryos to facilitate data analysis. We anticipate that the methodology described here will be broadly useful for the characterization of developmental phenotypes and will also provide a framework for large-scale screening approaches to systematically characterize genes required for embryonic development.

Lineaging-based methods to analyze development were pioneered by groups interested in understanding cell fate specification (Bao et al., 2006; Du et al., 2015; Du et al., 2014; Giurumescu et al., 2012; Ho et al., 2015; Murray et al., 2008; Murray et al., 2012; Santella et al., 2010). One outcome of this work has been a description of the regulatory network controlling founder cell specification in the embryo (Du et al., 2015; Du et al., 2014). However, while the power of lineaging-based approaches is clear, they also have limitations. In particular, lineaging is labor-intensive, requires expertise beyond that available in the majority of labs, is low throughput, and is not sufficient for the analysis of morphogenetic events. A major goal of our work was to develop a technically straightforward approach that, by virtue of the combinatorial expression of carefully selected fluorescent markers, could be implemented in any lab to provide a first-pass analysis of a broad spectrum of key events during embryogenesis. To demonstrate the utility of the method, we used it to monitor development in cohorts of embryos after knocking down a test set of 40 genes that act in diverse developmental events. The results show that the method generates distinct, reproducible phenotypic signatures that provide functional insight and enable the classification of genes across a broad spectrum of processes required for cell fate specification and tissue morphogenesis. In addition to being consistent with prior work on individual genes, our test set data provided *in vivo* evidence for a role for MBK-2 in mesoderm fate specification,

extending prior biochemical work (Nishi et al., 2008), and suggested a role for LET-381 in elongation.

In addition to providing a standardized means for the initial characterization of developmental phenotypes in the embryo, the method described here is well-suited for the systematic analysis of cohorts of embryos following RNAi-mediated knockdown of each of the ~2000 genes essential for embryonic development. An ongoing project is already generating a large dataset of 3D time-lapse sequences in the Germ Layer and Morphogenesis reporter strains following each gene knockdown. We expect that the *C. elegans* community will take advantage of the approach described here to characterize mutants, thereby providing additional data that will add to the RNAi-based dataset. An important future direction will be the development of automated methods to identify phenotypic features in time lapse data and to compare and classify early and late developmental phenotypes. Although manual analysis methods are sufficient for the analysis of small datasets, automated methods will be essential to ensure consistent data analysis at reasonable throughput across larger datasets.

METHODS

Strain construction

All transgenes except for the transgene expressing PHA-4::GFP (Fakhouri et al., 2010; Zhong et al., 2010), were generated using a transposon-based strategy (MosSCI; Frøkjær-Jensen et al., 2008). Promoter sequences can be found in the supplement. In the Germ Layer strain, ectoderm nuclei were marked by expressing mCherry::HIS-72 using a truncated *dlg-1* promoter (-4422 to -483 of *dlg-1* 5'UTR; epidermis) and a *cnd-1* promoter (3230 bp of *cnd-1* 5'UTR; ~40% embryonic neurons; (Murray et al., 2008)). The truncated *dlg-1* promoter deletes the 7th and most proximal GATA motif in the promoter region (P*dlg-1Δ7*; (Pauli et al., 2006)), and thus drives expression in the epidermis, but not in the pharynx or intestine at the embryonic stage (Pauli et al., 2006). A subset of the mesoderm nuclei (muscle cells) were marked yellow by expressing both GFP::HIS-72 and mCherry::HIS-72 under control of the *hlh-1* promoter (3345 bp of *hlh-1* 5'UTR). Nuclei in the endoderm (intestine) and pharynx (mixed lineage) were marked green by expressing a nuclear-localized transcription factor PHA-4::GFP under the control of endogenous *pha-4* regulatory elements (GFP inserted at the C-terminus of the PHA-4 coding sequence by fosmid recombineering; (Fakhouri et al., 2010; Zhong et al., 2010)). The two transgene modules encoding the red ectoderm nuclei, P*dlg-1Δ7::mCherry::his-72* and P*cnd-1::mCherry::his-72*, were cloned in tandem into pCFJ151 for insertion at *ttTi5605* on Chr II, while the two transgene modules encoding the yellow muscle nuclei, P*hlh-1::mCherry::his-72* and P*hlh-1::GFP::his-72*, were cloned in tandem into pCFJ178 for insertion at *cxTi10882* on Chr IV. Single copy transgenes were generated by injecting a mixture of the above transgene constructs, a plasmid encoding the Mos1 transposase (pCFJ601, P*eft-3::Mos1 transposase*, 50 ng/µL) and three plasmids encoding fluorescent markers for negative selection [pCFJ90 (P*myo-2::mCherry*, 2.5 ng/µL), pCFJ104 (P*myo-3::mCherry*, 5 ng/µL) and pGH8 (P*rab-3::mCherry*, 10 ng/µL)] into strains EG6429 (outcrossed from EG4322; *ttTi5605*, Chr II) or EG6250 (*cxTi10882*, Chr IV). After one week, moving worms without fluorescent markers were identified as candidates and transgene integration was confirmed in their progeny by PCR spanning both homology regions. After integration

transgenes were outcrossed 6 times into the wild type N2 worm to clean up the genetic background. The transgene expressing PHA-4::GFP (stIs10389) was isolated from RW10425 (obtained from CGC; (Fakhouri et al., 2010; Zhong et al., 2010)) by outcrossing 6 times into N2 and selecting for the PHA-4::GFP transgene. The three transgenes were then combined together to obtain the final Germ Layer strain (OD1719). To make the strain more sensitive to RNAi, two linked mutations [*nre-1(hd20) lin-15b(hd126)*; (Schmitz et al., 2007)] were introduced to obtain the sensitized Germ Layer strain (OD1854).

A second set of transgenes was generated to visualize the apical junctions of epidermis and the cell surface of neurons. The epithelial junctions were marked in the epidermis by expressing DLG-1::GFP using the P*dlg-1Δ7* promoter; this transgene was cloned into pCFJ352 for insertion at ttTi4348 on Chr I. The cell surface of neurons was marked by expressing an mCherry tagged plasma membrane marker (Audhya et al., 2005) using the *cnd-1* promoter (3230 bp of *cnd-1* 5'UTR; ~40% of embryonic neurons; (Murray et al., 2008)); this transgene was cloned into pCFJ151 for insertion at ttTi5605 on Chr II. Single copy transgenes were generated as described above and outcrossed 8x (P*dlg-1Δ7*::*dlg-1::GFP*) or 6x (P*cnd-1::mCherry::PH*) into N2 wild type worms. The two transgenes were then crossed together to generate the Morphogenesis strain (OD1689). To make the strain more sensitive to RNAi, two linked mutations [*nre-1(hd20) lin-15b(hd126)*; (Schmitz et al., 2007)] were introduced to obtain the sensitized Morphogenesis strain (OD1853). To ensure the RNAi enhancer mutations were introduced, OD1853 was backcrossed 4 times into the RNAi enhancer strain CZ8244 to obtain the final "morphogenesis sensitized strain" (OD2416).

RNA interference

Double stranded RNAs were generated by using the primers in Table S2.7 to amplify a 500-1000 bp coding region of the corresponding gene from genomic DNA. PCR reactions were cleaned (QiaQuick, Qiagen) and used as templates for T3 and T7 transcription reactions (Megascript T3 and T7 kits, Ambion), which were combined, cleaned (RNAeasy, Qiagen), and annealed by adding 3x Soaking buffer (32.7 mM Na2HPO4, 16.5 mM KH2PO4, 6.3 mM NaCl,

14.1 mM NH4Cl) to a final concentration of 1X and incubating the reactions at 68°C for 10 min followed by 37°C for 30 minutes.

RNAi was performed by injecting dsRNA into 12-18 L4 stage worms each from the Morphogenesis and Germ Layer strains. Injected worms were allowed to recover on OP50 seeded plates for 20-22 hours prior to dissection. Prior analysis of the expression of the genes in our test set (**Table S2.5**) suggests that genes required for cell fate specification and early morphogenesis generally exhibit either maternal or both maternal and zygotic expression, whereas genes whose inhibition results in later phenotypes tend to be zygotically expressed. Thus, getting good developmental phenotypes requires inhibition of both maternal and zygotic gene expression. In our experience, the key variable is the time between injection and embryo filming, which determines both the extent of maternal protein depletion and the amount of injected dsRNA loaded into the embryo. After the dsRNA is injected, the maternal mRNA corresponding to the gene is degraded. Depletion of the pre-existing protein then requires embryo production which ejects the pre-existing maternal protein from the germline by loading it into the forming embryos. The shortest incubation time that allows consistent maternal depletion is about 20 hours at 20°C and maternal depletion gets better at later times up to about 36-42 hours after injection. Preventing zygotic gene expression in the embryos depends on the amount of the injected dsRNA that is loaded into the embryo. The amount of RNA loaded is highest at about 5-10 hours after injection when embryos that have incorporated injected material first begin to be fertilized and then declines after this point. By 36-48 hours after injection, we have often observed that zygotic inhibition starts to be lost, presumably because insufficient RNA is loaded into the embryos produced at these late timepoints after injection. Consequently, we chose 20-22 hours after dsRNA injection as a convenient sweet spot where maternal protein depletion and inhibition of zygotic gene expression in the isolated embryos are both quite good.

Embryo dissection and image acquisition

For each RNAi condition, ~10 gravid adults were dissected in ice cold tetramisole hydrochloride (TMHC; 0.1 mg/ml) dissolved in M9, and 2 to 16-cell stage embryos were transferred by mouth pipet to individual wells containing 70µl of the same solution in a glass bottom 384 well Sensoplate (Greiner Bio-One), which was maintained on ice until dissection was completed for all conditions. For each overnight run, 14 conditions were prepared: a control for each strain (2 wells) and 6 RNAi conditions in both strain backgrounds (12 wells). Mouth pipets were switched and dissection equipment cleaned between dissection of the worms for each condition to prevent cross-contamination. Prior to imaging, the 384-well plate was spun for 1 minute at 600 x g to seat the embryos. Embryos were imaged in a CV1000 confocal scanner box equipped with a microlens enhanced dual Nipkow spinning disk (Yokogawa Electric Corporation), a 512x512 EM-CCD camera (Hamamatsu), a high-precision auto-XY-Stage (designated resolution 0.1µm) and motorized z-axis control (designated resolution 0.1µm). The room was maintained at 16°C to allow the instrument to maintain the imaging chamber between 21 and 23°C. Fields with suitable embryos were identified by performing a pre-scan of each well using a 10X 0.4 NA U-PlanApo objective. Two (controls) or four (RNAi conditions) fields, with between 1 and 5 embryos per field, were imaged. Overall, a total of 4 to 15 embryos from each strain for each condition were selected for high resolution imaging. Each field was imaged by using a 60X 1.35NA U-PlanApo objective to acquire 15 (initial screen) or 18 (nuclear counting) z-sections at 2 µm intervals every 20 minutes for 10 hours. Imaging conditions were as follows: (1) bright-field: 90% power, 25 ms, 20% gain, (2) 488nm: 100% power, 200 ms, 60% gain, (3) 568nm: 45% power, 150 ms, 60% gain. To assess embryonic lethality, wells were post-scanned 24 hours after the start of imaging using bright-field optics and a 10X 0.4 NAU-PlanApo objective. All image acquisition was performed using CellVoyager software.

Embryonic lethality scoring

Embryonic lethality and larval defects were scored in the post-run 10X scanned fields (16 fields per well). Hatched worms and unhatched embryos were counted for each well. All unhatched embryos were scored as embryonic lethal, with the exception of arrested 1-4 cell stage embryos, which were excluded from the count because we observed that embryos dissected from control worms that emerge from the mothers prior to the end of meiosis II, when the eggshell permeability barrier forms, frequently arrest prior to the 4-cell stage due to osmotic complications during the first two divisions. To circumvent this issue, we also avoid transferring one cell stage embryos into the imaging wells, which reduces the possibility of including embryos with partially formed eggshells. Partially hatched or fully hatched worms with body morphology or behavioral defects, such as dumpy or paralyzed, were scored as "abnormal larva". Embryonic lethality was assessed for controls in each run. In the rare event that embryonic lethality in control wells exceeded 20% lethality (two standard deviations from the mean for embryonic lethality percentage) the RNAi conditions for that run were repeated.

Automated Cropping

We provide a link to download our automated cropping software which includes straightforward instructions for setting it up on a PC or Mac (https://zenodo.org/record/1475442#.W9jvApNKiUl), along with the source code repository for the GUI, which also includes Python code that can crop embryos from larger data sets in batch format (https://github.com/renatkh/embryo_crop.git). Our automated cropping software detects individual embryos in a binary mask, generated from the bright-field images, sequentially crops them out, and orients each embryo (**Fig. 4C**). Prior to cropping, the software corrects for drift, subtracts background and performs depth attenuation correction in each fluorescence channel (customizable options selectable in the GUI). Each step is described in detail below.

Drift was calculated by applying a phase correlation method to the central plane bright field images; if calculated displacement between consecutive images was larger than 50 pixels, the correction was rejected. Background subtraction was performed on every image in every channel independently by applying a Gaussian filter (201 and 41-pixel sizes, respectively, for MS and GLS embryos) and subtracting the obtained background from the original image. Signal attenuation was corrected assuming a linear decay to 10% of signal strength within the 36 µm depth; these values were empirically determined.

Binary masks for cropping are generated from 8-bit bright-field images using the following steps: (1) bright-field images from a single z-stack are blurred using a Gaussian filter (sigma = 11 pixel), (2) edges are detected using a Canny edge detection algorithm ((Canny, 1986); high threshold set to 100, low threshold set to 30), (3) a maximum intensity projection is calculated from the 3 central planes of the edge detected z-stack, (4) the resulting image is transformed with a closing morphology transformation (11 pixel size), (5) holes smaller than 2000 square pixels are filled in and objects less than 10,000 square pixels are removed from the mask.

Individual embryos are detected by fitting a Cassini oval to a section of the binary mask outline (**Figs. 4C, S4G**) using the following procedure: 100 arcs are extended in both directions from random seed points on the mask surface. An arc is allowed to extend only until the maximum indentation point between the arc and the shortest convex path that encases the entire arc (α in **Fig. S4G)** is >30 pixels; arc extension is also terminated if the distance between the point of divergence of the arc from the convex path and the point where the arc and convex path re-converge (β in **Fig. S4G**) is greater than 30 pixels (i.e. if there is a long shallow indentation). When arc extension is terminated, the point of maximum indentation becomes the arc end. The arcs are extended to a maximum length of 1000 pixels. Each of the obtained arcs is fit to a Cassini oval and the oval with dimensions closest to 180x90 pixels is kept. This oval is cropped out from the mask, and the remaining mask is subjected to an open morphology transformation with a size of 11 pixels. Embryo detection and removal is repeated

until the remaining mask has no objects larger than 10,000 square pixels. Individual image files for each embryo were assembled into composite movies for each condition using a custom ImageJ script (https://zenodo.org/record/1475442#.W9jvApNKiUl).

Anterior-Posterior Orientation

The anterior-posterior orientation of each embryo was determined using maximum intensity projections of the signal in the red channel after attenuation correction as illustrated in Figure 4D. The ratio between the total fluorescence signal in the two halves of the embryos was calculated for three timepoints (6,8 and 10 for the Morphogenesis strain and 8, 10 and 12 for the Germ Layer strain) and embryos were oriented so that the average value of ratio of the fluorescence on the left side of the embryo to the average fluorescence on the right side of the embryo is greater than 1 (see schematic in **Fig. 4D**). If the ratio is less than one, the embryo is flipped. For the Germ Layer strain, if the ratio is between 0.8 and 1.25, we calculate the ratio for the red fluorescence after subtracting green fluorescence intensity and require this ratio (left/right) to be greater than 1.

Manual Analysis of Phenotypes

Movies were generated for each RNAi condition (available at https://datadryad.org/review?doi=doi:10.5061/dryad.kb79121) by using a custom FIJI script that combined the cropped sequences for individual embryos (available at https://zenodo.org/record/1475442#.W9jvApNKiUl). Processed and assembled images were scored for a collection of 18 specific defects as described in the text. For details on how each defect was scored see Figure S4 and Table S2.2. Some defects were scored in both strains and other defects were only scored in the Germ Layer or Morphogenesis strains. For a comparison of scored phenotypes with a summary of the expected phenotype reported in Wormbase, see Expected-Observed Phenotypes tab in Table S2. For the arrest point analysis in Figure 3E-F, the most prominent arrest point observed in at least 2 embryos was selected for each RNAi condition. If embryonic lethality counts were higher than 20% but no arrest point

at the 3-fold stage or earlier emerged, the condition was marked as “Other (EMB)”. If no phenotype was observed, but the RNAi condition scored above 20% abnormal larva, the condition was marked as “larval abnormal”. If none of these criteria were met, the condition was marked as WT**.**

Nuclear Counts

To measure the total number of nuclei for each germ layer (**Fig. 5B**), the Batch Coordinator x64 tool in Imaris (Bitplane) was used to segment the nuclear signal in the 4D image stacks of individually cropped embryos and partition identified nuclei between red, green, and yellow channels. To do this, we used an estimated nuclear diameter of 5μm to define “spots” for each channel. Nuclei were assigned to the red, green or yellow channels using the following intensity gate settings: (1) green (green > 7830 *and* red < 6695), (2) red (red > 6695 *and* green < 7830) and (3) yellow (red > 6695 *and* green > 7830). This allowed for tracking of nuclear counts for each germ layer at each time point. Because nuclear count data is not accurate once muscle contraction has initiated (due to rapid movement of the worm inside the eggshell), we averaged nuclear count information for the three timepoints prior to onset of movement; at this time (comma through 2-fold stage) the majority of cell divisions have been completed and cell fate has been specified. For conditions that exhibited an arrest prior to the onset of movement, the nuclear counts for the final three imaged timepoints were averaged. For the graph in Figure 5B, the average nuclear count values for green, red, and yellow nuclei in controls were subtracted from the values measured for each RNAi condition prior to plotting.

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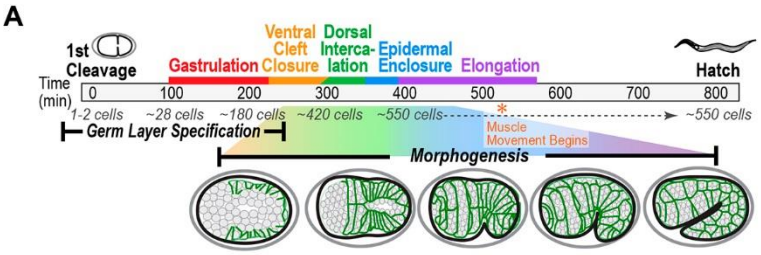
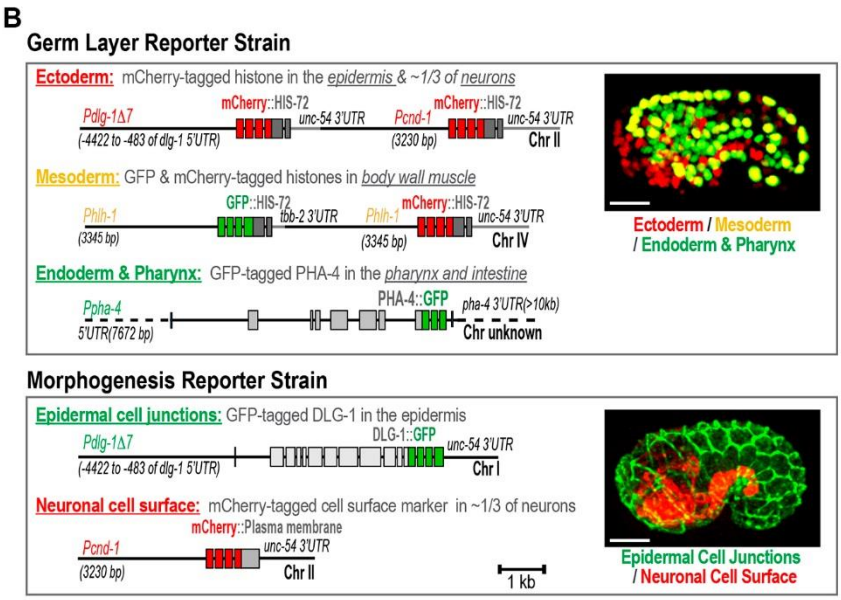
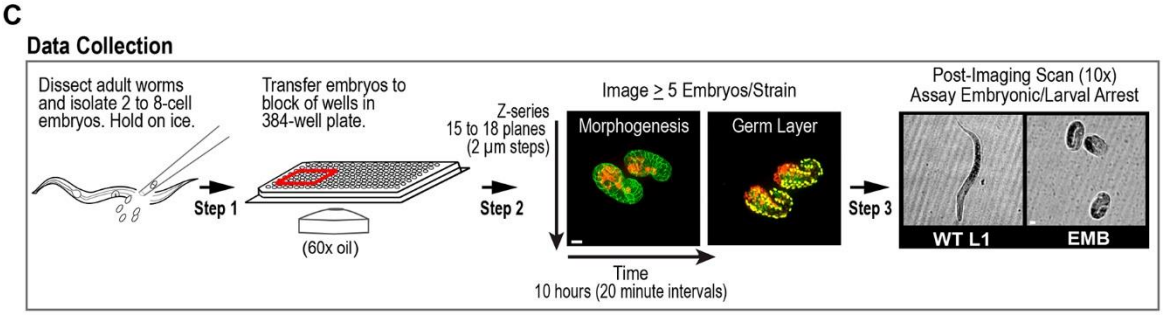
Figures







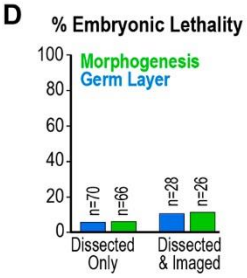


Figure 1. Strains and a data collection method for high-content imaging of events during *C. elegans* embryogenesis. *(***A**) Timeline of *C. elegans* embryonic development. Schematics illustrate morphogenetic events. (**B**) Schematics show transgenes in the Germ Layer (*top*) and Morphogenesis (bottom) strains. Images (*right*) show embryos from each strain during early elongation. (**C**) Schematics illustrate a data collection method that allows simultaneous monitoring of 80-100 embryos across multiple fields. (**D**) Graph plots embryonic lethality after the indicated manipulations. Scale bars, 10μm.

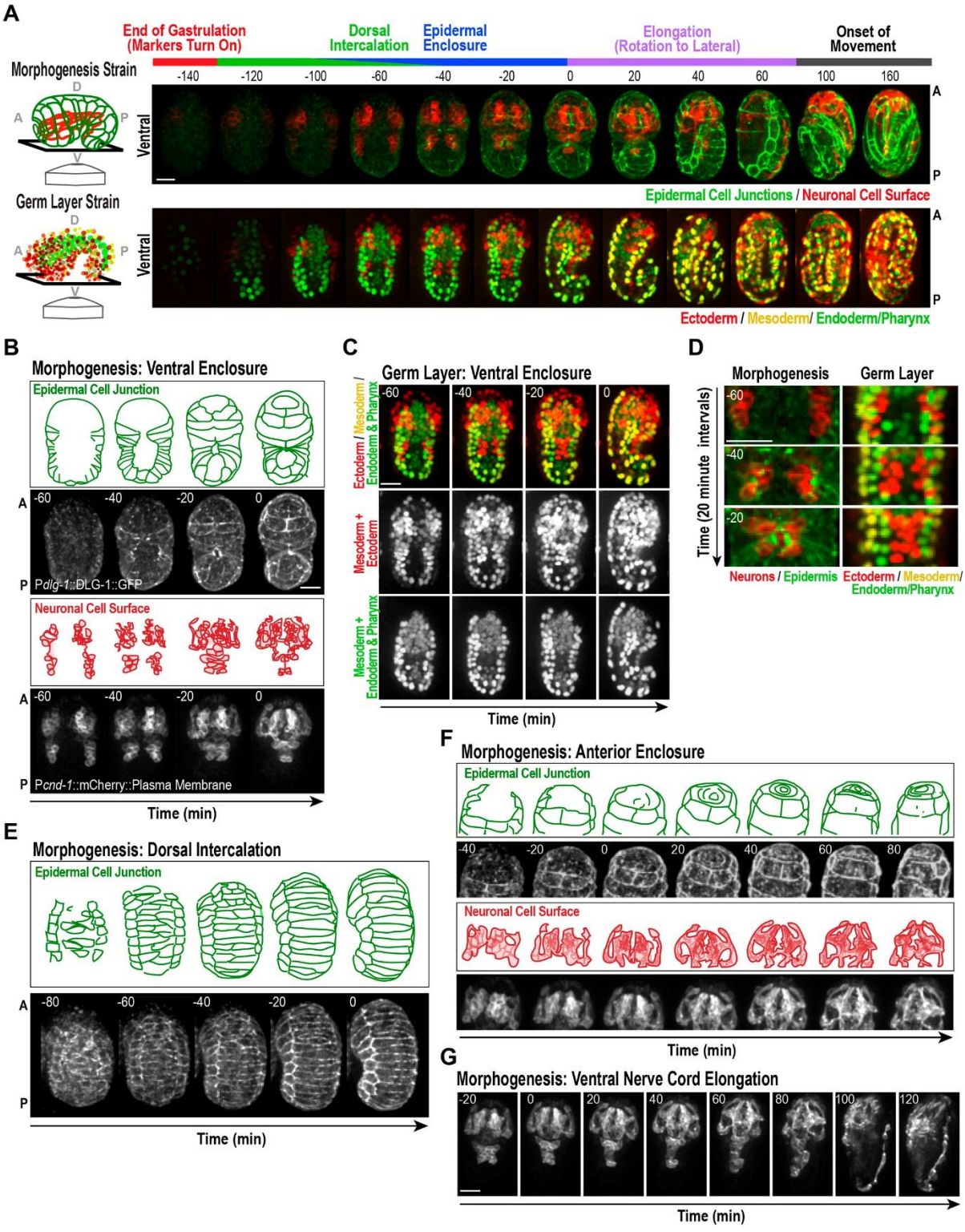


Figure 2. Visualization of key events during embryonic development in the Germ Layer and Morphogenesis strains. (**A**) Maximum intensity projections show a ventral view (illustrated in schematics) of embryonic development in the Morphogenesis (***top***) and Germ Layer (***bottom***) strains. Times are minutes relative to the comma stage (t=0). For a dorsal view see Figure S2A. (**B-G**) Panels illustrate events during embryonic development in the Morphogenesis and Germ Layer strains. Images are maximum intensity projections and times are minutes relative to the comma stage (t=0). (**B**) Schematics and grayscale images illustrating the dynamics of epidermal cell junctions (***top***) and marked neurons (***bottom***) during ventral enclosure (ventral view) in the morphogenesis strain. (**C**) Color and grayscale images showing movement of the ectoderm (red), endoderm/pharynx (green) and mesoderm (yellow, signal in both red and green channels), during ventral enclosure in the Germ Layer strain. (**D**) High magnification view of ventral enclosure in the Germ Layer and Morphogenesis strains. (**E**) Schematics (***top***) and grayscale images (***bottom***) illustrating epidermal cell junction dynamics during dorsal intercalation (dorsal view) in the morphogenesis strain. (**F**) Schematics and grayscale images illustrating epidermal layer dynamics during head enclosure (***top panels***), and the concurrent restructuring of the nervous system (***bottom panels***). (**G**) Grayscale images of marked neurons showing elongation of the ventral nerve cord (enclosure through 2-fold stage) in the Morphogenesis strain. Scale bars, 10μm.

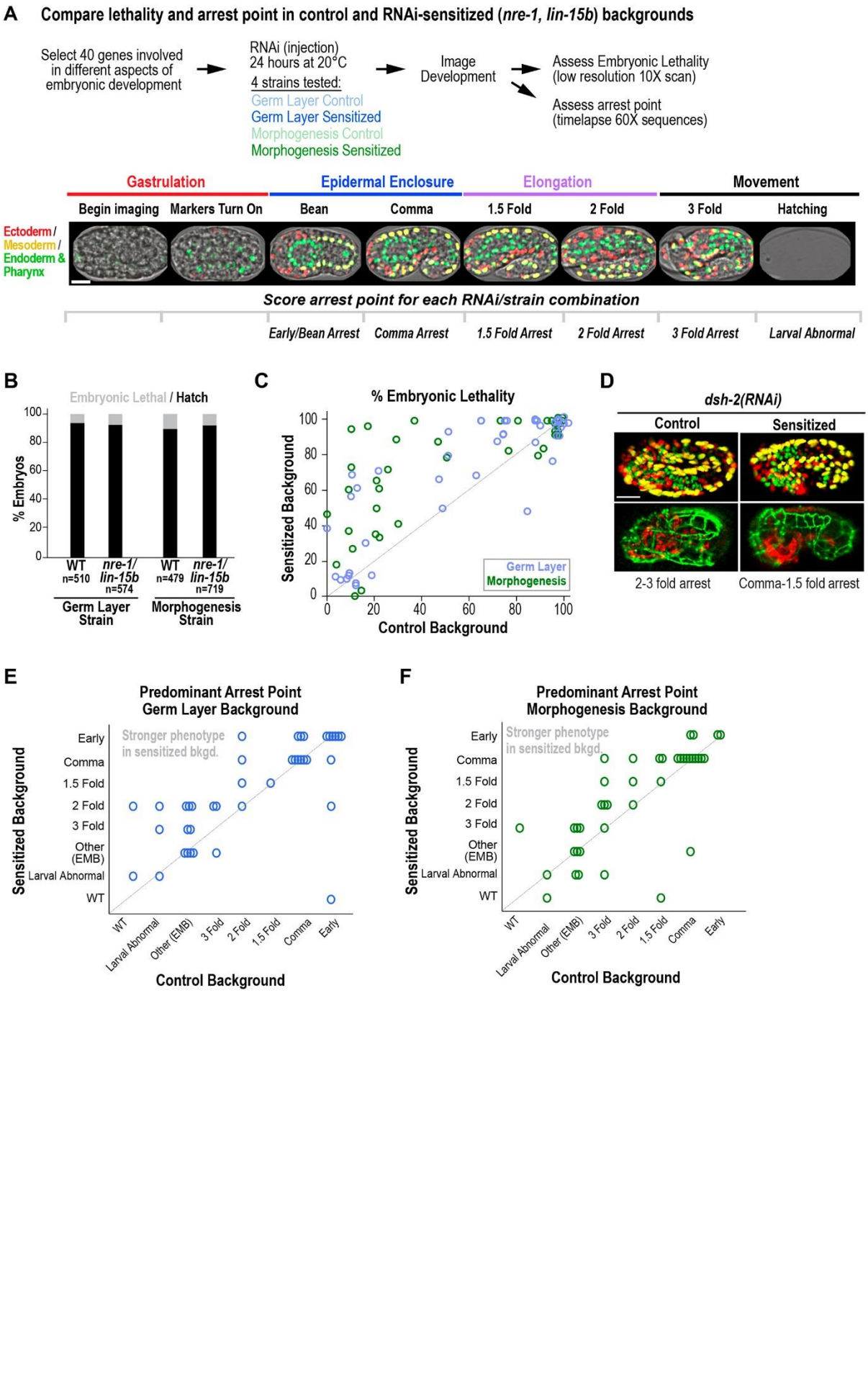


Figure 3. Penetrant phenotypes in RNAi-sensitized versions of the Germ Layer and Morphogenesis strains. (**A**)(***top***) Outline of experiment to compare embryonic lethality and arrest points in the Germ Layer and Morphogenesis strains in the presence and absence of RNAi-sensitizing mutations for 40 developmental genes. (***bottom***) Images (central z-sections from the germ-layer strain with bright-field images overlaid) illustrate major arrest points. (**B**) Stacked bar graph showing embryonic lethality (***gray***) and hatching (***black***) percentages for strains with and without RNAi-sensitizing mutations in the absence of RNAi. (**C**) Graph plots percent embryonic lethality in the control (***x-axis***) versus the RNAi-sensitized background (***y-axis***) for each RNAi condition in the Germ Layer (***blue***) and Morphogenesis (***green***) strains. Points above the diagonal represent RNAi conditions with higher embryonic lethality in the sensitized background compared to the control. For a list of genes in each region see Figure S3. (**D**) Representative images showing the terminal *dsh-2(RNAi)* phenotype in the Germ Layer (***top***) and Morphogenesis (***bottom***) strains without (***left***) and with (***right***) RNAi-sensitizing mutations. (**E, F**). Graphs plot the predominant arrest point in the control (***x-axis***) versus the RNAi-sensitized background (***y-axis***) for each RNAi condition in the Germ Layer (**E**) and Morphogenesis (**F**) strains. Points above the diagonal represent RNAi conditions with an earlier arrest point in the sensitized background compared to the control. Scale bars, 10μm.

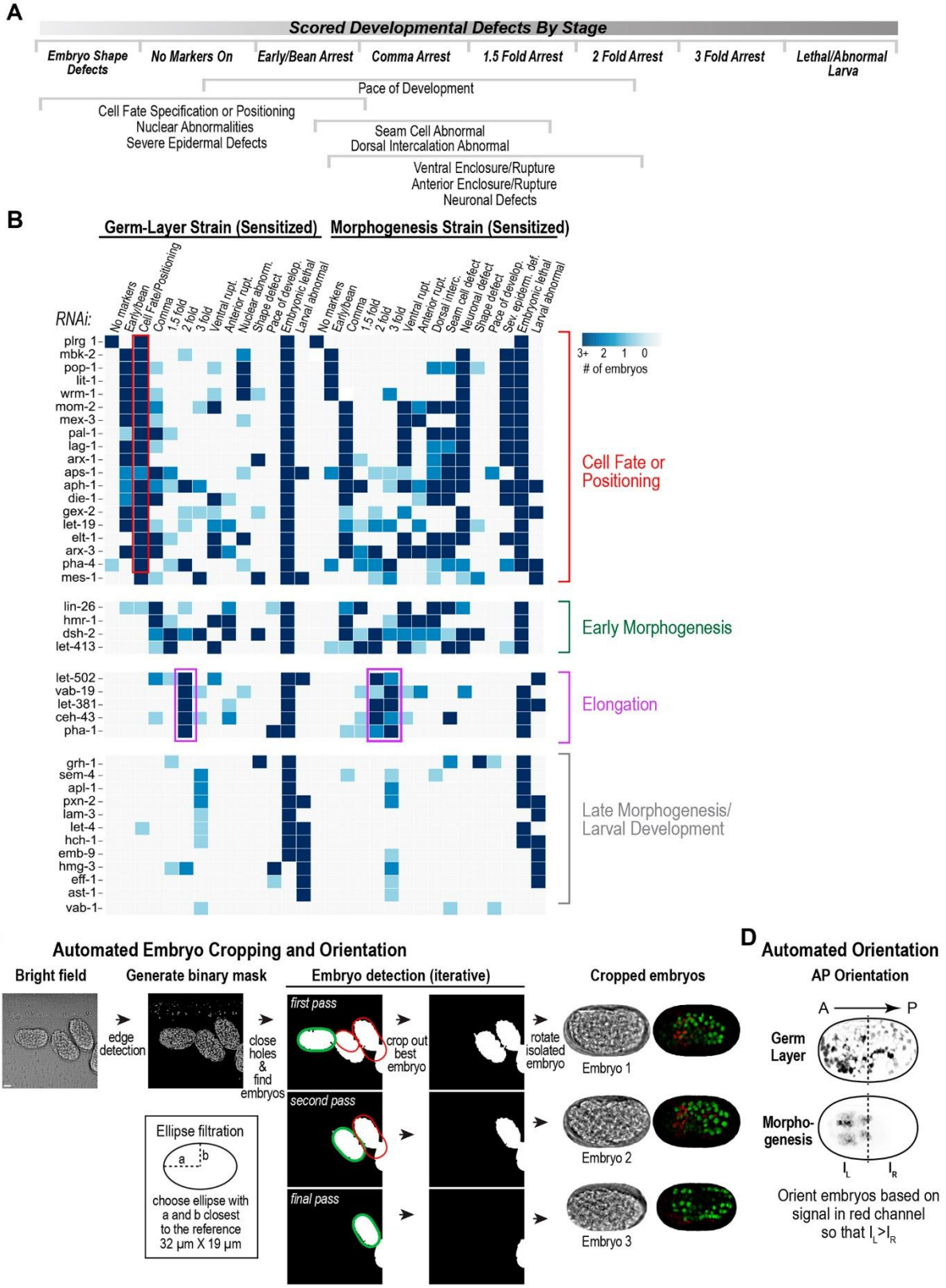


Figure 4. Manual annotation of defects in the Germ Layer and Morphogenesis strains partitions development genes into four broad classes. (**A**) Timeline illustrates arrest points and defects scored during the indicated time windows (brackets; see also **Figure S4** and **Table S2.2**). (**B**) Heatmap summarizes defects scored in the RNAi-sensitized Germ Layer and Morphogenesis strains. Color range indicates the number of embryos in which the defect was observed: lightest blue = 1 embryo to darkest blue = 3+ embryos. Dark blue coloring in the 'Embryonic lethal' and 'Larval abnormal' columns indicates > 20% incidence in a 10X whole-well scan. (**C**) Graphic summary of automated embryo cropping procedure. (**D**) Schematic illustrates method for orienting embryos along the anterior-posterior axis. Scale bar, 10μm.

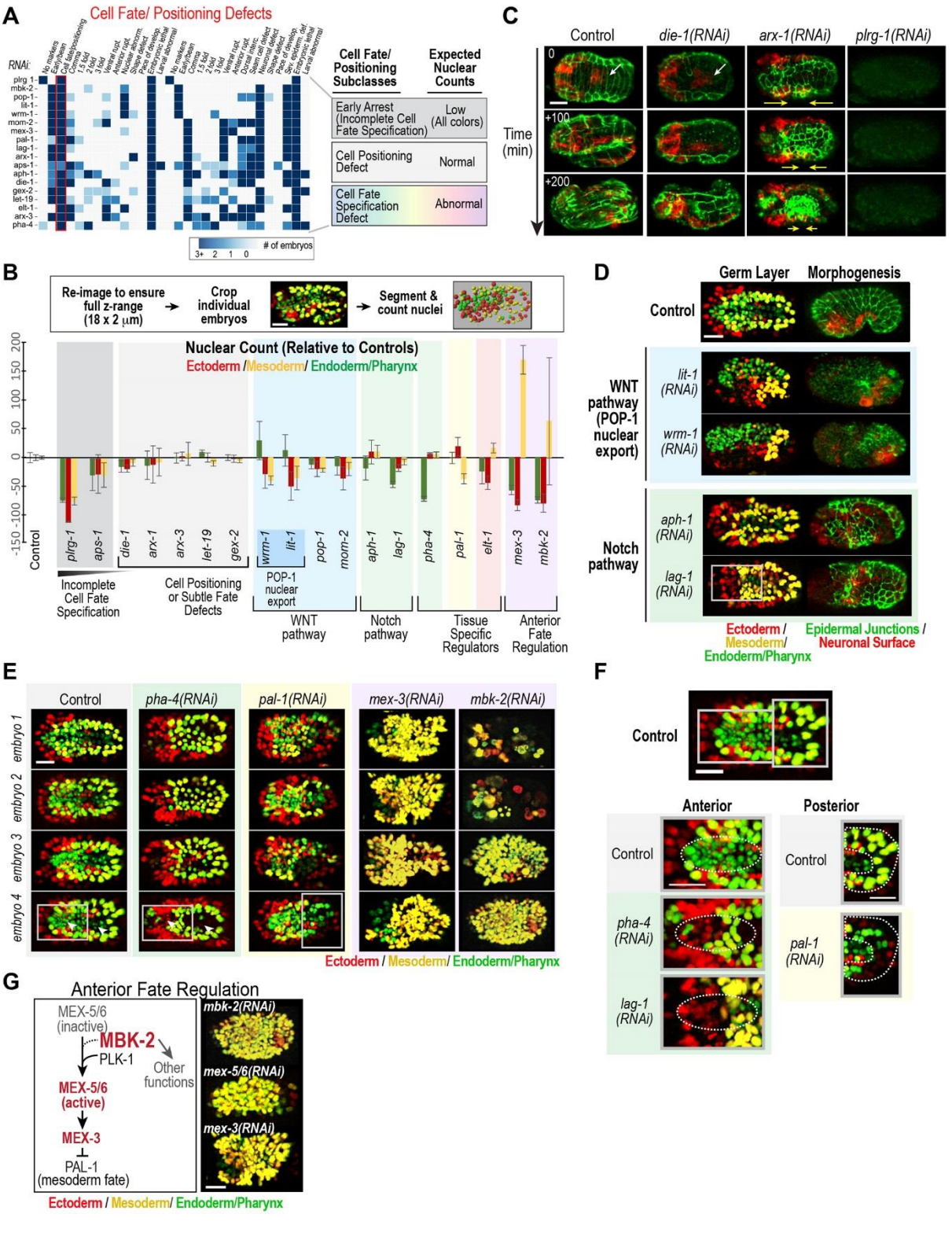


Figure 5. Imaging in the Germ Layer and Morphogenesis strains is an efficient means to functionally profile genes required for cell fate specification and/or cell positioning. (**A**) Genes required for cell fate and/or positioning (heatmap region reproduced from **Fig. 4B**) are partitioned into three subclasses based on nuclear counts. (**B**) (***top***) Schematics outline nuclear counting procedure. Graph plots number of nuclei in each germ layer for each condition after subtraction of the mean value for controls (mean number of embryos per condition = 6; n for controls = 9). Error bars = SD. (**C**) Representative time-lapse images showing a dorsal view in the RNAi-sensitized Morphogenesis strain for a control, one class 1 (*plrg-1*), and two class 2 (*arx-1*, *die-1*) genes. White arrows in the control and *die-1(RNAi)* images point to cell-cell boundaries on the dorsal side of the control embryo that are absent in *die-1(RNAi)* embryos. Yellow arrows track compression of the dorsal epidermis coincident with ventral rupture in the *arx-1(RNAi)* embryo. (**D**) Representative images show arrest point phenotypes in the Germ Layer strain following knockdown of two components of the WNT signaling pathway (*lit-1* and *wrm-1*, blue background) and two components of the Notch pathway (*lag-1* and *aph-1*, green background) compared to a comparably staged control embryo (***top***). The gray box marks a region shown at higher magnification in (F). (**E**) Four embryos are shown for each condition to illustrate embryo-to-embryo variation in phenotype. The top control panel is reproduced for comparison from Figure 5D. All phenotypes were highly consistent, with the exception of *mbk-2(RNAi)* which was bimodal, exhibiting either an early (***top***) or later (***bottom***) arrest. Arrowheads mark green PHA-4::GFP-labeled pharynx and endoderm nuclei that are missing in *pha-4(RNAi)* embryos. Gray boxes mark regions shown at higher magnification in (F). (**F**) (*top*) The bottom control embryo from (E) is shown with its anterior and posterior regions marked (gray boxes). (*bottom left*) Higher magnification view of the anterior regions of selected control, *pha-4(RNAi)* and *lag-1(RNAi)* embryos. White dashed ovals mark the region where the PHA-4::GFP expressing pharynx nuclei in control embryos are missing in *pha-4* and *lag-1(RNAi)* embryos. (*bottom right*) Higher magnification view of the posterior regions of a control and *pal-1(RNAi)* embryo. The region outlined with the white

dashed line marks the cup of mesoderm nuclei in the posterior of control embryo that is absent in the *pal-1(RNAi)* embryo. (**G**) Schematic (***left***) shows a simplified version of the pathway by which MEX-5/6 activates MEX-3, which inhibits PAL-1 to suppress mesoderm fate in the embryo anterior (Kuersten and Goodwin, 2003). Images (***right***) illustrate the similar Germ Layer strain phenotypes for *mbk-2(RNAi)*, *mex-5/6(RNAi)*, and *mex-3(RNAi)*. The *mbk-2(RNAi)* and *mex-3(RNAi)* images are reproduced from panel E for comparison. Scale bars, 10µm.

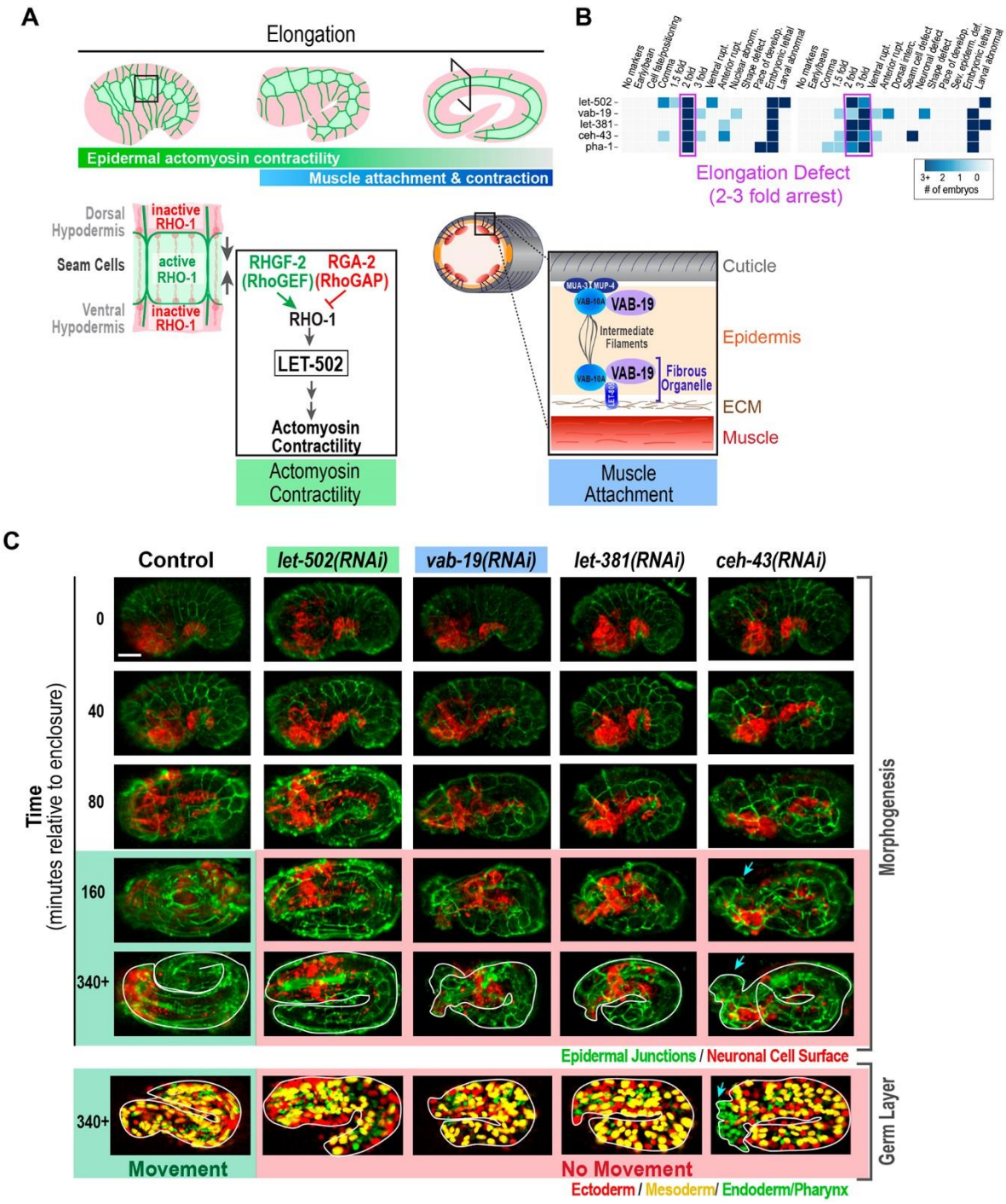


Figure 6: High-content imaging in the Germ Layer and Morphogenesis strains enables functional classification of genes required for elongation. (**A**) (***top***) Schematics illustrate cell shape changes in the seam (***green***) and dorsal/ventral (***red***) epidermal cells during

elongation, and the two temporally overlapping processes that drive elongation (adapted from (Vuong-Brender et al., 2016)). (***bottom***) Schematics outline two key pathways that contribute to elongation. (**B**) Genes required for elongation (heatmap region reproduced from **Fig. 4B**). (**C**) (***top***) Representative time-lapse images in the RNAi-sensitized Morphogenesis strain of a control embryo and knockdown embryos for 4 genes specifically required for elongation. (***bottom***) Representative images showing arrest point phenotypes in the RNAi-sensitized Germ Layer strain compared to a control embryo at a comparable stage. For more complete time-lapse series see Figure S5C. Elongating embryos were outlined to make embryo shape at arrest more visible. Inhibition of *ceh-43* (***blue arrowheads***) lead to irregularities in the anterior region. Green shading indicates successful elongation and movement within the eggshell, whereas red shading indicates arrest and failure to move. Scale bar, 10 μm.

SUPPLEMENTAL FIGURES

Wang, Ochoa, Khaliullin et al., Figure S1

	Promoter	Length	Reporter(s)	Expressing Tissue(s)	3' UTR	Allele(s)
Transgenes used in Germ Layer & Morphogenesis strains	*Pcnd-1*[1]	3230 bp	mCh::Plasma Membrane	Strong, specific expression in ~1/3 of neurons from embryonic stage	*unc-54*	*ltSi511*
	Pdlg-1Δ7[2]	3940 bp	DLG-1::GFP	Embryonic epidermis	*unc-54*	*ltSi249*
	Pcnd-1	3230 bp	mCh::histone (tandem with *Pdlg-1Δ7* mCh::histone)	Strong, specific expression in ~1/3 of neurons from embryonic stage	*unc-54*	*ltSi539*
	Pdlg-1Δ7	3940 bp	mCh::histone (tandem with *Pcnd-1* mCh::histone)	Embryonic epidermis	*unc-54*	*ltSi539*
	Phlh-1[3]	3345 bp	mCh::histone & GFP::histone (in tandem)	Body wall muscle	*tbb-2*	*ltSi507*
	pha-4 fosmid[4]	33505 bp	PHA-4::GFP	Intestine and pharynx	*pha-4*	*stIs10389*
Other transgenes generated for the project	*Pcnd-1*	3230 bp	GFP::histone (tandem with *Pdlg-1Δ7* GFP::histone)	Strong, specific expression in ~1/3 of neurons from embryonic stage	*unc-54*	*ltSi509*
	Pdlg-1Δ7	3940 bp	GFP::histone (tandem with *Pcnd-1* GFP::histone)	Embryonic epidermis	*unc-54*	*ltSi509*
	Pelt-2	2994 bp	mCh::histone & GFP::histone (in tandem)	Intestine	*tbb-2*	*ltSi506*
	Phlh-1	3345 bp	mCh::histone, GFP::histone (separate)	Body wall muscle	*unc-54*	*ltSi456, ltSi457*
	Ppha-4+intron1	6452 bp	mCh::histone (tandem with *Phlh-1* mCh::histone)	Weak pharynx, strong intestine	*tbb-2*	*ltSi504*
	Ppha-4	4157 bp	mCh::histone (tandem with *Phlh-1* mCh::histone)	Weak pharynx, variable intestine	*tbb-2*	*ltSi273*
	Prgef-1	4040 bp	mCh::Plasma Membrane	No embryonic expression Post-embryonic pan-neuronal	*unc-54*	*ltSi318*
	Punc-33	2003 bp	mCh::Plasma Membrane	All embryonic cells	*unc-54*	*ltSi316*
	Punc-119+intron1	4749 bp	mCh::Plasma Membrane	All embryonic cells	*unc-54*	*ltSi321*

Footnotes:

1. A HIS-24::mCherry reporter utilizing P*cnd-1* (-3266 to -10 bp) is expressed in a subset of AB progeny cells starting at about the 100-cell stage. These cells include 97 neurons, 17 glia cells and 13 non-nervous system cells (for a map of P*cnd-1* expression superimposed with the embryonic lineage see Murray and Bao et al., 2008). Note that the *C. elegans* embryo has 248 total neurons, so the P*cnd-1* promoter directs expression in about 40% of neurons. These neurons include ventral cord motor neurons and some neurons in the nerve ring ganglion, and do not overlap with UNC-86-expressing neurons (egg-laying neurons, mechanosensory neurons, and chemosensory interneurons; Hallam and Singer et al., 2000).

2. P*dlg-1Δ7* drives expression in all epidermal cells (Sheffield et al., 2007)

3. A lacZ reporter utilizing Phlh-1 (-3053 to + 70 bp) showed expression in all body wall muscle precursor cells with no ectopic expression (Krause et al., 1994). A HIS-24::mCherry reporter utilizing P*hlh-1* (-3053 to -4 bp) showed expression in all body wall muscle cells as well as some pharyngeal cells of the MS lineage (Murray and Bao et al., 2008; includes a map of expression superimposed with the embryonic lineage). In our case, P*hlh-1* (-3333 to +12 bp) reporter expression appears to be expressed in all body wall muscle precursors (Fig. S2B, bottom panels).

4. The fosmid recombineering transgene we used for PHA-4 is allele stIs10389 (Fakhouri et al.2010, Zhong, Niu et al. 2010). It was made by tagging PHA-4 at the C-terminus with GFP, a Ty1 peptide and 3xFLAG in the fosmid WRM0617dE06. This fosmid is 33505 bp in length and contains the entire *pha-4* genomic cassette (Fakhouri et al. 2010; Zhong et al., 2010). Consistent with our own observations, this transgene likely recapitulates the endogenous *pha-4* expression pattern, which includes all pharyngeal, midgut and rectal precursors (Horner et al., 1998).

Figure S1. List of transgenes constructed for this study. Table listing the promoter/reporter combinations used in the transgenes constructed for this study, along with notes on the tissue expression of each marker. The combinations in the top half of the table (***green background***) were used in the transgenes in the Germ Layer and Morphogenesis strains. The combinations in the bottom half of the table (***gray background***) were tried and are available but were not used in our final strains.

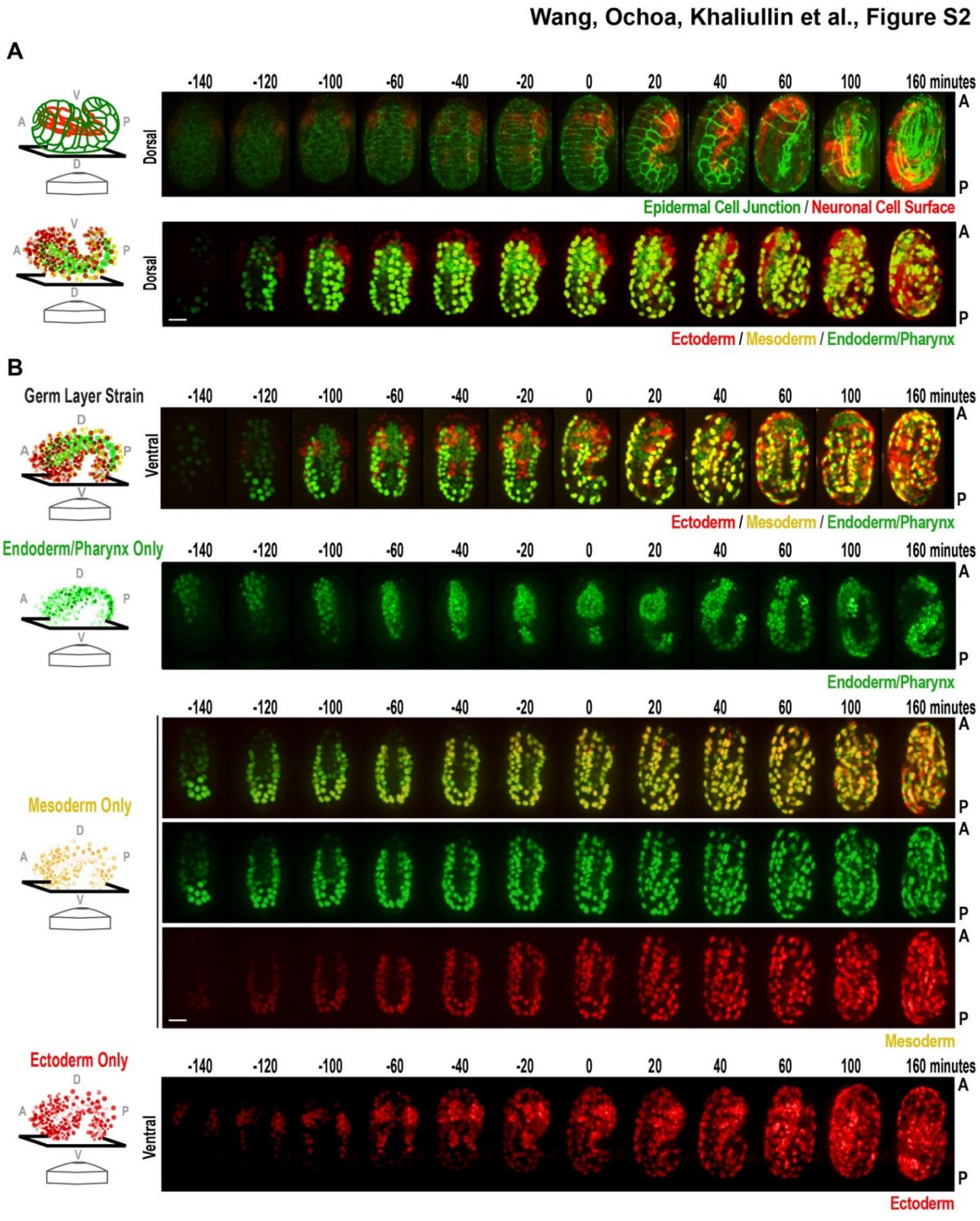


Figure S2. Images showing development from a dorsal view in the Germ Layer and Morphogenesis strains and in embryos individually expressing the three transgenes that make up the Germ Layer strain. (**A**) Maximum intensity projections showing a dorsal view (see schematics on left) of embryonic development in the Morphogenesis (***top***) and Germ Layer (***bottom***) strains acquired at the indicated timepoints relative to the comma stage (t=0 minutes). (**B**) Maximum intensity projections showing a ventral view (see schematics on left) of embryos from the Germ Layer strain (top panels; reproduced from Figure 2A for comparison) and strains individually expressing the three component transgenes that make up the Germ Layer strain (Ectoderm Only, strain OD1599; Endoderm/Pharynx Only, strain OD1598; and Mesoderm Only, strain OD1543). Scale bars are 10 µm.

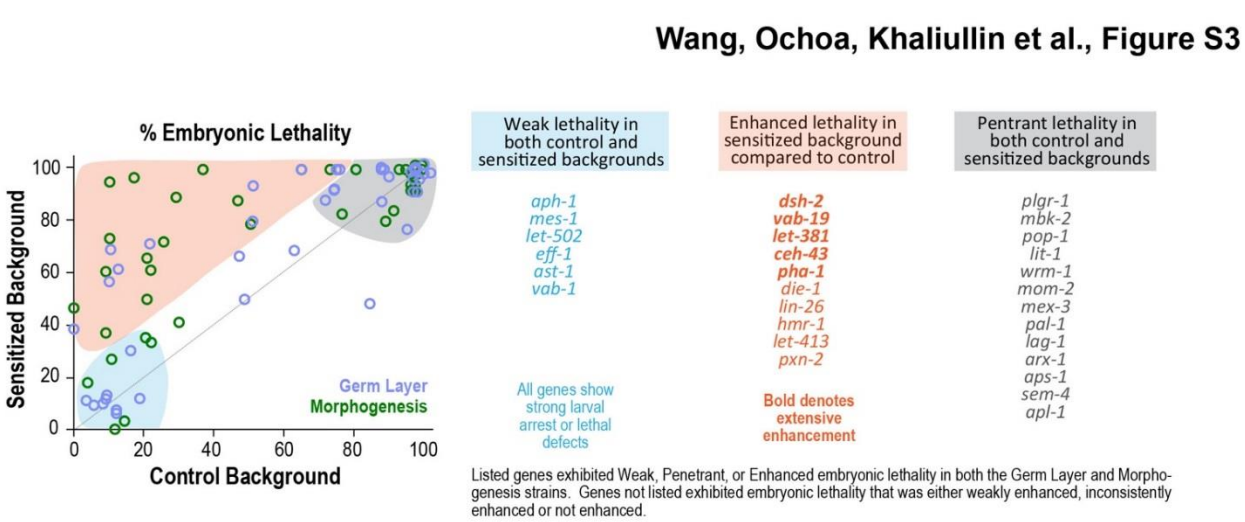


Figure S3. Comparison of embryonic lethality in the Germ Layer and Morphogenesis strains for Control and Sensitized backgrounds. (**A**) Graph (***left***) plots percent embryonic lethality in the control background (x-axis) versus percent embryonic lethality in the RNAi-sensitized background (y-axis) for each RNAi condition in the Germ Layer (***blue data points***) and Morphogenesis (***green data points***) strains. Shaded colored regions highlight the gene targets that exhibited weak embryonic lethality (<30%) in both backgrounds (***light blue***), penetrant embryonic lethality (>70%) in both backgrounds (***gray***), or showed enhanced embryonic lethality (20% or greater increase) in the sensitized background compared to the control background (***orange***). Genes in each of these color-coded cohorts are listed (***right***).

Wang, Ochoa, Khaliullin et al., Figure S4

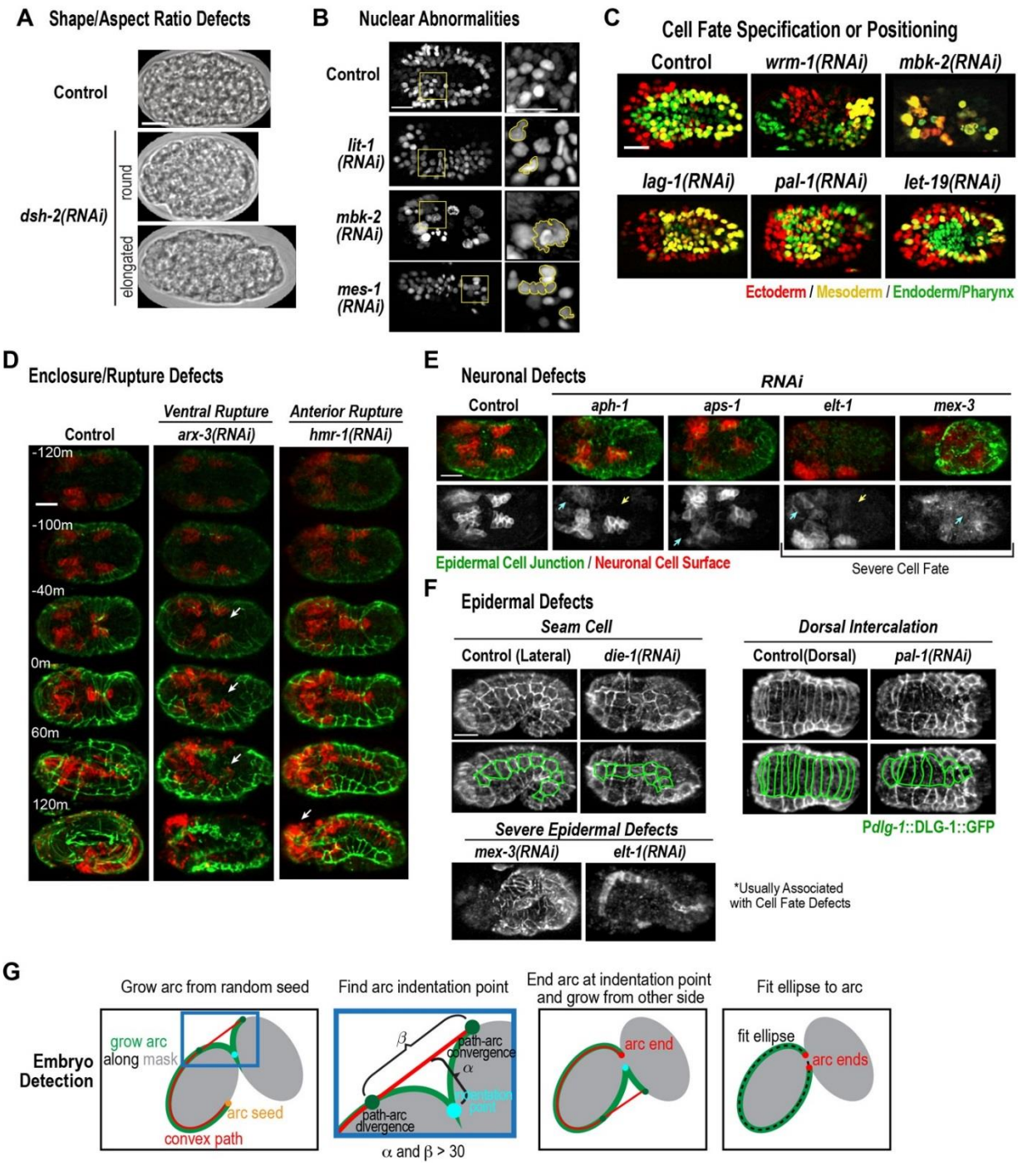


Figure S4. Scoring of phenotypic features in the Germ Layer and Morphogenesis strains and automated cropping. (**A-F**) Maximum intensity projections of fluorescence confocal images (**B-F**), or single plane brightfield images (**A**) of embryos from specific RNAi conditions scored with the indicated descriptors are shown to illustrate how each descriptor was scored. (**A**) Embryos were scored with a "shape/aspect ratio" defect if embryo shape deviated significantly different from controls. (**B**) Embryos (Germ Layer only) were scored with "nuclear abnormalities" if any nuclei were abnormal in shape, if micronuclei or lagging chromosomes were observed, or if nuclear marker intensity was uneven. (**C**) Germ Layer embryos were scored with a "cell fate specification or positioning" defect if the number or position of nuclei in the germ layers appeared to deviate from that in controls (based on visual assessment) prior to the onset of morphogenesis (comma stage). Control, *lag-1(RNAi), pal-1(RNAi)* and *mbk-2(RNAi)* images are reproduced for comparison from Figure 5D-E. (**D**) Embryos were scored for a "ventral enclosure defect/ventral rupture" if the ventral epidermal/ectodermal cells moved towards the midline but failed to completely close the ventral opening, or an "anterior closure defect/anterior rupture" if the epidermal/ectoderm cells failed to move anteriorly to completely cover the head. (**E**) Embryos (Morphogenesis only) were scored with "neuronal defect" if neuronal organization was altered prior to elongation. (**F**) Embryos (Morphogenesis only) were scored with a "seam cell defect" if the position, structure of number of lateral seam cells was abnormal between the comma and 1.5-fold stages, with "dorsal intercalation defect" if the dorsal hypodermal cells (either one or two to all) failed to properly intercalate or to fuse after intercalation, and with "severe epidermal defect" if the pattern of cells that expressed the markers was severely disorganized. (**G**) In our automated cropping algorithm, a binary mask was generated from 8-bit brightfield images and individual embryos were detected and cropped out. Schematics detail the procedure used to iteratively detect embryos in the binary mask. Scale bar is 10 µm.

Wang, Ochoa, Khaliullin et al., Figure S5

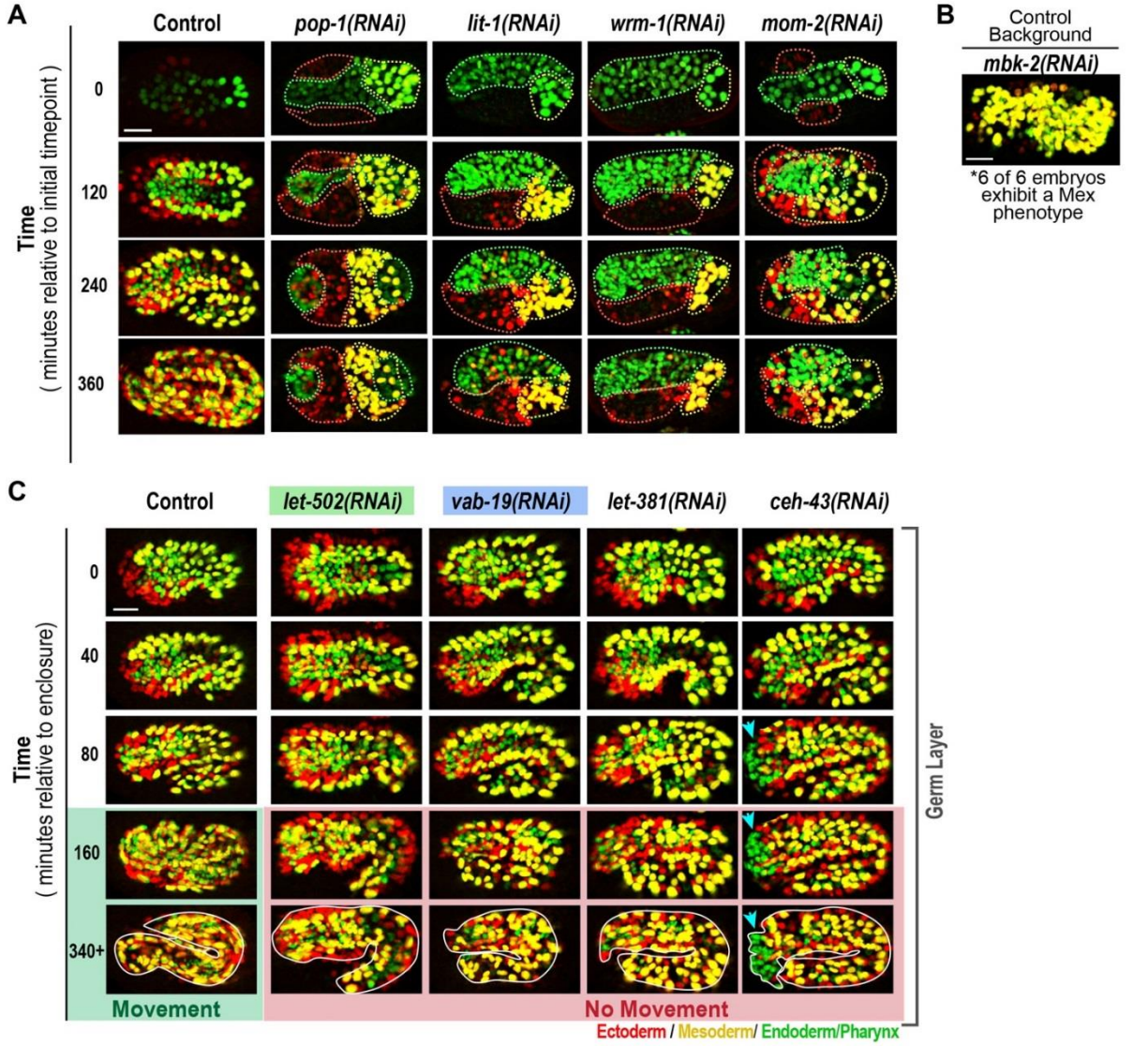


Figure S5. Germ Layer strain phenotypes for WNT pathway components and proteins required for embryo elongation. (**A**) Maximum intensity projections of confocal images of embryos from the Germ Layer strain with RNAi-sensitizing mutations after knockdown of genes encoding four components of the WNT signaling pathway. Dashed green, red and yellow outlines mark the locations where nuclei of the corresponding colors are observed. See text for phenotype descriptions. Final *wrm-1(RNAi)* and *lit-1(RNAi)* images are reproduced from Figure 5D. For the full sequences see movies available at Dryad repository: (https://datadryad.org/review?doi=doi:10.5061/dryad.kb79121). (**B**) Image shows the Germ Layer *mbk-2(RNAi)* phenotype in control background lacking the RNAi-sensitizing mutations. (**C**) Maximum intensity projections of confocal images of embryos from the Germ Layer strain after knockdown of four genes required for elongation past the 2-fold stage. Arrowheads in *ceh-43(RNAi)* point to the position where pharyngeal nuclei are leaking out of the head of the embryo. Elongating embryos were outlined in the final frame to make embryo shape at arrest more visible. In the *ceh-43(RNAi)* embryo, green pharyngeal nuclei are observed outside of the embryo. Green shading indicates successful elongation and movement of the embryo within the eggshell, whereas, red shading indicates arrest and failure to move. Scale bars are 10 µm.

Wang, Ochoa, Khaliullin et al., Figure S6

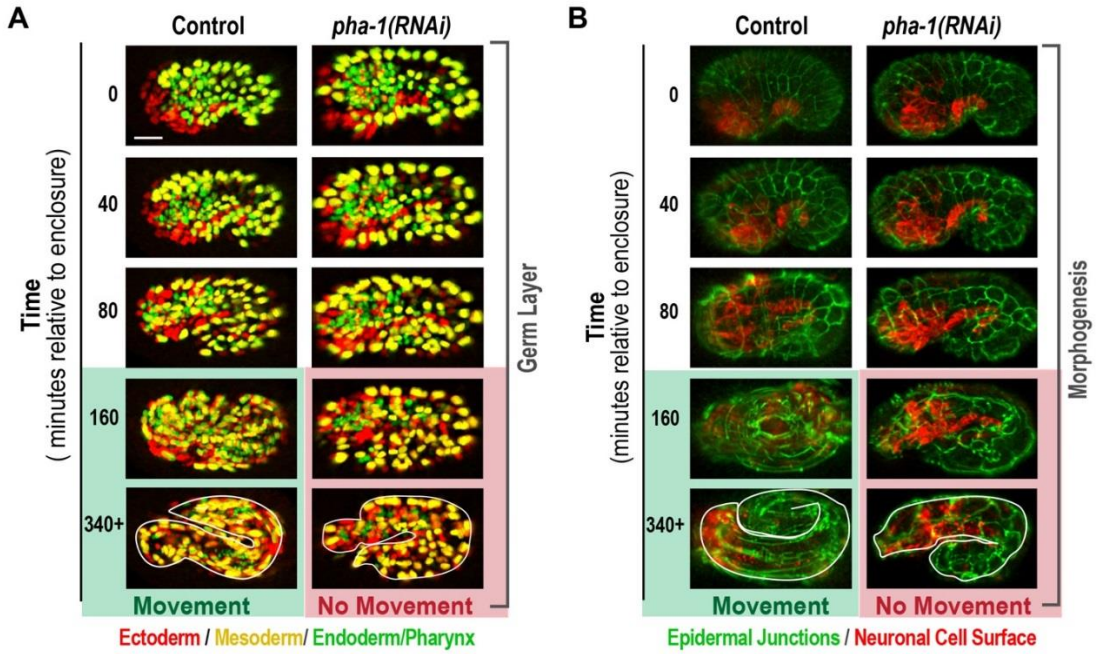
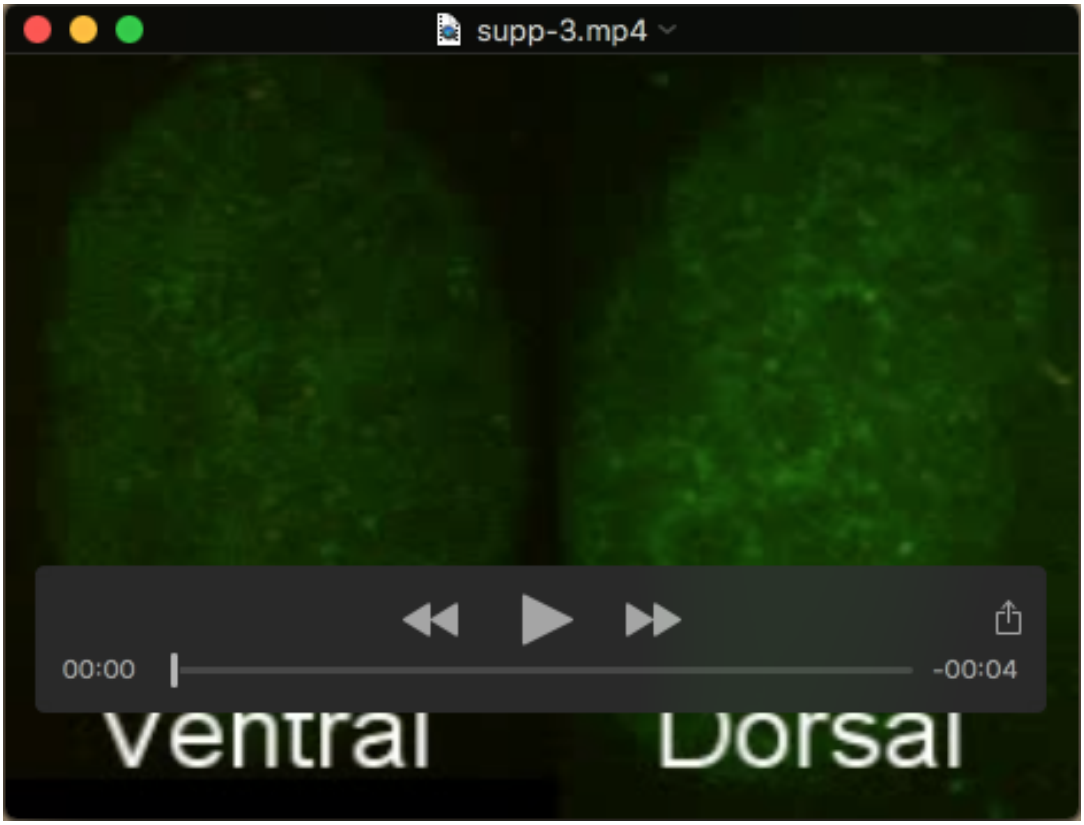


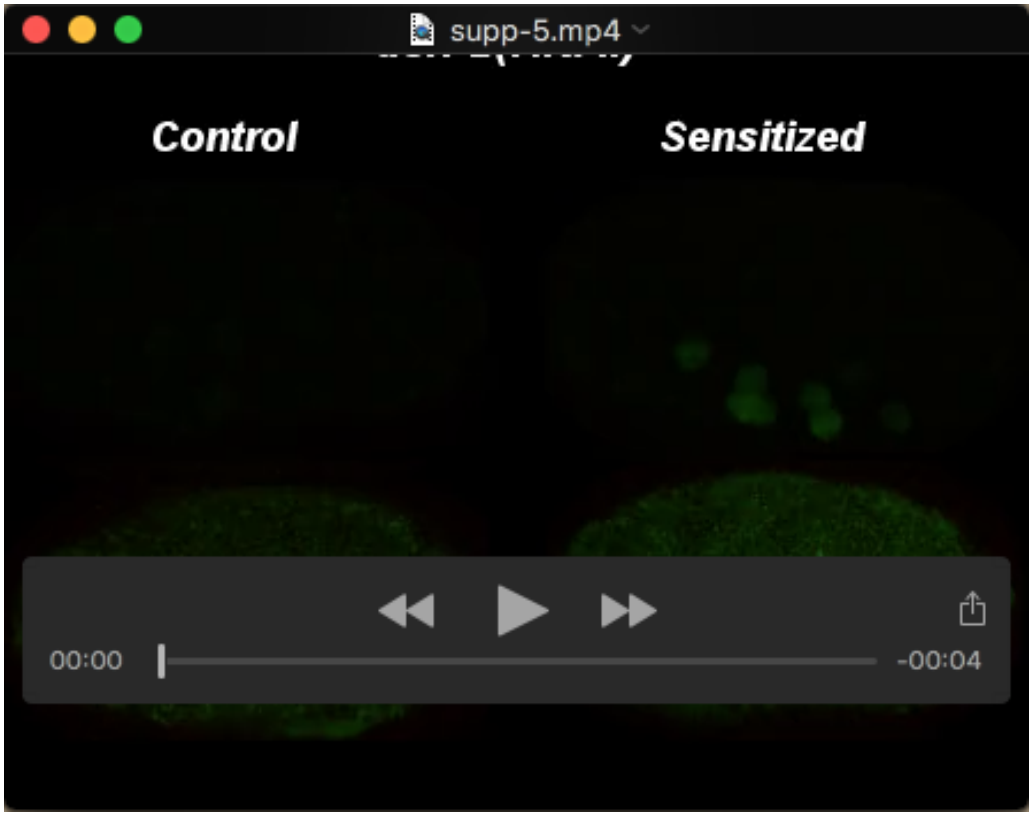
Figure S6. **Knockdown of *pha-1* leads to an elongation defect and a "pinched red head" phenotype.** (**A, B**) Maximum intensity projections of confocal images of embryos from the Germ Layer (A) and Morphogenesis (B) strains with RNAi-sensitizing mutations. Control embryo sequences are reproduced from Figure 6 and Figure S5 for comparison. PHA-1 is required for pharyngeal cells to attach to neighboring arcade cells to connect the pharynx to the buccal cavity, and *pha-1* mutants exhibit a penetrant Pun phenotype (Schnabel and Schnabel 1990, Fay 2004, Kuzmanov et al., 2014). Mosaic analysis suggested that PHA-1 may not be required in the pharynx but instead might promote pharyngeal development via a role in other cell types such as the epidermis (Kuzmanov et al., 2014). Interestingly, recent work has shown that, rather than being inherently required for development, PHA-1 is an antidote that counteracts the toxicity of a second protein called SUP-35 that is encoded with it on a selfish genetic element (Ben-David et al., 2017). Thus, *pha-1* knockdown phenotypes inform on the mechanism of action of the SUP-35 toxin. (**A**) In the Germ Layer strain, *pha-1* knockdown led to a "pinched redhead" phenotype, possibly due to the defect in pharyngeal morphogenesis/attachment (**also see Movie S7**). (**B**) In the morphogenesis strain, PHA-1 inhibition led to a crumpled epidermis elongation defect, which our analysis suggests can arise as a consequence of a defect in mouth-pharynx attachment (*ceh-43*) or the failure to form transepithelial muscle attachments (*vab-19*). Green shading indicates successful elongation and movement of the embryo within the eggshell, whereas, red shading indicates arrest and failure to move. Scale bars are 10 µm.

Movies

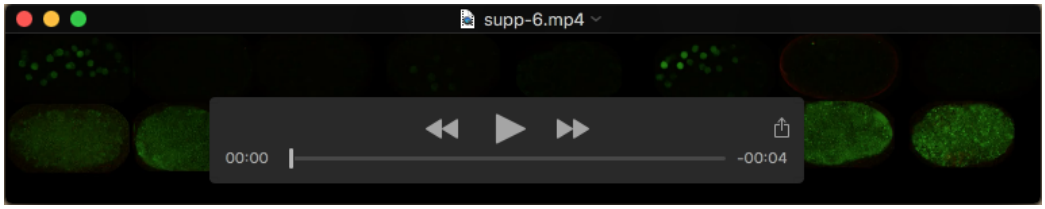


Movie 1. **Embryonic development imaged in the Morphogenesis strain.** Side-by-side ventral (***left***) and dorsal (***right***) views of development in control embryos from the Morphogenesis strain which has markers that localize to apical cell junctions in the epidermis (***green***) and the cell surface in about 1/3 of neurons (***red***). Images were acquired every 20 minutes. Playback is 7200X real time.

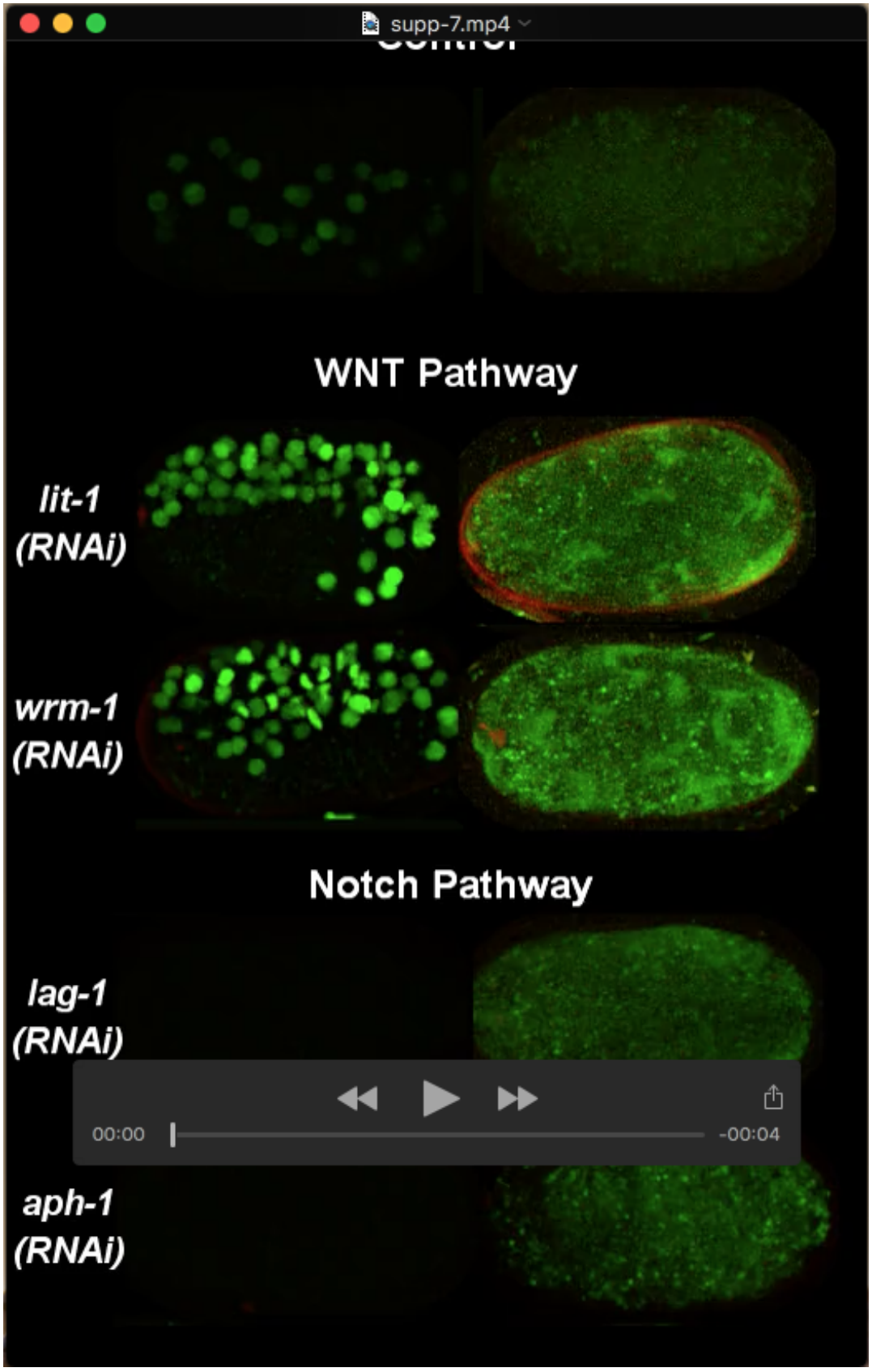
Movie 2. Embryonic development imaged in the Germ Layer strain. Side-by-side ventral (***left***) and dorsal (***right***) views of development in control embryos from the Germ Layer strain, which marks nuclei in the ectoderm (epidermis and ~1/3 of neurons; ***red***), mesoderm (***yellow***) and endoderm/pharynx (***green***). Images were acquired every 20 minutes. Playback is 7200X real time.



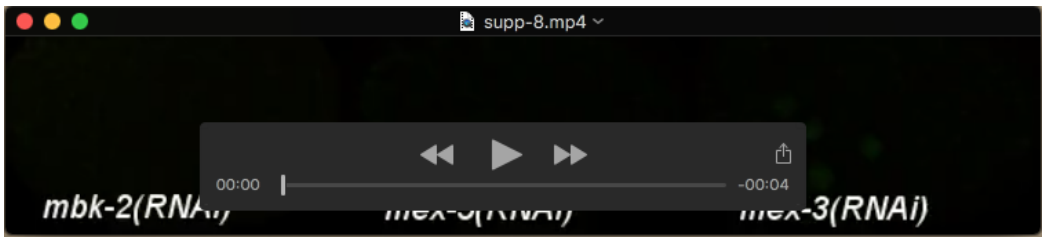
Movie 3. Example of phenotypic enhancement in the RNAi-sensitized versions of the Germ Layer and Morphogenesis strains. Side-by-side views showing the consequences of *dsh-2(RNAi)* in control (***left***) and RNAi-sensitized (***right***) versions of the Germ Layer (***top***) and Morphogenesis (***bottom***) strains. Images were acquired every 20 minutes. Playback is 7200X real time.



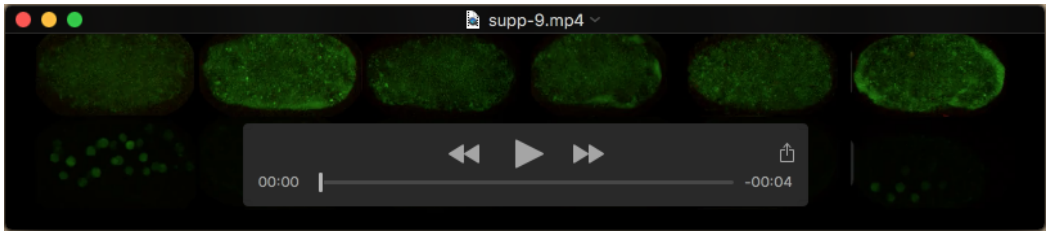
Movie 4. Knockdown of genes required for cell fate specification and/or positioning leads to diverse, distinct phenotypes in the Germ Layer and Morphogenesis strains. Genes scored as having a defect in cell fate and/or positioning were partitioned into three classes based on analysis of nuclear counts: (1) genes whose inhibition leads to incomplete cell fate specification due to arrest prior to the completion of cell division, (2) genes whose inhibition leads to an abnormal nuclear pattern despite normal numbers of each type of nuclei and (3) bona fide genes cell fate specification genes. This movie shows side-by-side views of the phenotypes in the first two classes in the Germ Layer (***top***) and Morphogenesis (***bottom***) strains. See text for description of the *plrg-1*, *arx-1*, *arx-3*, *gex-2* and *die-1* knockdown phenotypes. APS-1, is an adaptin complex subunit involved in the formation of intracellular transport vesicles (Boehm and Bonifacino, 2001); *aps-1(RNAi)* embryos typically arrested without rupture, but with severe defects in epidermal and nervous system morphology. LET-19 is a component of the mediator complex previously shown to modulate the transcription of several genes involved in development (Wang et al., 2004; Yoda et al., 2005); *let-19* knockdown led to defects in epidermal morphology that were frequently accompanied by rupture of the epidermis at the embryo anterior. Playback is 7200X real time.



Movie 5. Knocking down genes encoding WNT and NOTCH pathway components leads to distinct signature phenotypes in the Germ Layer and Morphogenesis strains. Movie presents side-by-side views of development in the Germ Layer (***left***) and Morphogenesis (***right***) strains for a control embryo (***top***) along with embryos in which genes encoding components of the WNT (*lit-1, wrm-1*) or NOTCH (*lag-1, aph-1*) signaling pathways have been knocked down. Images were acquired every 20 minutes. Playback is 7200X real time.



Movie 6. A Mex phenotype, similar to that in *mex-5/6* and *mex-3(RNAi)* embryos can be observed in *mbk-2* knockdown embryos. Movie presents side-by-side views of embryonic development in the Germ Layer strain after RNAi targeting *mbk-2* (***left***), *mex-5/mex-6* (***middle***) and mex-3 (***right***). Images were acquired every 20 minutes. Playback is 7200X real time.



Movie 7. Filming in the Germ Layer and Morphogenesis strains enables functional classification of genes required for elongation. Movie presents side-by-side views of development in the Morphogenesis (***top***) and Germ Layer (***bottom***) strains for a control embryo (***left***) and embryos in which five genes required for elongation have been knocked down. Images were acquired every 20 minutes. Playback is 7200X real time.

SUPPLEMENTAL TABLES

Table S1: Strains generated for this study

Table S1a. Available transgenic strains used in this study

Strain Name	Transgene(s)	Genotype
Composite Strains		
OD1689 (Morphogenesis Reporter Strain, control background)	GFP-tagged cell junctions (epidermis); mCherry-labeled cell surface (1/3 of neurons)	*ltSi249[pOD1274/pSW098; Pdlg-1delta7::dlg-1-GFP::unc-54-3'UTR; cb-unc-119(+)]I; ltSi511[pOD2983/pSW207; Pcnd-1::mCherry-PH::unc-54_3'UTR; cb-unc-119(+)]II*
OD2416 (Morphogenesis Reporter Strain, RNAi-sensitized background)	GFP-tagged cell junctions (epidermis); mCherry-labeled cell surface (1/3 of neurons); with RNAi-sensitizing mutations *nre-1(hd20)* & *lin-15b(hd126)*	*ltSi249[pOD1274/pSW098; Pdlg-1delta7::dlg-1-GFP::unc-54-3'UTR; cb-unc-119(+)]I; ltSi511[pOD2983/pSW207; Pcnd-1::mCherry-PH::unc-54_3'UTR; cb-unc-119(+)]II; nre-1(hd20)X; lin-15b(hd126)X*
OD1719 (Germ Layer Reporter Strain, control background)	mCherry-tagged histone (epidermis and 1/3 of neurons); yellow (GFP & mCherry) histone (muscle); GFP::PHA-4 (pharynx and intestine)	*stIs10389 [pha-4::TGF(3E3)::GFP::TY1::3xFLAG inserted into fosmid WRM0617dE06 as C-terminal protein fusion]; ltSi539[pOD1519/pSW224; Pdlg-1Δ7::mCherry::his-72::unc-54_3'UTR; Pcnd-1::mCherry::his-72::unc-54_3'UTR; cb-unc-119(+)]II; ltSi507[pOD1492/pSW201; Phlh-1::GFP::his-72::tbb-2_3'UTR, Phlh-1::mCherry::his-72::tbb-2_3'UTR; cb-unc-119(+)]IV*
OD1854 (Germ Layer Reporter Strain, RNAi-sensitized background)	mCherry-tagged histone (epidermis and 1/3 of neurons); yellow (GFP & mCherry) histone (muscle); GFP::PHA-4 (pharynx and intestine); with RNAi-sensitizing mutations *nre-*	*stIs10389 [pha-4::TGF(3E3)::GFP::TY1::3xFLAG inserted into fosmid WRM0617dE06 as C-terminal protein fusion]; ltSi539[pOD1519/pSW224; Pdlg-1Δ7::mCherry::his-72::unc-54_3'UTR; Pcnd-1::mCherry::his-72::unc-54_3'UTR; cb-unc-119(+)]II; ltSi507[pOD1492/pSW201; Phlh-1::GFP::his-72::tbb-2_3'UTR, Phlh-1::mCherry::his-72::tbb-2_3'UTR; cb-unc-119(+)]IV; nre-1(hd20)X; lin-15b(hd126)X*

	1(hd20) & lin-15b(hd126)		
Intermediate Strains			
OD1593	mCherry-labeled cell surface (1/3 of neurons)	*ltSi511[pOD2983/pSW207; Pcnd-1::mCherry-PH::unc-54_3'UTR; cb-unc-119(+)]II*	6x
OD1591	GFP-tagged cell junctions (epidermis)-also in pharynx and intestine at post-embryonic stage.	*ltSi249[pOD1274/pSW098; Pdlg-1delta7::dlg-1-GFP::unc-54-3'UTR; cb-unc-119(+)]I*	8x
OD1599	mCherry-tagged histone (epidermis and 1/3 of neurons)	*ltSi539[pOD1519/pSW224; Pdlg-1Δ7::mCherry::his-72::unc-54_3'UTR; Pcnd-1::mCherry::his-72::unc-54_3'UTR; cb-unc-119(+)]II*	6x
OD1592	yellow (GFP & mCherry) histone (muscle)	*unc-119(ed3)III; ltSi507[pOD1492/pSW201; Phlh-1::GFP::his-72::tbb-2_3'UTR, Phlh-1::mCherry::his-72::tbb-2_3'UTR; cb-unc-119(+)]IV*	6x
OD1598	GFP::PHA-4 (intestine & pharynx); previously generated insertion (Fakhouri et al., 2010 (PMID: 20714352)) that we backcrossed 6X	*stIs10389 [pha-4::TGF(3E3)::GFP::TY1::3xFLAG inserted into fosmid WRM0617dE06 as C-terminal protein fusion]*	6x

Table S1b. Available transgenic strains made for this study, but not used

Strain Name	Expressing Transgene(s)	Genotype	Out-crossed
Nuclear reporters			

OD1557	mCherry-tagged histone (post-embryonic pan-neuronal)	*ltSi517[pOD1489/pSW198; Prgef-1::mCherry::his-72::unc-54_3'UTR; cb-unc-119(+)]I; unc-119(ed3)III*	0x
OD1433	mCherry-tagged histone (body wall muscle)	*ltSi456[pOD1486/pSW195; Phlh-1::mCherry::his-72::unc-54_3'UTR; cb-unc-119(+)]I; unc-119(ed3)III*	0x
OD1434	GFP-tagged histone (body wall muscle)	*ltSi457[pOD1487/pSW196; Phlh-1::GFP::his-72::unc-54_3'UTR; cb-unc-119(+)]I; unc-119(ed3)III*	0x
OD1596	yellow (mCherry and GFP) histone (intestine)	*ltSi506[pOD1491/pSW200; Pelt-2::GFP::his-72::tbb-2_3'UTR, Pelt-2::mCherry::his-72::tbb-2_3'UTR; cb-unc-119(+)]IV*	6x
OD1595	GFP-tagged histone (epidermis and 1/3 of neurons)	*ltSi509[pOD2982/pSW203; Pdlg-1Δ7::GFP::his-72::unc-54_3'UTR; Pcnd-1::GFP::his-72::unc-54_3'UTR; cb-unc-119(+)]II*	6x
OD1007	mCherry-tagged histone (body wall muscle, pharynx, and intestine -- note pharynx expression is weak, and intestine is variable among lines; this line has strong intestine expression)	*ltSi273[pOD1272/pSW094; Phlh-1::mCherry::his-72::unc-54_3'UTR; Ppha-4::mCherry::his-72::tbb-2_3'UTR; cb-unc-119(+)]I; unc-119(ed3)III*	0x
OD1540	mCherry-tagged histone (body wall muscle, pharynx, and intestine -- note pharynx expression is weak)	*ltSi504[pOD1294/pSW108; Phlh-1::mCherry::his-72::unc-54_3'UTR; Ppha-4intron1::mCherry::his-72::tbb-2_3'UTR; cb-unc-119(+)]I; unc-119(ed3)III*	0x
Cell morphology reporters			

OD1085	mCherry-labeled cell surface (embryonic, all cells; post-embryonic, appears to be pan-neuronal)	*ltSi316[pOD1293/pSW101s; Punc-33::mCherry-PH(PLCdelta1)::unc-54_3'UTR; cb-unc-119(+)]II; unc-119(ed3)III*	0x
OD1087	mCherry-labeled cell surface (embryonic, no expression; post-embryonic, pan-neuronal)	*ltSi318[pOD1296/pSW126; Prgef-1::mCherry-PH(PLCdelta1)::unc-54_3'UTR; cb-unc-119(+)]II; unc-119(ed3)III*	0x
OD1106	mCherry-labeled cell surface (embryonic, all cells; post-embryonic, appears to be pan-neuronal)	*ltSi321[pOD1295/pSW125; Punc-119::mCherry-PH(PLCdelta1)::unc-54_3'UTR; cb-unc-119(+)]II unc-119(ed3)III*	0x
Composite reporters			
OD1639	GFP-tagged cell junctions (epidermis); mCherry-labeled cell surface (1/3 of neurons); yellow (GFP & mCherry) histone (muscle)	*ltSi249[pOD1274/pSW098; Pdlg-1delta7::dlg-1-GFP::unc-54-3'UTR; cb-unc-119(+)]I; ltSi511[pOD2983/pSW207; Pcnd-1::mCherry-PH::unc-54_3'UTR; cb-unc-119(+)]II; ltSi507[pOD1492/pSW201; Phlh-1::GFP::his-72::tbb-2_3'UTR, Phlh-1::mCherry::his-72::tbb-2_3'UTR; cb-unc-119(+)]IV*	6x

Table S2: Summary of phenotypic data for the 40-gene test set. This document contains an index and 7 additional tabs. **S2.1-Embryonic Lethality**: Summarizes embryonic lethality and larval defects for each RNAi condition in the 4 strain backgrounds. **S2.2-Defects Scored**: provides a description of each phenotypic defect and how it was scored. **S2.3-Phenotype Scoring**: Includes quantification of all manually scored phenotypic characteristics for each gene in the 4 tested strain backgrounds (Germ Layer strain with RNAi-sensitizing mutations, Morphogenesis strain with RNAi-sensitizing mutations, Germ Layer strain control background, and Morphogenesis strain control background). **S2.4-Expected-Observed Summary**: Provides a comparison of the embryonic lethality and phenotypic defects observed in this study with annotated embryonic lethality and observed phenotypes by RNAi or mutant allele previously cataloged on WormBase. **S2.5-Gene Expression-Phenotypic Group:** shows the previously described temporal gene expression profile plots (Levin et al. 2012) for the genes in the broad phenotypic groups defined by the phenotypic scoring in this study. **S2.6-Raw Embryonic Lethality Data:** raw data counts of embryonic lethality, abnormal hatched L1, normal hatched L1 for the four considered strains. **S2.7-Oligo list:** sequences of the forward and reverse oligos used for the production of dsRNAs targeting each of the tested genes.

Click here to Download Table S2

PROMOTER SEQUENCES

P*cnd-1* (a subset of neurons, 3230 bp):

cagctatgacacgtggctctagtaatacttgaaagttttcgcgcatacttgtagtgatacactgtatccatccggtctgtatttattcaga
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P*dlg-1Δ7* (embryonic epidermis, 3940 bp):

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P*elt-2* (intestine, 2994 bp):

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P*hlh-1* (body wall muscle, 3345 bp):

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P*pha-4* (intestine and weak pharynx, 4157 bp):

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P*pha-4* + intron1 (pharynx and intestine, 6452 bp):

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P*rgef-1* (post-embryonic pan-neuronal):

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P*unc-119* + intron1 (embryonic all cells, 4749 bp)

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P*unc-33* (embryonic all cells, 2003 bp):

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tctaagaaaaaaaacattagaaaaactaaaacattatttttttgaaaatacaatattttttgttgtatttttagatttttttcctgcaggaataa
gatttttagtctcgtgagagaattcacactaaaaattgctaaatttacataaaatatgtgtaaaaagccatttttttccgtagaaaaatttc
aaaatttatgaatttttctttaaaatttgattaaaccccctgaaaaaccactggaaatttttgttttttcccctcgaaagttcccccaaagtca
cattttgcaaatttctaaagaagatttaccaccgcaattaccatagttaatccgaaaattgaaatttttcccgtctttttttcctccaatcttca
tttttacctcttttttaacctaaaaataattgaaaaaaacccaatttttcgttacagaaagcgcgcaaaatacacatttatcactagagga
gggccaaatagttggcg

CROPPING PROGRAM INSTRUCTIONS

We provide two repositories that contain software:

1. **Zenodo repository** (*https://zenodo.org/record/1475442#.W9jvApNKiUl*), which contains:

    - **embryoCropUI_WINDOWS**- *PC compatible executable version of graphical user interface (GUI)* version of our software, which can accommodate *individual image stacks from a range of imaging platforms.* This crops, rotates anterior-posterior, processes for drift correction, background subtraction and attenuation correction for individual image stacks. Cropped images are automatically saved to a "crop" file in the location of the file selected to be cropped.
    - **embryoCropUI_MacOS**- *Mac compatible executable version of graphical user interface (GUI)* version of our software, which can accommodate *individual image stacks from a range of imaging platforms.* This crops, rotates anterior-posterior, processes for drift correction, background subtraction and attenuation correction for individual image stacks. Cropped images are automatically saved to a "crop" file in the location of the file selected to be cropped. (requires MacOS X10.11)
    - **Test_files**- Contains two test files: a multi-tif format file and a folder with a tif image series, which can be used to test the GUI on your system (details for testing can be found in the instructions)
    - **OpenAndCombine_embs.ijm**- FIJI script that enables assembly of maximum intensity projections for multiple embryos within one viewer. Requires **ImageJ**. *Note this works with our file structure and will need to be modified to accommodate your file structure (see instructions).*
    - **Instructions**- Instructions for installation and running GUI (embryoCropUI) and FIJI script (OpenAndCombine.ijm) (this file)

2. **Github repository (*https://github.com/renatkh/embryo_crop.git*), which contains source code for embryoCropUI (embryoCropUI.py) and screenCrop.py:**

    - **embryoCropUI.py:** This is the source code for the user-friendly executable version described above, which crops, rotates anterior-posterior, processes for drift correction, background subtraction and attenuation correction for individual image stacks.
    - **screenCrop.py**: This batch version of the embryoCrop.py program takes in multiwell, multipoint imaging data and crops, rotates anterior-posterior, processes for drift correction, and performs background subtraction and attenuation correction in batch. Requires **Python3, virtual environment, and .csv** file with the file format and condition specifications delineated. Saves cropped files to new "Cropped" folder with file structure as specified in .csv file. *This program is specific to our file structure and acquisition parameters, but can be modified to accommodate similarly structured data if users have Python expertise- see instructions below for key variables*.
    - **README file**- Instructions for cloning repository, setting up virtual environment and running embryoCrop.py., and configuring necessary requirements to run screenCrop.py.

****Instructions for all programs can be found within each repository and are reproduced below for completeness:**----------

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Zenodo repository

(*https://zenodo.org/record/1475442#.W9jvApNKiUl*)

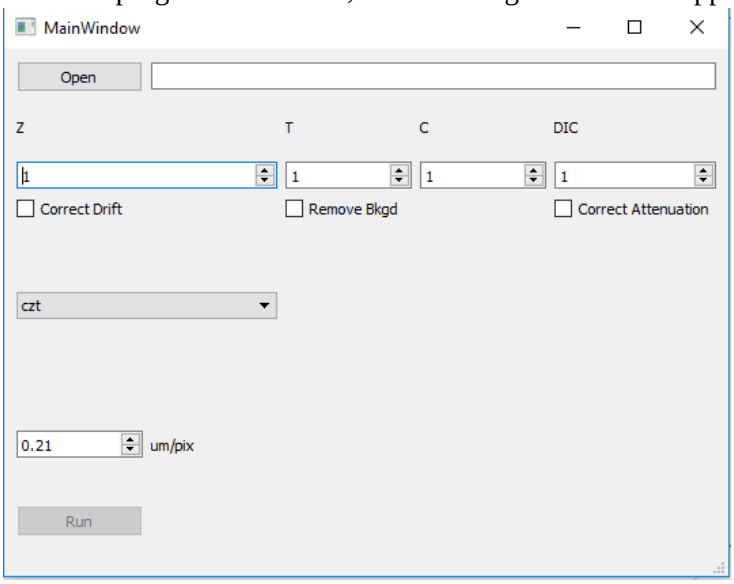
- **embryoCropUI_WINDOWS**
- **embryoCropUI_MacOS**-requires MacOS X10.11
- **Test_files**
- **OpenAndCombine_embs.ijm**
- **Instructions**

DETAILED INSTRUCTIONS

1. Running embryoCropUI.exe (MacOS and Windows)

1. Download Window or MacOS folder, unzip and navigate to find the embryoCropUI executable (…\embryoCropUI _WINDOWS\embryoCropUI\embryoCropUI.exe) or (…\embryoCropUI _MacOS\embryoCropUI\embryoCropUI.exe). Double click to launch (or chose 'open with'→ terminal) and run the embryoCropUI executable.
   Note- MacOS distribution is only compatible with Mac OS X 10.11 and higher.

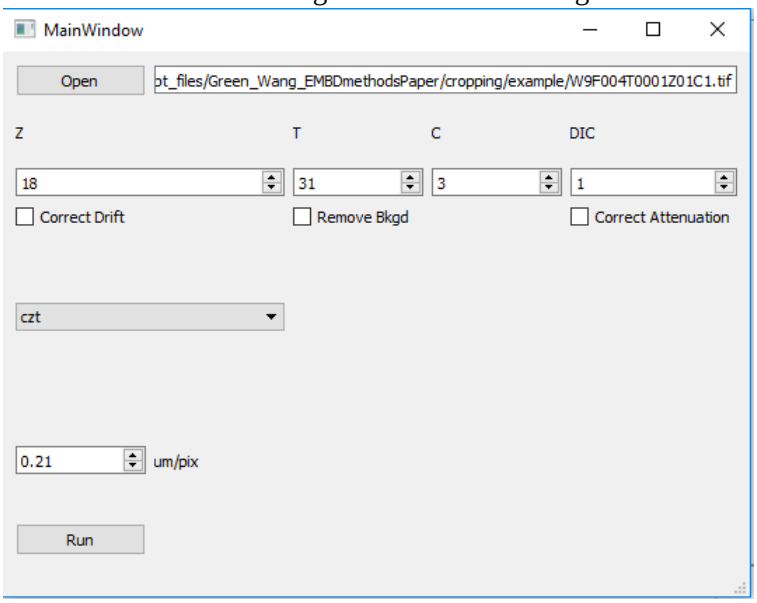
2. Once the program has started, the following window will appear:



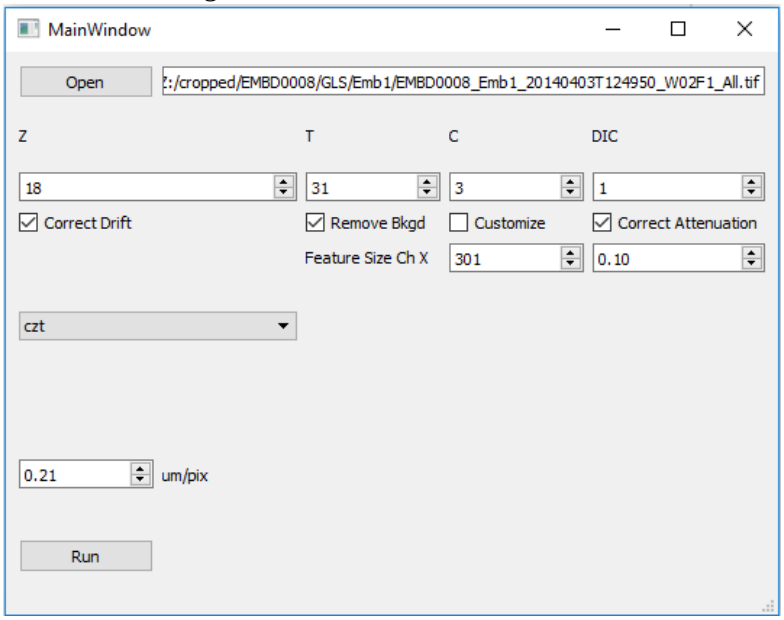
3. Select the **"Open"** button at the top of the window to load the specific image that you wish to crop. Should you be cropping an image series, with multiple dimensions (i.e. z, time, or channel), simply load the first image in the series within the folder. *Please make sure only images from one image series are present in this folder, otherwise the image series' will be loaded in tandem.*

4. Once you have loaded the desired images, you will need to specify the following information:
   a. Number of **Z slices (Z)**
   b. Number of **Time points (T)**
   c. Number of **Channels (C)**
   d. The channel that corresponds to **DIC or brightfield** (first=1, second=2, etc)

For example: our imaging protocol was 18 z-steps, imaged for 31 time-points in 3 channels with the DIC channel being the first channel imaged. The window should look like this:

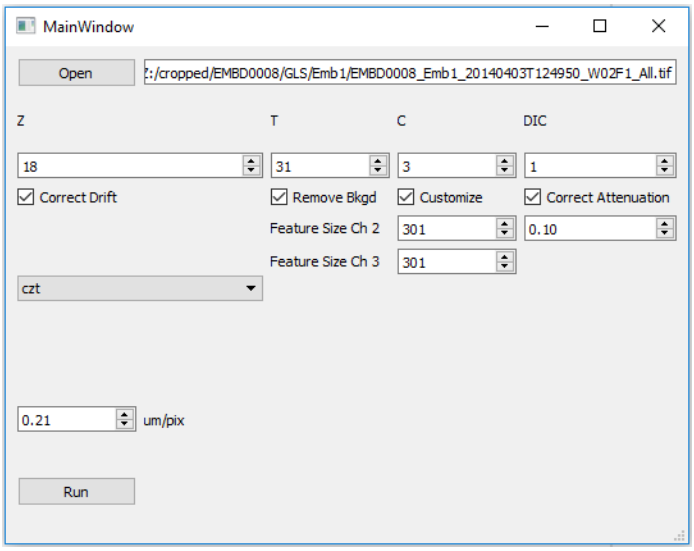


5. Now that you have your images loaded and specified the image parameters, you must choose what processing you would like to do alongside the embryo cropping. The program gives you the option to perform **Drift Correction, Background Subtraction, and Attenuation Correction**. Background Subtraction and Attenuation Correction must be done in conjunction with each other. The below image shows an image that will be going through Drift Correction, Background Subtraction, and Attenuation Correction.



   a. When selecting Background Subtraction and Attenuation Correction, specify parameters for each to guide the processing. For *Background Subtract*, define a feature size (*odd numbered*) that reflects the level of detail you wish to resolve, larger feature size equates to more detail. For *Attenuation Correction*, you need to input a value from 0-1. This value represents the percent of original intensity that remains at the furthest distance through the object being imaged.

b. As Shown below, you have even greater options to customize Background subtraction. By selecting Customize, you will be able to define a feature size for different channels.

c. 

7. Next, specify the **order** in which the images were collected (i.e. **channel-z-time (czt)**, or **z-channel-time (zct)**)
8. Specify the **microns per pixel of the camera being used** for the images.
   a. note that failure to properly define pixel size will result in poor image cropping!!
9. Select **Run** at the bottom left corner and the program
   When the cropping and processing of your images has completed, the cropped versions will be saved in a new subfolder labeled "crop" in the same folder as the uncropped images.

Two formats of **test files are available in the repository- download and unzip. We recommend testing one or both of these to ensure the program is functioning properly on your system:

i. TESTME2_BGLI140_1t_a1.tif- a compiled multi-tif format
   1. Load file in the 'open' field. Set Z=1, T=6, C=3, DIC=1 and use the default settings for all other fields. Click Run. If successful, a message will appear at the bottom of the GUI window that says 'embryos saved' and it will generate a folder in the same location as the test file labeled "crop"; this should contain 4 embryos.

ii. Test_field- a folder containing an image series
   1. Load the first image in the test_field folder into the 'open' window. Set Z=18, T=4, C=3, DIC=3, change the pixel size to 0.26um/pix. Click Run. a message will appear at the bottom of the GUI window that says 'embryos saved' and it will generate a folder in the same location as the test file labeled "crop"; this should contain 2 embryos.

**These files should crop in seconds to minutes, but larger image sequences may take some time. The bottom corner of the GUI window will read-out what the program is doing ("Loading images", "cropping", or "embryos saved"). If an error occurs, the message will appear here.

2. Running OpenandCombine_embs (Fiji processing for viewing):

1. This Fiji Script "OpenandCombine_embs" combines all the images for a specific condition and strain into one easy to view Fiji file. Requires installation of **ImageJ**. Note that this program runs according to our file structure and may need to be modified to work with your

file structure (see our file structure below as a guide). To prepare to run this script, you must know the following information:

a. The location where images where stored following cropping.
b. The desired location for saving the Fiji files after processing.
c. The Target Name for the specific condition you wish to process.
d. The Experiment Folder Name.

*for reference, our file location structure looks like this:

Z:\cropped\Target\Strain\Emb#\Target_Emb#_Experiment Folder Name_W##F#_T##_Z##_C#.tif

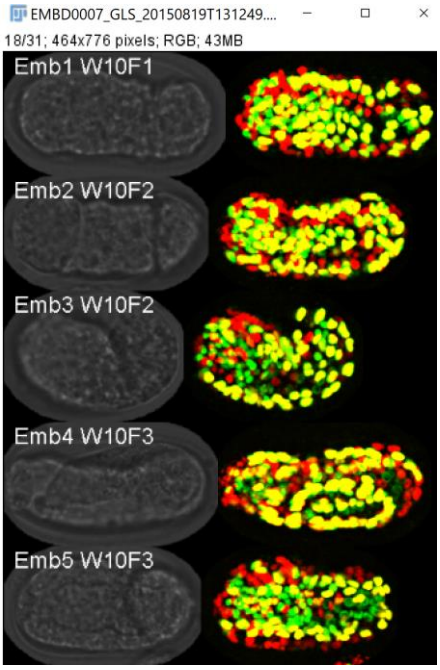
i.e. Z:\cropped\EMBD0002\MS\Emb1\EMBD0002_Emb1_20140402T140154_W06F2_T01_Z01_C1

2. Open ImageJ, drag our .ijm Script file to the ImageJ bar. Once the script is open, locate lines 28-31, they are shown below. Within these lines you will fill in the Information you gathered above.

```
27 }
28 RNAL = newArray("EMBD0000/","EMBD0052/","EMBD0053/","EMBD0054/");
29 date = newArray("20140110T153253");
30 folder = "Z:/cropped/";
31 folderOut2 = "Z:/EMBD_fiji_processed/";
32 fSize = 24;
33 setFont( "SansSerif", fSize);
34 for (r=0; r<date.length; r++){
35 for (l=0; l<RNAL.length; l++){
36     RNA = RNAL[l];
37     listStrains = getFileList(folder+RNA);
38     for (j = 0; j < listStrains.length; j++){
39         if (listStrains[j] == "GLS/"){
```

a. In Line 30, input the folder location of the images you wish to process in quotations.
b. In Line 31, input the desired save location in quotations.
c. In Line 28, input the Target name for the images you wish to process. Each Target name must be put in the format shown above, with a forward slash at the end and in quotations.
d. In Line 29, input the Image Folder Name in quotations.

3. Press Run at the bottom left corner of the script window. The program will then begin processing your images and compiling them into one file. Once the Program has finished the files will be open allowing you to review them. The files can be closed without saving, as the program has already saved them during processing.
Example:



Github Repository (*https://github.com/renatkh/embryo_crop.git*)

- **embryoCropUI.py**
- **screenCrop.py**
- **README file**

SETUP

Both programs (**embryoCropUI.py** and **screenCrop.py**) use specific versions of Python and Python modules, thus configuring an appropriate environment is essential for the programs to run. We recommend and provide instructions for installation of **Git**, **Anaconda (includes Python3)**, and **PyCharm** to enable proper environment configuration (detailed instructions below). ***Note- MacOS distribution of embryoCropUI.UI is only compatible with Mac OS X 10.11 and higher***

DETAILED INSTRUCTIONS

1. Configure your environment

Clone repository with GIT

1. If you don't already have GIT installed, go to **https://git-scm.com/download/** . You may need to enable security settings to be sure it will download.

2. Check install by going to terminal or command prompt and enter:

    > **git --version**

    **if installed a version will be listed in the terminal*

3. Clone repository:

    >**git clone https://github.com/renatkh/embryo_crop.git**

4. Check in your home directory to ensure that it was properly downloaded.

Install Visual Studio (WINDOWS ONLY):

1. Go to www.visualstudio.microsoft.com/downloads and **download Visual Studio**. This contains C++ tools, which are required for proper setup of the virtual environment with anaconda .yml files.
2. Select C++ tools
3. Install

Setup virtual environment with Anaconda

1. If you don't already have Anaconda, go to www.anaconda.com/download/ and Download **Anaconda3** (python3.7 version), launch anaconda setup and click through default options to install.

2. set environment variables and add conda to the path:

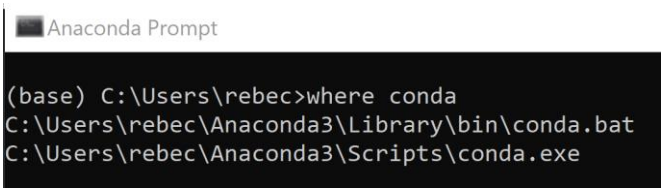
    a. Find **conda.exe** location in **Anaconda Prompt**:

WINDOWS: Go to **windows button-> Anaconda3-> Anaconda Prompt**

MacOS: **Anaconda3-> Anaconda Prompt**

At the prompt type in

> **where conda**



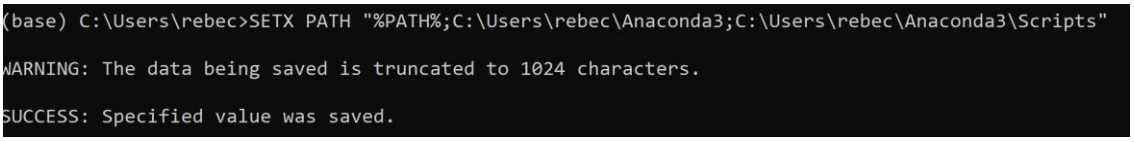
Find the location where conda.exe is located (ignore the .bat location) so you can add this location to environmental variables (this can be done within Anaconda Prompt).

In this case it is **C:\Users\rebec\Anaconda3\Scripts**
But obviously this will be specific to your system, so please edit the path appropriately!

For this example, we need to add both paths:
C:\Users\rebec\Anaconda3
C:\Users\rebec\Anaconda3\Scripts

b. Add to environment variables. To do this, type

> **SETX PATH "%PATH%;C:\Users\rebec\Anaconda3;C:\Users\rebec\Anaconda3\Scripts"**



3. Close **Anaconda Prompt**

3. Go to **system terminal or command prompt** (*not anaconda prompt*) and check to be sure that the **conda** command works.

> **conda**

this should return information about **conda functionality. If it does not, you have not successfully added path environmental variables.*

4. Configuring the environment in command line/terminal:

a. Navigate to the location where embryo_crop repository was saved.
i.e. for example, it is saved here: C:\Users\rebec\embryo_crop
so at the prompt:
> **cd C:\Users\rebec\embryo_crop**

b. Create new conda environment from .yml file

Once inside the directory, create the environment:

-for Windows: > **conda env create -f environment_win.yml**

-for MacOS:> **conda env create -f environment_mac.yml**

This step will take a few minutes to solve the environment……

```
C:\Users\rebec\embryo_crop>conda env create -f environment_win.yml
Solving environment: done
Preparing transaction: done
Verifying transaction: done
Executing transaction: done
Collecting pyqt5==5.11.3 (from -r C:\Users\rebec\embryo_crop\condaenv.lmfjd3fz.requirements.txt (
  Using cached https://files.pythonhosted.org/packages/a7/2d/d2c989006c86ae98ed230c28c3e0dd7fa037
Collecting pyqt5-sip==4.19.13 (from -r C:\Users\rebec\embryo_crop\condaenv.lmfjd3fz.requirements.
  Using cached https://files.pythonhosted.org/packages/46/86/0e35563d0c67c3f6b50e344624b87bfa7e72
Collecting tifffile==0.15.1 (from -r C:\Users\rebec\embryo_crop\condaenv.lmfjd3fz.requirements.tx
  Using cached https://files.pythonhosted.org/packages/1f/a1/4055cd679081cb4c1e40aa7648adb12574bf
Requirement already satisfied: numpy>=1.8.2 in c:\users\rebec\anaconda3\envs\embryocrop\lib\site-
Building wheels for collected packages: tifffile
  Running setup.py bdist_wheel for tifffile ... done
  Stored in directory: C:\Users\rebec\AppData\Local\pip\Cache\wheels\22\5b\8e\a9bc85b5dfc1cc91b84
Successfully built tifffile
mkl-random 1.0.1 requires cython, which is not installed.
Installing collected packages: pyqt5-sip, pyqt5, tifffile
  The scripts pylupdate5.exe, pyrcc5.exe and pyuic5.exe are installed in 'C:\Users\rebec\Anaconda
  Consider adding this directory to PATH or, if you prefer to suppress this warning, use --no-war
Successfully installed pyqt5-5.11.3 pyqt5-sip-4.19.13 tifffile-0.15.1
#
# To activate this environment, use:
# > activate embryocrop
#
# To deactivate an active environment, use:
# > deactivate
#
# * for power-users using bash, you must source
#
```

5. When finished, you can continue in command line to run embryoCropUI (below) or switch to an IDE to run screenCrop or embryoCropUI.

a. *To continue in command line (for embryoCropUI):*

Activate the environment according to the instructions listed in the terminal.

For Windows:

> **activate embryocrop**

For MacOS:

> **source activate embryocrop**

Once activated, you can run python programs by calling the program in command line

>**python embryoCropUI.py**

*this will launch the GUI window- please follow the instructions for the GUI use (in the readme file).

When finished:

Close GUI

>**deactivate**

Configure Environment in IDE (Setup in PyCharm)

To access the code directly, which is necessary for **screenCrop.py** functionality, open the environment in your favorite **Integrated Development Environment (IDE)**. There are many IDEs that are commonly used for coding in Python. An overview of features and limitations for popular options can be found here: https://stackoverflow.com/questions/81584/what-ide-to-use-for-python.

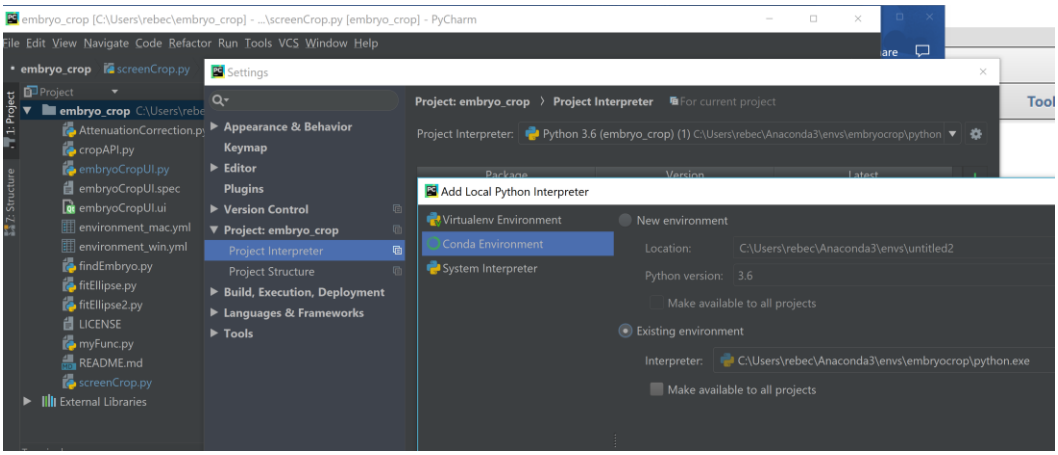
We include instructions for installing and running with **PyCharm**, however if you already have a different IDE that is compatible with Python (i.e. Eclipse) it should work fine. Note that Jupyter notebooks currently DOES NOT support GUIs, so our embryoCropUI will not run properly in this environment.

a. To get **PyCharm**, go to: https://www.jetbrains.com/pycharm/download/#section=windows. Select your operating system and click the black download button under "Community".
   i. For Windows- run the PyCharm-*.exe file and follow the installation wizard.
   ii. For MaOSc, open the PyCharm-*.dmg package and drag PyCharm to the Application folder.
b. When you start PyCharm for the first time, you will be prompted with **a complete installation** dialog box. Click **Do not import settings**.
c. Select your preferred user interface theme [Default or Darcula (black background)]
d. You will be prompted to install any additional plugins (not necessary)
e. Now you should be set up and greeted with a "welcome screen" where you can create, open, or check out a project.

In PyCharm:

1. **File > Open > embryo_crop**
2. Configure environment. Go to **file > settings > project:embryo_crop > project interpreter > add local** (select conda) **> existing environment >**

   select the newly generated conda env from within the Anaconda3 envs folder: **…Anaconda3\envs\embryocrop\python.exe**



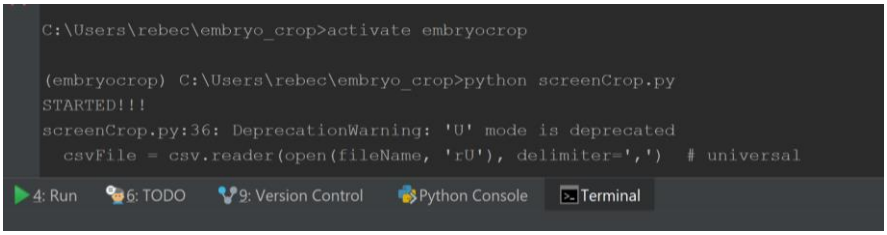
3. From here, you should be able to run programs using the PyCharm 'run' button (program instructions below). If this doesn't properly structure the environment, it may crash. If this happens, you can access the *terminal window within PyCharm* and activate the environment this way:
   a. For Windows:
      > **activate embryocrop**
   b. For MacOS:
      > **source activate embryocrop**

4. Once the environment is activated, you can run the program via the *terminal within PyCharm*:

   > **python screenCrop.py**

   or > **python embryoCropUI.py**

** note that the screenCrop program will need to be modified to work with your file structure!!**See instructions below**.



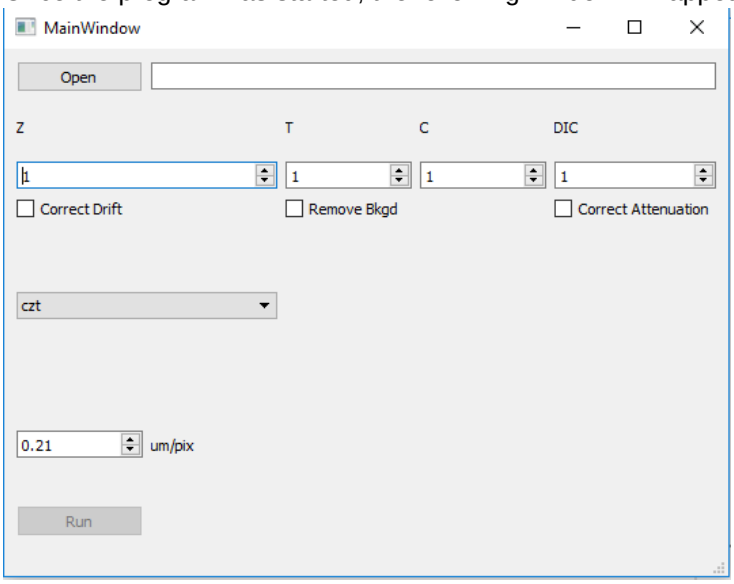
When finished:

>**deactivate**

2.Running embryoCropUI.py GUI with Python

1. In PyCharm (or your preferred IDE) double click on the **embryoCrop** folder and locate the file that says **embryoCropUI.py** (DO NOT open embryoCropUI.ui). The code will appear in the workspace.
2. If this is the only file open, go to the top right-hand corner and **click the green triangle** to start the run. If multiple files are open, right click and select '**Run embryoCropUI**' to ensure the proper program is run. Alternatively, activate the environment and run from PyCharm terminal, as outlined above.

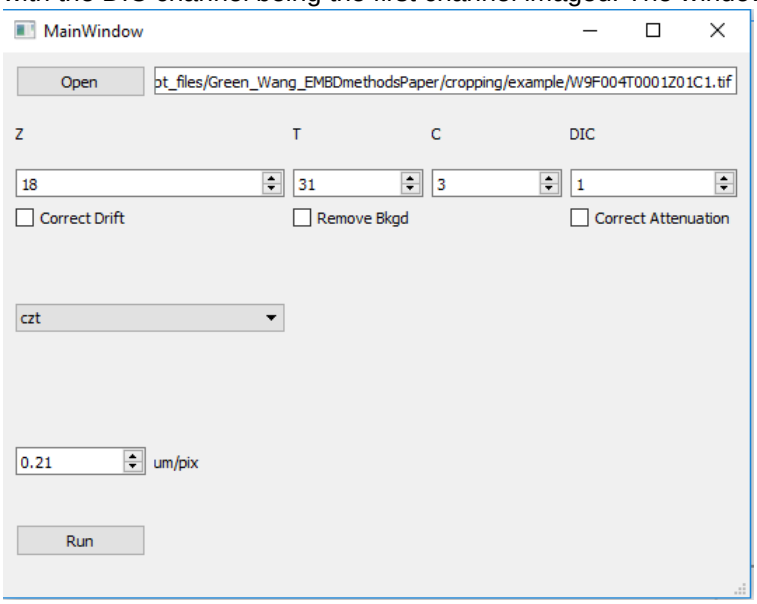
3. Once the program has started, the following window will appear:



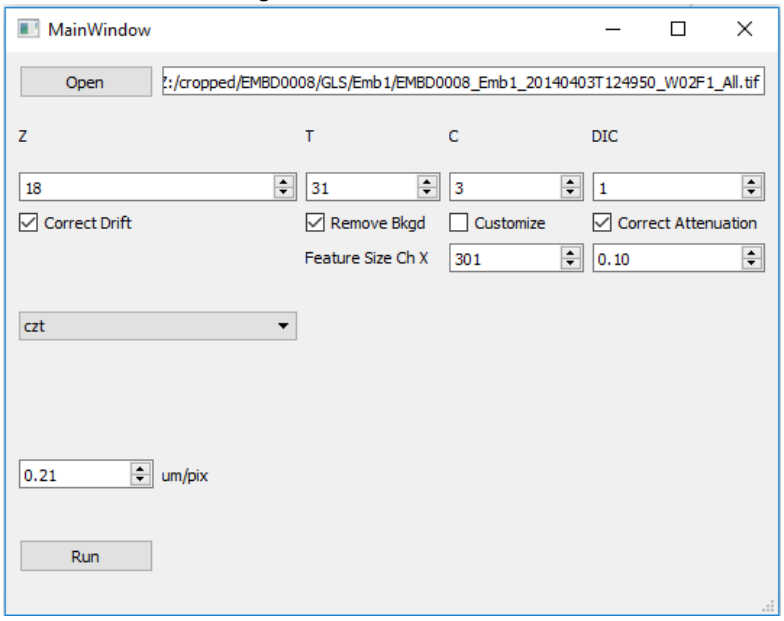
4. Select the **"Open"** button at the top of the window to load the specific image that you wish to crop. Should you be cropping an image series, with multiple dimensions (i.e. z, time, or channel), simply load the first image in the series within the folder. *Please make sure only images from one image series are present in this folder, otherwise the image series' will be loaded in tandem.*

5. Once you have loaded the desired images, you will need to specify the following information:
   e. Number of **Z slices (Z)**
   f. Number of **Time points (T)**
   g. Number of **Channels (C)**
   h. The channel that corresponds to **DIC or brightfield** (first=1, second=2, etc)

For example: our imaging protocol was 18 z-steps, imaged for 31 time-points in 3 channels with the DIC channel being the first channel imaged. The window should look like this:

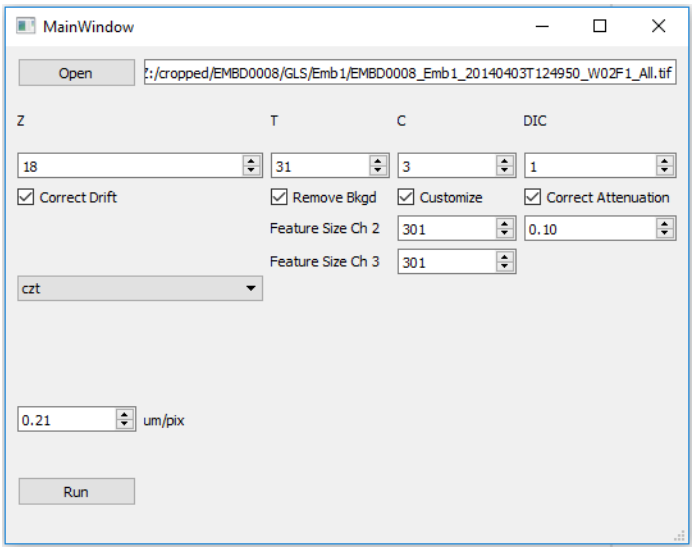


6. Now that you have your images loaded and specified the image parameters, you must choose what processing you would like to do alongside the embryo cropping. The program gives you the option to perform **Drift Correction, Background Subtraction, and Attenuation Correction**. Background Subtraction and Attenuation Correction must be done in conjunction with each other. The below image shows an image that will be going through Drift Correction, Background Subtraction, and Attenuation Correction.



   d. When selecting Background Subtraction and Attenuation Correction, specify parameters for each to guide the processing. For *Background Subtract*, define a feature size (*odd numbered*) that reflects the level of detail you wish to resolve, larger feature size equates to more detail. For *Attenuation Correction*, you need to input a value from 0-1. This value represents the percent of original intensity that remains at the furthest distance through the object being imaged.

   e. As shown below, you have even greater options to customize Background subtraction. By selecting Customize, you will be able to define a feature size for different channels.

f. 

7. Next, specify the **order** in which the images were collected (i.e. **channel-z-time (czt)**, or **z-channel-time (zct)**)
8. Specify the **microns per pixel of the camera being used** for the images.
   a. failure to properly define pixel size will result in poor image cropping!!
10. Select **Run** at the bottom left corner and the program
    When the cropping and processing of your images has completed, the cropped versions will be saved in a new subfolder labeled “crop” in the same folder as the uncropped images.

We make available two formats of **test files for testing embryoCropUI.py. This folder is too large for Github requirements and thus is stored on the Zenodo repository (https://zenodo.org/record/1475442#.W9jvApNKiUl). Download and unzip. We recommend testing one or both to ensure the program is functioning properly on your system:

iii. TESTME2_BGLI140_1t_a1.tif- a compiled multi-tif format
   1. Load file in the ‘open’ field. Set Z=1, T=6, C=3, DIC=1 and use the default settings for all other fields. Click Run. If successful, a message will appear at the bottom of the GUI window that says ‘embryos saved’ and it will generate a folder in the same location as the test file labeled “crop”; this should contain 4 embryos.

iv. Test_field- a folder containing an short image series
   1. Load the first image in the test_field folder into the ‘open’ window. Set Z=18, T=4, C=3, DIC=3, change the pixel size to 0.26um/pix. Click Run. a message will appear at the bottom of the GUI window that says ‘embryos saved’ and it will generate a folder in the same location as the test file labeled “crop”; this should contain 2 embryos.

**These files should crop in seconds to minutes, but larger image sequences may take some time. The bottom corner of the GUI window will read-out what the program is doing (“Loading images”, “cropping”, or “embryos saved”). If an error occurs, the message will appear here.

3.Running screenCrop.py

This software allows you to batch crop many files at once, but it is less user friendly and has not been optimized across platforms. *It was designed to function with data output from CV1000 imaging systems*; if you have a CV1000 and follow the instructions below, it should work seamlessly. If you have another system, modifications to the code will likely be required and someone with Python experience will be

needed. In the event that it is needed, we outline key elements of the code and our file structure to guide such efforts. Successful bulk cropping requires:

1. A **reference .csv** file that contains essential image information, which is called by our Python software
2. **Properly named files**

CSV:

The CSV file contains information that will be called on during processing or used to generate the cropped file path. Below is an example .csv file that is compatible with our programs. Formatting your .csv file in the same way will ensure your data will go through our programs with minimal issues.

Experiment	Experiment Folder Name	Post-scan folder name	Well designation	Target	strain	Plate Coordinate
EMBD_112013	20131120T160955	20131121T104921	Well001	EMBD0000	GL	C3
	20131120T160955	20131121T104921	Well005	EMBD0000	GLS	D3
	20131120T160955	20131121T104921	Well009	EMBD0000	M	E3
	20131120T160955	20131121T104921	Well013	EMBD0000	MS	F3
	20131120T160955	20131121T104921	Well002	EMBD0002	GL	C4
	20131120T160955	20131121T104921	Well006	EMBD0002	GLS	D4
	20131120T160955	20131121T104921	Well010	EMBD0002	M	E4
	20131120T160955	20131121T104921	Well014	EMBD0002	MS	F4
	20131120T160955	20131121T104921	Well003	EMBD0003	GL	C5
	20131120T160955	20131121T104921	Well007	EMBD0003	GLS	D5
	20131120T160955	20131121T104921	Well011	EMBD0003	M	E5
	20131120T160955	20131121T104921	Well015	EMBD0003	MS	F5
	20131120T160955	20131121T104921	Well004	EMBD0015	GL	C6
	20131120T160955	20131121T104921	Well008	EMBD0015	GLS	D6
	20131120T160955	20131121T104921	Well012	EMBD0015	M	E6
	20131120T160955	20131121T104921	Well016	EMBD0015	MS	F6

Rundown of .CSV file contents:

- Experiment: arbitrary name given to each experiment (not important for software functionality, but this column needs to be maintained)
- Experiment Folder Name: name of folder in where specific experiments images are stored. We prefer Date/Time file name, though any name will suffice (do not include spaces or disallowed characters, as the contents of this cell are added to the file path).
- Post-Scan Folder Name: name of post- scan (10x data) folder. Not important for software functionality, but this column needs to be maintained. You can populate this with 'empty'.
- Well Designation: Well numbers as determined by CV1000 software adhering to the Well### regime.
- Target: Experimental conditions (e.g. RNAi condition), we use a blinded, unique identifier system (EMBD####) for our experimental conditions, though this is not necessary. Output files will be saved according to this name, so do not include spaces or disallowed characters.
- Strain: Specific strain used in experiment. Scaling and background subtraction is applied differently depending on the strain used.
- Plate Coordinate: Coordinates from 384 well plate (for reference, not used by program).

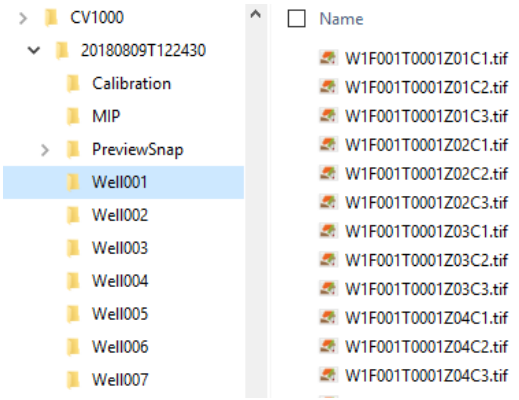
Properly naming files:

Input path:

The path to access the raw data files is referred to in **screenCrop.py**, based on the information in the CSV file (highlighted in red below). Our data is structured such that multiple point visits are contained within each well folder and all of the image files are listed within that well (not in separate subfolders per point visit). Image files have been automatically named according to CV1000 software image naming conventions, as follows:

Z:\CV1000\Experiment Folder Name\Well designation\W##F##T###Z##C#.tif

i.e. Z:\CV1000\20180809T122430\Well001\W1F001T0001Z01C1.tif.

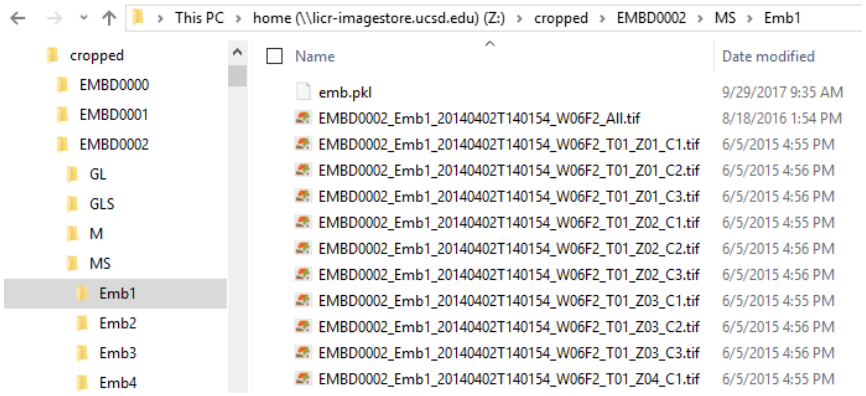


Output path:

The output path specified in **screenCrop.py** is also generated based on information in the CSV file (highlighted in red below). Our saving regime uses our naming scheme (Emb#) for outer folders representing data for each embryo and this contains individual tifs that are named as follows:

Z:\cropped\Target\Strain\Emb#\Target_Emb#_Experiment Folder Name_W##F#_T##_Z##_C#.tif

i.e. Z:\cropped\EMBD0002\MS\Emb1\EMBD0002_Emb1_20140402T140154_W06F2_T01_Z01_C1



Cropping Your Raw Images

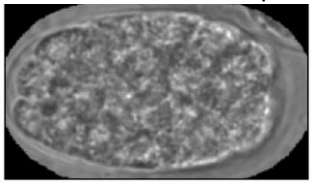
Using **ScreenCrop.py**, you will be able to crop all your images from a folder. The program crops each image by fitting an ellipse to each embryo at the fourth time point.

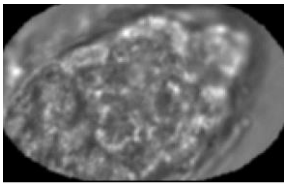
1. Open PyCharm, or other IDE, load the embryo_crop repository and locate the program screenCrop.py. It is important to know the following Information and fill it in at the specified line:
    a. *loadFolder (line 9):* The drive on which the files are stored (e.g. Z:/ , D:// etc.)
    b. *date (line 7):* this is the file referred to as Experiment Folder Name in the CSV
    c. *trackingFile (line 11)*: the path to the CSV file in which experiment information is stored
    d. *z (line 13)*: The number of z planes
    e. *nT (line14)*: Number of timepoints
    f. *nWells (line 19):* the number of wells used
    g. *pointVisits (line 20)*: the maximum number of point visits (per well)

```
4  Loads embryo images generated by CV1000 and crops/orients and saves separate embryos based on DIC (C3) central image
5  '''
6
7  date = '20170118T125754'
8
9  loadFolder = 'Z:/'
10 folderIn = loadFolder + 'CV1000/' + date #Input folder
11 trackingFile = 'Z:/Experiment_tracking_sheets/EMBD_fileNames_Tracking_Sheet.csv'
12 aspectRatioFile = 'Z:/cropped/aspects.csv'
13 z = 18 #number of z planes
14 nT = 31 #number of time points
15 corrDrift = True
16 removeBG = True
17 attCorrect = True
18 apRotate = True
19 nWells = 14#number of wells (14)
20 pointVisits = 4# number of point visits (4)
21
22 import glob, csv, cv2, os, shutil
23 import numpy as np
24 from findEmbryo import showIm
25 from myFunc import clearFolder
26 from cropAPI import cropEmbs
27 import tkMessageBox
28
29 debug = False # use to debug the program
30
31 def getConditions(date, fileName):
32     ''' loads RNAi strains for a specified date from a csv file '''
33     global RNAi, strains
34 #     csvFile = csv.reader(open(fileName, 'rb'), delimiter=',')
35     csvFile = csv.reader(open(fileName, 'rU'), delimiter=',') #universal
36     fileData=[]
37     for row in csvFile:
38         fileData.append(row[1:-1])
39     myDate = [s for s in fileData if s[0]==date]
40     myDate = sorted(myDate, key=lambda well: well[2])
41     RNAi = [s[3] for s in myDate]
42     strains =  [s[4] for s in myDate]
43     return
```

h. In line 10 find the location currently occupied by 'CV1000/' and input the outer folder used in your file path. To avoid issues, use the following convention: 'XXXXXXX/'.
i. In Line 12, input a valid file path for storing aspect ratio data for the cropped.
j. In Lines 15, 16, 17, and 18 input True/False for whether you would like your images to go through the following processing:
    i. Drift Correction (Line 15)
    ii. Background Subtract (Line 16), feature size should be defined as 41 for GLS strain and 201 for MS strain. Background Subtract must be done in conjunction with Attenuation Correction.
    iii. Attenuation Correction (Line 17)
    iv. AP Rotate (Line 18)

2. Once all the changes have been made to tailor the program for your data, you may begin cropping. This is done by selecting the green play icon in the toolbar, this will have a drop down menu where you select "Run As" and then "Python Run". Alternatively, activate the environment and run from PyCharm terminal (described above at the end of the PyCharm section).
3. The Program will then begin cropping your images, *this may take a few hours depending on the number of images that need to be processed*. Once completed, a series of small windows containing embryo images will open; this will allow you to curate the cropped data before saving (i.e. delete embryos that are cut off, out of focus, or poorly cropped can be deleted).

Good! 

Bad!! 

4. For each image you have three options:
    a. **Save:** If the image appears to be cropped properly with no areas of interest being cut off, press the space bar to save the image.
    b. **X:** If the image appears to have areas of interest cut off and you still wish to save the image, press X and the image will be saved with an X in front of the name to separate it from the others.
    c. **Delete:** If the image is not cropped properly or the embryo is not to your liking, press D to delete the cropped image.
5. Once you have gone through all your images and determined whether you wish to **save, x, or delete** them, the program will then begin to save your images. ***The images will be saved to a subfolder named "Cropped" in the Load Folder that was defined in Line 9 of the program.***

SUPPLEMENTAL FIGURES

Wang, Ochoa, Khaliullin et al., Figure S1

	Promoter	Length	Reporter(s)	Expressing Tissue(s)	3' UTR	Allele(s)
Transgenes used in Germ Layer & Morphogenesis strains	*Pcnd-1*[1]	3230 bp	mCh::Plasma Membrane	Strong, specific expression in ~1/3 of neurons from embryonic stage	*unc-54*	*ltSi511*
	Pdlg-1Δ7[2]	3940 bp	DLG-1::GFP	Embryonic epidermis	*unc-54*	*ltSi249*
	Pcnd-1	3230 bp	mCh::histone (tandem with *Pdlg-1Δ7* mCh::histone)	Strong, specific expression in ~1/3 of neurons from embryonic stage	*unc-54*	*ltSi539*
	Pdlg-1Δ7	3940 bp	mCh::histone (tandem with *Pcnd-1* mCh::histone)	Embryonic epidermis	*unc-54*	*ltSi539*
	Phlh-1[3]	3345 bp	mCh::histone & GFP::histone (in tandem)	Body wall muscle	*tbb-2*	*ltSi507*
	pha-4 fosmid[4]	33505 bp	PHA-4::GFP	Intestine and pharynx	*pha-4*	*stIs10389*
Other transgenes generated for the project	*Pcnd-1*	3230 bp	GFP::histone (tandem with *Pdlg-1Δ7* GFP::histone)	Strong, specific expression in ~1/3 of neurons from embryonic stage	*unc-54*	*ltSi509*
	Pdlg-1Δ7	3940 bp	GFP::histone (tandem with *Pcnd-1* GFP::histone)	Embryonic epidermis	*unc-54*	*ltSi509*
	Pelt-2	2994 bp	mCh::histone & GFP::histone (in tandem)	Intestine	*tbb-2*	*ltSi506*
	Phlh-1	3345 bp	mCh::histone, GFP::histone (separate)	Body wall muscle	*unc-54*	*ltSi456, ltSi457*
	Ppha-4+intron1	6452 bp	mCh::histone (tandem with *Phlh-1* mCh::histone)	Weak pharynx, strong intestine	*tbb-2*	*ltSi504*
	Ppha-4	4157 bp	mCh::histone (tandem with *Phlh-1* mCh::histone)	Weak pharynx, variable intestine	*tbb-2*	*ltSi273*
	Prgef-1	4040 bp	mCh::Plasma Membrane	No embryonic expression Post-embryonic pan-neuronal	*unc-54*	*ltSi318*
	Punc-33	2003 bp	mCh::Plasma Membrane	All embryonic cells	*unc-54*	*ltSi316*
	Punc-119+intron1	4749 bp	mCh::Plasma Membrane	All embryonic cells	*unc-54*	*ltSi321*

Footnotes:

1. A HIS-24::mCherry reporter utilizing P*cnd-1* (-3266 to -10 bp) is expressed in a subset of AB progeny cells starting at about the 100-cell stage. These cells include 97 neurons, 17 glia cells and 13 non-nervous system cells (for a map of P*cnd-1* expression superimposed with the embryonic lineage see Murray and Bao et al., 2008). Note that the *C. elegans* embryo has 248 total neurons, so the P*cnd-1* promoter directs expression in about 40% of neurons. These neurons include ventral cord motor neurons and some neurons in the nerve ring ganglion, and do not overlap with UNC-86-expressing neurons (egg-laying neurons, mechanosensory neurons, and chemosensory interneurons; Hallam and Singer et al., 2000).

2. P*dlg-1Δ7* drives expression in all epidermal cells (Sheffield et al., 2007)

3. A lacZ reporter utilizing Phlh-1 (-3053 to + 70 bp) showed expression in all body wall muscle precursor cells with no ectopic expression (Krause et al., 1994). A HIS-24::mCherry reporter utilizing P*hlh-1* (-3053 to -4 bp) showed expression in all body wall muscle cells as well as some pharyngeal cells of the MS lineage (Murray and Bao et al., 2008; includes a map of expression superimposed with the embryonic lineage). In our case, P*hlh-1* (-3333 to +12 bp) reporter expression appears to be expressed in all body wall muscle precursors (Fig. S2B, bottom panels).

4. The fosmid recombineering transgene we used for PHA-4 is allele stIs10389 (Fakhouri et al.2010, Zhong, Niu et al. 2010). It was made by tagging PHA-4 at the C-terminus with GFP, a Ty1 peptide and 3xFLAG in the fosmid WRM0617dE06. This fosmid is 33505 bp in length and contains the entire *pha-4* genomic cassette (Fakhouri et al. 2010; Zhong et al., 2010). Consistent with our own observations, this transgene likely recapitulates the endogenous *pha-4* expression pattern, which includes all pharyngeal, midgut and rectal precursors (Horner et al., 1998).

Figure S1. List of transgenes constructed for this study. Table listing the promoter/reporter combinations used in the transgenes constructed for this study, along with notes on the tissue expression of each marker. The combinations in the top half of the table (***green background***) were used in the transgenes in the Germ Layer and Morphogenesis strains. The combinations in the bottom half of the table (***gray background***) were tried and are available but were not used in our final strains.

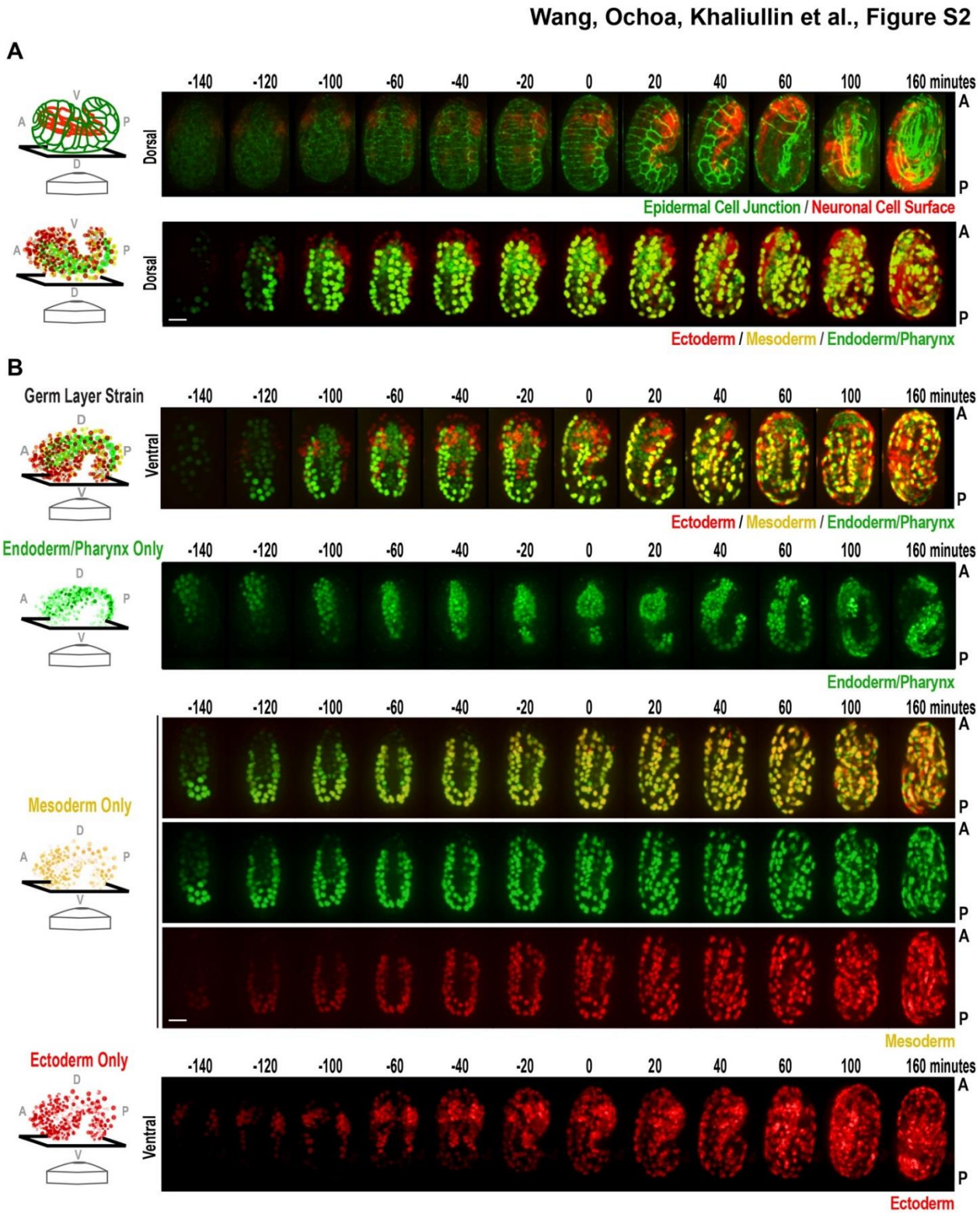


Figure S2. Images showing development from a dorsal view in the Germ Layer and Morphogenesis strains and in embryos individually expressing the three transgenes that make up the Germ Layer strain. (**A**) Maximum intensity projections showing a dorsal view (see schematics on left) of embryonic development in the Morphogenesis (***top***) and Germ Layer (***bottom***) strains acquired at the indicated timepoints relative to the comma stage (t=0 minutes). (**B**) Maximum intensity projections showing a ventral view (see schematics on left) of embryos from the Germ Layer strain (top panels; reproduced from Figure 2A for comparison) and strains individually expressing the three component transgenes that make up the Germ Layer strain (Ectoderm Only, strain OD1599; Endoderm/Pharynx Only, strain OD1598; and Mesoderm Only, strain OD1543). Scale bars are 10 µm.

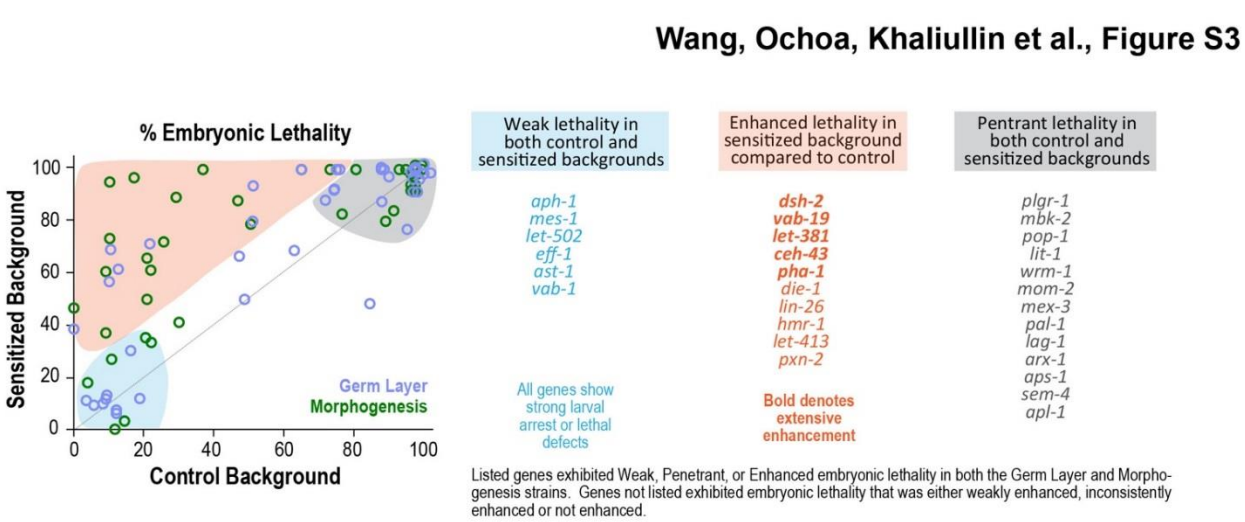


Figure S3. Comparison of embryonic lethality in the Germ Layer and Morphogenesis strains for Control and Sensitized backgrounds. (**A**) Graph (***left***) plots percent embryonic lethality in the control background (x-axis) versus percent embryonic lethality in the RNAi-sensitized background (y-axis) for each RNAi condition in the Germ Layer (***blue data points***) and Morphogenesis (***green data points***) strains. Shaded colored regions highlight the gene targets that exhibited weak embryonic lethality (<30%) in both backgrounds (***light blue***), penetrant embryonic lethality (>70%) in both backgrounds (***gray***), or showed enhanced embryonic lethality (20% or greater increase) in the sensitized background compared to the control background (***orange***). Genes in each of these color-coded cohorts are listed (***right***).

Wang, Ochoa, Khaliullin et al., Figure S4

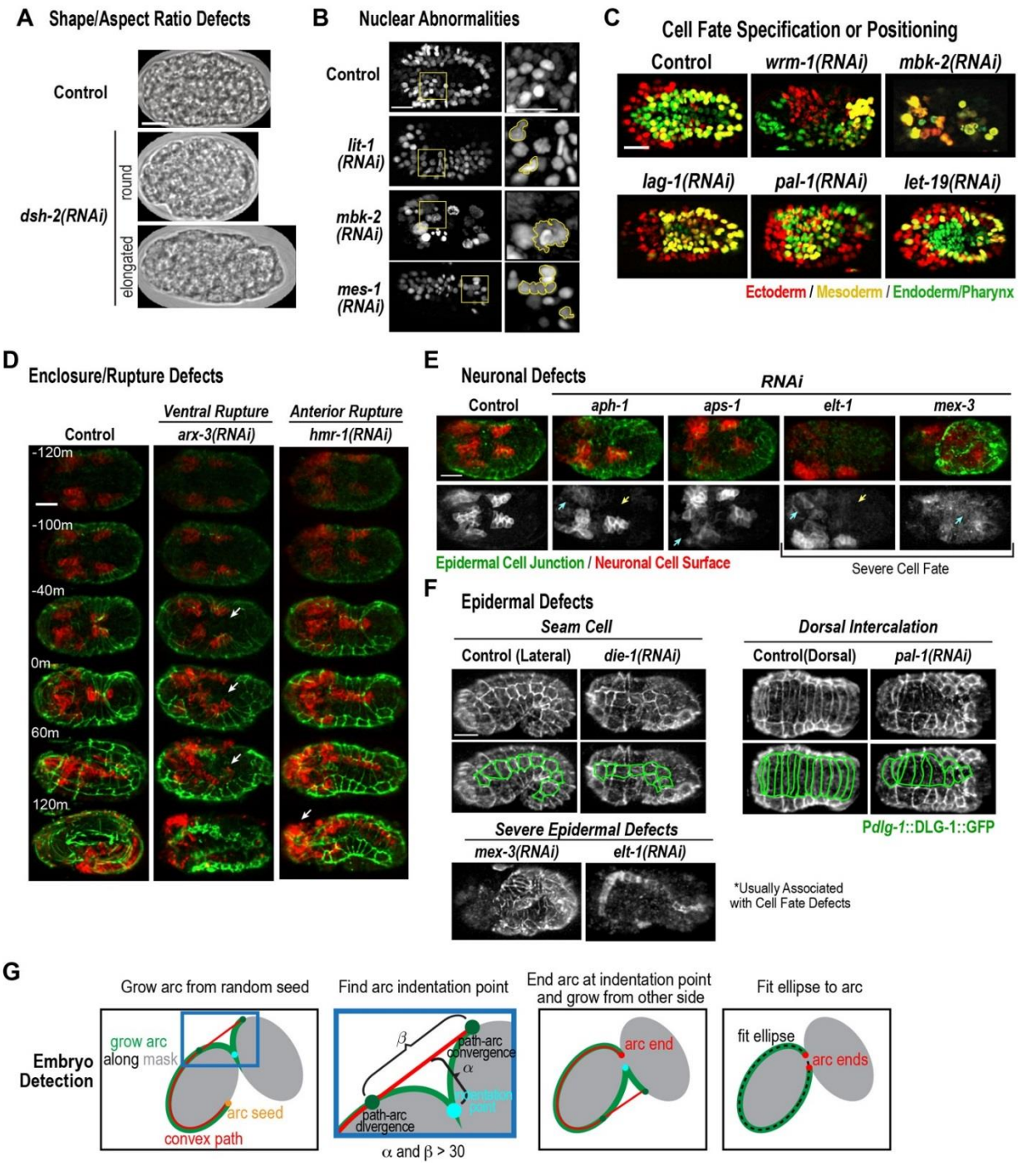


Figure S4. Scoring of phenotypic features in the Germ Layer and Morphogenesis strains and automated cropping. (**A-F**) Maximum intensity projections of fluorescence confocal images (**B-F**), or single plane brightfield images (**A**) of embryos from specific RNAi conditions scored with the indicated descriptors are shown to illustrate how each descriptor was scored. (**A**) Embryos were scored with a "shape/aspect ratio" defect if embryo shape deviated significantly different from controls. (**B**) Embryos (Germ Layer only) were scored with "nuclear abnormalities" if any nuclei were abnormal in shape, if micronuclei or lagging chromosomes were observed, or if nuclear marker intensity was uneven. (**C**) Germ Layer embryos were scored with a "cell fate specification or positioning" defect if the number or position of nuclei in the germ layers appeared to deviate from that in controls (based on visual assessment) prior to the onset of morphogenesis (comma stage). Control, *lag-1(RNAi), pal-1(RNAi)* and *mbk-2(RNAi)* images are reproduced for comparison from Figure 5D-E. (**D**) Embryos were scored for a "ventral enclosure defect/ventral rupture" if the ventral epidermal/ectodermal cells moved towards the midline but failed to completely close the ventral opening, or an "anterior closure defect/anterior rupture" if the epidermal/ectoderm cells failed to move anteriorly to completely cover the head. (**E**) Embryos (Morphogenesis only) were scored with "neuronal defect" if neuronal organization was altered prior to elongation. (**F**) Embryos (Morphogenesis only) were scored with a "seam cell defect" if the position, structure of number of lateral seam cells was abnormal between the comma and 1.5-fold stages, with "dorsal intercalation defect" if the dorsal hypodermal cells (either one or two to all) failed to properly intercalate or to fuse after intercalation, and with "severe epidermal defect" if the pattern of cells that expressed the markers was severely disorganized. (**G**) In our automated cropping algorithm, a binary mask was generated from 8-bit brightfield images and individual embryos were detected and cropped out. Schematics detail the procedure used to iteratively detect embryos in the binary mask. Scale bar is 10 µm.

Wang, Ochoa, Khaliullin et al., Figure S5

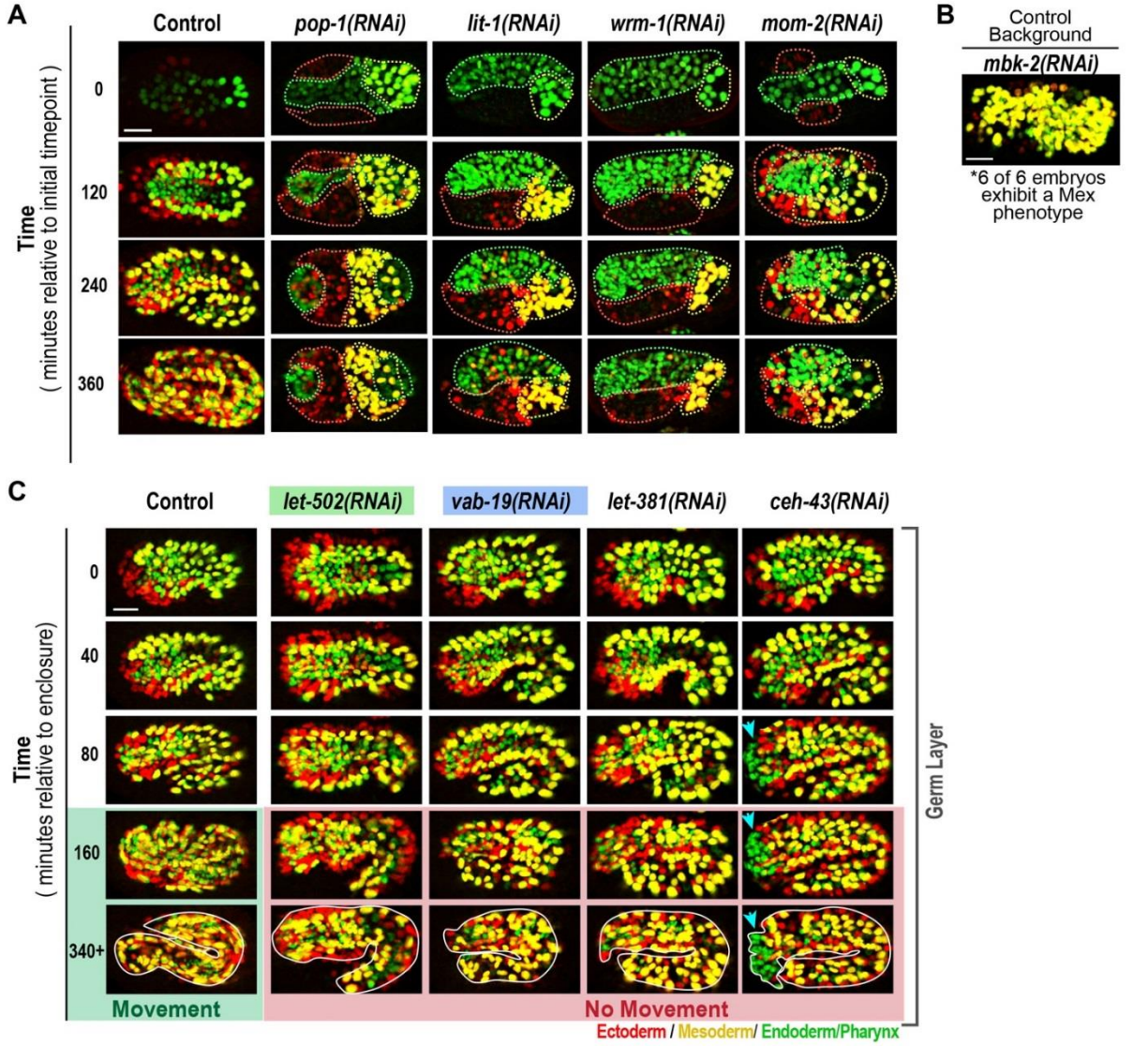


Figure S5. Germ Layer strain phenotypes for WNT pathway components and proteins required for embryo elongation. (**A**) Maximum intensity projections of confocal images of embryos from the Germ Layer strain with RNAi-sensitizing mutations after knockdown of genes encoding four components of the WNT signaling pathway. Dashed green, red and yellow outlines mark the locations where nuclei of the corresponding colors are observed. See text for phenotype descriptions. Final *wrm-1(RNAi)* and *lit-1(RNAi)* images are reproduced from Figure 5D. For the full sequences see movies available at Dryad repository: (https://datadryad.org/review?doi=doi:10.5061/dryad.kb79121). (**B**) Image shows the Germ Layer *mbk-2(RNAi)* phenotype in control background lacking the RNAi-sensitizing mutations. (**C**) Maximum intensity projections of confocal images of embryos from the Germ Layer strain after knockdown of four genes required for elongation past the 2-fold stage. Arrowheads in *ceh-43(RNAi)* point to the position where pharyngeal nuclei are leaking out of the head of the embryo. Elongating embryos were outlined in the final frame to make embryo shape at arrest more visible. In the *ceh-43(RNAi)* embryo, green pharyngeal nuclei are observed outside of the embryo. Green shading indicates successful elongation and movement of the embryo within the eggshell, whereas, red shading indicates arrest and failure to move. Scale bars are 10 µm.

Wang, Ochoa, Khaliullin et al., Figure S6

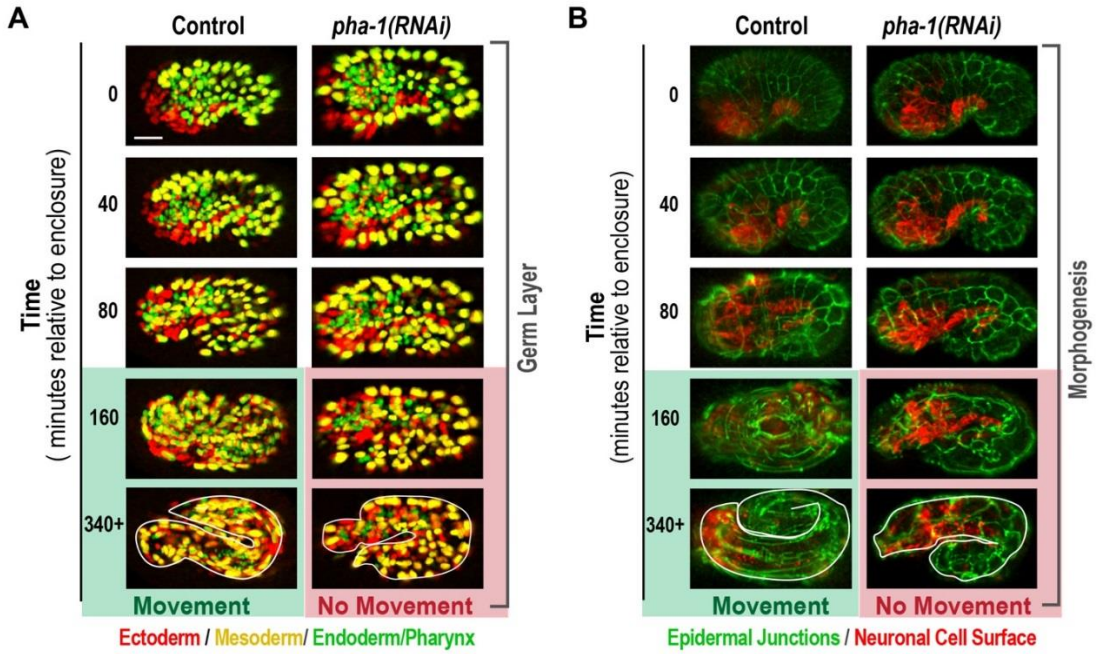
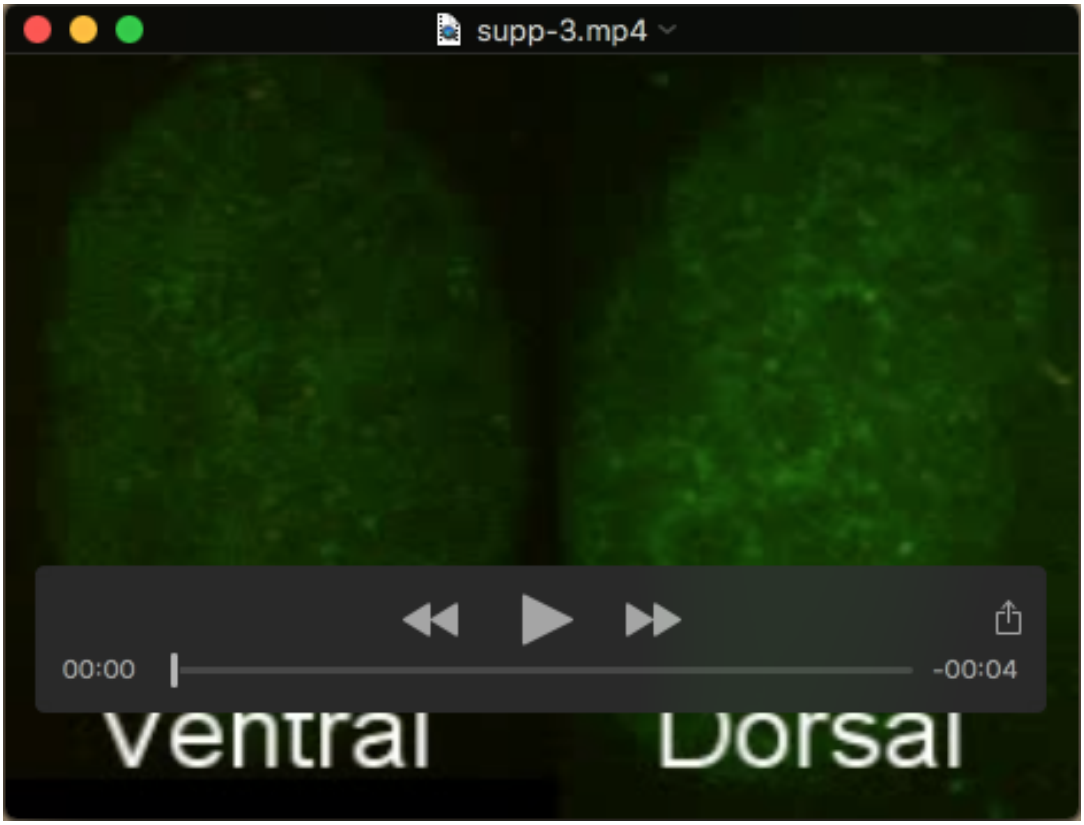


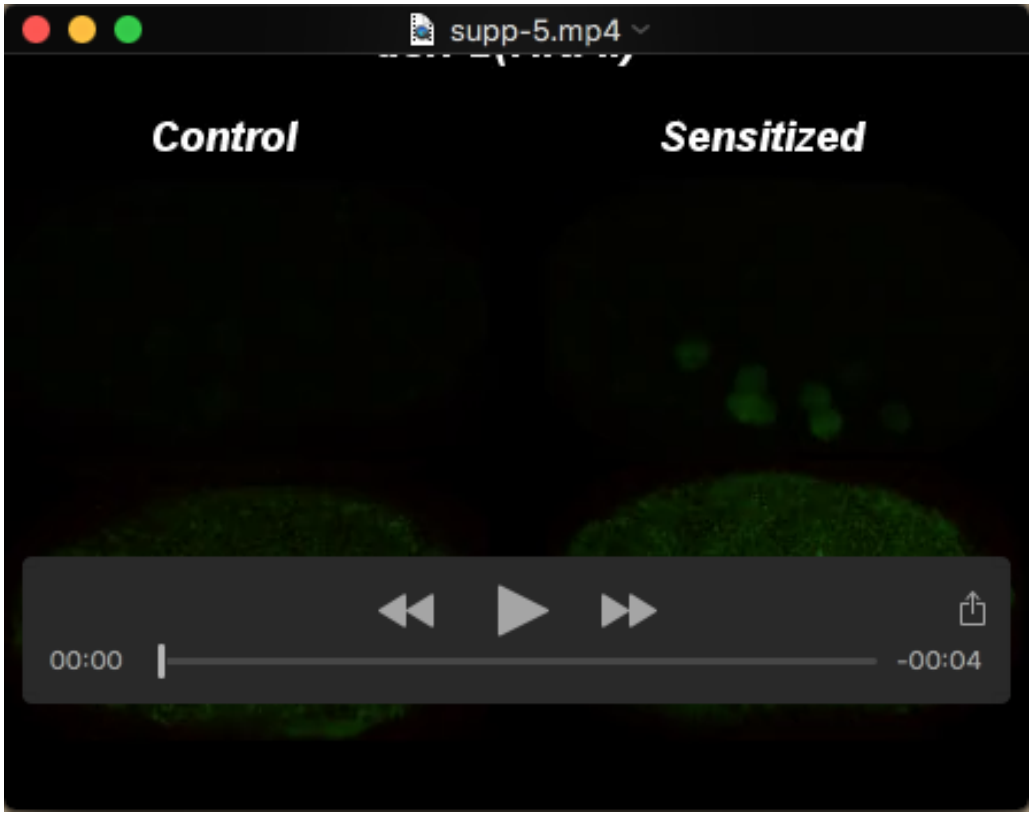
Figure S6. **Knockdown of *pha-1* leads to an elongation defect and a "pinched red head" phenotype.** (**A, B**) Maximum intensity projections of confocal images of embryos from the Germ Layer (A) and Morphogenesis (B) strains with RNAi-sensitizing mutations. Control embryo sequences are reproduced from Figure 6 and Figure S5 for comparison. PHA-1 is required for pharyngeal cells to attach to neighboring arcade cells to connect the pharynx to the buccal cavity, and *pha-1* mutants exhibit a penetrant Pun phenotype (Schnabel and Schnabel 1990, Fay 2004, Kuzmanov et al., 2014). Mosaic analysis suggested that PHA-1 may not be required in the pharynx but instead might promote pharyngeal development via a role in other cell types such as the epidermis (Kuzmanov et al., 2014). Interestingly, recent work has shown that, rather than being inherently required for development, PHA-1 is an antidote that counteracts the toxicity of a second protein called SUP-35 that is encoded with it on a selfish genetic element (Ben-David et al., 2017). Thus, *pha-1* knockdown phenotypes inform on the mechanism of action of the SUP-35 toxin. (**A**) In the Germ Layer strain, *pha-1* knockdown led to a "pinched redhead" phenotype, possibly due to the defect in pharyngeal morphogenesis/attachment (**also see Movie S7**). (**B**) In the morphogenesis strain, PHA-1 inhibition led to a crumpled epidermis elongation defect, which our analysis suggests can arise as a consequence of a defect in mouth-pharynx attachment (*ceh-43*) or the failure to form transepithelial muscle attachments (*vab-19*). Green shading indicates successful elongation and movement of the embryo within the eggshell, whereas, red shading indicates arrest and failure to move. Scale bars are 10 µm.

Movies

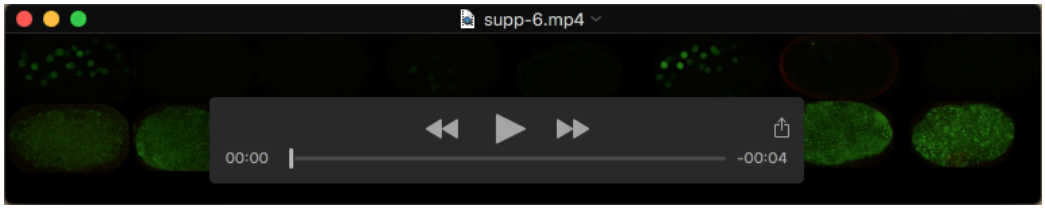


Movie 1. **Embryonic development imaged in the Morphogenesis strain.** Side-by-side ventral (***left***) and dorsal (***right***) views of development in control embryos from the Morphogenesis strain which has markers that localize to apical cell junctions in the epidermis (***green***) and the cell surface in about 1/3 of neurons (***red***). Images were acquired every 20 minutes. Playback is 7200X real time.

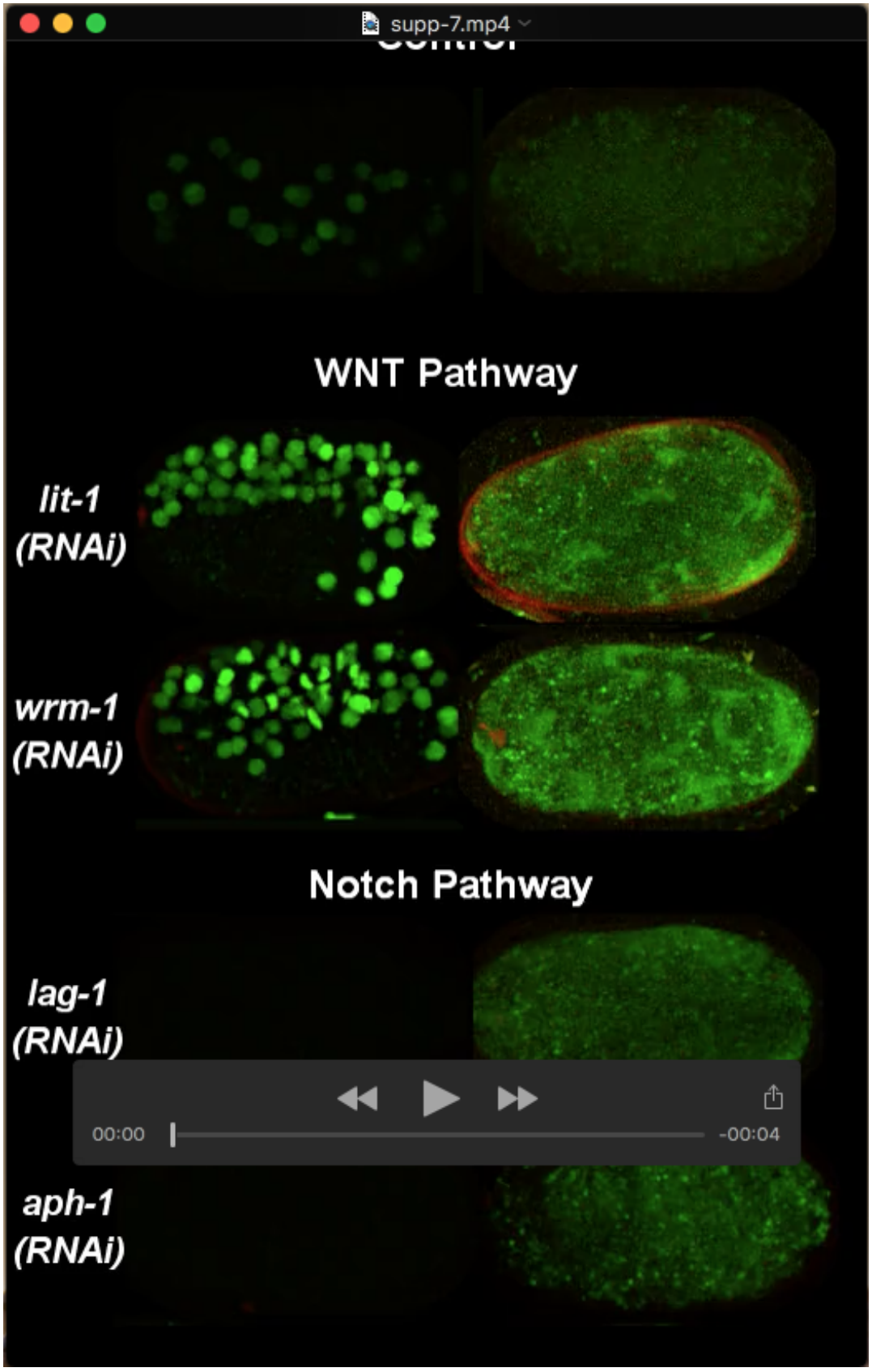
Movie 2. Embryonic development imaged in the Germ Layer strain. Side-by-side ventral (***left***) and dorsal (***right***) views of development in control embryos from the Germ Layer strain, which marks nuclei in the ectoderm (epidermis and ~1/3 of neurons; ***red***), mesoderm (***yellow***) and endoderm/pharynx (***green***). Images were acquired every 20 minutes. Playback is 7200X real time.



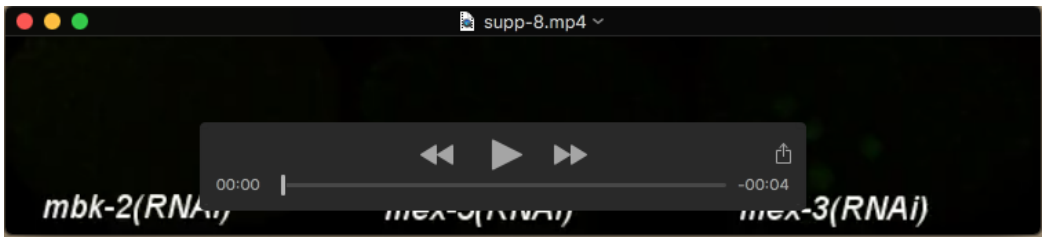
Movie 3. Example of phenotypic enhancement in the RNAi-sensitized versions of the Germ Layer and Morphogenesis strains. Side-by-side views showing the consequences of *dsh-2(RNAi)* in control (***left***) and RNAi-sensitized (***right***) versions of the Germ Layer (***top***) and Morphogenesis (***bottom***) strains. Images were acquired every 20 minutes. Playback is 7200X real time.



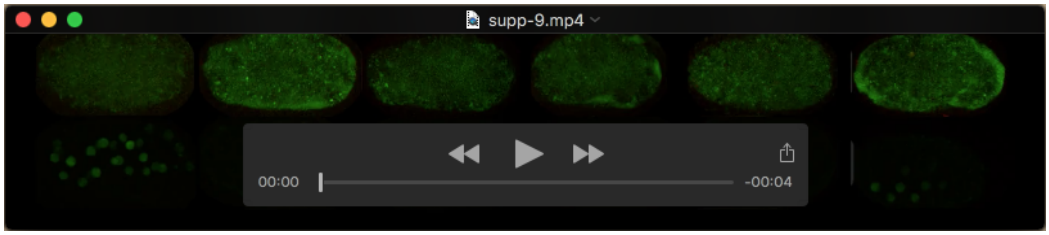
Movie 4. Knockdown of genes required for cell fate specification and/or positioning leads to diverse, distinct phenotypes in the Germ Layer and Morphogenesis strains. Genes scored as having a defect in cell fate and/or positioning were partitioned into three classes based on analysis of nuclear counts: (1) genes whose inhibition leads to incomplete cell fate specification due to arrest prior to the completion of cell division, (2) genes whose inhibition leads to an abnormal nuclear pattern despite normal numbers of each type of nuclei and (3) bona fide genes cell fate specification genes. This movie shows side-by-side views of the phenotypes in the first two classes in the Germ Layer (***top***) and Morphogenesis (***bottom***) strains. See text for description of the *plrg-1*, *arx-1*, *arx-3*, *gex-2* and *die-1* knockdown phenotypes. APS-1, is an adaptin complex subunit involved in the formation of intracellular transport vesicles (Boehm and Bonifacino, 2001); *aps-1(RNAi)* embryos typically arrested without rupture, but with severe defects in epidermal and nervous system morphology. LET-19 is a component of the mediator complex previously shown to modulate the transcription of several genes involved in development (Wang et al., 2004; Yoda et al., 2005); *let-19* knockdown led to defects in epidermal morphology that were frequently accompanied by rupture of the epidermis at the embryo anterior. Playback is 7200X real time.



Movie 5. Knocking down genes encoding WNT and NOTCH pathway components leads to distinct signature phenotypes in the Germ Layer and Morphogenesis strains. Movie presents side-by-side views of development in the Germ Layer (***left***) and Morphogenesis (***right***) strains for a control embryo (***top***) along with embryos in which genes encoding components of the WNT (*lit-1, wrm-1*) or NOTCH (*lag-1, aph-1*) signaling pathways have been knocked down. Images were acquired every 20 minutes. Playback is 7200X real time.



Movie 6. A Mex phenotype, similar to that in *mex-5/6* and *mex-3(RNAi)* embryos can be observed in *mbk-2* knockdown embryos. Movie presents side-by-side views of embryonic development in the Germ Layer strain after RNAi targeting *mbk-2* (***left***), *mex-5/mex-6* (***middle***) and mex-3 (***right***). Images were acquired every 20 minutes. Playback is 7200X real time.



Movie 7. Filming in the Germ Layer and Morphogenesis strains enables functional classification of genes required for elongation. Movie presents side-by-side views of development in the Morphogenesis (***top***) and Germ Layer (***bottom***) strains for a control embryo (***left***) and embryos in which five genes required for elongation have been knocked down. Images were acquired every 20 minutes. Playback is 7200X real time.

SUPPLEMENTAL TABLES

Table S1: Strains generated for this study

Table S1a. Available transgenic strains used in this study

Strain Name	Transgene(s)	Genotype
Composite Strains		
OD1689 (Morphogenesis Reporter Strain, control background)	GFP-tagged cell junctions (epidermis); mCherry-labeled cell surface (1/3 of neurons)	*ltSi249[pOD1274/pSW098; Pdlg-1delta7::dlg-1-GFP::unc-54-3'UTR; cb-unc-119(+)]I; ltSi511[pOD2983/pSW207; Pcnd-1::mCherry-PH::unc-54_3'UTR; cb-unc-119(+)]II*
OD2416 (Morphogenesis Reporter Strain, RNAi-sensitized background)	GFP-tagged cell junctions (epidermis); mCherry-labeled cell surface (1/3 of neurons); with RNAi-sensitizing mutations *nre-1(hd20)* & *lin-15b(hd126)*	*ltSi249[pOD1274/pSW098; Pdlg-1delta7::dlg-1-GFP::unc-54-3'UTR; cb-unc-119(+)]I; ltSi511[pOD2983/pSW207; Pcnd-1::mCherry-PH::unc-54_3'UTR; cb-unc-119(+)]II; nre-1(hd20)X; lin-15b(hd126)X*
OD1719 (Germ Layer Reporter Strain, control background)	mCherry-tagged histone (epidermis and 1/3 of neurons); yellow (GFP & mCherry) histone (muscle); GFP::PHA-4 (pharynx and intestine)	*stIs10389 [pha-4::TGF(3E3)::GFP::TY1::3xFLAG inserted into fosmid WRM0617dE06 as C-terminal protein fusion]; ltSi539[pOD1519/pSW224; Pdlg-1Δ7::mCherry::his-72::unc-54_3'UTR; Pcnd-1::mCherry::his-72::unc-54_3'UTR; cb-unc-119(+)]II; ltSi507[pOD1492/pSW201; Phlh-1::GFP::his-72::tbb-2_3'UTR, Phlh-1::mCherry::his-72::tbb-2_3'UTR; cb-unc-119(+)]IV*
OD1854 (Germ Layer Reporter Strain, RNAi-sensitized background)	mCherry-tagged histone (epidermis and 1/3 of neurons); yellow (GFP & mCherry) histone (muscle); GFP::PHA-4 (pharynx and intestine); with RNAi-sensitizing mutations *nre-*	*stIs10389 [pha-4::TGF(3E3)::GFP::TY1::3xFLAG inserted into fosmid WRM0617dE06 as C-terminal protein fusion]; ltSi539[pOD1519/pSW224; Pdlg-1Δ7::mCherry::his-72::unc-54_3'UTR; Pcnd-1::mCherry::his-72::unc-54_3'UTR; cb-unc-119(+)]II; ltSi507[pOD1492/pSW201; Phlh-1::GFP::his-72::tbb-2_3'UTR, Phlh-1::mCherry::his-72::tbb-2_3'UTR; cb-unc-119(+)]IV; nre-1(hd20)X; lin-15b(hd126)X*

	1(hd20) & lin-15b(hd126)		
Intermediate Strains			
OD1593	mCherry-labeled cell surface (1/3 of neurons)	*ltSi511[pOD2983/pSW207; Pcnd-1::mCherry-PH::unc-54_3'UTR; cb-unc-119(+)]II*	6x
OD1591	GFP-tagged cell junctions (epidermis)-also in pharynx and intestine at post-embryonic stage.	*ltSi249[pOD1274/pSW098; Pdlg-1delta7::dlg-1-GFP::unc-54-3'UTR; cb-unc-119(+)]I*	8x
OD1599	mCherry-tagged histone (epidermis and 1/3 of neurons)	*ltSi539[pOD1519/pSW224; Pdlg-1Δ7::mCherry::his-72::unc-54_3'UTR; Pcnd-1::mCherry::his-72::unc-54_3'UTR; cb-unc-119(+)]II*	6x
OD1592	yellow (GFP & mCherry) histone (muscle)	*unc-119(ed3)III; ltSi507[pOD1492/pSW201; Phlh-1::GFP::his-72::tbb-2_3'UTR, Phlh-1::mCherry::his-72::tbb-2_3'UTR; cb-unc-119(+)]IV*	6x
OD1598	GFP::PHA-4 (intestine & pharynx); previously generated insertion (Fakhouri et al., 2010 (PMID: 20714352)) that we backcrossed 6X	*stIs10389 [pha-4::TGF(3E3)::GFP::TY1::3xFLAG inserted into fosmid WRM0617dE06 as C-terminal protein fusion]*	6x

Table S1b. Available transgenic strains made for this study, but not used

Strain Name	Expressing Transgene(s)	Genotype	Out-crossed
Nuclear reporters			

OD1557	mCherry-tagged histone (post-embryonic pan-neuronal)	*ltSi517[pOD1489/pSW198; Prgef-1::mCherry::his-72::unc-54_3'UTR; cb-unc-119(+)]I; unc-119(ed3)III*	0x
OD1433	mCherry-tagged histone (body wall muscle)	*ltSi456[pOD1486/pSW195; Phlh-1::mCherry::his-72::unc-54_3'UTR; cb-unc-119(+)]I; unc-119(ed3)III*	0x
OD1434	GFP-tagged histone (body wall muscle)	*ltSi457[pOD1487/pSW196; Phlh-1::GFP::his-72::unc-54_3'UTR; cb-unc-119(+)]I; unc-119(ed3)III*	0x
OD1596	yellow (mCherry and GFP) histone (intestine)	*ltSi506[pOD1491/pSW200; Pelt-2::GFP::his-72::tbb-2_3'UTR, Pelt-2::mCherry::his-72::tbb-2_3'UTR; cb-unc-119(+)]IV*	6x
OD1595	GFP-tagged histone (epidermis and 1/3 of neurons)	*ltSi509[pOD2982/pSW203; Pdlg-1Δ7::GFP::his-72::unc-54_3'UTR; Pcnd-1::GFP::his-72::unc-54_3'UTR; cb-unc-119(+)]II*	6x
OD1007	mCherry-tagged histone (body wall muscle, pharynx, and intestine -- note pharynx expression is weak, and intestine is variable among lines; this line has strong intestine expression)	*ltSi273[pOD1272/pSW094; Phlh-1::mCherry::his-72::unc-54_3'UTR; Ppha-4::mCherry::his-72::tbb-2_3'UTR; cb-unc-119(+)]I; unc-119(ed3)III*	0x
OD1540	mCherry-tagged histone (body wall muscle, pharynx, and intestine -- note pharynx expression is weak)	*ltSi504[pOD1294/pSW108; Phlh-1::mCherry::his-72::unc-54_3'UTR; Ppha-4intron1::mCherry::his-72::tbb-2_3'UTR; cb-unc-119(+)]I; unc-119(ed3)III*	0x
Cell morphology reporters			

OD1085	mCherry-labeled cell surface (embryonic, all cells; post-embryonic, appears to be pan-neuronal)	*ltSi316[pOD1293/pSW101s; Punc-33::mCherry-PH(PLCdelta1)::unc-54_3'UTR; cb-unc-119(+)]II; unc-119(ed3)III*	0x
OD1087	mCherry-labeled cell surface (embryonic, no expression; post-embryonic, pan-neuronal)	*ltSi318[pOD1296/pSW126; Prgef-1::mCherry-PH(PLCdelta1)::unc-54_3'UTR; cb-unc-119(+)]II; unc-119(ed3)III*	0x
OD1106	mCherry-labeled cell surface (embryonic, all cells; post-embryonic, appears to be pan-neuronal)	*ltSi321[pOD1295/pSW125; Punc-119::mCherry-PH(PLCdelta1)::unc-54_3'UTR; cb-unc-119(+)]II unc-119(ed3)III*	0x
Composite reporters			
OD1639	GFP-tagged cell junctions (epidermis); mCherry-labeled cell surface (1/3 of neurons); yellow (GFP & mCherry) histone (muscle)	*ltSi249[pOD1274/pSW098; Pdlg-1delta7::dlg-1-GFP::unc-54-3'UTR; cb-unc-119(+)]I; ltSi511[pOD2983/pSW207; Pcnd-1::mCherry-PH::unc-54_3'UTR; cb-unc-119(+)]II; ltSi507[pOD1492/pSW201; Phlh-1::GFP::his-72::tbb-2_3'UTR, Phlh-1::mCherry::his-72::tbb-2_3'UTR; cb-unc-119(+)]IV*	6x

Table S2: Summary of phenotypic data for the 40-gene test set. This document contains an index and 7 additional tabs. **S2.1-Embryonic Lethality**: Summarizes embryonic lethality and larval defects for each RNAi condition in the 4 strain backgrounds. **S2.2-Defects Scored**: provides a description of each phenotypic defect and how it was scored. **S2.3-Phenotype Scoring**: Includes quantification of all manually scored phenotypic characteristics for each gene in the 4 tested strain backgrounds (Germ Layer strain with RNAi-sensitizing mutations, Morphogenesis strain with RNAi-sensitizing mutations, Germ Layer strain control background, and Morphogenesis strain control background). **S2.4-Expected-Observed Summary**: Provides a comparison of the embryonic lethality and phenotypic defects observed in this study with annotated embryonic lethality and observed phenotypes by RNAi or mutant allele previously cataloged on WormBase. **S2.5-Gene Expression-Phenotypic Group:** shows the previously described temporal gene expression profile plots (Levin et al. 2012) for the genes in the broad phenotypic groups defined by the phenotypic scoring in this study. **S2.6-Raw Embryonic Lethality Data:** raw data counts of embryonic lethality, abnormal hatched L1, normal hatched L1 for the four considered strains. **S2.7-Oligo list:** sequences of the forward and reverse oligos used for the production of dsRNAs targeting each of the tested genes.

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PROMOTER SEQUENCES

P*cnd-1* (a subset of neurons, 3230 bp):

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P*hlh-1* (body wall muscle, 3345 bp):

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P*pha-4* (intestine and weak pharynx, 4157 bp):

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P*pha-4* + intron1 (pharynx and intestine, 6452 bp):

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P*rgef-1* (post-embryonic pan-neuronal):

gcggtcaactagtgtatgattcctcgaatgtattgaacttttcttctgtctcgaatttccagtgatataaacacattctcgtgattgttccttt
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agt

P*unc-119* + intron1 (embryonic all cells, 4749 bp)

atggccgactggaatcaatacgtggaattaattgaaacatatcacttcttattttatttatttttcatatgaatatgtgtatttattgatttttttag
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P*unc-33* (embryonic all cells, 2003 bp):

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CROPPING PROGRAM INSTRUCTIONS

We provide two repositories that contain software:

1. **Zenodo repository** (*https://zenodo.org/record/1475442#.W9jvApNKiUl*), which contains:

    - **embryoCropUI_WINDOWS**- *PC compatible executable version of graphical user interface (GUI)* version of our software, which can accommodate *individual image stacks from a range of imaging platforms.* This crops, rotates anterior-posterior, processes for drift correction, background subtraction and attenuation correction for individual image stacks. Cropped images are automatically saved to a "crop" file in the location of the file selected to be cropped.
    - **embryoCropUI_MacOS**- *Mac compatible executable version of graphical user interface (GUI)* version of our software, which can accommodate *individual image stacks from a range of imaging platforms.* This crops, rotates anterior-posterior, processes for drift correction, background subtraction and attenuation correction for individual image stacks. Cropped images are automatically saved to a "crop" file in the location of the file selected to be cropped. (requires MacOS X10.11)
    - **Test_files**- Contains two test files: a multi-tif format file and a folder with a tif image series, which can be used to test the GUI on your system (details for testing can be found in the instructions)
    - **OpenAndCombine_embs.ijm**- FIJI script that enables assembly of maximum intensity projections for multiple embryos within one viewer. Requires **ImageJ**. *Note this works with our file structure and will need to be modified to accommodate your file structure (see instructions).*
    - **Instructions**- Instructions for installation and running GUI (embryoCropUI) and FIJI script (OpenAndCombine.ijm) (this file)

2. **Github repository (*https://github.com/renatkh/embryo_crop.git*), which contains source code for embryoCropUI (embryoCropUI.py) and screenCrop.py:**

    - **embryoCropUI.py:** This is the source code for the user-friendly executable version described above, which crops, rotates anterior-posterior, processes for drift correction, background subtraction and attenuation correction for individual image stacks.
    - **screenCrop.py**: This batch version of the embryoCrop.py program takes in multiwell, multipoint imaging data and crops, rotates anterior-posterior, processes for drift correction, and performs background subtraction and attenuation correction in batch. Requires **Python3, virtual environment, and .csv** file with the file format and condition specifications delineated. Saves cropped files to new "Cropped" folder with file structure as specified in .csv file. *This program is specific to our file structure and acquisition parameters, but can be modified to accommodate similarly structured data if users have Python expertise- see instructions below for key variables*.
    - **README file**- Instructions for cloning repository, setting up virtual environment and running embryoCrop.py., and configuring necessary requirements to run screenCrop.py.

****Instructions for all programs can be found within each repository and are reproduced below for completeness:**----------

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Zenodo repository

(*https://zenodo.org/record/1475442#.W9jvApNKiUl*)

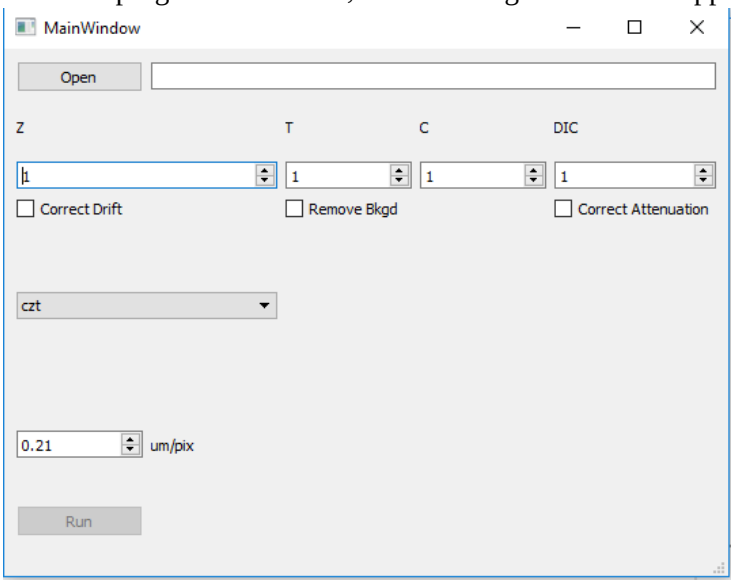
- **embryoCropUI_WINDOWS**
- **embryoCropUI_MacOS**-requires MacOS X10.11
- **Test_files**
- **OpenAndCombine_embs.ijm**
- **Instructions**

DETAILED INSTRUCTIONS

1. Running embryoCropUI.exe (MacOS and Windows)

1. Download Window or MacOS folder, unzip and navigate to find the embryoCropUI executable (…\embryoCropUI _WINDOWS\embryoCropUI\embryoCropUI.exe) or (…\embryoCropUI _MacOS\embryoCropUI\embryoCropUI.exe). Double click to launch (or chose 'open with'→ terminal) and run the embryoCropUI executable.
   Note- MacOS distribution is only compatible with Mac OS X 10.11 and higher.

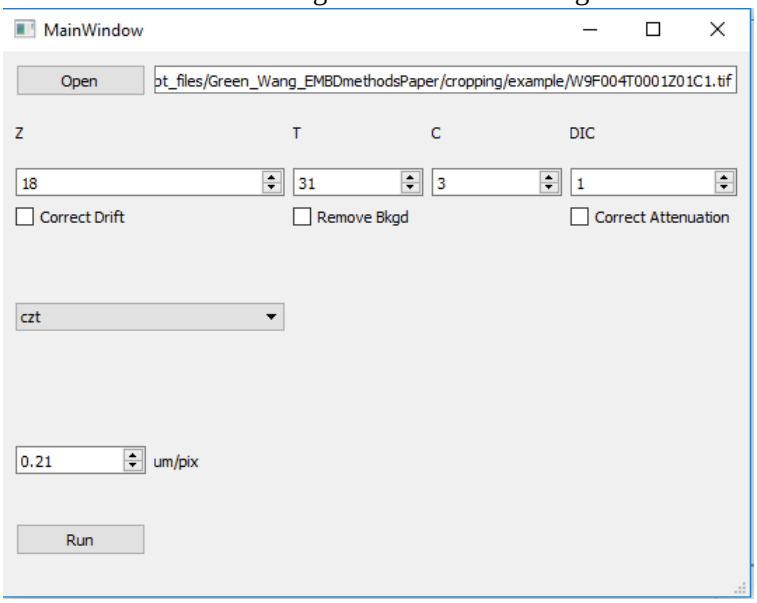
2. Once the program has started, the following window will appear:



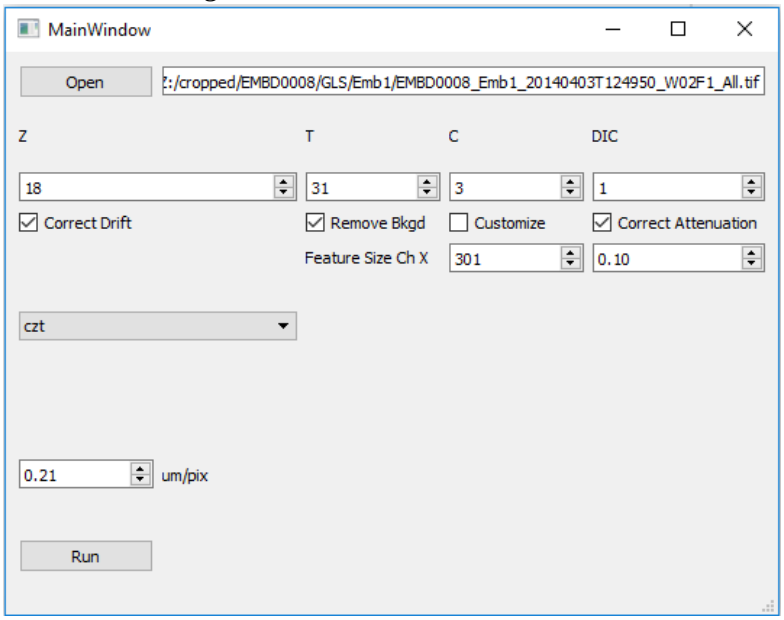
3. Select the **"Open"** button at the top of the window to load the specific image that you wish to crop. Should you be cropping an image series, with multiple dimensions (i.e. z, time, or channel), simply load the first image in the series within the folder. *Please make sure only images from one image series are present in this folder, otherwise the image series' will be loaded in tandem.*

4. Once you have loaded the desired images, you will need to specify the following information:
   a. Number of **Z slices (Z)**
   b. Number of **Time points (T)**
   c. Number of **Channels (C)**
   d. The channel that corresponds to **DIC or brightfield** (first=1, second=2, etc)

For example: our imaging protocol was 18 z-steps, imaged for 31 time-points in 3 channels with the DIC channel being the first channel imaged. The window should look like this:

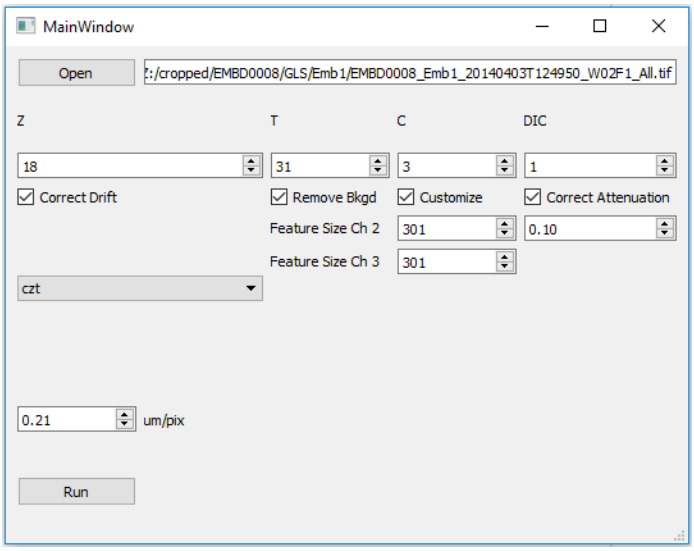


5. Now that you have your images loaded and specified the image parameters, you must choose what processing you would like to do alongside the embryo cropping. The program gives you the option to perform **Drift Correction, Background Subtraction, and Attenuation Correction**. Background Subtraction and Attenuation Correction must be done in conjunction with each other. The below image shows an image that will be going through Drift Correction, Background Subtraction, and Attenuation Correction.



   a. When selecting Background Subtraction and Attenuation Correction, specify parameters for each to guide the processing. For *Background Subtract*, define a feature size (*odd numbered*) that reflects the level of detail you wish to resolve, larger feature size equates to more detail. For *Attenuation Correction*, you need to input a value from 0-1. This value represents the percent of original intensity that remains at the furthest distance through the object being imaged.

b. As Shown below, you have even greater options to customize Background subtraction. By selecting Customize, you will be able to define a feature size for different channels.

c. 

7. Next, specify the **order** in which the images were collected (i.e. **channel-z-time (czt)**, or **z-channel-time (zct)**)
8. Specify the **microns per pixel of the camera being used** for the images.
   a. note that failure to properly define pixel size will result in poor image cropping!!
9. Select **Run** at the bottom left corner and the program
   When the cropping and processing of your images has completed, the cropped versions will be saved in a new subfolder labeled "crop" in the same folder as the uncropped images.

Two formats of **test files are available in the repository- download and unzip. We recommend testing one or both of these to ensure the program is functioning properly on your system:

i. TESTME2_BGLI140_1t_a1.tif- a compiled multi-tif format
   1. Load file in the 'open' field. Set Z=1, T=6, C=3, DIC=1 and use the default settings for all other fields. Click Run. If successful, a message will appear at the bottom of the GUI window that says 'embryos saved' and it will generate a folder in the same location as the test file labeled "crop"; this should contain 4 embryos.

ii. Test_field- a folder containing an image series
   1. Load the first image in the test_field folder into the 'open' window. Set Z=18, T=4, C=3, DIC=3, change the pixel size to 0.26um/pix. Click Run. a message will appear at the bottom of the GUI window that says 'embryos saved' and it will generate a folder in the same location as the test file labeled "crop"; this should contain 2 embryos.

**These files should crop in seconds to minutes, but larger image sequences may take some time. The bottom corner of the GUI window will read-out what the program is doing ("Loading images", "cropping", or "embryos saved"). If an error occurs, the message will appear here.

2. Running OpenandCombine_embs (Fiji processing for viewing):

1. This Fiji Script "OpenandCombine_embs" combines all the images for a specific condition and strain into one easy to view Fiji file. Requires installation of **ImageJ**. Note that this program runs according to our file structure and may need to be modified to work with your

file structure (see our file structure below as a guide). To prepare to run this script, you must know the following information:

a. The location where images where stored following cropping.
b. The desired location for saving the Fiji files after processing.
c. The Target Name for the specific condition you wish to process.
d. The Experiment Folder Name.

*for reference, our file location structure looks like this:

Z:\cropped\Target\Strain\Emb#\Target_Emb#_Experiment Folder Name_W##F#_T##_Z##_C#.tif

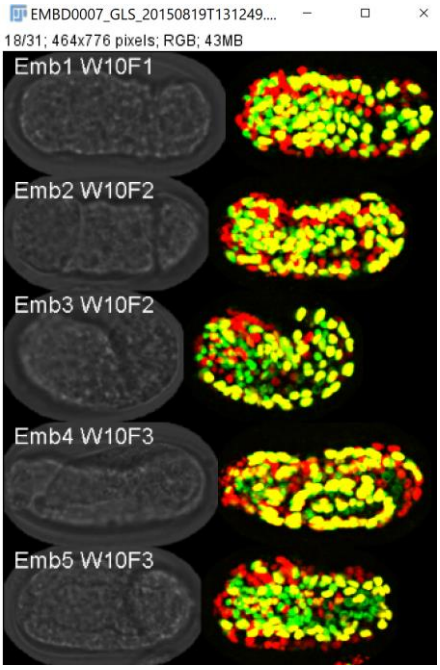
i.e. Z:\cropped\EMBD0002\MS\Emb1\EMBD0002_Emb1_20140402T140154_W06F2_T01_Z01_C1

2. Open ImageJ, drag our .ijm Script file to the ImageJ bar. Once the script is open, locate lines 28-31, they are shown below. Within these lines you will fill in the Information you gathered above.

```
27 }
28 RNAL = newArray("EMBD0000/","EMBD0052/","EMBD0053/","EMBD0054/");
29 date = newArray("20140110T153253");
30 folder = "Z:/cropped/";
31 folderOut2 = "Z:/EMBD_fiji_processed/";
32 fSize = 24;
33 setFont( "SansSerif", fSize);
34 for (r=0; r<date.length; r++){
35 for (l=0; l<RNAL.length; l++){
36     RNA = RNAL[l];
37     listStrains = getFileList(folder+RNA);
38     for (j = 0; j < listStrains.length; j++){
39         if (listStrains[j] == "GLS/"){
```

a. In Line 30, input the folder location of the images you wish to process in quotations.
b. In Line 31, input the desired save location in quotations.
c. In Line 28, input the Target name for the images you wish to process. Each Target name must be put in the format shown above, with a forward slash at the end and in quotations.
d. In Line 29, input the Image Folder Name in quotations.

3. Press Run at the bottom left corner of the script window. The program will then begin processing your images and compiling them into one file. Once the Program has finished the files will be open allowing you to review them. The files can be closed without saving, as the program has already saved them during processing.
Example:



Github Repository (*https://github.com/renatkh/embryo_crop.git*)

- **embryoCropUI.py**
- **screenCrop.py**
- **README file**

SETUP

Both programs (**embryoCropUI.py** and **screenCrop.py**) use specific versions of Python and Python modules, thus configuring an appropriate environment is essential for the programs to run. We recommend and provide instructions for installation of **Git**, **Anaconda (includes Python3)**, and **PyCharm** to enable proper environment configuration (detailed instructions below). ***Note- MacOS distribution of embryoCropUI.UI is only compatible with Mac OS X 10.11 and higher***

DETAILED INSTRUCTIONS

1. Configure your environment

Clone repository with GIT

1. If you don't already have GIT installed, go to **https://git-scm.com/download/** . You may need to enable security settings to be sure it will download.

2. Check install by going to terminal or command prompt and enter:

    > **git --version**

    **if installed a version will be listed in the terminal*

3. Clone repository:

    >**git clone https://github.com/renatkh/embryo_crop.git**

4. Check in your home directory to ensure that it was properly downloaded.

Install Visual Studio (WINDOWS ONLY):

1. Go to www.visualstudio.microsoft.com/downloads and **download Visual Studio**. This contains C++ tools, which are required for proper setup of the virtual environment with anaconda .yml files.
2. Select C++ tools
3. Install

Setup virtual environment with Anaconda

1. If you don't already have Anaconda, go to www.anaconda.com/download/ and Download **Anaconda3** (python3.7 version), launch anaconda setup and click through default options to install.

2. set environment variables and add conda to the path:

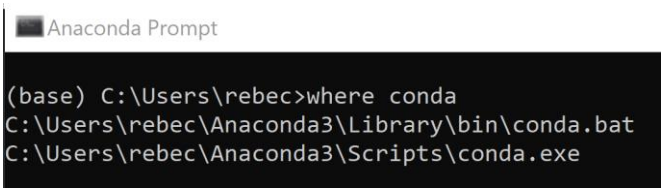
    a. Find **conda.exe** location in **Anaconda Prompt**:

WINDOWS: Go to **windows button-> Anaconda3-> Anaconda Prompt**

MacOS: **Anaconda3-> Anaconda Prompt**

At the prompt type in

> **where conda**



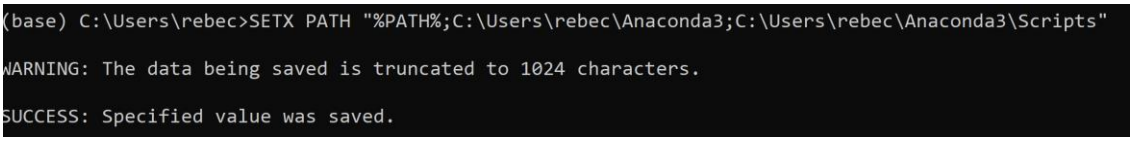
Find the location where conda.exe is located (ignore the .bat location) so you can add this location to environmental variables (this can be done within Anaconda Prompt).

In this case it is **C:\Users\rebec\Anaconda3\Scripts**
But obviously this will be specific to your system, so please edit the path appropriately!

For this example, we need to add both paths:
C:\Users\rebec\Anaconda3
C:\Users\rebec\Anaconda3\Scripts

b. Add to environment variables. To do this, type

> **SETX PATH "%PATH%;C:\Users\rebec\Anaconda3;C:\Users\rebec\Anaconda3\Scripts"**



3. Close **Anaconda Prompt**

3. Go to **system terminal or command prompt** (*not anaconda prompt*) and check to be sure that the **conda** command works.

> **conda**

this should return information about **conda functionality. If it does not, you have not successfully added path environmental variables.*

4. Configuring the environment in command line/terminal:

a. Navigate to the location where embryo_crop repository was saved.
i.e. for example, it is saved here: C:\Users\rebec\embryo_crop
so at the prompt:
> **cd C:\Users\rebec\embryo_crop**

b. Create new conda environment from .yml file

Once inside the directory, create the environment:

-for Windows: > **conda env create -f environment_win.yml**

-for MacOS:> **conda env create -f environment_mac.yml**

This step will take a few minutes to solve the environment……

```
C:\Users\rebec\embryo_crop>conda env create -f environment_win.yml
Solving environment: done
Preparing transaction: done
Verifying transaction: done
Executing transaction: done
Collecting pyqt5==5.11.3 (from -r C:\Users\rebec\embryo_crop\condaenv.lmfjd3fz.requirements.txt (
  Using cached https://files.pythonhosted.org/packages/a7/2d/d2c989006c86ae98ed230c28c3e0dd7fa037
Collecting pyqt5-sip==4.19.13 (from -r C:\Users\rebec\embryo_crop\condaenv.lmfjd3fz.requirements.
  Using cached https://files.pythonhosted.org/packages/46/86/0e35563d0c67c3f6b50e344624b87bfa7e72
Collecting tifffile==0.15.1 (from -r C:\Users\rebec\embryo_crop\condaenv.lmfjd3fz.requirements.tx
  Using cached https://files.pythonhosted.org/packages/1f/a1/4055cd679081cb4c1e40aa7648adb12574bf
Requirement already satisfied: numpy>=1.8.2 in c:\users\rebec\anaconda3\envs\embryocrop\lib\site-
Building wheels for collected packages: tifffile
  Running setup.py bdist_wheel for tifffile ... done
  Stored in directory: C:\Users\rebec\AppData\Local\pip\Cache\wheels\22\5b\8e\a9bc85b5dfc1cc91b84
Successfully built tifffile
mkl-random 1.0.1 requires cython, which is not installed.
Installing collected packages: pyqt5-sip, pyqt5, tifffile
  The scripts pylupdate5.exe, pyrcc5.exe and pyuic5.exe are installed in 'C:\Users\rebec\Anaconda
  Consider adding this directory to PATH or, if you prefer to suppress this warning, use --no-war
Successfully installed pyqt5-5.11.3 pyqt5-sip-4.19.13 tifffile-0.15.1
#
# To activate this environment, use:
# > activate embryocrop
#
# To deactivate an active environment, use:
# > deactivate
#
# * for power-users using bash, you must source
#
```

5. When finished, you can continue in command line to run embryoCropUI (below) or switch to an IDE to run screenCrop or embryoCropUI.

a. *To continue in command line (for embryoCropUI):*

Activate the environment according to the instructions listed in the terminal.

For Windows:

> **activate embryocrop**

For MacOS:

> **source activate embryocrop**

Once activated, you can run python programs by calling the program in command line

>**python embryoCropUI.py**

*this will launch the GUI window- please follow the instructions for the GUI use (in the readme file).

When finished:

Close GUI

>**deactivate**

Configure Environment in IDE (Setup in PyCharm)

To access the code directly, which is necessary for **screenCrop.py** functionality, open the environment in your favorite **Integrated Development Environment (IDE)**. There are many IDEs that are commonly used for coding in Python. An overview of features and limitations for popular options can be found here: https://stackoverflow.com/questions/81584/what-ide-to-use-for-python.

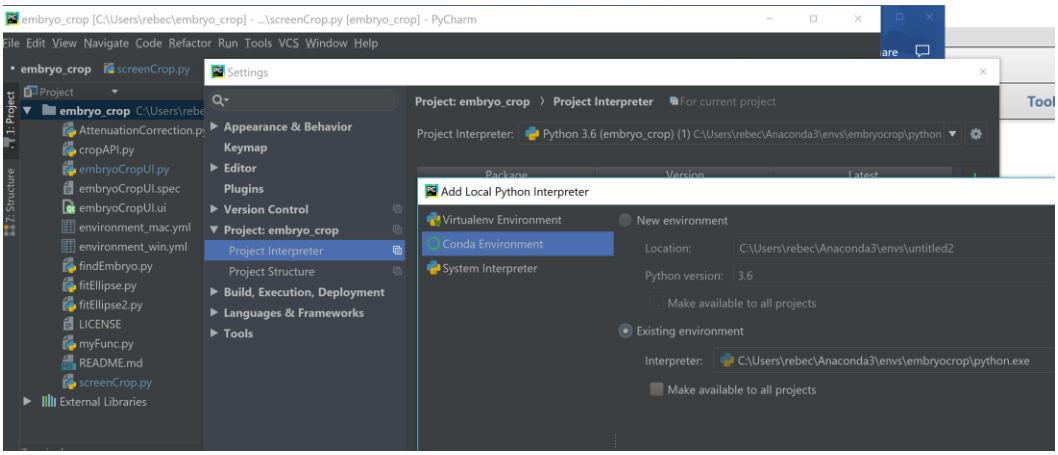
We include instructions for installing and running with **PyCharm**, however if you already have a different IDE that is compatible with Python (i.e. Eclipse) it should work fine. Note that Jupyter notebooks currently DOES NOT support GUIs, so our embryoCropUI will not run properly in this environment.

a. To get **PyCharm**, go to: https://www.jetbrains.com/pycharm/download/#section=windows. Select your operating system and click the black download button under "Community".
   i. For Windows- run the PyCharm-*.exe file and follow the installation wizard.
   ii. For MaOSc, open the PyCharm-*.dmg package and drag PyCharm to the Application folder.
b. When you start PyCharm for the first time, you will be prompted with **a complete installation** dialog box. Click **Do not import settings**.
c. Select your preferred user interface theme [Default or Darcula (black background)]
d. You will be prompted to install any additional plugins (not necessary)
e. Now you should be set up and greeted with a "welcome screen" where you can create, open, or check out a project.

In PyCharm:

1. **File > Open > embryo_crop**
2. Configure environment. Go to **file > settings > project:embryo_crop > project interpreter > add local** (select conda) **> existing environment >**

   select the newly generated conda env from within the Anaconda3 envs folder: **…Anaconda3\envs\embryocrop\python.exe**



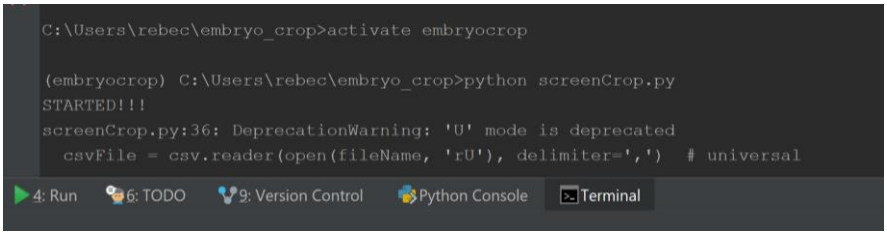
3. From here, you should be able to run programs using the PyCharm 'run' button (program instructions below). If this doesn't properly structure the environment, it may crash. If this happens, you can access the *terminal window within PyCharm* and activate the environment this way:
   a. For Windows:
      > **activate embryocrop**
   b. For MacOS:
      > **source activate embryocrop**

4. Once the environment is activated, you can run the program via the *terminal within PyCharm*:

   > **python screenCrop.py**

   or > **python embryoCropUI.py**

** note that the screenCrop program will need to be modified to work with your file structure!!**See instructions below**.



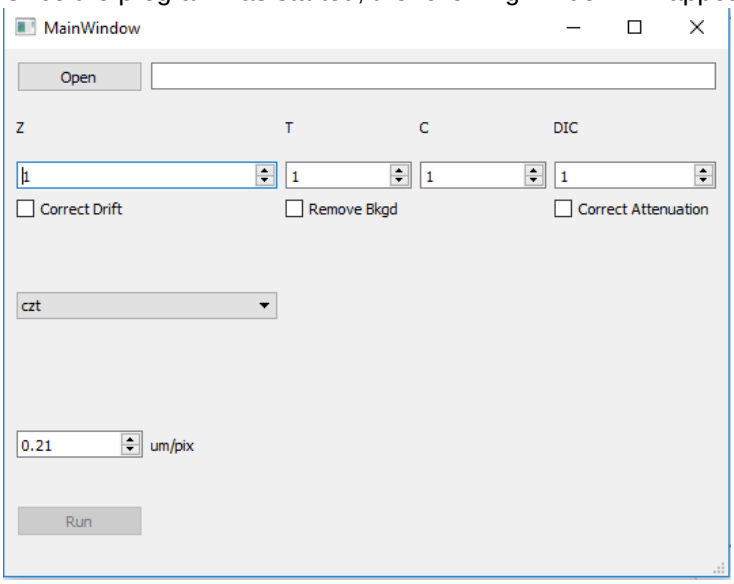
When finished:

>**deactivate**

2.Running embryoCropUI.py GUI with Python

1. In PyCharm (or your preferred IDE) double click on the **embryoCrop** folder and locate the file that says **embryoCropUI.py** (DO NOT open embryoCropUI.ui). The code will appear in the workspace.
2. If this is the only file open, go to the top right-hand corner and **click the green triangle** to start the run. If multiple files are open, right click and select '**Run embryoCropUI**' to ensure the proper program is run. Alternatively, activate the environment and run from PyCharm terminal, as outlined above.

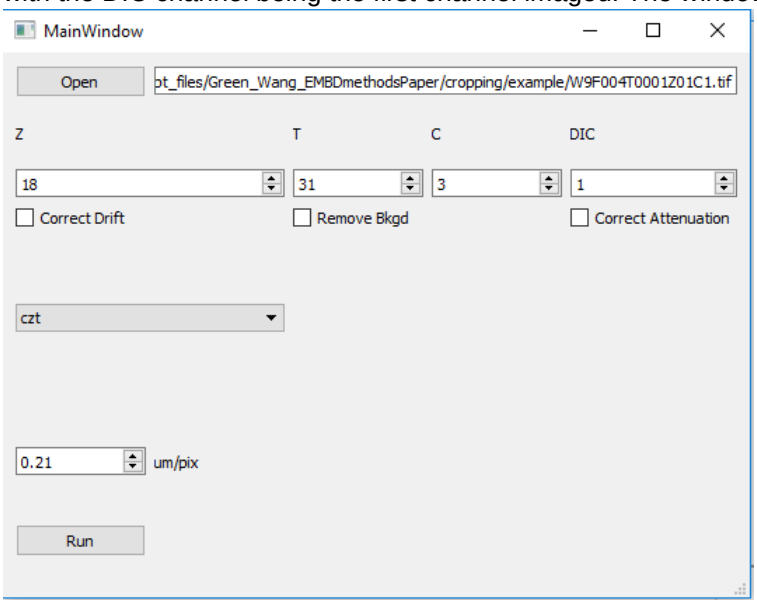
3. Once the program has started, the following window will appear:



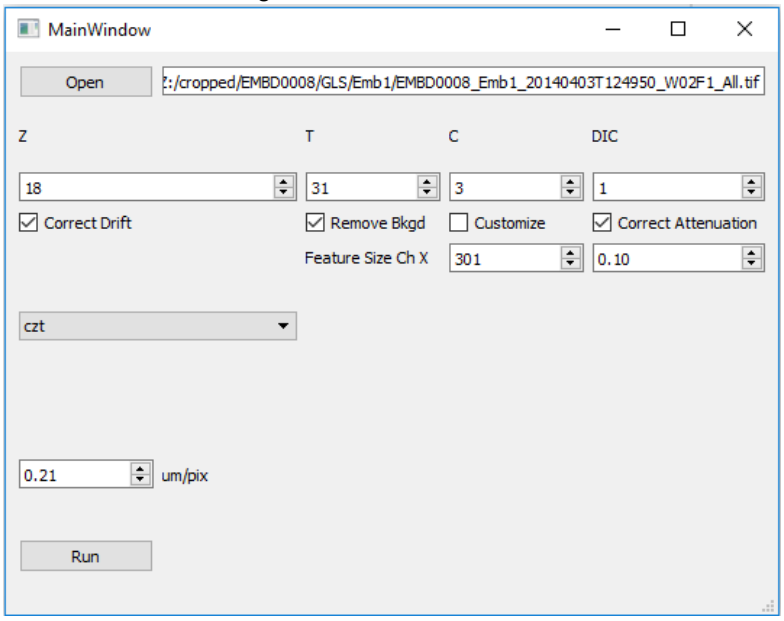
4. Select the **"Open"** button at the top of the window to load the specific image that you wish to crop. Should you be cropping an image series, with multiple dimensions (i.e. z, time, or channel), simply load the first image in the series within the folder. *Please make sure only images from one image series are present in this folder, otherwise the image series' will be loaded in tandem.*

5. Once you have loaded the desired images, you will need to specify the following information:
   e. Number of **Z slices (Z)**
   f. Number of **Time points (T)**
   g. Number of **Channels (C)**
   h. The channel that corresponds to **DIC or brightfield** (first=1, second=2, etc)

For example: our imaging protocol was 18 z-steps, imaged for 31 time-points in 3 channels with the DIC channel being the first channel imaged. The window should look like this:

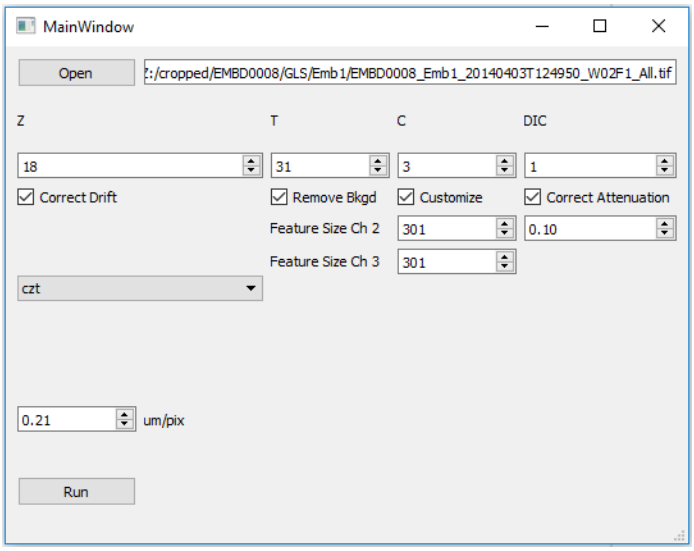


6. Now that you have your images loaded and specified the image parameters, you must choose what processing you would like to do alongside the embryo cropping. The program gives you the option to perform **Drift Correction, Background Subtraction, and Attenuation Correction**. Background Subtraction and Attenuation Correction must be done in conjunction with each other. The below image shows an image that will be going through Drift Correction, Background Subtraction, and Attenuation Correction.



   d. When selecting Background Subtraction and Attenuation Correction, specify parameters for each to guide the processing. For *Background Subtract*, define a feature size (*odd numbered*) that reflects the level of detail you wish to resolve, larger feature size equates to more detail. For *Attenuation Correction*, you need to input a value from 0-1. This value represents the percent of original intensity that remains at the furthest distance through the object being imaged.

   e. As shown below, you have even greater options to customize Background subtraction. By selecting Customize, you will be able to define a feature size for different channels.

f. 

7. Next, specify the **order** in which the images were collected (i.e. **channel-z-time (czt)**, or **z-channel-time (zct)**)
8. Specify the **microns per pixel of the camera being used** for the images.
   a. failure to properly define pixel size will result in poor image cropping!!
10. Select **Run** at the bottom left corner and the program
    When the cropping and processing of your images has completed, the cropped versions will be saved in a new subfolder labeled “crop” in the same folder as the uncropped images.

We make available two formats of **test files for testing embryoCropUI.py. This folder is too large for Github requirements and thus is stored on the Zenodo repository (https://zenodo.org/record/1475442#.W9jvApNKiUl). Download and unzip. We recommend testing one or both to ensure the program is functioning properly on your system:

iii. TESTME2_BGLI140_1t_a1.tif- a compiled multi-tif format
   1. Load file in the ‘open’ field. Set Z=1, T=6, C=3, DIC=1 and use the default settings for all other fields. Click Run. If successful, a message will appear at the bottom of the GUI window that says ‘embryos saved’ and it will generate a folder in the same location as the test file labeled “crop”; this should contain 4 embryos.

iv. Test_field- a folder containing an short image series
   1. Load the first image in the test_field folder into the ‘open’ window. Set Z=18, T=4, C=3, DIC=3, change the pixel size to 0.26um/pix. Click Run. a message will appear at the bottom of the GUI window that says ‘embryos saved’ and it will generate a folder in the same location as the test file labeled “crop”; this should contain 2 embryos.

**These files should crop in seconds to minutes, but larger image sequences may take some time. The bottom corner of the GUI window will read-out what the program is doing (“Loading images”, “cropping”, or “embryos saved”). If an error occurs, the message will appear here.

3.Running screenCrop.py

This software allows you to batch crop many files at once, but it is less user friendly and has not been optimized across platforms. *It was designed to function with data output from CV1000 imaging systems*; if you have a CV1000 and follow the instructions below, it should work seamlessly. If you have another system, modifications to the code will likely be required and someone with Python experience will be

needed. In the event that it is needed, we outline key elements of the code and our file structure to guide such efforts. Successful bulk cropping requires:

1. A **reference .csv** file that contains essential image information, which is called by our Python software
2. **Properly named files**

CSV:

The CSV file contains information that will be called on during processing or used to generate the cropped file path. Below is an example .csv file that is compatible with our programs. Formatting your .csv file in the same way will ensure your data will go through our programs with minimal issues.

Experiment	Experiment Folder Name	Post-scan folder name	Well designation	Target	strain	Plate Coordinate
EMBD_112013	20131120T160955	20131121T104921	Well001	EMBD0000	GL	C3
	20131120T160955	20131121T104921	Well005	EMBD0000	GLS	D3
	20131120T160955	20131121T104921	Well009	EMBD0000	M	E3
	20131120T160955	20131121T104921	Well013	EMBD0000	MS	F3
	20131120T160955	20131121T104921	Well002	EMBD0002	GL	C4
	20131120T160955	20131121T104921	Well006	EMBD0002	GLS	D4
	20131120T160955	20131121T104921	Well010	EMBD0002	M	E4
	20131120T160955	20131121T104921	Well014	EMBD0002	MS	F4
	20131120T160955	20131121T104921	Well003	EMBD0003	GL	C5
	20131120T160955	20131121T104921	Well007	EMBD0003	GLS	D5
	20131120T160955	20131121T104921	Well011	EMBD0003	M	E5
	20131120T160955	20131121T104921	Well015	EMBD0003	MS	F5
	20131120T160955	20131121T104921	Well004	EMBD0015	GL	C6
	20131120T160955	20131121T104921	Well008	EMBD0015	GLS	D6
	20131120T160955	20131121T104921	Well012	EMBD0015	M	E6
	20131120T160955	20131121T104921	Well016	EMBD0015	MS	F6

Rundown of .CSV file contents:

- Experiment: arbitrary name given to each experiment (not important for software functionality, but this column needs to be maintained)
- Experiment Folder Name: name of folder in where specific experiments images are stored. We prefer Date/Time file name, though any name will suffice (do not include spaces or disallowed characters, as the contents of this cell are added to the file path).
- Post-Scan Folder Name: name of post- scan (10x data) folder. Not important for software functionality, but this column needs to be maintained. You can populate this with 'empty'.
- Well Designation: Well numbers as determined by CV1000 software adhering to the Well### regime.
- Target: Experimental conditions (e.g. RNAi condition), we use a blinded, unique identifier system (EMBD####) for our experimental conditions, though this is not necessary. Output files will be saved according to this name, so do not include spaces or disallowed characters.
- Strain: Specific strain used in experiment. Scaling and background subtraction is applied differently depending on the strain used.
- Plate Coordinate: Coordinates from 384 well plate (for reference, not used by program).

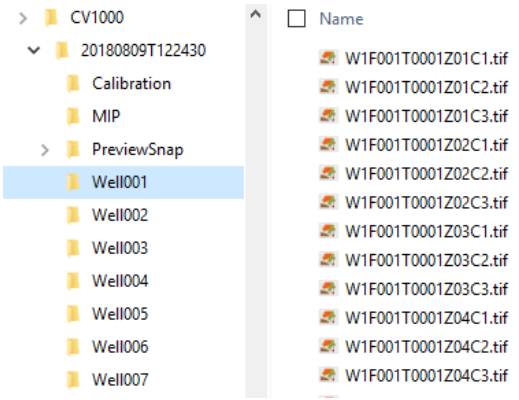
Properly naming files:

Input path:

The path to access the raw data files is referred to in **screenCrop.py**, based on the information in the CSV file (highlighted in red below). Our data is structured such that multiple point visits are contained within each well folder and all of the image files are listed within that well (not in separate subfolders per point visit). Image files have been automatically named according to CV1000 software image naming conventions, as follows:

Z:\CV1000\Experiment Folder Name\Well designation\W##F##T###Z##C#.tif

i.e. Z:\CV1000\20180809T122430\Well001\W1F001T0001Z01C1.tif.

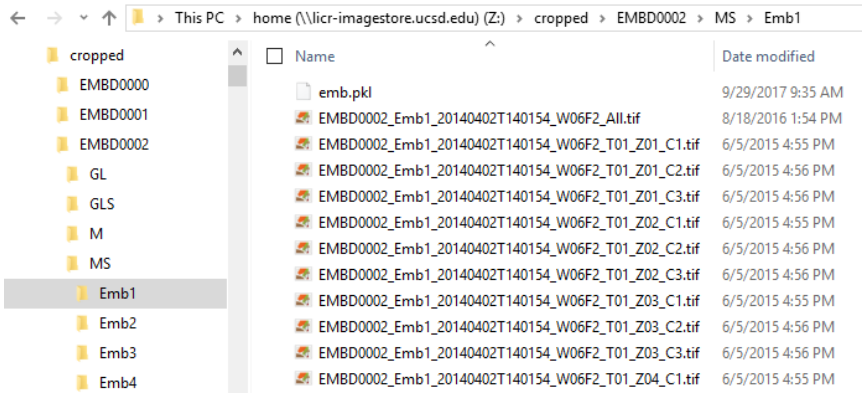


Output path:

The output path specified in **screenCrop.py** is also generated based on information in the CSV file (highlighted in red below). Our saving regime uses our naming scheme (Emb#) for outer folders representing data for each embryo and this contains individual tifs that are named as follows:

Z:\cropped\Target\Strain\Emb#\Target_Emb#_Experiment Folder Name_W##F#_T##_Z##_C#.tif

i.e. Z:\cropped\EMBD0002\MS\Emb1\EMBD0002_Emb1_20140402T140154_W06F2_T01_Z01_C1



Cropping Your Raw Images

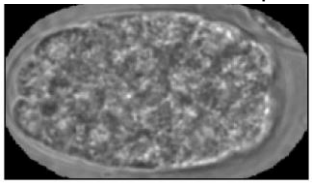
Using **ScreenCrop.py**, you will be able to crop all your images from a folder. The program crops each image by fitting an ellipse to each embryo at the fourth time point.

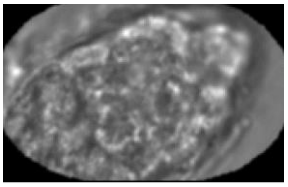
1. Open PyCharm, or other IDE, load the embryo_crop repository and locate the program screenCrop.py. It is important to know the following Information and fill it in at the specified line:
    a. *loadFolder (line 9):* The drive on which the files are stored (e.g. Z:/ , D:// etc.)
    b. *date (line 7):* this is the file referred to as Experiment Folder Name in the CSV
    c. *trackingFile (line 11)*: the path to the CSV file in which experiment information is stored
    d. *z (line 13)*: The number of z planes
    e. *nT (line14)*: Number of timepoints
    f. *nWells (line 19):* the number of wells used
    g. *pointVisits (line 20)*: the maximum number of point visits (per well)

```
4  Loads embryo images generated by CV1000 and crops/orients and saves separate embryos based on DIC (C3) central image
5  '''
6
7  date = '20170118T125754'
8
9  loadFolder = 'Z:/'
10 folderIn = loadFolder + 'CV1000/' + date #Input folder
11 trackingFile = 'Z:/Experiment_tracking_sheets/EMBD_fileNames_Tracking_Sheet.csv'
12 aspectRatioFile = 'Z:/cropped/aspects.csv'
13 z = 18 #number of z planes
14 nT = 31 #number of time points
15 corrDrift = True
16 removeBG = True
17 attCorrect = True
18 apRotate = True
19 nWells = 14#number of wells (14)
20 pointVisits = 4# number of point visits (4)
21
22 import glob, csv, cv2, os, shutil
23 import numpy as np
24 from findEmbryo import showIm
25 from myFunc import clearFolder
26 from cropAPI import cropEmbs
27 import tkMessageBox
28
29 debug = False # use to debug the program
30
31 def getConditions(date, fileName):
32     ''' loads RNAi strains for a specified date from a csv file '''
33     global RNAi, strains
34 #     csvFile = csv.reader(open(fileName, 'rb'), delimiter=',')
35     csvFile = csv.reader(open(fileName, 'rU'), delimiter=',') #universal
36     fileData=[]
37     for row in csvFile:
38         fileData.append(row[1:-1])
39     myDate = [s for s in fileData if s[0]==date]
40     myDate = sorted(myDate, key=lambda well: well[2])
41     RNAi = [s[3] for s in myDate]
42     strains =  [s[4] for s in myDate]
43     return
```

h. In line 10 find the location currently occupied by 'CV1000/' and input the outer folder used in your file path. To avoid issues, use the following convention: 'XXXXXXX/'.
i. In Line 12, input a valid file path for storing aspect ratio data for the cropped.
j. In Lines 15, 16, 17, and 18 input True/False for whether you would like your images to go through the following processing:
    i. Drift Correction (Line 15)
    ii. Background Subtract (Line 16), feature size should be defined as 41 for GLS strain and 201 for MS strain. Background Subtract must be done in conjunction with Attenuation Correction.
    iii. Attenuation Correction (Line 17)
    iv. AP Rotate (Line 18)

2. Once all the changes have been made to tailor the program for your data, you may begin cropping. This is done by selecting the green play icon in the toolbar, this will have a drop down menu where you select "Run As" and then "Python Run". Alternatively, activate the environment and run from PyCharm terminal (described above at the end of the PyCharm section).
3. The Program will then begin cropping your images, *this may take a few hours depending on the number of images that need to be processed*. Once completed, a series of small windows containing embryo images will open; this will allow you to curate the cropped data before saving (i.e. delete embryos that are cut off, out of focus, or poorly cropped can be deleted).

Good! 

Bad!! 

4. For each image you have three options:
    a. **Save:** If the image appears to be cropped properly with no areas of interest being cut off, press the space bar to save the image.
    b. **X:** If the image appears to have areas of interest cut off and you still wish to save the image, press X and the image will be saved with an X in front of the name to separate it from the others.
    c. **Delete:** If the image is not cropped properly or the embryo is not to your liking, press D to delete the cropped image.
5. Once you have gone through all your images and determined whether you wish to **save, x, or delete** them, the program will then begin to save your images. ***The images will be saved to a subfolder named "Cropped" in the Load Folder that was defined in Line 9 of the program.***