Protein Phosphatase 1ß Limits Ring Canal Constriction during Drosophila Germline Cyst Formation

Shinya Yamamoto, Vafa Bayat, Hugo J. Bellen, Change Tan

Introduction

The first step in sexual reproduction is the formation of functional male and female gametes. A key feature of gamete formation in many organisms is incomplete cytokinesis (IC), in which contractile rings during cytokinesis constrict, but do not fully close and generate cysts [1–8]. The arrested contractile rings are then modified to form stable intercellular bridges, also known as ring canals, whose diameters increase at later stages of gametogenesis [9–15].

We have recently shown that germline cyst formation in D. melanogaster females serves as a good model to study IC [9] (Figure 1A–B). In the germarium, a germline stem cell (GSC) divides asymmetrically via complete cytokinesis to form another GSC and a cystoblast (Figure 1B). The cystoblast then undergoes four-round mitotic divisions, via IC, forming a cyst with 16-interconnected cystocytes. Each IC proceeds through five distinct stages, with the four mitotic divisions being: (1) stages Ia to Ie, (2) IIa to Ile, (3) IIIa to IIIe, and (4) IVa to IVe. Then, the 16-cell cyst develops via nine additional stages, four in region 2a, four in region 2b, and one in region 3, resulting in a stage 1 egg chamber. The stage 1 egg chamber then leaves the gerarium and continues to develop in the vitellarium through 13 stages, forming a mature stage 14 egg (Figure 1A). IC staging is based on the levels of the continuous ER network, and is required for their formation [9–15]. ER localizes to the contractile ring, ring canal, and/or nuclei, with levels and distribution dependent on the cell cycle and the cyst age [9–15].

In a previous study, we identified the Drosophila Myosin Phosphatase Targeting Protein (DMYPT) as a critical regulator of IC [20]. DMYPT is highly enriched in cells undergoing IC. Loss of DMYPT in germ cells results in over-constriction of contractile rings and ring canals during IC, especially after the fourth mitotic division and prior to ring canal growth (Figure 1C). As a result, minute ring canals form in DMYPT mutants that prevent intracellular nurse cell cytoplasm transport, resulting in...
Figure 1. Germline cyst formation during *D. melanogaster* oogenesis. (A) A schematic drawing of an ovariole. An ovariole is composed of an anterior germarium (boxed) and a posterior vitellarium containing an array of developing egg chambers. The germarium is subdivided into regions 1, 2a, 2b, and 3. The cyst in region 3 is also referred to as a stage 1 egg chamber. The vitellarium is subdivided into stages 2–14 with stage 14 being a mature egg. Ring canals start to grow in size in region 2b, reach their maximal sizes at stage 10, and degenerate after nurse cell dumping, a rapid phase of transporting the nurse cell cytoplasm into the oocyte. A schematic representation of some ring canals is shown in the vitellarium. (B) Stages of *D. melanogaster* germline cyst formation. *D. melanogaster* germ cells divide in a fixed pattern. Each mitotic division is characterized by five distinct stages (a-e). Following the final mitotic division, there are eight additional distinct stages in region 2 (IVf–nona-2b4) and a final one in region 3. Numbers beside ring canals (blue) indicate their mitotic origins. Note that ring canals are organized along the fusome (yellow). (C) Top: Regulation of non-muscle myosin II. Myosin II is a hexameric enzyme consisting of two heavy chains (MHC/Zip), two regulatory light chains (MRLC/Sqh), and two essential light chains (MELC) (only three subunits are shown here to simplify the image, the fly homologs are shown after “/”). The activity of myosin II is regulated by the phosphorylation of MRLC/Sqh. Myosin light chain kinase and several other kinases phosphorylate MRLC and activate myosin. In
contrast, myosin phosphatase (MLCP) dephosphorylates phospho-MRLC and inactivates myosin. The myosin phosphatase is composed of three subunits, the myosin binding subunit MYPT (DMYPT in D. melanogaster), the catalytic subunit PP1cB (Flw in D. melanogaster), and a small subunit M20 of unknown function (no M20 has been identified in D. melanogaster). The DMYPT has been shown being required for IC, but whether Flw or M20 functions during IC is unclear. Bottom: A schematic view of incomplete cytokinesis and ring canal formation. An M1 ring canal is shown as an example. The same principle applies to M2, M3, and M4 ring canals but with different starting points. For each ring canal, its starting point is its birth mitotic division. The units for time and ring sizes are arbitrary. Neither the ring size nor the time is to scale. In wild-type flies, during each germline cytoocyte mitotic division a contractile ring constricts and is then arrested when it reaches its maximal constriction point. A fusome plug forms in the arrested contractile ring and marks the conversion of the contractile ring into a ring canal. The fusome plug then fuses with the fusome from earlier mitotic divisions and grows to form a mature fusome. The ring canal does not change in size during the subsequent mitotic divisions. When all four mitotic divisions are finished, the fusome begins to degrade, and eventually disappears. Ring canals start to grow at stage nona-2b1, after a slight constriction. Similar events occur in DMYPT heterozygotes. In the homozygous DMYPT mutants, contractile rings constrict to a greater degree than those in heterozygotes resulting in smaller nascent ring canals. The ring canals remain at that size until the fusome starts to degrade. Although ring canals constrict only slightly after the final mitotic division in the presence of DMYPT, they constrict dramatically in its absence. Figure 1A are adapted and modified from [9], while 1B and 1C from [20].
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Results

A Germline Mosaic Screen of X-linked Essential Genes Identifies One Complementation Group, with Defects in Incomplete Cytokinesis Similar to DMYPT Mutants

To identify novel genes required for IC, we performed an unbiased genetic screen (Figure S1) of a collection of about 2,000 lethal mutations on the X-chromosome [29–31]. We reasoned that a mutation interfering with IC would cause oogenesis failure, leading to infertility, and the resultant ring canals would be small, as seen in DMYPT mutations, or be oversized, opposite to the phenotype seen in DMYPT mutants. Thus, we first screened for mutations laying no or only non-functional and deformed eggs under a dissecting microscope and then screened these oogenesis mutations for ones that affected ring canal morphogenesis with phalloidin staining analyses under a compound microscope. After that we investigated which mutants had IC defects by immunostaining and confocal microscopic analysis.

In the primary screen, we generated females carrying homozygous germline clones (GLCs) of the mutations using the Flippase-dominant female sterile (FLP-DFS) technique [40]. 1,798 of the crosses generated female offspring with the desired genotype. These mutant lines differed greatly in their ability to produce eggs (fecundity), with egg production ranging from 0 to over 40/animal/day. Eggs from some mutant lines also had highly aberrant morphology. Consistent with previous reports that most essential genes affect oogenesis [41,42], we found that 1,073 mutations (60%) are associated with oogenesis defects with either diminished fecundity (laying less than 3 eggs/animal/day) or leading to eggs with morphological deformities.

In a secondary screen, we screened these 1,073 mutant lines with oogenesis defects for aberrant actin ring canal morphology. We generated flies carrying GLCs marked by the absence of red fluorescence protein (RFP) and stained their ovaries for F-actin with phalloidin. Five independent mutant lines exhibiting small actin ring canals throughout oogenesis were identified (Figure 2A-B and data not shown). No small ring canal phenotype was observed when the homozygous clones were only in the somatic follicle cells (Figure 2C), demonstrating that the gene is required in the germ cells to control ring canal morphogenesis. Homozygous GLCs of these mutants produced small, non-fertile eggs (Figure 2D, homozygous XE55E on the left and heterozygous on the right).

Interestingly, all 5 mutants failed to complement each other’s lethality, indicating that they are alleles of the same complementation group. We named this complementation group XE55, and

small, non-functional eggs. DMYPT mutations have no effect on the number of mitotic divisions and do not affect cell fate determination of germ cells.

How DMYPT functions during IC is still unclear. Several studies have shown that MYPT can form a tripartite myosin light chain phosphatase (MLCP) with a catalytic serine/threonine Protein Phosphatase 1 (PP1) β (also known as PP1B in vertebrates) and a small subunit M20, and together the three inactivate myosin II by dephosphorylating phosphorylated myosin II regulatory light chain, encoded by the *spinigerous (spg)* gene in *Drosophila* (Figure 1C) [reviewed in [21] and [22]]. Thus, one hypothesis is that the *D. melanogaster* PP1B, encoded by *flapwing* (flw) [23], functions during IC with DMYPT. However, Vereshchagina and colleagues found that *flw* played no role during early oogenesis, but instead was required for ring canal growth in late stages of oogenesis [24]. Recently, Sun and colleagues found that *flw* functions in follicle cells to control oocyte polarization, but they did not investigate the role of *flw* in the germ cells during IC [25]. *D. melanogaster* has three other PP1s, named according to their isotypes and cytological locations: PP1α567B, PP1α13CG, and PP1α396A, as well as another DMYPT-like molecule, MYPT-75D [26,27]. Flw binds both DMYPT and MYPT-75D, but unlike MYPT-75D, DMYPT also binds PP1α076B. Furthermore, vertebrate MYPT has been identified from cell culture studies as an interaction platform for a broad range of proteins [22]. A recent large-scale proteomic and interactomic study using Parallel Affinity Capture coupled to mass spectrometry found that Flw also has many binding partners [28]. Thus, whether other DMYPT-interacting proteins play regulatory roles during IC needs further investigation.

To identify additional players in IC, we performed an unbiased FLP/FRT-mediated germline mosaic screen on a collection of Ethyl Methane Sulfonate (EMS)-induced lethal mutations on the X chromosome [29–31]. Through screening of ~1,800 X-linked recessive lethal stocks, we identified five mutations that show an IC phenotype, very similar to that seen in DMYPT mutants. The ring canals are severely over-constricted after the fourth mitotic division and before ring canal growth, although the constriction of contractile rings is arrested during the mitotic divisions of germ cells. Through genetic and molecular mapping of these mutants, all five mutations turned out to be alleles of *flw*. We found that *flw* was expressed in germ cells undergoing incomplete cytokinesis, completely colocalized with DMYPT. Our genetic interaction data suggests that *flw* and DMYPT function together to negatively regulate myosin II activity. The discrepancy between this study and the earlier study by Vereshchagina et al. seems to have resulted from the difference in the allelic strengths used in the studies. The identification of two subunits of myosin phosphatase as the first two main players required to regulate ring canal constriction indicates that tight regulation of myosin activity is essential for germ cell development.
Figure 2. Homozygous XE55E germline clones, but not follicle clones, lead to formation of minute actin-ring canals and small eggs. (A–C) Stage 10 egg chambers with heterozygous XE55E (A), homozygous XE55E in germ cells (B), or homozygous XE55E in somatic follicle cells (C). Actin phalloidin-staining on the left and nuclear RFP images on the right. The genotypes of the germ cells and somatic cells of the egg chambers are as indicated in the figure. The heterozygous XE55E is XE55E/+ P{ovoD1} y FRT19A hsflp. The inserts are magnified views of the ring canals marked with arrows in each panel. Note that small ring canals formed only when the egg chamber contains homozygous XE55E in its germ cells. (D) Stage 14 eggs with homozygous XE55E (I) or heterozygous (II) XE55E in germ cells. White field image on the left and nuclear RFP image on the right. Scale bars: 10 μm for A–C, 100 μm for D.

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refer to the alleles as XE55A-E in the order of identification. Being the first two mutations identified, XE55A and XE55B were used in most of the characterization experiments described later. The identification of only five mutations showing the same phenotype in a collection of about 2,000 mutations indicated that the screen was highly specific.

To determine whether the small actin ring canals of GLCs of the XE55 mutations result from a disruption of IC, we immunostained ovaries carrying RFP-marked GLCs with antibodies against Anillin, a marker for both contractile rings and early ring canals (Figure 3, green), and z-spectrin, a fusome marker (Figure 3, red). Ring canals in a heterozygous XE55 mutant (Figure 3A, and data not shown) are indistinguishable from those of wild-type (00B) flies (Figure 5B, also compare movies S1 and S2). As expected, all XE55 mutations disrupted IC, resulting in tiny Anillin-staining ring canals caused by over-constriction of contractile rings and ring canals (Figure 3C-G and movie S3). Some of the ring canals (marked with asterisks) were so small that no center void could be detected with light microscopy and classified as unmeasurable because their diameters were too small to be measured for quantitative analysis of the ring canal size for the following analyses. Such unmeasurable ring canals are extremely rare in wild-type cysts or in heterozygous XE55 or heterozygous DMYPT mutant cysts. The phenotype of the XE55 mutations is very similar to that of DMYPT mutants (Movies S1–3 and [20]). Surprisingly, we found that a strong lethal mutation of flw from Sun and colleagues [25] also produced the same IC defect (Figure 3H and Movie S4), contrary to the report by Vereshchagina and colleagues [24].

It is worth noting that in the XE55 mutant GLCs, cystoblasts proceeded through the normal set of four mitotic divisions with IC to form 16 interconnected cells, with one differentiating into an oocyte (Figure 2B and data not shown). Fusome formation and distribution were also unaffected (movies S1–3). Thus, these 5 mutations do not affect the number of mitotic divisions and the determination of cell fate and, thus, are all genuine IC mutations.

To further characterize the precise defects occurring during IC in XE55 mutant GLCs, we examined the 15 ring canals from a stage IVg homozygous XE55A cyst (Figure 4A) and compared the ring canal diameter to those of a same-age heterozygous cyst (Figure 4B). The ring canals in the homozygous XE55A cyst are obviously smaller; in fact, four of its 15 ring canals are unmeasurable (Figure 4A arrows).

To determine at which stage the mutant phenotype becomes severe, we assessed the phenotypes of XE55 mutant cysts at different stages. We found that XE55 phenotypes were more severe after the four mitotic divisions were complete than during those divisions (Figure 5, movies S1–3). For instance, at stages II or III, the ring canals of homozygous XE55A mutant clones (Figure 5B and D and movie S3) appeared similar to those of heterozygous (Figure 5A and C and movie S2) or wild-type (movie S1) cysts. However, at stage IVg, the sixth stage of the fourth mitotic division and before ring canal growth, the ring canals of homozygous XE55A mutant clones (Figure 5F and movie S5) were obviously smaller than those of heterozygous or wild-type cysts (Figure 5E and movies S1–2). This is in contrast to the behavior of a normal ring canal, which constricts only slightly after the fourth mitotic division, before they increase in size (movie S1 and [20]).

This observation was confirmed by a comparison of ring canal diameters of heterozygous and homozygous XE55A mutant cysts at two stages, stage II and stage IVg (Figure 5 G–H). Significant ring canal diameter differences were detected between heterozygous (grey) and homozygous (dotted). XE55A mutants at stage IVg but not at stage II (Figure 5G). Figure 5H shows the percentage of unmeasurable ring canals at stage IVg. These data show that the constriction of contractile rings in homozygous XE55 mutants were arrested, albeit constricted further than in heterozygote or wild-type flies, and that the newly formed ring canals do not change in size greatly while the cysts are actively dividing but over-shrink after mitotic divisions are finished, a phenotype reminiscent of DMYPT mutants (Figure 1C and [20]).

**XE55 Complementation Group Maps to flapwing, Encoding the Drosophila Homolog of Protein Phosphatase 1B**

Through duplication mapping, involving flies carrying large X chromosomal segments translocated to the Y chromosome [43] and molecularly defined P[acman] BAC duplications [36], we mapped the lethality of XE55 mutations to a region containing the four genes, flw, Rhab2, CG12640, and CG32683 (Table 1 and Figure S2). Since flw encodes a predicted subunit of the myosin phosphatase, and since mutants for DMYPT, a regulatory subunit of myosin phosphatase, have similar IC defects [20], we hypothesized that XE55 mutants are allelic to flw, even though this hypothesis was contrary to a previous report [24]. Indeed, XE55 mutants failed to complement two previously reported semi-lethal flw mutations: flw<sup>α</sup>, an EMS-induced mutation, and flw<sup>β</sup>, a mutation caused by a P-element insertion [23,24]. In addition, transheterozygotes for XE55 and flw<sup>α</sup>, a viable, EMS-induced point mutation that when homozygous results in flightless flies [23], were also flightless. Furthermore, the lethality of XE55 mutants (except XE55C) could be rescued by Ga4/UA5-AIS-induced expression of flw cDNA transgene in somatic cells (Table 1) [23,24,44]. Finally, an independently identified, strong flw mutation caused similar IC defect (Figure 3H above and movie S4). These data indicate that XE55 are alleles of flw, and thus we renamed XE55A-E flw<sup>XE55A-E</sup>.

To identify the responsible molecular lesions, we isolated genomic DNA from hemizygous flw<sup>XE55A-E</sup> larvae and sequenced the flw open reading frame. flw is predicted to produce two transcripts through alternative splicing, a long (Flw-pA) and a short isoform (Flw-pB) [45]. The Flw-pA isoform has an extra, Drosophila genus-specific, coding exon compared to Flw-pB. As a consequence, Flw-pA is 131 amino acids longer than Flw-pB. We identified unique single point mutations in the flw coding region for each of the 5 alleles (Figure 6A). flw<sup>XE55A-E</sup> carries a missense mutation (Flw-pB R93W; Flw-pA R226W), while alleles flw<sup>XE55A</sup>–<sup>XE55D</sup> carry various nonsense mutations. All flw mutations, including the five identified in our screens and those previously reported [23–25,28] are shown in Figure 6. flw<sup>XE55A-E</sup>, flw<sup>XE55A</sup>, and flw<sup>XE55D</sup>, and flw<sup>XE55C</sup> may affect both the active site and DMYPT binding or lead to a protein with low stability due to the truncation. Since flw<sup>XE55A</sup> survives only to larval stages, while flw<sup>XE55A</sup>–<sup>XE55D</sup> die at pupal stages (Table 1), flw<sup>XE55D</sup> may be a null allele or a strong hypomorph. Note that it was previously reported that flw<sup>PP1</sup> also die as larvae, while 1% of flw<sup>α</sup> and 2% of flw<sup>β</sup> hemizygous males survive to adult stages [23–25].

**Flw and DMYPT Colocalize in Cells Undergoing Incomplete Cytokinesis**

To determine whether Flw is expressed in the right place to play a role during IC, we obtained two independent Flw-yellow fluorescence protein (YFP) fusion protein-trap lines: flw-YFP-159 and flw-YFP-284 (Figure 6A and [28]). Both Flw-YFP fusion proteins were expressed in the germarium and enriched in the
Figure 3. Homozygous GLCs of XE55 mutations cause over-constriction of Anillin-stained ring canals during IC. Confocal images of part of germaria co-immunostained with antibodies against anillin (green) and α-spectrin (red). All cysts are at stage IVg, the sixth stage of the 4th
Figure 8C and D). On the other hand, the extent as the homozygous (upward slash) produced over-constricted ring canals to the same multiplication, and determined the ring canal sizes at two different stages, double heterozygous flies generated few (caused by halving DMYPT dosage (Figure 8B). Interestingly, the number of unmeasurable ring canals was further supported by an increase in the germline in both wild-type and fly. Hence, DMYPT mutants enhanced the phenotype of a hypomorphic fly mutation and the two genes interact genetically. This conclusion was further supported by an increase in unmeasurable ring canals caused by halving DMYPT dosage (Figure 8B). Interestingly, the double heterozygous flies generated few (<3%) cysts with unmeasurable ring canals, and this did not change with cyst age (compare the percentage of unmeasurable ring canals at early stage IVg on the left of Figure 8B and stage 2b on the right). In contrast, unmeasurable ring canals increased with time in homozygous flyXEs5A germline cysts (Figure 8B, compare the grey column at early stage IVg on the left with that at stage 2b on the right); by the time a homozygous flyXEs5A cyst reaches region 2b, about half of its ring canals had become unmeasurable (Figure 8B right). Removing a copy of DMYPT significantly increased the frequency of unmeasurable ring canals at both stages and in homozygous as well as homozygous flies (Figure 8B).

The consequences of the ring canal phenotypes are reflected in the eggs formed at the end. Heterozygous flyXEs5A germline cysts produce normal eggs (Figure 8C and D). On the other hand, homozygous flyXEs5A germline cysts formed small, non-functional eggs (Figure 8E), which were made even smaller by reducing DMYPT dosage (Figure 8F and G). These small eggs were likely the result of failure to transport nurse cell cytoplasm into the egg (Figure 8H). The presence of the TM3 balancer chromosome had no obvious effect on the phenotypes of the fliesXEs5A mutation (Figure 8, compare I with C and J with E). The morphological observations of the eggs were substantiated by a comparison of the egg length of flies with different levels of flyXEs5A and DMYPT (Figure 8K).

Our comparisons of ring canal diameters of homozygous DMYPTXEs5A cysts with one copy of sqh<sup>-</sup> and a null mutation of the myosin II regulatory light chain, or flyXEs5A suggest that Sqh may be one of the targets of Flw during IC (Figure 8L). For ring canal size comparisons, we generated three kinds of cysts, all DMYPTXEs5A homozygous (Figure 8L). Group I cysts (black) contained a copy of sqh<sup>-</sup> and thus should have the lowest level of active myosin II. Group II cysts (dark grey) contained an FMR balancer and should have a medium level of active myosin II. Group III cysts (light grey) contained a copy of flyXEs5A so that they should have the highest level of active myosin II. Ring canals in Group I cysts were normally the largest, while those in Group III cysts were the smallest. While we didn’t observe a statistically significant (<p>0.05) difference when DMYPT homozygous mutants alone were compared to DMYPT homozygous mutant in combination with fly/sqh alleles, we consistently observed a trend in which fly mutations enhanced, while sqh mutations suppressed DMYPT mutant phenotypes (Figure 8L). This conclusion is further supported by an increase or decrease in the number of unmeasurable ring canals from removing a copy of DMYPT or sqh, respectively (Figure 8L, insert). These genetic interactions suggest that Flw and DMYPT negatively regulate contractile ring constriction, while Sqh facilitates contractile ring constriction.

To determine whether phospho-Sqh (p-Sqh) levels were altered in fly mutant germlaria, we performed immunostaining using several p-Sqh antibodies. However, the p-Sqh signal is very weak in the germline in both wild-type and fly mutant cells, while we observed that, in follicle cells, fly mutations had increased p-Sqh (Figure 8S). Therefore, while the genetic interaction data suggests that Sqh is a potential target of Flw, the low endogenous p-Sqh levels in the germlaria prevents us from conclusively stating that Sqh phosphorylation is altered in fly mutant cells during IC due to technical reasons.

Discussion

Using an unbiased genetic screen of lethal mutants on the X chromosome, we have uncovered a second player mediating IC, Flw, encoding the Drosophila homolog of serine/threonine Protein Phosphatase 1B. We identified six independent alleles of fly that exhibits similar defects in IC, five from our screen and another from an independent forward genetic screen carried out by Sun et al. [25]. All six alleles had over-constricted ring canals similar to DMYPT mutants. Consequently, small ring canals were formed and the cytoplasmic transfer from nurse cells to oocyte was impaired, resulting in small nonfunctional eggs. Both colocalization and genetic interaction studies suggest that fly functions together with DMYPT, possibly to negatively regulate myosin activity of Sqh. Interestingly, the number of mitotic divisions and cell fates were unaffected, suggesting that fly is specifically required for regulation of IC.

Protein Phosphatase 1B and Incomplete Cytokinesis
they found that ring canals in a flw mutant (flw6) are initially normal but failed to grow, leading to formation of small F-actin-staining ring canals at stage 10, we found that the ring canals of homozygous GLCs of flw6/E-AK are small throughout oogenesis. More importantly, we show that homozygous flw6/E-AK mutant GLCs show IC defects very early on, at a stage Vereshchagina and colleagues did not analyze in their studies. The discrepancy between their study and ours may be due to the different allelic strengths used, which is supported by our lethal phase analysis as well as the rescue results (Table 1). Furthermore, although we have not been able to directly investigate the effect of the flw6 allele they used in their IC studies because the mutant stocks obtained from two sources have become viable and fertile (presumably due to the loss of flw6 or the accumulation of suppressors), we found that GLCs of homozygous flw6/E-AK, an independently isolated, strong allele [25], also produced over-constriction of contractile rings and ring canals, similar to XE55 alleles (Figure 3H and movie S4). The data that six different alleles of flw isolated independently from two laboratories caused IC defects strongly supports our conclusion that flw is indeed a gene necessary for successful IC. Since Flw and PPI8 are highly conserved, we speculate that PPI8 may function as a common factor mediating germline cyst formation in other organisms.

Several lines of evidence suggest that Flw and DMYPT form a bona fide myosin phosphatase during IC. Firstly, the mutations of flw and of DMYPT exhibited similar phenotypes [this study and [20]]. Secondly, even though there are four PPI1 encoding genes in D. melanogaster, the flw mutation alone is sufficient to cause a strong IC phenotype. This suggests that the role played by Flw during IC cannot be substituted by any other endogenous PPIs, either because they are not expressed in the germ cells undergoing IC or they function differently. Consistently, two of the four D. melanogaster PPIs, PPI1a/13C and PPI1a/19C, are not required for fertility [26,27], and thus are unlikely to function during IC, at least not by themselves alone. The role of PPI1a/19C during oogenesis remains unknown, and we did not observe any germline clones with a null allele of PPI1a/19C, indicating that it may be essential for germline cell viability and have a more general housekeeping role (data not shown). Thirdly, mutations in DMYPT alone are sufficient to cause an IC defect [20], though another myosin-binding regulatory subunit, MYP3T-75D, is present in the fly genome. DMYPT is most similar to human MYPT1, with both containing leucine zipper motifs at their C-termini, and highly conserved, inhibitory, Rho kinase phosphorylation sites in their central regions [46]. In contrast, Drosophila MYPT-75D is most similar to human MYPT3, lacking the Rho regulatory phosphorylation sites. Instead, MYPT-75D has SH3 sites and the C-terminal prenylation motif CAAX. Both MYP3T-75D and DMYPT can bind p-Sqh and inactivate myosin II [24,47]. Based on immunoprecipitation assays, DMYPT binds PPI1a/19C, as well as Flw, while MYP3T-75D only binds Flw [24]. The physiological relevance of these interactions is unclear. No MYP3T-75D mutants have yet been identified to date. Although we cannot rule out the possibility that MYP3T-75D may also be involved in IC, it is unlikely that DMYPT and MYP3T-75D have redundant functions in IC, as mutations in DMYPT alone are sufficient to cause IC defects [20]. Since the IC defects found in flw mutants are identical to DMYPT mutants, it is likely that Flw forms a complex with DMYPT during IC to negatively regulate myosin activity. This is supported by the colocalization of Flw and DMYPT in cells undergoing IC, as well as the enhancement of the flw mutation-caused oogenesis defects by DMYPT mutations and vice versa.

The involvement of flw and DMYPT during IC does not exclude their potential roles in later stages of oogenesis or in other tissues.
In fact, both *flw* and *DMYPT* are known to be highly pleiotropic. For example, *flw* and/or *DMYPT* mutations in follicle cells affect oocyte polarity, egg chamber shape, and border cell migration and their mutations also interfere with other developmental processes [25,37,47–50]. Nonetheless, unlike homozygous *flw* mutations in germ cells, homozygous *flw* mutations in follicle cells do not affect ring canal morphogenesis (Figure 2G). Thus, Flw activity in germ cells is essential for IC and ring canal formation in a cell autonomous manner.

Figure 5. The *XE55* IC phenotype is more severe after all four mitotic divisions than during those divisions. (A–F) Confocal images of anillin (green) and α-spectrin (red) immunostained germ line cysts at stages IId (A–B), IIId (C–D), and IVg (E–F). Genotypes of the germ cells housing the ring canals of interest are heterozygous *XE55A* for panels A, C, and E and homozygous *XE55A* for panels B, D, and F. Homozygous germine mosaic clones, marked by the absence of RFP (not shown), are outlined. The numbers mark the mitotic origins of ring canals. Two normal M4 ring canals from heterozygous *XE55A* (F, right) were also labeled for comparison; the top M4 ring canal appeared small because the current focal plane does not reveal its real diameter. All images have the same magnification. Scale bar: 2 μm. (G) Average diameters of ring canals of cysts with heterozygous (grey) or homozygous (dotted) *XE55A*. Stage II is on the left and stage IVg on the right. (H) Percentage of ring canals too small to be measured. Error bars are standard error of the means. Numbers in panels G and H are ring canals measured or counted. The ring canal diameters that are significantly different (P<0.05) are marked with asterisks. 14 germaria were analyzed. Data shown is from a single representative experiment. doi:10.1371/journal.pone.0070502.g005
In conclusion, using an unbiased genetic screen, we have identified a novel role for Flw during IC, unexpected from previous studies on the roles of flw during D. melanogaster oogenesis [24,25]. Flw is expressed in cells undergoing incomplete cytokinesis, completely colocalized with DMYPT, and alleles of flw cause over-constricted ring canals during germline cyst formation. The identification of critical roles for both the catalytic subunit and the regulatory subunit in the process of incomplete cytokinesis during germline cyst formation is relevant for both the study of the cyclin-dependent kinase (CDK) complex and the myosin II binding subunit, which is involved in myosin phosphatase activity during IC.

Materials and Methods

Fly Strains

The fly strains we used include the following: OreR, P(w1118) y FRT19A hsFlp from David Bilder (UC Berkeley [32,33]), flw FRT19A/FM7 from Trudi Schupbach (Princeton University [25]), sqhAX3/FM7 from Roger Karczew (Université Paris Diderot [34,35]), y w FRT19A; zyg1/Cyo, Df(1)[s]-L15, y' w/ C(1)DX, y' w' f'; Dp(1;2) y 75d/+; flw y/FM7, flw y/FM7, actin5C-GAL4 hsFlp122, FRT19A, DMYPT03802/TM3 Sb and P[acman] BAC duplication lines [36] from the Bloomington Drosophila Stock Center (BDSC), flo-u FLP-159 (w1118) P(bac.681.P.FSVS-1) flw CPT1001360 and flo-u FLP-284 (w1118) P(bac.681.P.FSVS-1) flw CPT1001360 [28] were obtained from the Drosophila Genetic Resource Center (DGRC: Kyoto, Japan).

Fly husbandry and crosses

Fly stocks were maintained on standard cornmeal-glucose food. Newly eclosed flies were fed with active yeast daily for optimal oogenesis until dissection. Germline clones (GLCs) were generated according to [37] using progeny of y w mutant FRT19A/FM7; Kr-GFP crossed with P[w1118] y FRT19A hsFlp/Y [32,33] for the primary screen, and withubi-RFP y hsFlp122 FRT19A/Y for the remaining experiments.

Lethal Phase Analysis

Eggs laid by y w flw XE55 FRT19A/FM7; Ki-GFP females were grown on standard grape juice-agar plates with yeast paste at room temperature. GFP-negative hemizygous male larvae were transferred onto new plates, and raised until they died. The stages the animals reached at the time of lethality were documented, and classified as “embryonic lethal”, “larval lethal”, “pupal lethal”, “semi-lethal (some survive to adulthood)” or “viable”.

Genetic Interactions

To determine the genetic interactions between flw and DMYPT, we either fixed the copy number of DMYPT and altered the levels of flw or sqh, or fixed the copy number of flw and changed the levels of DMYPT. For the former, we crossed females of sqhAX3/FM7; DMYPT03802 FRT2A/TM3 (or y w flw XE55A FRT19A/FM7; DMYPT03802 FRT2A/TM3) with hs-DMYPT; DMYPT03802/TM3 males and heat-shocked their offspring for 30 minutes in a 37°C water bath each day until eclosion. Flies carrying homozygous hypomorphic DMYPT03802 mutation were generated by rescuing the mutants with heat-shock-induced ectopic expression of DMYPT (hs-DMYPT). After a four-day depletion of the heat-shock-induced DMYPT, homozygous DMYPT03802 flies were dissected and their ovaries immunostained. For the latter, y w flw XE55A FRT19A/FM7; Kr-GFP were crossed withubi-RFP y hsFlp122 FRT19A/Y; DMYPT03802/TM3. Homozygous germline clones were induced by heat-shock their offspring at 37°C at the third instar larval stage for two consecutive days, two hours each day. Two days after eclosion, females were placed into yeasted vials for two days with FM7 males, and then dissected.

Table 1. Lethal phases of flw alleles and rescue data.

| Allele Name | Lethal Phase | Rescue | Duplication | UAS5-Pp1ß9C |
|-------------|--------------|--------|-------------|-------------|
| XE55A       | Pupa         | Yes    | N/A         | N/A         |
| XE55B       | Pupa         | Yes    | N/A         | N/A         |
| XE55C       | Pupa         | Yes/no | N/A         | N/A         |
| XE55D       | Pupa         | Yes    | N/A         | N/A         |
| XE55E       | Larval       | Yes    | N/A         | N/A         |
| flw xe      | Pupa/Adult escapers | Yes | N/A         | N/A         |
| flw xe     | Larval       | Yes    | N/A         | N/A         |

Note: Lethal phase is defined as the maximum stage the animals can reach. Some animals die at earlier stages, and if the culture is too crowded, the lethal phase can be earlier. The lethal phases of flw xe, flw xe, and flw xe are listed as already published [23-25]. In addition, since the eggs for lethal phase analysis were from heterozygous females, maternal contributions were included. Maternal contributions cannot be avoided because Flw is required for oogenesis. All alleles except XE55C were rescued to fertile adults with an flw-carrying duplication Dp(1;3)DC224.
Figure 6. Mapping of XE55 mutations. (A) Molecular nature of various flw mutations. Genomic annotation of flw is shown on the top, with the exons boxed. flw7 is a P-element insertion in the 5’ untranslated region. flw-YFP-159 and flw-YFP-284 are two intronic PiggyBac yellow fluorescence protein trap lines. All other mutations are EMS-induced coding mutations. The three non-coding mutations are shown in the genomic map, while the coding mutations are shown in the annotated proteins. Protein regions shared between Flw-pA and Flw-pB are in blue. The Flw-pA-specific region is in grey. Amino acids are named with single letters. Positions according to the short isoform Flw-pB are in parentheses. Nonsense mutations are in red. flw1, flw6, flw7, flw-YFP-159, flw-YFP-284, and flwFP41 have been described previously [23–25,28]. Flw translation start and stop codons are indicated on the genomic map with green and red lines, respectively. The enzyme active site and the DMYPT binding site of Flw predictions are based on the Conserved Domain Database, which consists of a collection of well-annotated multiple sequence alignment models for full-length proteins (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). (B) Flw mutations alter conserved residues. Protein sequence alignment of the short peptide pB of D. melanogaster Flw with PP1ß from Zebrafish (Danio rerio), Frog (Xenopus tropicalis), Mouse (Mus musculus), Dog (Canis lupus familiaris) and Human (Homo sapiens). The amino acids mutated in Flw are boxed. Amino acid substitutes are included below each corresponding mutation, with “*”s indicate stop codons. Amino acids common to the majority of organisms are shown above the position lines and represented with dots in the alignment. Note that PP1ß is highly conserved across phylogeny, with only one amino acid difference between human PP1ß and Xenopus PP1ß or mouse PP1ß. The alignment was done using DNAStar software.
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Figure 7. Flw is co-expressed with DMYPT in the germarium and flw mutations have no effect on the expression and localization of DMYPT. (A–B) Germaria of two independent Flw-YFP protein trap lines were stained with anti-GFP and DMYPT antibodies. Both Flw-YFP proteins colocalize with DMYPT signal in regions of the germaria where IC is taking place. (A) A germarium of flw-YFP-159 line. (B) A germarium of flw-YFP-284 line. (C–D) Germaria were immunostained with antibodies against DMYPT (green) and α-spectrin (red). Homozygous germline mosaic clones are marked by the absence of RFP (white). (C) A germarium with heterozygous flwX55B mutation. (D) A germarium with homozygous flwX55B germline clones. The boundaries of homozygous clones are outlined. A and B have the same magnification, so do C and D. Scale bars: 10 μm (A–B), 5 μm (C–D).

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Figure 8. Genetic interaction of flw, DMYPT, and sqh during oogenesis. Data shown for each panel is from a single experiment, though all experiments were repeated. Numbers in the columns of the histograms are ring canals or eggs measured or counted. Error bars are standard error of the means. The values that are significantly different are marked with asterisks (**p<0.05, ***p<0.01, ****p<0.0001). (A) Average diameters of ring canals of heterozygous flw^{RESA} with the balancer TM3 (black) or a copy of DMYPT^{RESA} (upward slash), or homozygous flw^{RESA} germline clones with TM3 (grey) or a copy of DMYPT^{RESA} (downward slash). Stage II is on the left and stage IV on the right. 24 and 28 germaria were analyzed for those with TM3 and those without, respectively. (B) Percentage of unmeasurable ring canals at early stage IVg (left) and stage 2b (right) of the same germaria analyzed in panel A. (C–J) Stage 14 eggs developed from germline cysts with various levels of Flw and DMYPT. (C) Heterozygous flw^{RESA}, (D) Heterozygous flw^{RESA} with heterozygous DMYPT^{RESA}, (E) Homozygous flw^{RESA}, (F–H) Homozygous flw^{RESA} with heterozygous DMYPT^{RESA}, (I) Heterozygous flw^{RESA} with TM3, (J) Homozygous flw^{RESA} with TM3. All homozygous flw^{RESA} eggs were germline clones generated in the corresponding flw^{RESA} heterozygous females. All images have the same magnification. Scale bar: 100 μm. Eggs in panels F and H are slightly younger than those in other panels and still contain undegraded nurse cells. Nurse cells and the oocyte in panel H were outlined with dashed and solid lines, respectively. (K) Average length of mature eggs with various levels of Flw and DMYPT. (L) Average diameters of ring canals of homozygous DMYPT^{RESA} cysts with a copy of null mutation of sqh (sqh^{RESA}, black), or the FM7 balancer (dark grey), or a copy of flw^{RESA} (light grey). Stage II is on the left and early stage IV on the right. Insert: percentage of unmeasurable ring canals. Germinaria analyzed: seven for sqhAX3-carrying flies, nine for FM7-carrying, and eight for flw^{RESA}-carrying.

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Immunohistochemistry

Immunostaining was performed according to [20]. F-actin staining with fluorescently labeled Phalloidin was performed as in [37]. The following primary antibodies were used: rabbit anti-DMYPT antibody, preadsorbed with fly embryos, 1:100 [20], rabbit anti-Anillin, 1 μg/ml (a gift from Christine Field [10]), mouse anti-α-spectrin 1:100 [Developmental Studies Hybridoma Bank (DSHB) [38]], mouse anti-GFP (1:500) [Invitrogen, mAb 11E5 and mAb 3E6], and several antibodies for phosphorylated Sqq: rabbit anti-phospho-MLC2 S19 1:250 and rabbit anti-phospho-NLP-MLC2 T108S19 1:250 from Cell Signaling Technology, rabbit anti-phospho-Sqq 1:400 from Luke Alphey [24], guinea pig anti-Sqh 1P 1:300 and rat anti-Sqh 2P 1:3000 from Robert Ward [39]. Secondary antibodies [Invitrogen] used were Alexa488, Alexa546, or Alexa647 anti-rabbit, mouse, rat, or guinea pig (highly cross-adsorbed if available) (1:500).

Images in Figure 2 and Figure 8C–J were taken using a Leica DM5000 microscope. All others were captured using a Zeiss LSM 510 META NLO (63X oil C-Apochromat objective, zoom 2x, Z step size 0.5 μm) and analyzed using the LSM Image Browser. All figures were prepared using PowerPoint (Microsoft) and Photoshop (Adobe).

Statistics

Statistics were performed using GraphPad Prism v. 6 for Mac (GraphPad Software, La Jolla, CA, USA): Figure 5G, as well as Figure 8A, K, and L: multiple comparisons using Ordinary one-way ANOVA nonparametric tests, Figure 5H, Figure 8B, and the insert of Figure 8L: pair-wise comparisons using two-sided Fisher’s exact tests.

Supporting Information

Figure S1 A flowchart of the procedures to isolate mutations causing defective incomplete cytokinesis. IC mutations were identified in four steps: screening the 1,796 lethal lines for mutations disrupting oogenesis, screening the 1,073 oogenesis mutations for those affecting actin ring canal morphogenesis, screening the small ring canal mutations for IC mutations, and mapping of the IC mutations.

Figure S2 Mapping of XE55 mutations. (A) Duplication mapping of XE55s between X chromosome bands 9B1 and 9F2. Duplication were shown as boxes below a drawing (by C.B. Bridges) of part of a polytene X chromosome. The duplication that rescued the mutation is labeled in pink, while those did not rescue are in grey. Same is true for panel B. (B) Refinement of the locations of the mutations with P[acman] BAC duplications to a region containing four genes. Above the duplications is a gene annotation of the relevant chromosomal region (adapted from http://flybase.org).

Figure S3 Mutations of flw in follicle cells caused an increase of phosphorylated Sqh. Immunostaining of a stage 10 egg chamber from a XE55A/abs-REFP^{RESA} hsFlp122 FRT19A fly with a phospho-myoosin light chain 2 (Ser19) antibody (Cell Signaling Technology, Inc. #3671), which recognizes Sqh phosphorylated at Ser21 (red). Panels A and B are two different focal planes of the same egg chamber. Homozygous clones (boxed with dashed lines) were marked by the absence of RFP (white). Note the increase of phosphorylated Sqh in all the clones. Scale bar: 10 μm.

Figure S4 A confocal Z-stack of a germinaria from an Osr^{fkh} fly immunostained with antibodies against anillin (green) and α-spectrin (red).

Figure S5 A confocal Z-stack of a germinaria carrying heterozygous XE55A mutant. Green: anillin, red: fusome, white: RFP. Note that the heterozygous cysts are similar to the wild type cysts shown in Movie 1.

Figure S6 A confocal Z-stack of a germinaria carrying homozygous XE55A mutant clones. Green: anillin, red: fusome, white: RFP. Homozygous clones are RFP negative. Note that the homozygous clones are similar to the heterozygous cysts in region 1 but contain abnormally small ring canals in region 2.

Figure S7 A confocal Z-stack of a germinaria carrying homozygous flw^{FP41} mutant clones. Green: anillin, red: fusome, white: RFP. Homozygous clones are RFP negative. Note that the homozygous clones are similar to the heterozygous cysts in region 1 but contain abnormally small ring canals in region 2, similar to flw^{RESA}.

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Author Contributions
Conceived and designed the experiments: CT SY. Performed the experiments: CT SY VB. Analyzed the data: CT SY VB. Contributed reagents/materials/analysis tools: CT HB. Wrote the paper: CT SY VB HB.

References
1. Gondos B (1987) Comparative studies of normal and neoplastic ovarian germ cells. 2. Ultrastructure and pathogenesis of dysgerminoma. Int J Gynecol Pathol 6: 124–131.
2. Gondos B (1995) Ultrastructure of developing and malignant germ cells. Eur Urol 23: 68–74; discussion 75.
3. Robinson DN, Coady L (1996) Stable intercellular bridges in development: the cytoskeleton lining the tunnel. Trends Cell Biol 6: 474–479.
4. Guo QQ, Zheng GC (2004) Hypotheses for the functions of intercellular bridges in germ cell development and its cellular mechanisms. J Theor Biol 229: 139–146.
5. Greenbaum MP, Ma L, Matuszk MM (2007) Conversion of midbodies into germ cell intercellular bridges. Dev Biol 305: 389–396.
6. Greenbaum MP, Yan W, Wu MH, Lin YN, Agno JE, et al. (2006) TEX14 is essential for intercellular bridges and fertility in male mice. Proc Natl Acad Sci U S A 103: 4982–4987.
7. Swiatk P, Kubaekiewicz J, Klaj J (2009) Formation of germ-line cysts with a central cytoplasmic core is accompanied by specific orientation of mitotic spindles and partitioning of existing intercellular bridges. Cell Tissue Res 337: 137–148.
8. Hagland K, Neis IP, Stemmark H (2011) Structure and functions of stable intercellular bridges formed by incomplete cytokinesis during development. Commun Integr Biol 4: 359–362.
9. Ong S, Tan C (2010) Germline cyst formation and incomplete cytokinesis during Drosophila melanogaster oogenesis. Dev Biol 337: 84–98.
10. Field CM, Alberts BN (1995) Anillin, a contractile ring protein that cycles from the nucleus to the cell cortex. J Cell Biol 131: 165–178.
11. Straight AF, Field CM, Mitchison TJ (2005) Anillin binds nonmuscle myosin II and regulates the contractile ring. Mol Biol Cell 16: 193–201.
12. Hagland K, Neis IP, Lemus D, Grabbe C, Wescje J, et al. (2010) Cntfr interacts with anillin to control cytokinesis in Drosophila melanogaster. Curr Biol 20: 944–950.
13. Pickney AJ, Ghotzer M (2008) Anillin is a scaffold protein that links RhoA, actin, and myosin during cytokinesis. Curr Biol 18: 30–36.
14. D’Avino PP (2009) How to scaffold the contractile ring for a safe cytokinesis – lessons from Anillin-related proteins. J Cell Sci 122: 1071–1079.
15. Goldbach P, Wong R, Briese N, Sarpal R, Trimble WS, et al. (2010) Stabilization of the actomyosin ring enables spermatoocyte cytokinesis in Drosophila. Mol Biol Cell 21: 1492–1499.
16. de Cuevas M, Spradling AC (1998) Morphogenesis of the Drosophila fusome and its implications for oocyte specification. Development 125: 2781–2789.
17. Lin H, Yue L, Spradling AC (1994) The Drosophila fusome, a germline-specific organelle, contains membrane skeletal proteins and functions in cyst formation. Development 120: 947–956.
18. de Cuevas M, Lee JK, Spradling AC (1996) alpha-spectrin is required for germ cell division and differentiation in the Drosophila embryo. Dev Biol 172: 1395–1398.
19. Snapp EL, Lida T, Frescas D, Lippincott-Schwartz J, Lilly MA (2004) The fusome mediates intercellular endoplasmic reticulum connectivity in Drosophila ovarian cysts. Mol Biol Cell 15: 4512–4521.
20. Ong S, Foote C, Tan C (2010) Mutations of DMYP cause over contraction of contractile rings and ring canals during Drosophila germline cyst formation. Dev Biol 346: 161–169.
21. Grassie ME, Mofiat LD, Walsh MP, MacDonald JA (2011) The myosin phosphatase targeting protein (MYPT) family: a regulated mechanism for achieving substrate specificity of the catalytic subunit of protein phosphatase type I delta. Arch Biochem Biophys 510: 147–159.
22. Matsumaru F, Haraumhe IJ (2000) Myosin phosphatase target subunit: Many roles in cell function. Biochem Biophys Res Commun 269: 149–156.
23. Raghavan S, Williams L, Asham H, Thomas D, Szoer B, et al. (2000) Protein phosphatase Ibeta is required for the maintenance of muscle attachments. Curr Biol 10: 269–272.
24. Verschooriana N, Bennett D, Szoer B, Bicukjer C, Gross S, et al. (2004) The essential role of PPIbeta in Drosophila is to regulate nonmuscle myosin. Mol Biol Cell 15: 4935–4945.
25. Sun Y, Yan Y, Derew N, Sculptchbach T (2011) Regulation of somatic myosin activity by protein phosphatase Ibeta controls Drosophila oocyte polarization. Development 138: 1991–2001.