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Lauren R. Lym April 8, 2018

Germ cell-less and the formation of primordial germ cells in *Drosophila melanogaster*

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Abstract

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Impaired fertility affects 1 in 25 men, and though the specific underlying cause of impaired fertility is unknown in most cases, recent research has linked human male sterility to a gene called *Germ cell-less like protein 1 (GMCL1*), a homolog of *Drosophila germ cell-less* (*gcl*) (Kleiman et. al, 2003). Gcl is most well-known for its role in the proper formation of primordial germ cells (PGCs), the precursor cells to adult germ cells, or gametes (Jongens et al., 1992). The early separation of PGCs from the soma is a widely-conserved process that protects PGCs from somatic signaling during rapid development in other parts of the embryo, maintaining the integrity of PGC stem cell character late into development (Strome and Lehmann, 2007). In the syncytial *Drosophila* embryo, posterior nuclei surrounded by the germ plasm, a collection of nuclear determinants that specify PGCs (Mahowald, 2001), will bud early and cellularize before somatic cells form. *gcl* controls PGC formation by regulating the centrosome segregation that allows PGC nuclei to cellularize (Lerit et. al., 2017). However, the mechanism behind *gcl* controlled centrosome segregation is currently unknown. To investigate *gcl* controlled centrosome segregation, we evaluated potential Gcl binding partners that have known roles in centrosome segregation. Eb1 is a conserved protein found on the plus-ends of growing microtubules that functions in the centrosome segregation required to form the mitotic spindle (Rogers et. al., 2002). Eb1 was also previously identified as a potential interacting partner of Gcl in a high-throughput screen (Giot, 2003). We identified similar PGC reduction phenotypes and centrosome defects in *gcl* and *Eb1* knock-out mutants, and we observed an increase in phenotype severity in double knock-out mutants that may indicate negative epistasis. Through the

completion of these studies, we aim to clarify the mechanism behind germ cell development to better understand the role *gcl* plays in sterility.

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Table of Contents

Index of Figures

Background and Motivation

Lay abstract

1 in 25 men are affected by impaired fertility, but the specific underlying cause is unknown in most cases (Kleiman et. al, 2003). Recent research, however, has linked a gene called *Germ cell-less like protein 1 (GMCL1*) to human male sterility (Kleiman et. al, 2003). *GMCL1* is a highly-conserved gene, meaning that the gene and protein sequences are nearly the same across many species. *GMCL1* was originally identified in *Drosophila*, however, as *germ cell-less* (*gcl*), and is required for proper formation of primordial germ cells (PGCs), the stem cells which will later form gametes (Jongens, 1992). Early in development, PGCs separate from body cells to maintain their stem cell character during rapid growth and the specialization of body tissues within the embryo (Strome and Lehmann, 2007). The rapid development, high level of genetic conservation, rich genetic tools, ease of use, and relatively low cost make *Drosophila* a valuable model to study human health pathologies, such as sterility. During the first two hours of development, the *Drosophila* embryo is a syncytium, meaning that it is one cell with many nuclei (Foe and Alberts, 1983). PGCs are the first cells that form from the syncytial embryo. PGC formation is instructed by a specialized cytoplasm, the germ plasm, which is a collection of molecules that instruct the germline fate (Mahowald, 2001). *Gcl* controls PGC formation by inducing the separation of centrosomes (Lerit et. al., 2017), which organize filaments of the cytoskeleton called microtubules (Urbani and Stearns, 1999). Models suggest that centrosome separation serves to transmit force against the embryo membrane, effectively generating a budding protrusion that sequesters the associated PGC nucleus into a new cell. Nonetheless, the molecular mechanism controlling centrosome separation in PGCs is currently unknown (Lerit et.

al., 2017). To investigate this mechanism, we evaluated Eb1, a protein found on growing microtubules, as a potential binding partner of Gcl protein (Rogers et. al., 2002). When flies lacked either protein, we identified a reduced number of PGCs and a failure to fully separate centrosomes. Additionally, we observed an increase in severity when both proteins were depleted. We hypothesize that Gcl and Eb1 proteins work together to promote the centrosome separation that facilitates cell formation. Through a clearer understanding of the role *gcl* plays in germ cell development, we may gain insight into the specific underlying causes of impaired fertility.

Germ cell-less and male infertility

Idiopathic infertility in humans is typically attributed to genetic causes, though the specific underlying cause is unknown in most cases (Kleiman et. al, 2003). However, a gene called *Germ cell-less like protein 1 (GMCL1*) has recently been linked to human male sterility and defective sperm motility through its function in spermatogenesis (Kleiman et. al, 2003). *GMCL1* is a homolog of a previously identified *Drosophila* gene called *germ cell-less* (*gcl*), (Kleiman et. al, 2003), which is most well-known for its role in embryogenesis (Jongens et al. 1992). Maternal Gcl protein is required for proper formation of primordial germ cells (PGCs), the precursor cells to adult germ cells, or gametes (Jongens et al. 1992). Germline formation is easy to study in *Drosophila* because embryos are laid for development and PGCs collect on the exterior of the embryo, and *Drosophila* is an ideal model system for cellular study due to the large amount of resources and information available from previous research.

An overview of *Drosophila* embryogenesis

In the first two hours post-fertilization, the embryo functions as a syncytium, undergoing fourteen nucleic division cycles (Foe and Alberts, 1983). As the embryo nears nuclear cycle ten, nuclei migrate to the cortex of the embryo (Foe and Alberts, 1983). After the fourteenth nuclei division, simultaneous cellularization occurs for the somatic nuclei (Foe and Alberts, 1983). Maternal transcripts are the primary source of mRNA during the syncytial period (Foe and Alberts, 1983). During oogenesis, nurse cells dump maternally transcribed mRNA into the oocyte, many of which then localize to specific regions of the oocyte pre-fertilization to pattern the embryo, establishing the dorsal-ventral and anterior-posterior axes (Manseau and Schupbach, 1989). Zygotic transcription begins at nuclear cycle six, but significant amounts of zygotic mRNA are not observed until the fourteenth nuclear cycle and cellularization (Farrell and O'Farrell, 2014). Once cellularization is complete, the embryo enters gastrulation and morphogenesis ensues (Foe and Alberts, 1983).

PGCs are a subset of cells that form early during the syncytial period, separating precociously from the soma (Jongens et. al., 1994). PGCs bud off the embryonic posterior pole at nuclear cycle 9-10 (Jongens et. al., 1994). At the onset of gastrulation, the PGC cluster migrates internally to contact somatic gonadal precursor cells, which will form the supportive structures of the reproductive system, allowing gonadogenesis to proceed (Jongens et. al., 1994). Early germ cell specification and germ cell migration are widely conserved features of embryogenesis across species, largely because early specification of germ cells maintains their status as stem cells much further into development, shielding them from other differentiation signaling and protecting their integrity during rapid growth in other parts of the embryo (Jongens et. al., 1994).

PGC nuclei are posterior pole nuclei that have been specified by the presence of germ plasm, a collection of germline fate determinants that are maternally derived (Mahowald, 2001). Components of the germ plasm are highly conserved across species, which is unsurprising as the processes of germ cell early specification and migration are widely conserved as well (Mahowald, 2001). During oogenesis, *nanos*, an axis-patterning and germ plasm factor that serves as a posterior marker, anchors and recruits other germ plasm components to the posterior pole (Mahowald, 2001). During PGC formation at nuclear cycle 9-10, the proximity of germ plasm to migrating nuclei and their associated centrosomes induces budding of the posterior membrane and encapsulation of the germ plasm (Lerit and Gavis, 2011). Centrosomes nucleate microtubules and serve as an organization center for the cytoskeleton, functioning in cell division, cell motility, and intracellular transport (Urbani and Stearns, 1999). In budding PGCs, growing microtubules push the associated centrosomes apart, segregating them to opposite sides of the nucleus (Lerit et al. 2017), and then protrude into the syncytial membrane until the contraction of an actin-derived anillin ring completes the early cellularization of PGCs (Cinalli and Lehmann, 2013).

Germ cell-less is required for germ cell formation

gcl is a germ plasm component that is required for the proper formation of PGCs (Jongens, 1992). In healthy embryos, Gcl is expressed in the oocyte and at the posterior pole of the embryo, remaining detectable in PGCs until early gastrulation (Jongens, 1992). *Gcl* was identified in flies by the Jongens lab in a screen for grandchild-less mutants, indicating that the sterility phenotype is maternal effect. This means that the mother's genotype controls the offspring's phenotype because the active transcript in the embryo is maternal. A heterozygous fly

with only one non-functional copy of *gcl* can produce healthy, fertile female offspring that are homozygous for the null *gcl* allele (*Δgcl*) (Jongens, 1992). These *Δgcl* mothers then fail to provide a functioning *gcl* transcript to their oocytes, producing sterile offspring that lack germ cells. Though Gcl protein has been identified in other tissues later in development, the null phenotype appears to be specific to germ cell development (Jongens, 1992). Though the progeny of *Δgcl* mothers are sterile, they are otherwise morphologically normal and appear healthy (Jongens, 1992). Additionally, *gcl* is a particularly interesting target for study because embryos with highly reduced levels of *gcl* mRNA show normal germ plasm assembly (Jongens et al., 1992), whereas embryos lacking other identified germ plasm components fail to appropriately assemble germ plasm and therefore fail to form germ cells entirely (Mahowald, 2001). *gcl* is the only previously-identified component of the germ plasm where the embryos of knockout flies fail to produce PGCs appropriately despite normal germ plasm assembly (Jongens et al., 1992).

Previous research has shown that *gcl* controls PGC formation by regulating the centrosome segregation that is required for early PGC budding (Lerit et. al., 2017). *Δgcl* embryos fail to properly migrate the centrosomes associated with the nuclei fated to become PGCs, which likely explains the failure to bud and overall reduction in PGC number. However, the mechanism through which Gcl regulates centrosome dynamics is currently unknown. In previous experiments, Gcl protein has only been localized to the nuclear envelope (Robertson et. al., 1991), not the centrosomes, suggesting that *gcl* may regulate centrosome dynamics indirectly. To investigate this mechanism, we have chosen to look at potential interacting proteins that would allow Gcl to indirectly regulate centrosome migration.

Eb1 as a potential interacting partner of Gcl

One notable interacting partner is a conserved microtubule protein called Eb1, which is found on the plus-ends of growing microtubules (Rogers et. al., 2002). In early experiments, *14-3-3*, a microtubule associated protein that is localized to PGCs functions in both PGC migration and gonadogensis (Tsigkari et. al., 2012), was also tested for a PGC reduction phenotype. However, *14-3-3* knock-down embryos showed wildtype budding, where *Eb1* knock-down embryos showed a reduction in PGC number, demonstrating phenotype specificity toward *Eb1*. Eb1 was previously identified as an interacting partner with Gcl in a high-throughput yeast two-hybrid screen (Giot, 2003), and it is a good candidate for a PGC formation complex due to both its functions in the cytoskeleton and the presence of maternal *Eb1* mRNA in early embryos (Rodgers et al. 2002). Eb1 plays an integral role in the facilitation of microtubule growth and is required for mitotic spindle positioning (Chen et. al., 2014). The separation of centrosomes necessary for mitotic spindle assembly is similar to the separation of centrosomes necessary for PGC budding, and Eb1 depletion has been shown to affect microtubule dynamics, specifically in actively dividing cells like those of an embryo (Buttrick et. al., 2019). We hypothesize that a Gcl-Eb1 protein complex induces PGC budding. By investigating the role of a Gcl-Eb1 complex in PGC formation, we aim to clarify the process of germ cell development and gain new understanding about cell fate specification, which in turn will allow us to better understand the role *gcl* plays in sterility.

Specific Aims

Experimental objective

Our specific objective is to determine the mechanism through which *gcl* regulates centrosome dynamics to promote PGC budding. We will achieve this by investigating potential interacting partners of Gcl, first verifying the PGC reduction and centrosome segregation phenotypes found in embryos with a *Δgcl* maternal genotype and then identifying these defects in other genotypic backgrounds. Using co-localization, immunohistochemistry, and immunoprecipitation, we will verify the existence of a genetic and/or biochemical interaction between Gcl and a binding partner in early *Drosophila* embryos.

Hypothesis

We hypothesize that an interaction between Gcl and Eb1 coordinates germ cell development and is required for proper PGC formation. At nuclear cycle 10, Gcl, a germ plasm protein, binds to Eb1, a microtubule protein, signaling centrosomes localized to posterior nuclei to begin PGC formation.

Methods

Drosophila strains

We have raised wildtype flies for males, as the proteins of interest are maternal effect and therefore the zygotic genotype does not affect emergence of the phenotype. *Δgcl* is a null allele (Robertson, et al. 1999). Eb1 RNAi knock down was achieved in two strains, *UAS-Eb1* insertion on the second chromosome (*Eb1* II RNAi, Bloomington *Drosophila* Stock Center) and *UAS-Eb1* insertion on the third chromosome (*Eb1* III RNAi, Bloomington *Drosophila* Stock Center). As an RNAi control, we used a *UAS-RFP* line (*mCherry* RNAi, Bloomington *Drosophila* Stock Center). For specificity, we controlled with a *UAS-14-3-3z* line (*14-3-3* RNAi, Bloomington *Drosophila* Stock Center). RNAi expression is driven in the germline through a maternal *nanos-UAS-GAL4* driver (*nosGAL4*, Van Doren et al., 1998). We first crossed heterozygous RNAi males with *nosGAL4* virgin females and then bred the F1 generation with wildtype males, collecting F2 embryos for experimentation. All strains used are detailed in **Table 1**.

Early results indicated that *Eb1* III RNAi flies produce a more severe phenotype then *Eb1* II RNAi flies. All following experiments using flies with an *Eb1* RNAi knockout have therefore been performed with the third chromosome insertion. We have also bred double mutant knockout flies that lack *gcl* and *Eb1* through a series of genetic crosses. We first used *maternal 67C Tubulin-GAL4* (*matTubGAL4*, Lee et al., 2001) to cross a *matTubGAL4* driver into a heterozygotic balanced *Δgcl* stock (Robertson, et al. 1999). We then collected virgin females and crossed them to *Eb1* RNAi males to obtain *Δgcl*; *Eb1 RNAi* mutant embryos that lack expression of both genes.

Immunohistochemistry of PGCs

Embryos were collected from wildtype, *mCherry* RNAi, *14-3-3* RNAi, *Δgcl*, *Eb1* RNAi, and *Δgcl*; *Eb1* RNAi flies. For our PGC reduction phenotypic screen, we have used embryos at nuclear cycle 14 stained with a rat anti-Vasa (DSHB, 1:10) antibody and counter-stained with DAPI (Invitrogen, 1:1000) and fluorescently-conjugated goat anti-rat antibodies (Invitrogen, 1:500). Images were obtained on a Nikon TiE spinning disk confocal microscope outfitted with a Yokagawa CSU-22 spinning disk head and a Hamamatsu Orca Flash 4.0 cMOS camera. Our system is fitted with 3 high-end objectives. PGC count images were taken on a 40X NA 1.49 oil immersion lens. Images of the PGC cluster were taken in a z-stack format with a step size of 1.5 um, and individual cells were counted by hand in ImageJ or Nikon Elements software. PGCs were defined as posterior, Vasa positive cells that contain a nucleus and have fully undergone cytokinesis from the syncytium. Unusual morphology was not considered, provided a nucleus was clearly visible within the cell. Blebs containing Vasa but devoid of nuclei are not considered PGCs and were therefore not counted.

For our centrosome segregation phenotypic screen, embryos at nuclear cycle 9-10 were stained using guinea pig anti-Asterless (1:4000, gift from Nasser Rusan) to mark centrosomes, rabbit anti-Vasa (1:2000, gift from Paul Lasko) to mark germ plasm, mouse anti-phospho-Tyrosine (pTyr; 1:1000, Milipore: 05-321) to mark the cortex, and counter-stained with DAPI and fluorescently-conjugated goat anti-guinea pig and anti-mouse antibodies. Images were obtained on a 100X NA 1.49 oil immersion lens. PGC buds were staged morphologically and identified as in nuclear cycle 10 prophase when large, round nuclei form protrusions into the cortex at the posterior of the embryo. Further replicates for centrosome separation quantification will be stained with anti-phospho-Histone 3 (1:1000, Milipore: 05-570), which binds

phosphorylated Histone 3 at serine 10 during prophase (Hans and Dimitrov, 2001), for better accuracy in staging.

For co-localization experiments, we have used gifted, noncommercial antibodies. We received a rabbit anti-Gcl antibody (1:4000) from the Lasko Lab (Thomson and Lasko, 2004), which we have pre-absorbed with *Δgcl* fly tissue to reduce non-specific binding. Additionally, we have received a rabbit anti-Eb1 antibody (1:1000) from the Rogers Lab (Rodgers, et. al. 2002). Images were obtained on a 100X NA 1.49 oil immersion lens.

Immunoblotting

Gifted antibodies were verified through standard Western blotting techniques. Embryos were lysed in RBC complete buffer (50 mM HEPES, 150 mM NaCl, 2.5 mM $MgCl_2$, 0.2% Triton X-100, 250 mM sucrose) and centrifuged due to the high lipid content of embryonic tissue. The supernatant was aliquoted and diluted to 10 μL in SDS-loading buffer, and then run in a 7.5% SDS-PAGE Tris-HCL (Bio-Rad) gel. The stacking gel was run at 80 mV and the separating gel was run at 140 mV. Protein samples were then transferred to a nitrocellulose membrane by current application in a Bio-Rad Trans-Blot Turbo transfer system. Nitrocellulose membranes were blocked in 5% milk diluted in TBST buffer (0.2 M Tris base and 1.5 M NaCl at pH 7.5, 0.05% Tween-20). Nitrocellulose membranes were incubated in rabbit anti-Gcl antibody (Lasko, 1:2000) or rabbit anti-Eb1 antibody (Rogers, 1:2000), and a mouse anti-gamma tubulin antibody (Sigma: T6557, 1:2000) was used as a loading control. Primary antibodies were conjugated to Horseradish peroxidase secondary antibodies (Thermo Scientific anti-rabbit 31460 and anti-mouse 31430, 1:5000 in 5% milk in TBST), and membranes were visualized using

chemiluminescence on a Bio-Rad ChemiDoc™ MP imaging system. Optimal exposure lengths and intensities varied by primary antibody due to differences in signal strength.

Immunoprecipitation

Wildtype embryonic lysate from 0-2 hour collections was used to affinity purify Eb1 and Gcl proteins bound to anti-Gcl antibodies conjugated to Pierce™ Protein A/G magnetic agarose beads from Thermo-Fisher Scientific. Under the appropriate biochemical conditions (e.g., 150 mM NaCl), binding partners of Gcl will elute from the beads, as detected by SDS-PAGE and immunoblotting. Control beads were either conjugated to rabbit anti-GFP antibody (Fisher Scientific, A11122) or no antibody. Further, wildtype embryonic lysate from 0-2 hour collections was affinity purified by anti-Eb1 antibodies conjugated to magnetic beads, such that binding partners of Eb1 will elute from the beads, as detected by SDS-PAGE and immunoblotting.

Statistical methods

Data were plotted and statistical analysis was performed using GraphPad Prism software. PGC count data are displayed as mean ± standard deviations (SD). To calculate significance, a non-normal distribution was assumed and a one-way ANOVA test was run. Data were then analyzed by a multiple comparisons test for significance.

Results

PGC reduction phenotype

We began to test our hypothesize of a Gcl-Eb1complex that coordinates germ cell development by signaling posterior centrosomes to begin PGC budding by using immunohistochemistry to assay whether the loss of either *gcl* or *Eb1* resulted in PGC reduction. Where a complete failure to form PGCs indicates failure to assemble germ plasm, a reduction in the number of PGCs indicates budding error (Mahowald, 2001). Embryos at nuclear cycle 14, immediately after cellularization but before gastrulation has occurred, were labeled with Vasa, a germ plasm marker (Thomson and Lasko, 2004), and counterstained with DAPI to visualize nuclei. Vasa is a germ plasm protein that we have chosen for initial phenotype screening due to its reliability as a germ plasm and PGC marker and the availability of an inexpensive commercial antibody (Liu et al., 2003). Only PGCs that demonstrated full cytokinesis from the syncytium, contained a Vasa-rich cytoplasm and a nucleus, and were distinct single cells were quantified. PGC count experiments were performed in triplicate and showed consistent results.

Wildtype PGC budding was shown in mCherry RNAi control flies, as mCherry is not expressed in *Drosophila*, thus its depletion should not alter the production of PGCs (**Figure 1**). On average, we note that control embryos show n=31.2±7.9 PGCs. To indicate specificity of the phenotype, analysis of *14-3-3* RNAi flies was conducted, and *14-3-3* RNAi flies showed wildtype PGC budding (**Figure 1**). However, other alleles of 14-3-3 may be informative in determining phenotype specificity. Images of *Δgcl* embryos verify the mutant phenotype found by Jongens, showing an average of n=8.2±7.5 PGCs. We show that *Eb1* RNAi flies show a less severe PGC reduction phenotype than *Δgcl* flies, averaging at n=23.0±8.4 PGCs (**Figure 2**).

Despite the reduction in cell number, however, *Eb1* RNAi flies did not show the distinct morphology defects shown in *Δgcl* flies, such as cytoplasmic blebs, flattened cell shape, and uneven Vasa distribution (**Figure 2**). Images of *Δgcl*; *Eb1* RNAi embryos show an increased severity of the phenotype compared to both *Δgcl* embryos and *Eb1* RNAi embryos, showing n=0.8±1.9 PGCs. (**Figure 2**). Though double knock-outs for genes acting in the same pathway typically show a phenotype that mimics that of the downstream knockout, this increase in phenotype severity may be a function of negative epistasis (Wolf, 2000). This would then indicate that both genes are involved in PGC budding and likely cooperate within the same functional pathway.

Centrosome segregation defects

Having identified a similar loss-of-function phenotype in *Eb1* RNAi embryos as in *Δgcl* embryos, and established that these genes appear to genetically interact synergistically, we next characterized the phenotypic defects behind the reduction in PGC number. Embryos at nuclear cycles 9 and 10, as PGCs are just beginning to protrude from the syncytial membrane but have not yet undergone cytokinesis, were labeled with Asterless, a known centrosome marker (Varmark et. al., 2007), rabbit anti-Vasa, to mark germ plasm (Thomson and Lasko, 2004), mouse anti-phospho-Tyrosine to mark the cortex. These embryos were also counterstained with DAPI to visualize nuclei. The distance between centrosomes in prophase, nuclear cycle 10 budding nuclei was observed in Nikon Elements and Image J software. Though most individual nuclei show appropriate separation, both *Δgcl* embryos and *Eb1* RNAi embryos show segregation defects in some nuclei (**Figure 3**). Typically, this defect presents as a shortened distance between centrosomes with a spindle that is not parallel to the cortex of the embryo,

which would inhibit PGC budding. To quantify centrosome separation in prophase, we will perform replicates that includes anti-Asterless and anti-phospho-Histone 3, which binds phosphorylated Histone 3 at serine 10 during prophase (Hans and Dimitrov, 2001). With this replicate data, we will be able to more accurately identify prophase nuclei at nuclear cycle 10 and measure the distance between centrosomes.

Co-localization of Gcl and Eb1 proteins in various genetic backgrounds

Our genetic interaction data suggest that Gcl and Eb1 may functionally interact as a complex required for PGC formation, which suggests that the loss of either protein may affect the localization of the other. To examine this possibility, we have tested whether loss of either *gcl* or *Eb1* results in altered protein localization. Analyzing embryos at nuclear cycles 9 and 10 via immunofluorescence shows that Gcl localization is not affected by the absence of Eb1 protein (**Figure 4**). Gcl is observed at the nuclear envelope, but is also observed in brighter puncta dispersed around the nucleus. This localization of Gcl has not been previously identified, however, and so further replicates are necessary as these data are unexpected. Additionally, Eb1 protein localization appears to be more diffuse at the posterior pole in a null-Gcl background (**Figure 4**). However, further replicates and quantification of signal intensities are needed to confirm these results. These data hint that a complex may form between Eb1 and Gcl, affecting function and possibly localization of Eb1.

Immunoprecipitation of Gcl and Eb1 protein

To test the hypothesis that Gcl and Eb1 form a biochemical complex, we performed immunoprecipitation experiments. We first verified protein content of our fly strains via Western

blot using a noncommercial Gcl antibody (Thomson and Lasko, 2004) and a noncommercial Eb1 antibody (Rodgers, et. al. 2002) against an anti-actin loading control (1:2000, DHSB: JLA20) (**Figure 5**). Our noncommercial Gcl antibody demonstrated a clear lack of Gcl protein in *Δgcl* embryos, indicating the specificity of this antibody. Our noncommercial Eb1 antibody showed consistent, non-specific binding at approximately 50 kDa that can be used as a loading control. Eb1 RNAi knock-down (Figure 5B, lane 3) shows a partial reduction of Eb1 levels, indicative of RNAi-mediated partial gene silencing. Future work will measure the extent of Eb1 depletion through quantification of additional anti-Eb1 Western blots.

We began immunoprecipitation experiments with anti-Gcl antibodies to validate the existence of a biochemical Gcl-Eb1 complex. Anti-Gcl antibodies were immobilized on an affinity matrix consisting of magnetic beads, which were then be incubated with 0-2 hour wildtype embryonic extract. To control for nonspecific binding, we incubated magnetic beads either conjugated rabbit anti-GFP antibodies (Fisher Scientific, A11122) (Figure 6A, lane 3, 6, 6B, lane 3) or no antibody (Figure 6A, lane 4, 7, 6B, lane 4) in 0-2 hour wildtype embryonic extract. Western blotting was used to verify that Gcl protein specifically elutes from these beads (Figure 6A, lane 2), while immunoblotting for anti-Eb1 (Figure 6B, lane 2) currently fails to validate the presence of a Gcl-Eb1 complex in wildtype embryos (**Figure 6**). As the verification of a binding interaction between Gcl and Eb1 would provide evidence of a functional complex formed by these proteins, we will attempt to isolate Gcl from lysate washed over magnetic beads conjugated to anti-Eb1 antibody, as well.

Discussion

Through this research, we have specifically identified centrosome defects that affect microtubule organization. When either Gcl, Eb1, or both proteins are not present, embryos fail to adequately produce PGCs. We suspect that these centrosome segregation defects are related to the extension of microtubules, a process which pushes apart the centrosomes that nucleate them. Other research on highly mitotic cells has shown that the dissociation of Eb1 from microtubules slows their extension, resulting in a decreased segregation phenotype that negatively affects mitotic spindle formation (Buttrick et. al., 2019). We suspect that Eb1, as an interacting partner of Gcl, facilitates the physical pushing of centrosomes apart, inducing PGC budding (**Figure 7**). Our data shows that similar phenotypes exist in *gcl* and *Eb1* mutant backgrounds, which indicates that these proteins may work within the same pathway.

Double mutant backgrounds show an increased phenotype severity, which may indicate negative epistasis. Though traditional genetics would suggest that a double knock out mutant for epistatic genes will mimic the phenotype of the upstream gene's knock out mutant, negative epistasis, or a decrease in fitness in a double knock out background for epistatic genes, has been previously identified in other pathways (Wolf, 2000). We suspect that mutants lacking both *gcl* and *Eb1* have such a significant decrease in cytoskeletal stability at nuclear cycle ten that cellularization is not possible. Future work using live imaging may be useful to test this model. *Eb1* knock down mutants already display centrosome segregation defects, and combining those defects with a lack of PGC induction signaling seems to inhibit any ability to form PGCs.

The association of centrosome defects with failure to form PGCs is unsurprising, as previous research has also shown that centrosomes are necessary and sufficient for PGC budding

(Raff and Glover, 1989). In the absence of nuclear replication, centrosomes will independently migrate to the cortex of the embryo, reorganize cytoskeletal elements, and attempt to facilitate PGC budding despite the complete lack of posterior nuclei (Raff and Glover, 1989). This indicates that germ plasm components associate with centrosomes independently of the nucleus, which supports the idea that Gcl may have cytoplasmic action independent of its localization to the nuclear envelope. It has been shown that centrosomes associated with posterior nuclei induce germ plasm release from the cortex and mediate active transport of germ plasm components away from the cortex and toward nuclei, and ultimately toward centrosomes, which is sufficient to produce PGCs (Lerit and Gavis, 2011). This demonstrates a highly active role of the centrosome in PGC budding, and further suggests the importance of interaction between centrosomes and the germ plasm. A Gcl-Eb1 complex that functions to promote microtubule growth at nuclei approaching the posterior would enhance the release of germ plasm from the cortex and its recruitment to posterior nuclei. Though initial germ plasm assembly is unaffected in *Δgcl* embryos, a failure to appropriately release germ plasm from the cortex could cause a reduction in proper PGC formation.

Our data suggests one indirect mechanism of action for Gcl in centrosome segregation that may connect to other proposed models for Gcl action in the cytoskeleton. Cinalli and Lehmann link Gcl to the contractile anillin ring that completes PGC cellularization, demonstrating premature somatic cellularization when Gcl is mislocalized and proposing a spindle-independent function of Gcl in PGC budding (Cinalli and Lehmann, 2013). While this model provides evidence of additional functions of Gcl, it model fails to account the centrosome segregation defects in *Δgcl* embryos that link Gcl to the cleavage spindle. These models are not mutually exclusive, however, as Gcl protein may have multiple functions that promote PGC

budding. Centrosome segregation must occur before anillin ring contraction, and it is plausible that Gcl remains active in the cytosol during the entire PGC budding process, coming to its final localization at the nuclear envelope after PGC formation is complete.

Moving forward, we aim to expand our understanding of this mechanistic pathway by investigating the relationship of an Eb1 associated protein kinase, Akt. Centrosome segregation defects identified in mitotic cells in an Akt mutant background mimic phenotypes seen in mitotic cells in Eb1 mutant backgrounds (Buttrick et. al., 2019). The model proposed for Akt's mechanism shows that Akt facilitates microtubule anchoring by Eb1 by assisting in the recruitment of Eb1's protein complex (Buttrick et. al., 2019). We believe that Gcl may have a similar effect, binding with Eb1 to assist in the recruitment or formation of the Eb1 microtubule anchoring complex. The similarity in *Δgcl* and *Eb1* RNAi identified phenotypes leads us to believe that these proteins are working within the same pathway, but the increased severity of these phenotypes in a double knock out background suggests the interaction is not solely an activation or repression. This increased phenotype severity, and the previous identification of Gcl-Eb1 interaction (Giot, 2003), suggest the formation of an active complex that exhibits negative epistasis. It is possible that other factors, such as Akt, also functionally cooperate with Gcl to mediate PGC formation.

Clarifying the molecular function of *gcl* gives mechanistic context to the phenotypes linked to its loss-of-function and increases our understanding of early germ cell development in *Drosophila*. In the future, we plan to further validate an Eb1-Gcl complex through immunoprecipitations of Eb1 in wildtype vs. null-Gcl tissue and Gcl in wildtype vs. null-Eb1 tissue. We will also continue to replicate centrosome separation experiments and localization experiments to increase our statistical power. Further, we will investigate a potential role for Akt

in PGC budding through genetic cross experiments and phenotype identification. Due to the high conservation of genes like *gcl* and *Eb1*, mechanistic understanding of these pathways in *Drosophila* will offer insight into germ cell development in humans. A better understanding of the protein-protein interactions necessary for germ cell development can provide avenues for the development of specific drug therapies and targeted treatments for infertility. As sterility-linked mutations in *gcl* have been identified in men (Kleiman et. al, 2003), and spermatogenesis occurs consistently throughout an adult male's life, we have the potential to administer treatment at any point after sexual maturation. The identification of a novel Gcl-Eb1 protein complex may be of broad interest for infertility research.

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Figures

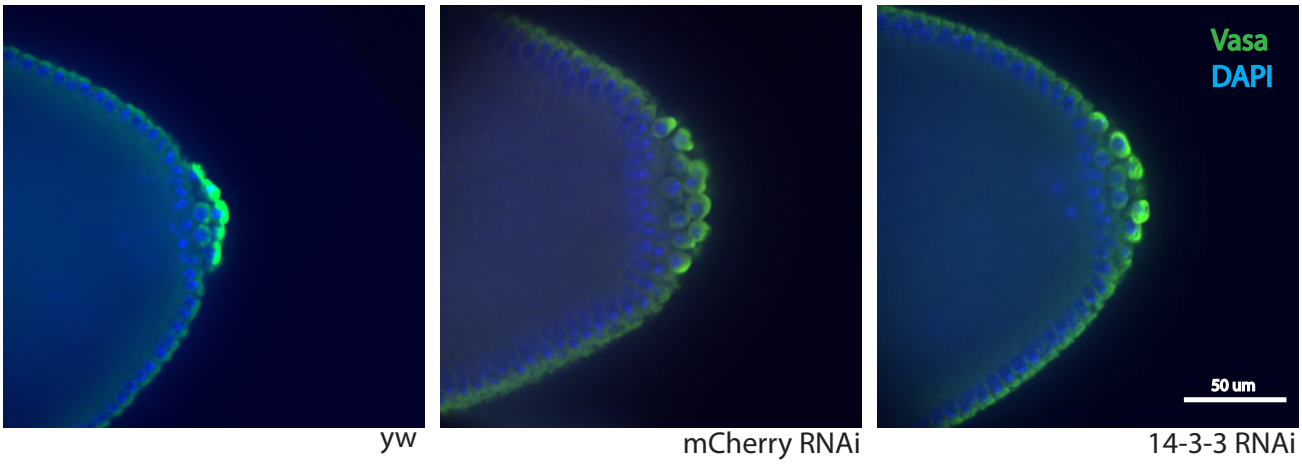
Table 1: *Drosophila* strains and genotypes

Fly Line	Genotype	Source
Δgcl	*Δgcl*	Robertson, et al. 1999
Eb1 II RNAi	*UAS-Eb1* (TRiP.HMS01568}attP40)	Bloomington *Drosophila* Stock Center, 36680
Eb1 III RNAi	*UAS-Eb1* (TRiP.GL00559}attP2)	Bloomington *Drosophila* Stock Center, 36599
14-3-3 RNAi	*UAS-14-3-3z* (TRiP.GL01310}attP40)	Bloomington *Drosophila* Stock Center, 41878
mCherry RNAi	*UAS-RFP* (VALIUM20-mCherry}attP2)	Bloomington *Drosophila* Stock Center, 35785
nosGAL4	GAL4::VP16-nos.UTR}MVD2	Bloomington *Drosophila* Stock Center, 7303
matTubGAL4	*maternal 67C Tubulin-GAL4*	Lee et al., 2001
Δgcl; *matTubGAL4*	*Δgcl; maternal 67C Tubulin-GAL4*	Bred in lab
Δgcl; *Eb1* RNAi	*Δgcl; UAS-Eb1* (TRiP.GL00559}attP2)	Bred in lab

Table 1 *Drosophila* strains used and their sources.

Figure 1: Wildtype PGC budding shown in RNAi controls

A



B

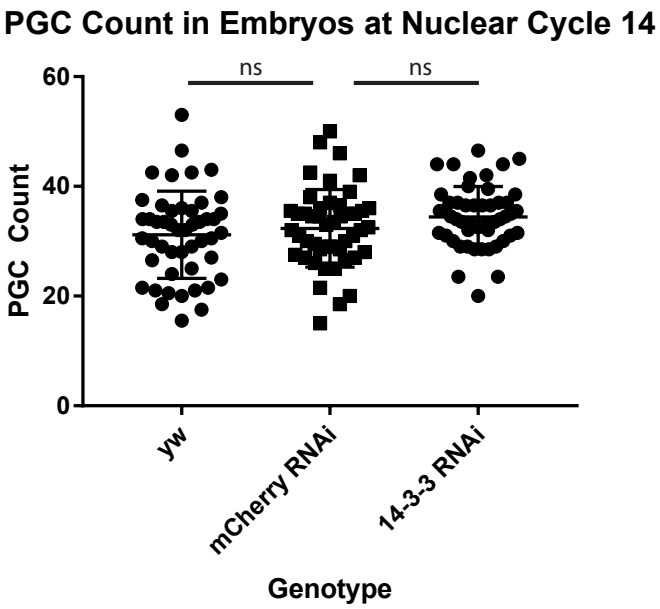
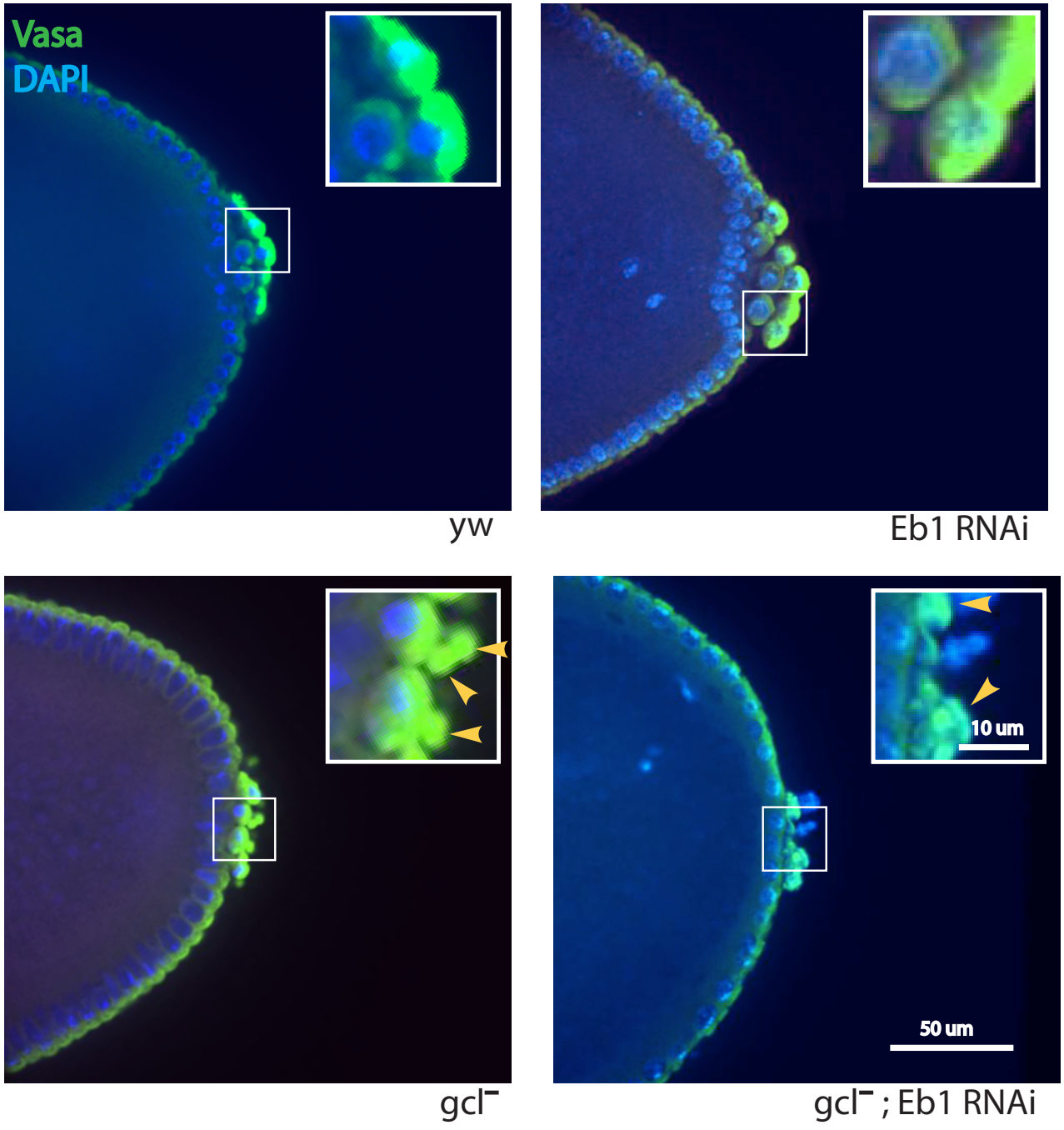


Figure 1 *yw* embryos are used as a wildtype control. Normal budding is shown in *mCherry* RNAi, our negative control, and *14-3-3* RNAi, indicating PGC reduction phenotype specificity to Eb1. (**A**) Representative images of mature PGCs from the indicated genotypes. Vasa is shown in green, nuclei are blue. Scale bar, 50 um. Shown is data from n=3 biological replicates. (**B**) PGC quantification, showing no significant difference in PGC number. *yw*: n= 48 embryos.

Average PGC number=31.2±7.9 PGCs. *mCherry* RNAi: n= 48 embryos. Average PGC number=32.3±7.1 PGCs. *14-3-3* RNAi: n=51 embryos. Average PGC number=34.4±5.5 PGCs. A one-way ANOVA test showed no significant differences between genotypes, p=0.0601. A multiple comparisons test showed no significant difference was shown between genotypes. *yw* vs. *mCherry* RNAi, p=0.6853. *yw* vs. *14-3-3* RNAi, p=0.0515. *mCherry* RNAi vs. *14-3-3* RNAi, p=0.2872.

Figure 2: PGC defects shown in knock-down embryos

A



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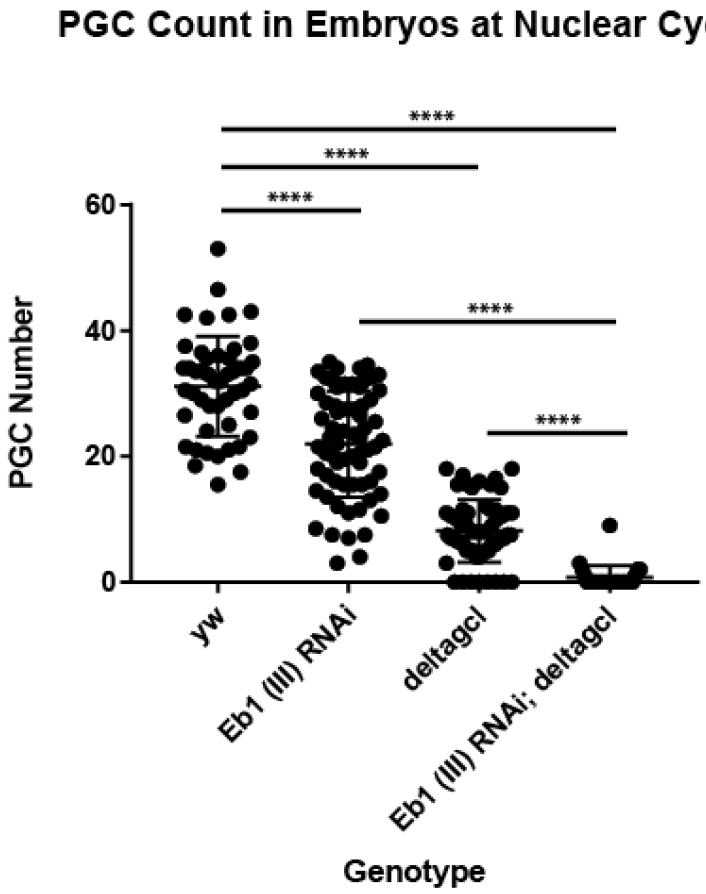

Figure 2 Defects in PGC budding show a reduction phenotype in embryos lacking Gcl of Eb1. *yw* embryos are used as a wildtype control. Embryos lacking both proteins show an increase in phenotype severity. (**A**) Representative images of mature PGCs from the indicated genotypes. Vasa is shown in green; nuclei are blue. Arrowheads indicate blebs, which do not contain a nucleus and would not count as independent PGCs. Scale bar, 50 um. Inset scale bar, 10 um. Shown is data from n=3 technical replicates. (**B**) PGC count quantification, showing significant differences in PGC number between genotypes. *yw*: n= 48 embryos. Average PGC number=31.2±7.9 PGCs. *Eb1* RNAi: n= 66 embryos. Average PGC number=22.0±8.4 PGCs. *Δgcl*: n=59 embryos. Average PGC number=8.2±5.0 PGCs. *Δgcl*; *Eb1* RNAi: n= 26 embryos. Average PGC number=0.8±1.9 PGCs. A one-way ANOVA was performed, showing a

significant difference in PGC count, $p<0.0001$. A multiple comparisons test showed a significant difference in PGC number between each genotype. *yw* vs. *Eb1* RNAi, $p<0.0001$. *yw* vs. *Δgcl*, $p<0.0001$. *yw* vs. *Δgcl*; *Eb1* RNAi, $p<0.0001$. *Δgcl* vs. *Δgcl*; *Eb1* RNAi, $p<0.0001$. *Eb1* RNAi vs. *Δgcl*; *Eb1* RNAi, $p<0.0001$.

Figure 3: Centrosome separation defects shown in knock-down embryos

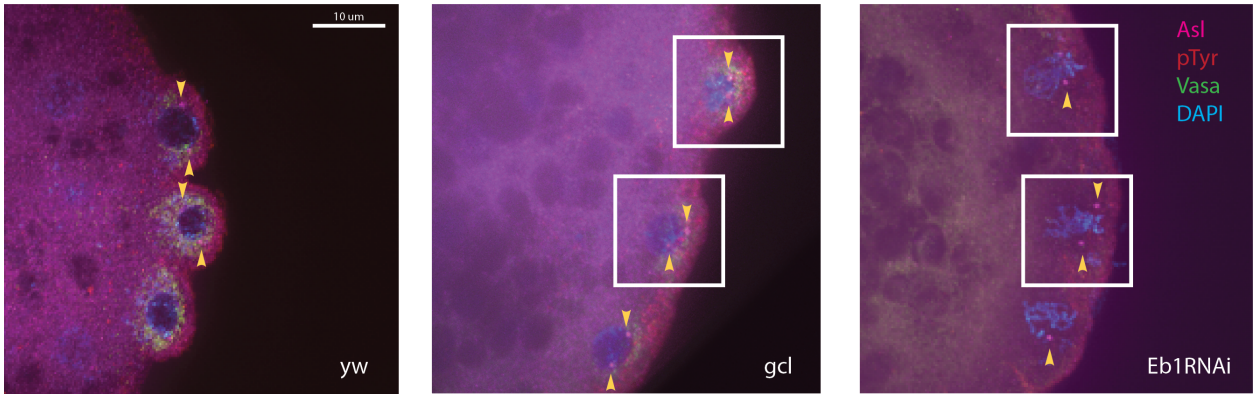

Figure 3 *Δgcl* and *Eb1* RNAi embryos show centrosome segregation defects that appear to misalign the spindle during PGC budding and occasionally shorten the distance between centrosomes. Embryos shown in representative images are stained for centrosomes in magenta (Asl), germ plasm in green (Vasa), and the cortex in red (pTyr). Nuclei are blue (DAPI). Boxes indicate defected nuclei and arrowheads indicate centrosomes. Scale bar, 10 um. Shown is data from *yw*: n=7, *Δgcl*: n=10 and *Eb1* RNAi: n=2 biological replicates. Further staining will include phosopho-histone 3 (pH3), which marks mitotic nuclei, for quantification of centrosome separation.

Figure 4: Localization phenotypes by genetic background

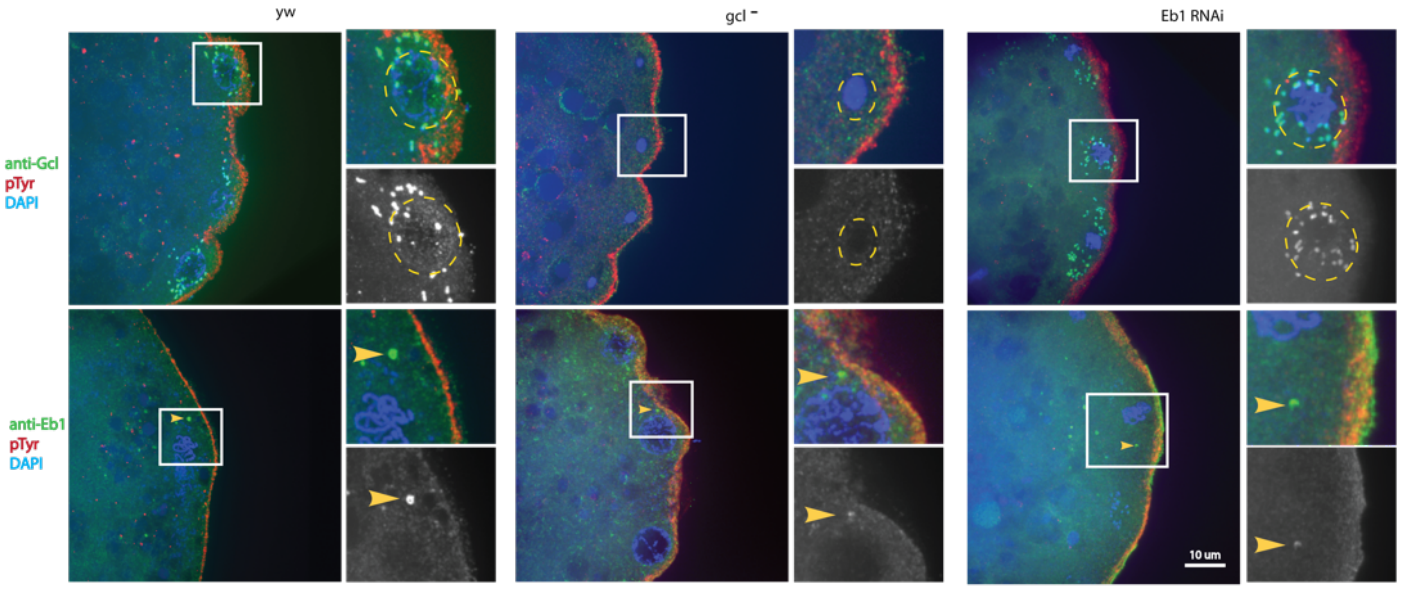

Figure 4 Gcl shows localization at the nuclear envelope, as expected, but also shows bright puncta around posterior nuclei that do not seem to be disrupted by the lack of Eb1. This data is unexpected, and further replicates will help us identify the relevance of these puncta. Eb1 shows localization at the centrosomes, and appears by eye to be more diffuse in Gcl-null embryos. Proteins of interest, Gcl and Eb1, are shown in green in the indicated images. Insets show proteins of interest, Gcl or Eb1, in grayscale. The cortex is marked in red (pTyr), and nuclei are blue (DAPI). Scale bar, 10 um. Shown is data from n=1 technical replicates.

Figure 5: Antibody verification via Western blot in wildtype embryonic tissue

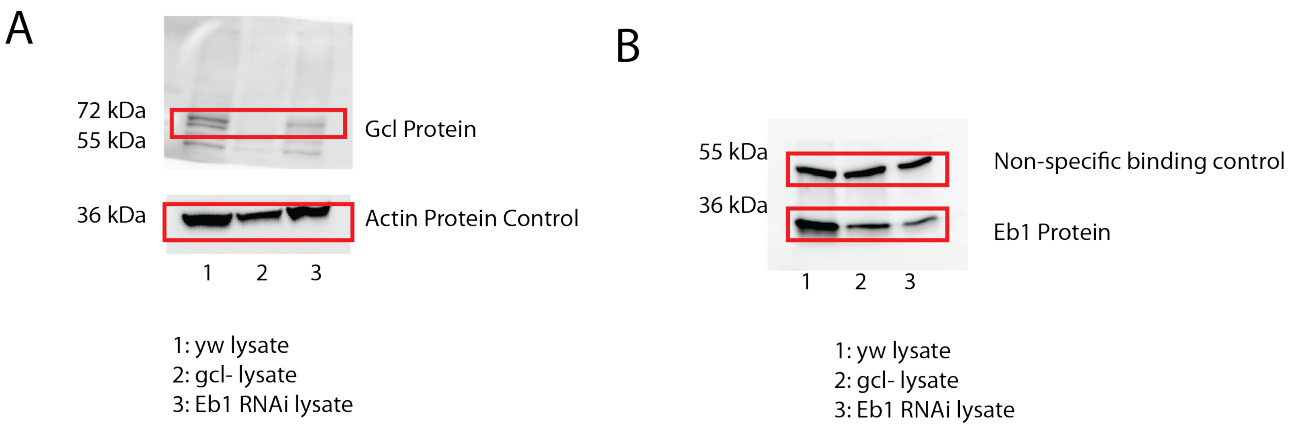

Figure 5 Western blot analysis of non-commercial anti-Gcl and anti-Eb1 antibodies. **(A)** Analysis of our anti-Gcl antibody verifies our genotype, showing a total lack of Gcl protein in *Δgcl* embryos (lane 2). Gcl content appears reduced in *Eb1*RNAi tissue by eye (lane 3), and we plan to quantify that reduction in the future. Gcl is predicted to run at 65 kDa (Jongens et. al., 1992), and observed to run at approximately 70 kDa. Shown is data from n=3 technical replicates. **(B)** The anti-Eb1 antibody we have received shows significant non-specific binding, however that binding occurs at a much lower molecular weight than Eb1 protein (lanes 1, 2, 3). Eb1 protein content also appears reduced in *Δgcl* embryos (lane 2), which we will verify with future quantification. The protein content shown in Eb1 RNAi embryos (lane 3) is due to the knock-down nature of RNAi. RNAi, as a silencing technique often fails to fully knock-out the target protein. Eb1 is predicted to run at 32 kDa (Rogers et. al., 2002) and observed to run at approximately 30 kDa.

Figure 6: Immunoprecipitation results via Western blot

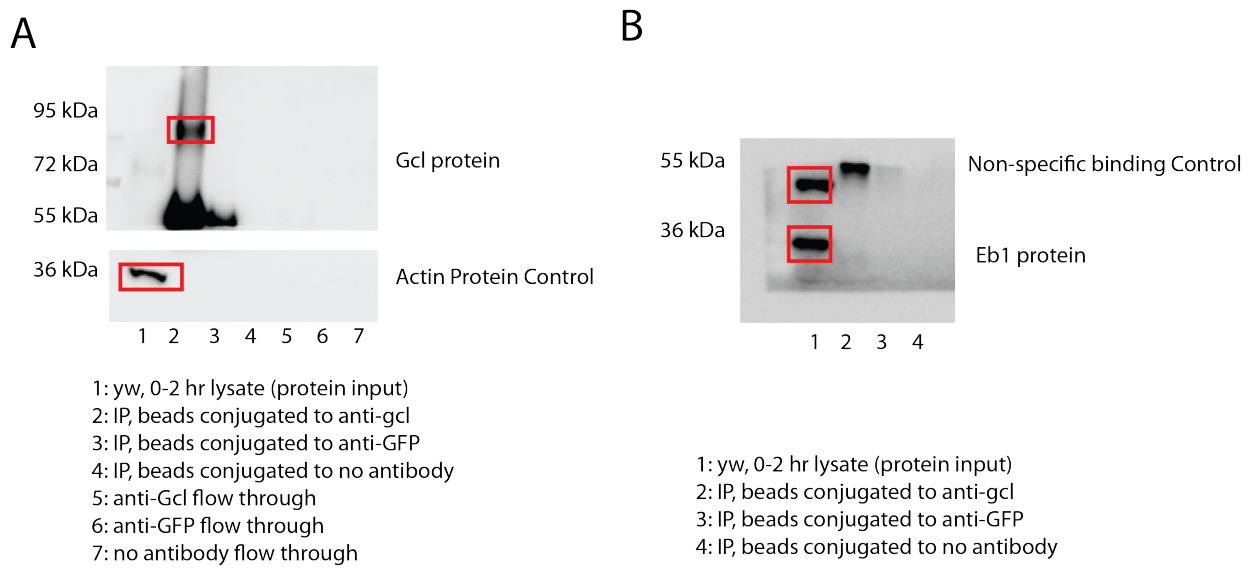

Figure 6 Western blot analysis of our immunoprecipitation does not identify the presence of a Gcl-Eb1 complex in wildtype, 0-2 hour embryos. Anti-Gcl antibodies were conjugated to magnetic beads, which successfully pulled Gcl out of wildtype embryonic lysate. Shown is data from n=2 technical replicates. **(A)** Gcl is highly enriched on the beads after immunoprecipitation (lane 2) and our anti-Gcl antibody is not washing out in the flow through (lanes 5, 6, 7). Non-specific binding is observed at a significantly lower molecular weight than Gcl (lanes 2, 3, 4). Gcl is predicted to run at 65 kDa (Jongens et. al., 1992), and observed to run at approximately 80 kDa. **(B)** Our data does not yet show that Eb1 is found specifically in a Gcl immunoprecipitation sample. Samples shown in B are from the same lysates as samples shown in A. We intend to re-run this IP, attempting to pull Gcl out using anti-Eb1 antibody conjugated to magnetic beads. Eb1 is predicted to run at 32 kDa (Rogers et. al., 2002) and observed to run at approximately 30 kDa.

Figure 7: Model of potential Gcl-Eb1 protein complex mechanism

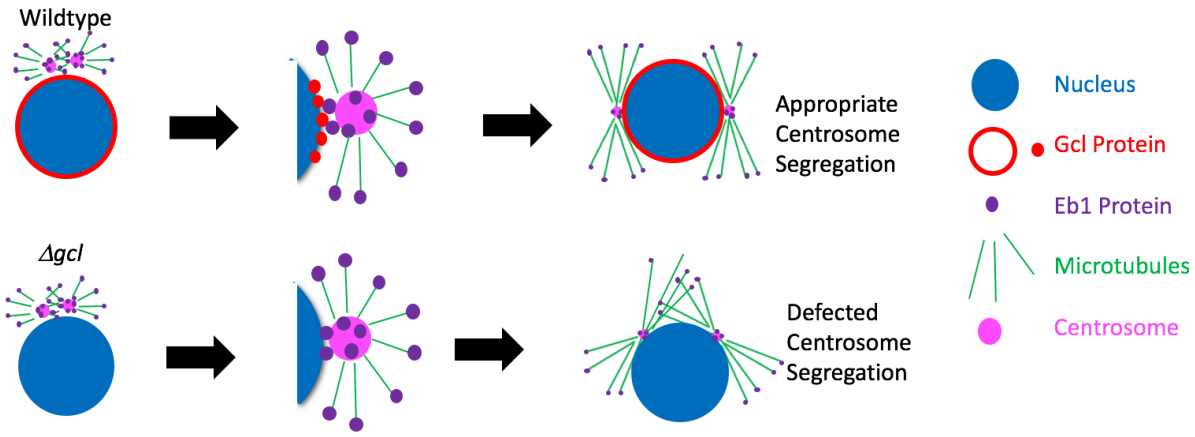

Figure 7 Our model suggests that, in the presence of Gcl, Eb1 facilitates early microtubule growth in PGC nuclei and promotes the centrosome segregation that physically pushes the nuclei into the cortex of the embryo. We propose that Eb1, at the centrosome, forms a complex with Gcl, at the nuclear envelope, which promotes centrosome segregation as Eb1 then localizes to the plus-end of the growing microtubule. Without Gcl protein, Eb1 does not push microtubules to extend fully and centrosomes fail to fully separate.