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A role for *Drosophila* Cyclin J in oogenesis revealed by genetic interactions with the piRNA pathway

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Abstract

Cyclin J (CycJ) is a poorly characterized member of the Cyclin superfamily of cyclin-dependent kinase regulators, many of which regulate the cell cycle or transcription. Although CycJ is conserved in metazoans its cellular function has not been identified and no mutant defects have been described. In Drosophila, *CycJ* transcript is present primarily in ovaries and very early embryos, suggesting a role in one or both of these tissues. The CycJ gene (*CycJ*) lies immediately downstream of *armitage* (*armi*), a gene involved in the Piwi-associated RNA (piRNA) pathways that are required for silencing transposons in the germline and adjacent somatic cells. Mutations in *armi* result in oogenesis defects but a role for *CycJ* in oogenesis has not been defined. Here we assessed oogenesis in *CycJ* mutants in the presence or absence of mutations in *armi* or other piRNA pathway genes. *CycJ* null ovaries appeared normal, indicating that *CycJ* is not essential for oogenesis under normal conditions. In contrast, *armi* null ovaries produced only two egg chambers per ovariole and the eggs had severe axis specification defects, as observed previously for *armi* and other piRNA pathway mutants. Surprisingly, the *CycJ armi* double mutant failed to produce any mature eggs. The double null ovaries generally had only one egg chamber per ovariole and the egg chambers frequently contained an overabundance of differentiated germline cells. Production of these compound egg chambers could be suppressed with *CycJ* transgenes but not with mutations in the checkpoint gene *mnk*, which suppress oogenesis defects in *armi* mutants. The *CycJ* null showed similar genetic interactions with the germline and somatic piRNA pathway gene *piwi*, and to a lesser extent with *aubergine* (*aub*), a member of the germline-specific piRNA

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pathway. The strong genetic interactions between *CycJ* and piRNA pathway genes reveal a role for *CycJ* in early oogenesis. Our results suggest that *CycJ* is required to regulate egg chamber production or maturation when piRNA pathways are compromised.

Background

Cyclin J (CycJ) is a poorly characterized member of the cyclin superfamily of proteins. Cyclins are eukaryotic proteins that contain a cyclin box, a domain that interacts with cyclin-dependent kinases (Cdks) (Hadwiger et al., 1989; Jeffrey et al., 1995). Many cyclins are known to have conserved roles in regulating the cell cycle. In metazoan species from Drosophila to human, for example, A and B cyclins regulate mitotic events, D cyclins regulate progression through G1, and E cyclins regulate entry into S phase (Minshull et al., 1989; Murray, 2004). Other cyclins have conserved roles in regulating transcription or other cellular processes (Lim and Kaldis, 2013). CycJ is conserved in all metazoans, yet it has only been studied in Drosophila were it was originally identified as a Cdk-interacting protein (Finley and Brent, 1994; Finley et al., 1996). The RNA expression pattern of *CycJ* is unique among Drosophila cyclins and suggests a possible role in oogenesis or embryogenesis. *CycJ* mRNA is present almost exclusively in ovaries and early embryos, whereas all other cyclins are expressed in multiple tissues and stages of development (Figure S1) (Arbeitman et al., 2002; Chintapalli et al., 2013; Finley et al., 1996; Graveley et al., 2011). A potential role for CycJ in embryogenesis was suggested in a study showing that injection of syncytial embryos with CycJ-inhibitory antibodies or peptide aptamers resulted in delays of the early nuclear division cycles (Kolonin and Finley, 2000). In another study, however, Althoff et al. examined embryos from *CycJ* null females and observed no obvious cell cycle defects (Althoff et al., 2009). That study also failed to detect CycJ protein expression in embryos using a genomic *CycJ* transgene fused to the green fluorescent protein gene, *GFP*. In contrast, the *GFP-CycJ* fusion could be detected in ovaries in all germline cells. In this study we set out to determine whether CycJ plays a role in ovaries where both the RNA and protein appear to be maximally expressed.

Oogenesis in *Drosophila* takes place in a series of parallel tubular structures called ovarioles, each of which is divided into an anterior region called the germarium and a posterior chain of developing egg chambers (Figure S2). Oogenesis is initiated when one of the two or three germline stem cells (GSCs) located at the anterior tip of a germarium undergoes mitotic division giving rise to a new stem cell and a differentiating daughter cell called a cystoblast (Schupbach et al., 1978; Spradling, 1993; Wieschaus and Szabad, 1979). The new stem cell remains in the stem cell niche at the anterior tip of the germarium where signaling from neighboring somatic terminal filament and cap cells leads to repression of differentiation factors (King and Lin, 1999; King et al., 2001; Song et al., 2004; Xie and Spradling, 1998, 2000). The cystoblast undergoes exactly four rounds of division with incomplete cytokinesis to give rise to 16 cystocytes interconnected by structural cell-cell connections known as fusomes and ring canals. The 16-cell cysts migrate toward the posterior region of the germarium where follicle cells encapsulate them to form egg chambers. One of the germline cells undergoes meiosis and becomes the oocyte while the other 15 undergo endoreduplication to become nurse cells that eventually donate their

cytoplasm to the oocyte through the ring canals. Ovarioles consist of long chains of egg chambers that mature and increase in size during posterior migration culminating in the formation of a mature stage 14 oocyte.

Several aspects of oogenesis depend on gene silencing pathways that involve the ~25-nucleotide small non-coding RNAs called PIWI-associated RNAs (piRNAs), which are synthesized from longer genome-encoded transcripts (Guzzardo et al., 2013; Khurana and Theurkauf, 2010; Thomson and Lin, 2009). piRNAs associate with the PIWI subfamily of argonaut proteins (Piwi, Aub, and Ago3) to silence transposons in the germline and adjacent somatic cells. The piRNA pathways silence transposons either by affecting chromatin structure or by targeting specific transposon RNAs for destruction (Brennecke et al., 2007; Peng and Lin, 2013; Vagin et al., 2006). Over 20 genes are known to be involved in the biogenesis and function of piRNAs, and many additional candidate piRNA pathway genes have been identified by large-scale screens (Czech et al., 2013; Handler et al., 2011; Muerdter et al., 2013). Mutations in several of the piRNA pathway genes (e.g., *armi*, and *aub*) result in transposon derepression accompanied by DNA damage accumulation (Haase et al., 2010; Khurana et al., 2010; Klattenhoff et al., 2007). The DNA damage activates checkpoint kinases that lead to disruption of the dorsal-ventral and anterior-posterior patterning of the oocyte (axis specification). The piRNA pathway also regulates germline stem cell maintenance both cell autonomously and from adjacent somatic cells by mechanisms that are not well understood (Juliano et al., 2011; Kirilly and Xie, 2007; Smulders-Srinivasan et al., 2010).

Here we set out to determine whether or not *CycJ* plays a role in oogenesis. We generated a deletion of the genomic region containing *armi* and *CycJ*, and by adding back individual transgenes, created null mutants for each gene. We show that while oogenesis is normal in the *CycJ* null, the *armi* null produces few egg chambers and mature eggs, all of which have axis specification defects. Surprisingly, in the *armi-CycJ* double null there was a further decrease in the number of egg chambers per ovariole, a drastic increase in the number of differentiated germline cells in each egg chamber, and no mature eggs. The *armi* null defects could be suppressed by mutation in the Chk2 checkpoint kinase gene as shown previously, but the *armi-CycJ* double null defects could not. We observed a similar genetic interaction between *CycJ* and two other piRNA pathway genes, *piwi* and *aub*, suggesting that *CycJ* plays a nonredundant role in oogenesis when the piRNA pathways are compromised.

Results

Construction of CycJ and armi null mutants

We set out to generate a *CycJ* null mutant by first creating a deletion of *armi*, *CycJ* and an uncharacterized gene, *CG14971*, and then replacing each of the three genes with genomic transgenes either individually or in combinations (Atikukke, 2009). We used flippase (FLP) induced recombination between two transposon insertion alleles, XPd07385 and RBe01160 that contain FLP recombination target (FRT) sites (Thibault et al., 2004). The resultant deletion strain, hereafter referred to as *Df(3L)armi-J*, eliminated the genomic region corresponding to all coding and noncoding exons of *armi*, *CycJ* and *CG14971* (Figure 1). The deletion boundaries were confirmed by sequencing (Materials and Methods). The same

three-gene deletion was created independently in other studies (Althoff et al., 2009; Olivieri et al., 2010), but oogenesis defects were not characterized in the individual and double mutants.

The homozygous *Df(3L)armi-J* mutant flies were viable indicating that *armi*, *CycJ* and *CG14971* are not essential for viability or development to adulthood, a conclusion that was also reached in previous studies (Althoff et al., 2009; Olivieri et al., 2010). The deletion, however, resulted in complete male and female sterility, and the females did not lay any eggs (Table 1). As described in detail below, the three-gene deletion resulted in major defects in oogenesis. To analyze the contributions of individual genes to these phenotypes, we constructed transgenic lines containing genomic clones of each gene (Figure 1) individually and in different combinations and tested their ability to modify the *Df(3L)armi-J* phenotypes. Several lines of evidence suggest that all of the oogenesis defects that we observed with *Df(3L)armi-J* are due to loss of either *armi*, *CycJ*, or both genes and not to loss of *CG14971* or disruption of the immediate upstream gene, *eIF5B*. First, *Df(3L)armi-J* complemented a lethal deficiency (*Df(3L)Excel6094*) (Figure 1) that removes *eIF5B*, which is an essential gene (Carrera et al., 2000), suggesting that *eIF5B* is not affected in the mutant that we generated. Transheterozygous *Df(3L)armi-J* / *Df(3L)Excel6094* males and females are viable and fertile, as are animals with *Df(3L)Exel6094* over a deficiency (*Df(3L)Exel6095*) that removes *CycJ*, *armi*, and several downstream genes (Figure 1). Furthermore, ovaries from Df(3L)6094/Df(3L)armi-J females or Df(3L)6094/Df(3L)6095 females showed show no signs of oogenesis defects (Figure S6). Second, we found that addition of the *CG14971* transgene into *Df(3L)armi-J* females either in the presence or absence of the *CycJ* and *armi* transgenes had no effect on any of the phenotypes that we observed (Table 1). Finally, the combination of both *armi* and *CycJ* transgenes was sufficient to fully suppress all of the oogenesis defects described below, suggesting that *CG14971* does not play a role in these phenotypes. Thus, we refer to either homozygous *Df(3L)armi-J* or transheterozygous *Df(3L)armi-J/Df(3L)Exel6095* animals as *armi-CycJ* double null.

Loss of both armi and CycJ results in severely disorganized ovarioles containing compound egg chambers

To look for oogenesis defects that may explain the lack of eggs from *armi-CycJ* double null females, we analyzed ovaries at various time points during the first week after eclosion. We determined that well-fed 3-day old flies were at the optimal age to allow observation of developmental abnormalities in the ovarioles before they became devoid of developing egg chambers. This analysis revealed that *armi-CycJ* double null females have oogenesis defects that were different from either single null fly (described below). Germaria from *armi-CycJ* double null animals were disorganized and the ovarioles generally contained only one egg chamber with many more than the normal number of germline cells (Figure 2B-C). The single egg chamber usually had a terminal filament (Figure 2C-D), suggesting that the germarium failed to package individual cysts and became the only egg chamber. The germline nuclei were of different sizes suggesting that they have undergone different numbers of endoreplicative cycles. Many egg chambers also showed an intense punctate DAPI staining pattern that increased as the females aged beyond 3 days, suggestive of

increasing cell death (arrows in Figure 2E and F). We observed identical phenotypes in ovaries from animals homozygous for *Df(3L)armi-J or* transheterozygous for *Df(3L)armi-J* and the deficiency (*Df(3L)Exel6095*) that uncovers *armi*, *CycJ*, and a number of additional genes (Figure 2B-C). Ovaries from *Df(3L)armi-J* mutants that were over 1 week old contained ovarioles with disorganized germaria, no developing egg chambers, and few visible nuclei (Figure 2G). These observations suggest that as females age, further development ceases and the few egg chambers that initially developed are eliminated, leaving only disorganized germaria.

To determine whether the germline cells in homozygous *Df(3L)armi-J* were differentiated we stained ovaries with antibodies against Hts-RC, a component of the ring canals that connect cystocytes (Robinson et al., 1994), and Orb, an oocyte-specific marker (Lantz et al., 1994). In wild-type ovaries, each cystoblast undergoes four rounds of cell division with incomplete cytokinesis resulting in 16 cystocytes that are connected by 15 ring canals (Figure 3A-B) (Spradling, 1993). One of the two cystocytes with four ring canals eventually adopts the oocyte fate, as is evident from Orb staining, while the other 15 cystocytes undergo endoreduplication to become nurse cells (Figure 3E, H). In the *Df(3L)armi-J* mutant, ring canals appear to develop normally at least initially, though there are far more than the normal number in each egg chamber (Figure 3C-D). Orb staining revealed that the process of oocyte selection takes place, though again there were two or more Orb-staining cells in each egg chamber of the *armi-CycJ* double null, suggesting that multiple cysts are enclosed in a single follicular layer (Figure 3F-G). Combined, these results indicate that in the *armi-CycJ* double null, cystoblast division and differentiation occur, giving rise to several groups of germline cells consisting of nurse cells and an oocyte all contained in a single compound egg chamber.

CycJ transgenes suppress oogenesis defects in the *armi-CycJ* double mutant

To determine whether the absence of *CycJ* contributes to defects observed in the armi-CycJ null, we introduced *CycJ* transgenes into *Df(3L)armi-J* homozygotes and *Df(3L)armi-J/ Df(3L)*Exel6095 transheterozygotes. We used two different genomic *CycJ* transgenes and obtained similar results with each. One transgene contained *CycJ* and its upstream region including *armi* exon 9 and part of *armi* exon 8 (Figure 1). The other transgene included the same region but had stop codons introduced into *armi* exon 8 to guard against the possible expression of a C-terminal fragment of *armi*. We found that introduction of the *CycJ* transgenes significantly suppressed the *armi-CycJ* double null phenotypes (Figure 4C and D). Unlike *Df(3L)armi-J*, the *Df(3L)armi-J* females containing a *CycJ* transgene were capable of producing fully developed stage 14 egg chambers and of laying eggs, albeit at a dramatically reduced rate compared to heterozygotes (Table 1). Introduction of the *CycJ* transgenes also resulted in a dramatic decrease in the frequency of compound egg chambers (Figures 5 and S4). We observed similar results with the two different *CycJ* transgenes and with multiple independent insertion lines. Germline expression of a myc-tagged Cyclin J in the *Df(3L)armi-J* mutant (Figure S5) also reduced the frequency of compound egg chambers (Figure 5 and S4). This indicates that it is likely the coding region of *CycJ* that modifies the *armi-CycJ* phenotypes and suggests that *CycJ* can function in the germline. A *CG14971* transgene did not modify the oogenesis phenotypes in any background. Combined, these

results indicate that the oogenesis defects observed in *Df(3L)armi-J* are due to the absence of both *armi* and *CycJ*.

These results also suggest that the defects observed in the *Df(3L)armi-J* animals that contain a *CycJ* transgene are due to the complete loss of *armi*; i.e., they represent the phenotype of an *armi* null. These *armi* null females produced mature eggs that were often collapsed and that had dorso-ventral axis establishment defects (Table 1), a phenotype previously observed for a strong *armi* loss-of-function allele, $armi^{72.1}$ (Cook et al., 2004). The *armi* null ovaries had significantly fewer egg chambers per ovariole compared to wild-type (Figure S4). *armi* null ovaries also showed frequent mislocalization of oocytes within egg chambers (Figure S3). Rather than being positioned in the posterior end of each egg chamber as seen in wild-type ovarioles, oocytes were found located at random positions in a majority of the *armi* null egg chambers. This phenotype was originally identified in mutants of *spindle C (spn-C)* and was later observed for other *spindle* class genes including *spn-A, spn-B, spn-D* and *spn-E* (Gonzalez-Reyes et al., 1997; Gonzalez-Reyes and St Johnston, 1994). While this severe patterning defect was not reported for the previous *armi* loss of function mutant, it is consistent with the established role for *armi* in axis specification (Cook et al., 2004).

CycJ is not essential for oogenesis

The ability of P{*CycJ*} transgenes to rescue the production of egg chambers with normal numbers of germline cells shows that *CycJ* plays a role in egg chamber formation, packaging, or maturation, at least in an *armi* loss-of-function background. To assess whether *CycJ* is important in the presence of wild-type *armi* we introduced a genomic *armi* transgene (P{*armi*}) into the *Df(3L)armi-J* background to generate a *CycJ* null. Previously it was shown that an *armi* transgene was able to restore fertility to animals with the same three-gene deletion (Althoff et al., 2009). We further found that P*{armi}* completely suppressed all of the specific oogenesis defects associated with the *Df(3L)armi-J* deletion mutant or with the *armi* null (Figures 4B and S4). All oocyte developmental stages appeared normal in the P*{armi}*-complemented *Df(3L)armi-J* ovaries. Specifically, cysts developed with the appropriate number of nuclei and ring canals, the size of nurse cell nuclei suggested normal differentiation and endoreplication, and each ovariole had the normal number of egg chambers with a single oocyte located at the posterior end (Figures 4, 5, and S3). The *P{armi}; Df(3L)armi-J/Df(3L)armi-J* females were fertile and laid eggs with normal dorsal appendages, though only 30% of the eggs hatched and developed normally (Table 1). Providing a *CycJ* transgene along with the *armi* transgene in *Df(3L)armi-J/Df(3L)armi-J* or *Df(3L)armi-J*/*Df(3L)Exel6095* significantly improved the hatching rate to 73 - 76% (Table 1). The introduction of a *CG14971* transgene had no effect on any of the oogenesis phenotypes or hatching rates (Table 1). Thus, while the failure to rescue hatching rates to 100% of wild-type suggests that at least one of the three transgenes we introduced fails to fully complement for embryogenesis functions, all of the oogenesis defects that we describe are accounted for by loss of either *armi* or both *armi* and *CycJ*.

CycJ genetically interacts with multiple members of the piRNA pathways

The strong genetic interaction between *CycJ* and *armi* suggested that *CycJ* may be required when a piRNA pathway is compromised. To further test this possibility we examined the

effect of removing *CycJ* in the background of mutants in two other piRNA pathway genes, *piwi* and *aub* (Figure 4). First, we examined a piwi loss-of-function mutant *piwi[06843]* over a deficiency *Df(2L)BSC145* lacking *piwi*; we refer to *piwi[06843]/ Df(2L)BSC145* as the *piwi* mutant in this study. *piwi* mutant ovaries were examined between zero and two days post eclosion because beyond two days they frequently contained agametic germaria and lacked egg chambers. Similar to the *armi* null, the *piwi* mutant produced only one to two egg chambers per ovariole and occasional compound egg chambers (Figures 4E and S4). Significantly, the *piwi-CycJ* double mutants exhibited a dramatic increase in the frequency of compound egg chambers to a level identical to that seen in the *armi-CycJ* double null (Figures 4F and 5). Addition of a *CycJ* transgene to the *piwi-CycJ* double mutant decreased the frequency of compound egg chambers to the levels seen in *piwi* mutants (Figure 5). These results define a strong genetic interaction between *CycJ* and a second piRNA pathway member.

Next we tested for a genetic interaction between *CycJ* and *aub. aub* is required for synthesis of piRNAs and for silencing of transposons specifically in germline cells (Aravin et al., 2004; Li et al., 2009; Malone et al., 2009; Vagin et al., 2006). *aub* mutants are similar to *armi* hypomorphs with respect to egg morphology, axis defects, accumulation of DNA double strand breaks, and checkpoint activation (Klattenhoff et al., 2007; Schupbach and Wieschaus, 1991). To test for a genetic interaction between *aub* and *CycJ* we generated females that were *CycJ* null and transheterozygous for the *aub* alleles, aub^{HN} and aub^{QC42} (hereafter referred to as *aub* mutants). While the *aub* mutants exhibited a modest decrease in egg laying capacity (75% of wild-type), the *aub-CycJ* double mutants laid no eggs (Table 1). Ovaries from the *aub* mutants had fully developed ovarioles with egg chambers at all stages of development (Figure 4G). In contrast, oocyte development in the *aub-CycJ* double mutant was arrested, with egg chambers rarely advancing beyond stage 7 or 8 (Figure 4H). The *aub-CycJ* ovaries had a significant number of compound egg chambers with multiple oocytes and more than 15 nurse cell nuclei and ring canals (Figures 5 and S3). Unlike the *armi-CycJ* and *piwi-CycJ* double mutants, the compound egg chambers in *aub-CycJ* ovaries often included a disorganized layer of follicle cells nearly separating two fully developed cysts (Figures S3, arrow). The *aub-CycJ* double mutant phenotypes could be fully suppressed by introduction of a *CycJ* transgene (Figures 5 and S4). These observations define a genetic interaction between *CycJ* and *aub*, similar to the genetic interactions of *CycJ* with *armi* and *piwi*. The phenotypes of the respective double mutants have several similarities such that those of *armi-CycJ* and *piwi-CycJ* could be interpreted as more severe than those of *aub-CycJ*; e.g., *armi-CycJ* and *piwi-CycJ* egg chambers arrested at earlier stages of development and produced more compound egg chambers compared to the *aub-CycJ* mutants.

A checkpoint mutation partially suppresses loss of armi function but not the combined loss of armi and CycJ

Theurkauf and colleagues (Klattenhoff et al., 2007), have shown that strong loss-of-function *armi* alleles result in increased and persistent female germline DNA damage and activation of a DNA damage checkpoint dependent on the Chk2 kinase gene (known as *loki* or *mnk* in Drosophila) (Abdu et al., 2002; Masrouha et al., 2003). Activation of this checkpoint appears to be responsible for the axial patterning defect in eggs from *armi* mutants, since

loss of *mnk* function significantly suppresses these *armi* mutant defects. To test if any of the phenotypes observed in the *armi* null or *armi-CycJ* double mutants are also due to checkpoint activation, we looked for suppression by a *mnk* mutant, using the mnk^{p6} allele (Brodsky et al., 2004; Takada et al., 2003). As previously demonstrated, the *mnk* mutation suppressed the patterning defects of the *armi* hypomorph, $armi^{72.1}$ (Table 1) (Klattenhoff et al., 2007). Whereas none of the eggs laid by $armi^{72.1}$/*Df(3L)armi-J* mothers had the normal number (two) of dorsal appendages, 85% of those from mnk^{P6} $armi^{72.1}$/*Df(3L)armi-J* double mutant mothers were normal. The *mnk* mutation also suppressed the patterning defect in the *armi* null. None of the eggs from the *armi* null had normal dorsal appendages, while 77% of the eggs from the mnk^{P6} *armi* null had normal egg morphology (Table 1). Interestingly, the *mnk* mutation also partially suppressed the egg production defects of both the *armi* hypomorph and null mutants. Whereas the *armi* null produced only rare eggs (<1% of the number laid by heterozygous *Df(3L)armi-J* mothers), introduction of the *mnk* mutation resulted in a >17-fold increase in egg production (Table 1). The *mnk* mutant also rescued egg production in the *armi* hypomorph by >2-fold (Table 1). These results suggest that checkpoint activation contributes to not only the patterning defects but also the decrease in oocyte maturation associated with loss of *armi* function. In contrast, the *mnk* mutation did not suppress the developmental abnormalities of the *armi-CycJ* double null mutants. The mnk^{P6}; *Df(3L)armi-J* females produced no eggs and had disorganized ovarioles essentially indistinguishable from those of *Df(3L)armi-J* (Table 1). Thus, while the checkpoint pathway clearly mediates the axis specification functions of *armi*, the *armi-CycJ* double mutant reveals functions for *armi* and *CycJ* that may be downstream or independent of the Chk2 checkpoint pathway.

Discussion

Cyclin J is one of a small handful of cyclins that remain poorly characterized despite their conservation in all metazoans. Cyclin J was originally identified in Drosophila where its mRNA was detected in adult females and embryos prior to cellularization but at no other stages (Finley et al., 1996). This pattern has been confirmed and further refined in several transcriptome studies, which have invariably shown that the *CycJ* message is highest in ovaries and early embryos and virtually absent in other tissues and developmental stages (Arbeitman et al., 2002; Chintapalli et al., 2013; Graveley et al., 2011), unlike all other cyclins that have broader expression patterns (Figure S1). Interestingly, the mosquito (*Aedes aegypti*) ortholog of *CycJ* is also expressed exclusively in ovaries and early embryos (Akbari et al., 2013). The conserved sequence and expression pattern suggested that Cyclin J may play an important role in oogenesis or early embryogenesis. Surprisingly, however, *CycJ* null Drosophila have no obvious defects and are fertile, albeit at a somewhat reduced rate compared to heterozygous controls. This could be explained if Cyclin J has an important function that is redundant with that of another protein, such as another cyclin. Althoff et al. explored this possibility by testing for genetic interactions between *CycJ* and *CycE*, *CycA*, *CycB*, and *CycB3* (Althoff et al., 2009). They observed no genetic interactions, though their results could not exclude the possibility of redundancy with one of these or another cyclin. Instead of being fully redundant with another gene, it may be that *CycJ* is required only under specific conditions. Such condition-specific requirements have been observed for

many genes in a number of organisms, most notably in yeast where only 17% of the genes are essential for growth in standard rich medium, whereas 97% of genes have been shown to be required under one or more specific environmental conditions (Giaever et al., 1999; Hillenmeyer et al., 2008; Winzeler et al., 1999). Our finding that a *CycJ* null mutant modifies the phenotypes of piRNA pathway mutants is consistent with a nonredundant role for Cyclin J in oogenesis under specific conditions; i.e., when some aspect of a piRNA pathway is compromised.

We have shown that mutation of *CycJ* alters the mutant phenotypes of *armi*, *piwi*, and *aub*. In the case of *armi*, the *armi-CycJ* double mutants have dramatic oogenesis defects, including only one egg chamber per ovariole, egg chambers with excess germline cells (compound egg chambers), and a complete failure to produce fully developed eggs. These defects can be partially rescued by introduction of *CycJ* transgenes, including a *CycJ* open reading frame driven by a germline promoter. These observations define a genetic interaction between *CycJ* and *armi* and indicate that *CycJ* plays some role in oogenesis. We observed a similar genetic interaction with *piwi*, where *piwi-CycJ* double mutants produced egg chambers with excess germline cells and this phenotype was rescued with *CycJ* transgenes back to levels observed in *piwi*. The genetic interaction with *aub* was also dramatic. While *aub* or *CycJ* mutant mothers produced eggs at 75% and 65% the rate of wild type, respectively, the double mutant produced no eggs.

The disruption of a piRNA pathway has a number of consequences, any of which could create a requirement for *CycJ*. *piwi* and *armi* are required for production of primary piRNAs in both the germline and associated somatic cells (Malone et al., 2009; Muerdter et al., 2013; Olivieri et al., 2010). Germline cells also possess a unique piRNA production mechanism known as ping-pong amplification that uses the germline-specific argonaut proteins Aub and Ago3 (Aravin et al., 2007; Li et al., 2009; Siomi et al., 2011). The requirement for *CycJ* may result from defects that these distinct pathways have in common since *CycJ* genetically interacts with members of each (e.g., *armi* and *piwi*, or *aub*). For example, loss of function mutations in members of either pathway result in increased transposon activity that can be accompanied by DNA double strand breaks in the germline (Klattenhoff et al., 2007). The DNA damage results in activation of checkpoint kinases such as Chk2 (encoded by *loki/mnk*) that in turn lead to defects in the localization of axis determinants. While any of these conditions may create a requirement for *CycJ*, our finding that a *mnk* mutant can suppress the *armi* null but not the *armi-CycJ* double mutant suggests that *CycJ* is not required specifically as a result of Chk2 activation. A similar analysis will be needed to test whether *CycJ* is required when other DNA damage responses are activated. Alternatively, it is possible that the genetic interactions we observe are a consequence of misregulation of genes other than transposon genes. The piRNA pathways, for example, have been shown to repress a small set of cellular genes (Sienski et al., 2012), any one of which could be responsible for uncovering a requirement for *CycJ*.

A common feature of the double mutants of *CycJ* and *armi*, *piwi*, or *aub* is the increased frequency of compound egg chambers. In both *armi-CycJ* and *piwi-CycJ* ovarioles there is often only one egg chamber and it has a terminal filament (Figure 4D and F), suggesting that packaging of individual egg chambers has completely failed and the germarium has itself

become an egg chamber. These egg chambers contain an excess of differentiated germline cells including multiple oocytes, indicating that germline differentiation has occurred (Figure 3 and S3). Thus, they are unlike mutants that affect germline differentiation, which lead to germaria with an excess of undifferentiated GSC-like cells (Chen and McKearin, 2003; McKearin and Ohlstein, 1995). Proper egg chamber packaging requires several signaling pathways including the Notch and Hedgehog pathways. For example, a mutation in *Delta* (Bender et al., 1993), the main receptor for Notch signaling in the ovary, results in loss of stalk cells, which leads to fusion of adjacent egg chambers and formation of a compound egg chamber similar to those produced in *aub-CycJ* mutants. Mutation of *Hedgehog* (Forbes et al., 1996) results in failure of germline cyst encapsulation by follicle cells, which produces a single compound egg chamber located in the germarium, similar to those seen in *armi-CycJ* and *piwi-CycJ*. Previous studies have suggested that piRNA pathways may also be involved in proper egg chamber packaging, though the mechanisms are not known. For example, mutations of the piRNA pathway member *maelstrom* (Sato et al., 2011) or the piRNA-producing locus *flamenco* (Mevel-Ninio et al., 2007) were shown to produce compound egg chambers. We also observed a low frequency of compound egg chambers in the *piwi* mutants, *aub* mutants, and *armi* nulls (Figure 5). Thus, it appears that mutation of *CycJ* exacerbates a preexisting packaging defect seen in these piRNA pathway mutants. How the piRNA pathway and *CycJ* may affect the signaling pathways that are required for normal egg chamber packaging remains to be determined.

In conclusion, we have demonstrated strong genetic interactions between *CycJ* and three piRNA pathway members, *armi*, *piwi*, and *aub*. *CycJ* is not required for oogenesis under normal conditions but its role was uncovered in the *armi*, *piwi*, and *aub* mutants. The double mutants of *CycJ* and each of these genes have similar phenotypes that can be suppressed with *CycJ* transgenes. Taken together, these data suggest a nonredundant function for *CycJ* in regulating oogenesis when the piRNA pathways are compromised. The double mutant phenotypes suggest that *CycJ* may contribute to the role of the piRNA pathways in egg chamber production or maturation.

Methods

Drosophila strains

All animals were raised at 25°C on standard food. We carried out all crosses at 25°C unless otherwise stated. w^{1118} was used for wild type in all of the control experiments. FRT-bearing transposon insertion alleles, RBe01160 and XPd07385 (Thibault et al., 2004) were obtained from the Exelixis collection at Harvard Medical School (Artavanis-Tsakonas, 2004). The *armi* strong loss-of-function mutant, $armi^{72.1}$ (Cook et al., 2004), was kindly provided by William E. Theurkauf. An allele of the *Drosophila* Chk2 gene, $mnkP^{6}$ (Brodsky et al., 2004; Takada et al., 2003), was kindly provided by Andrew Swan. Stocks for w^{1118}, P{hsFLP}1, y^{1} w^{1118}; Dr^{Mio}/*TM3*, *ry** Sb^{1}, two of the *aub* mutants, aub^{HN} cn^{1} bw^{1} and w^{1118}; aub^{QC42} cn^{1} bw^{1} (Schupbach and Wieschaus, 1991), *VP16::nos-Gal4*, Df(3L)Exel6064, Df(3L)Exel6065, balancer strains, y[1] M{vas-int.Dm}ZH-2A w[*]; M{3xP3-RFP.attP'}ZH-51C, P{ry[+t7.2]=PZ}piwi[06843] cn[1]/CyO; ry[506], and w[1118] ; Df(2L)BSC145/CyO were obtained from the Bloomington *Drosophila* Stock

Center. The P1 clone DS01105 and the BAC clone B22N9 were obtained from the Berkeley *Drosophila* Genome Project (BDGP) resource center.

Generation of CycJ and armi null alleles

We followed the procedure described by Parks et al., for generating a deletion (deficiency) mutant (Parks et al., 2004). We selected transposon elements, RB01160 and XP07385 with insertion sites flanking the region to be deleted (Figure 1). Males carrying RB01160 were mated with w^{1118}, P{hsFLP}1, y^1 w^{1118}; Dr^{Mio}/*TM3*, *ry** Sb^1 females that carry a heat shock inducible FLP recombinase transgene. Progeny males carrying both the RB01160 and FLP were then mated to females carrying XP07385 to generate progeny that contained FLP and the two FRT-bearing elements in *trans*. After 2 days, we subjected vials to a 1-hour heat shock by placing the vials into a 37°C water bath. After a total of 72 hours of egg-laying time, we removed the parents and subjected the vials to daily 1-hour heat shocks for 4 more days. We raised progeny to adulthood, collected virgin females and crossed them to males containing marked balancer chromosomes. Individual white-eyed progeny males having the putative deletion were then crossed pair-wise to virgin females to generate additional progeny for further confirmation by PCR and to make balanced stocks in an isogenic background. The deletion was confirmed by PCR from single flies using transposon-specific primer pairs and multiple pairs of gene-specific primers representing the genes deleted as well as the immediate upstream (*eIF5B*) and downstream (*CG32267*) genes. The resultant deletion mutant, referred to as *Df(3L)armi-J*, eliminated the genomic region corresponding to 670 bases upstream of the *eIF5B* start codon to 180 bases downstream of the stop codon of *CG32267* (Figure 1). Animals bearing deficiencies were verified by PCR using the following primer pairs. *Df(3L)Exel6094*: Ex94L (5' CGA GGC CGG AAC CAA GGA GC) and TnLeft (5' TAC TAT TCC TTT CAC TCG CAC TTA TTG); Ex94R (5' CCG CAA TCC GGA AAG TTT TTC G) and TnRight (5' TTT ACT CCA GTC ACA GCT TTG); *Df(3L)Exel6095:* Ex95L (5' GCC AAG TTG GCA GGT GGG CA) and TnLeft; Ex95R (5' AGC GCC TAA GCA GTT GCA GCA) and TnRight. *Df(3L)armi-J*: PA_Df(3L)armiJ_R (5' TAA CTG TCG AGC GAA TGG AAG CGA) and RB (5' GCA TCA AAG AAC AAG CCG GCC AAG).

Transgenic constructs for rescue experiments

Independent transgenic lines carrying genomic copies of *armi*, *CycJ*, or *CG14971* were generated by cloning the genomic region containing each individual gene along with the immediate upstream and downstream regions into the P-element vector, pCaSpeR (Thummel, 1992). To construct pCaSpeR-*CycJ*, we isolated a 4kb *Bgl*II fragment from genomic clone DS01105 and inserted it into the *Bam*HI site of pCaSpeR1. This fragment contained from 1.1 kb upstream of the *CycJ* ATG to 1.9 kb downstream of the *CycJ* stop codon, and includes the last exon of *armi*, which encodes a part of the helicase domain but without regulatory elements, and 400 bp downstream of the *CG14971* stop codon. pCaSpeR-*HZ14-CycJ* was constructed by cutting pCaSpeR-*CycJ* with SpeI, which uniquely digests in *armi* exon 8, filling in with Klenow (New England Biolabs), and religating. This introduced a frame shift 12 codons into the *armi* coding region in exon 8. To construct pCaSpeR-*armi*, a 6.3 kb genomic region containing *armi* and the neighboring region from 695 bp upstream of the *armi* ATG to 310 bp downstream of the *armi* stop codon, which

excludes coding regions from the neighboring gene, was amplified from BAC clone B22N9 using the primers 5'GAATCTACGAGCGGCCGCCGATCACTAGGGTATTTATGG3' and 5'CCATGGACGCCTAGGCCATTGTATCGAAATTGAATGC3' (RA30 and RA39) that also introduced *Not*I and *Avr*II restriction sites, respectively. The PCR product was digested with *Not*I and *Avr*II and ligated to pCaSpeR2 cut with *Not*I and *Xba*I. To construct pCaSpeR-*CG14971*, a 4 kb genomic region corresponding to *CG14971* from 820 bp upstream of the ATG to 897 bp downstream of the stop codon, excluding any of the neighboring gene coding regions, was amplified from P1 clone DS01105 using the primers, 5'CCATGGACGCTCTAGACGGCGTAGAAGAAAAAATGATCG3' and 5'GAATCTACGAGCGGCCGCCAAAGATTAGTAAAAGGG3' (RA09 and RA10) that introduced the restriction sites *Not*I and *Xba*I, respectively. The PCR product was digested with *Not*I and *Xba*I and ligated to pCaSpeR2 cut with *Not*I and *Xba*I to create pCaSpeR-*CG14971*. High fidelity polymerase, (Herculase, Stratagene Inc.) was used for all PCR and the resultant constructs were verified by DNA sequencing. Constructs were microinjected into w^{1118} *Drosophila* embryos to induce P-element mediated germline transformation as described (Rubin and Spradling, 1982). Injections were performed by the Model System Genomics facility at Duke University. Individual progeny bearing the transgenes as identified by eye color and verified by PCR were selected and mated with flies carrying marked balancer chromosomes to obtain balanced stocks. Chromosomes containing the transgenes are referred to as P{*CycJ*}, P{HZ14*CycJ*}, P{*armi*} and P{*CG14971*}. To express the CycJ open reading frame (ORF) from a UAS we used pHZ12 (Mairiang et al.), a vector derived from pUASattB (Bischof et al.) that contains an attB site, the mini-white gene, and a UAS driving expression of ORFs with an N-terminal 6His-3Myc tag. The *CycJ* ORF was amplified from a yeast two-hybrid clone (Stanyon et al., 2004) using recombination tag primers 5RT and 3RT (Parrish et al., 2004) and recombined into the 5RT and 3RT sites of vector pHZ12 as described (Parrish et al., 2004) to create pUAS-Myc-CycJ. The *CycJ* ORF in pUAS-Myc-CycJ was verified by sequencing and includes from the ATG to the stop codon of Cyclin J isoform A. pUAS-Myc-CycJ was inserted into the attP site at 51C in Drosophila line y[1] M{vas-int.Dm}ZH-2A w[*]; M{3xP3-RFP.attP'}ZH-51C (Bischof et al., 2007).

Immunohistochemistry

We followed the Cooley lab protocol (http://info.med.yale.edu/cooley/Protocol) (Verheyen and Cooley, 1994; Xue and Cooley, 1993) with slight modifications. We dissected ovaries from appropriately aged, well-fed, virgin females in EBR (Ringers solution; 100mM phosphate buffer pH6.8, 450mM KCl, 150mM NaCl, 20mM $MgCl_2$) on ice, fixed in 100μl of devitellinizing buffer (6% formaldehyde, 16.7mM KH_2PO_4/K_2HPO_4 [pH 6.8], 75mM KCl, 25mM NaCl, 3.3mM $MgCl_2$) and 600μl heptane for 10 minutes with gentle agitation, washed with PBS (150mM NaCl, 20mM K_2HPO_4/KH_2PO_4 [pH 7.4]) for 30 minutes and then with PBT (1X PBS, 0.3% Triton X-100 and 0.5% BSA) for an additional 10 minutes. After this, the ovaries were incubated with primary antibody for 1 hour at room temperature and then at 4°C overnight. Following this, the ovaries were washed with PBT for 1 hour, incubated with secondary antibody for 2 hours at room temperature, then washed with PBT for 1 hour, rinsed with PBS, and finally equilibrated with PBS: Glycerol (1:1) for at least 20 minutes. The following antibodies were used. Antibodies against hts-RC (Robinson et al.,

1994), Orb 6H4 and 4H8 (Lantz et al., 1994), and c-myc 9E10 were obtained from the Developmental Studies Hybridoma Bank, University of Iowa. Primary antibodies were used at 1:50 dilution. Goat anti-mouse IgG FITC conjugate (Sigma) or DyLight 550 and Dylight 388 (Thermo Scientific), were used as the secondary antibodies at 1:200 dilution. For DAPI staining, fixed egg chambers were incubated with 1μg/ml DAPI (Sigma) in PBT for 30 minutes followed by several washes with PBT and then with PBS. Stained ovaries were mounted in 90% Glycerol containing 2.33% w/v DABCO (Sigma) for imaging. Fluorescence micrographs were obtained at room temperature using a Zeiss Axio imager upright microscope.

Quantification of phenotypes and rescue

To assess embryo hatching rates and morphology, equal numbers of newly emerged virgin females of each genotype were mated with w^{1118} males and the eggs were collected on apple juice plates and grape juice plates every day for 4 days. The number of eggs hatched was determined by counting the number of eggs that failed to hatch after aging for 24 hours at 25°C. Eggs were counted and categorized based on dorsal appendage morphology. At least 200 eggs were examined for each genotype except the *armi* null for which repeated collections provided 50 eggs to examine. To quantify oogenesis defects, females were ages for 2-4 days post eclosion and ovaries from at least 10 flies per genotype were extracted and stained with DAPI as described above. *piwi* mutant females were only aged for 0-2 days post eclosion due to rapid egg chamber loss. All ovarian material was transferred to a slide and ovarioles were mechanically separated from one another with forceps or insulin syringes for analysis. Number of mature oocytes per fly, number of egg chambers per ovariole, and percent of compound egg chambers per fly were quantified and averaged for each genotype. Statistical differences between single and double mutants for each phenotype were calculated using a type 3 student's T-test, which accounts for unequal sample sizes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- Cyclin J is a highly conserved cyclin without a described function.
- Unlike all other cyclins, Drosophila Cyclin J is expressed specifically in ovaries
- We characterized a Cyclin J null along with piRNA pathway mutants in oogenesis
- The Cyclin J null is normal but modifies oogenesis defects in piwi and armi mutants
- Cyclin J contributes to egg chamber development with piRNA pathway genes

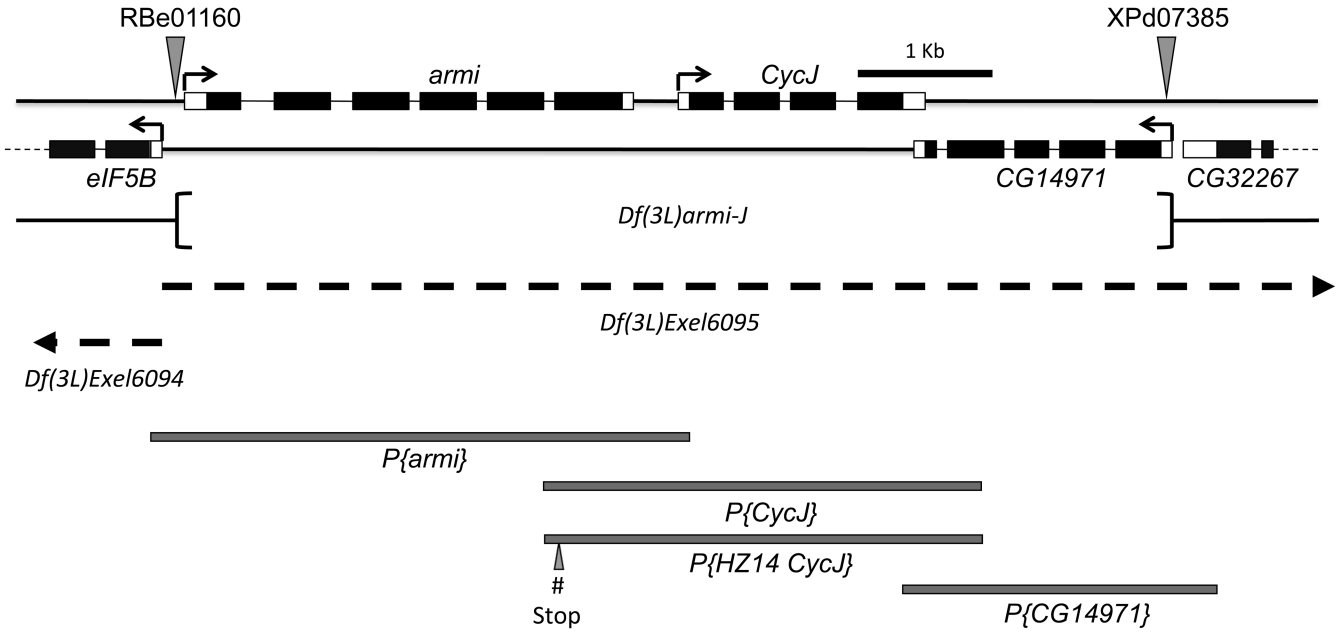

Figure 1.

Schematic representation of the genomic region corresponding to *CycJ*, *armi*, and the neighboring genes on chromosome 3L. The region shown corresponds to estimated cytological band 63E1. Black boxes represent coding regions and open boxes represent 5' and 3' untranslated regions. *armi* and *CycJ* are transcribed left to right while all other genes shown are transcribed right to left (arrows). FRT-bearing transposon insertions RBe00161 and XPd07385 (shown by triangles) were used to delete the intervening genomic region. The deleted genomic region is represented as *Df(3L)armi-J*. Rescue experiments were conducted by using independent transgenes, *P{armi}*, *P{CycJ}*, and *P{CG14971}* that were generated using the indicated genomic regions (shaded boxes). Rescue experiments also used a second *CycJ* genomic transgene, *P{HZ14CycJ}*, that introduced stop codons (# stop) in the *armi* coding region eliminating *armi* coding potential. Regions missing in the deficiency chromosomes *Df(3L)Exel6094* and *Df(3L)Exel6095* are indicated by dashed lines and extend beyond the region shown (arrowheads).

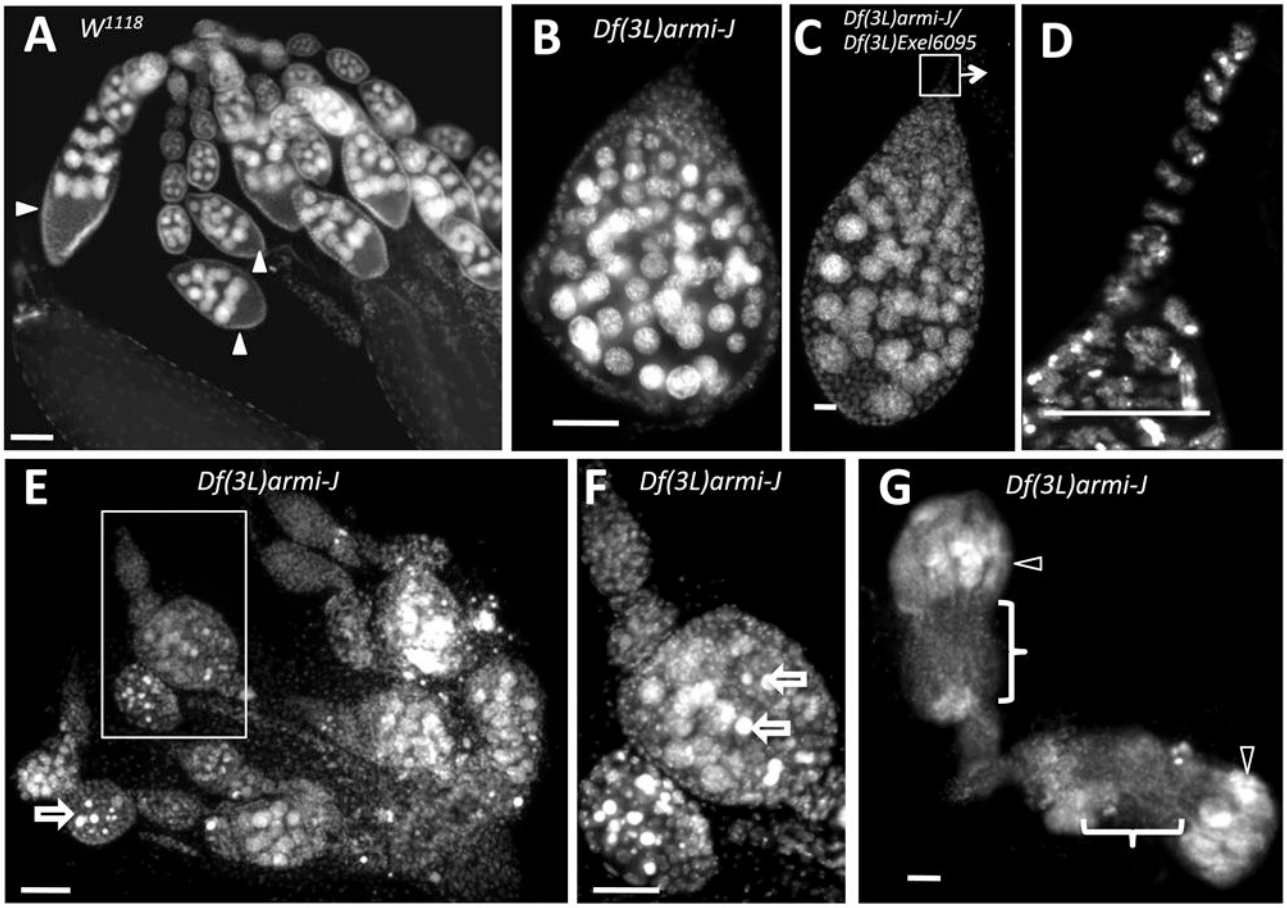

Figure 2.

Deletion of both *armi* and *CycJ* results in accumulation of egg chambers with excess germline cells. Ovaries from wild type (w^{1118}) and *Df(3L)armi-J* mutant females stained with DAPI to visualize nuclei. (A) Wild-type ovarioles from 3-day old or 8-day old females (not shown) consist of chains of developing egg chambers (arrows). Each egg chamber has 15 nurse cells undergoing endoreplication (large nuclei) and a single oocyte located at the posterior end (arrowheads). (B) A typical cyst from 3-day old *Df(3L)armi-J* females has many more than 16 nuclei undergoing endoreplicative cycles along with pycnotic nuclei. (C) An ovariole from a *Df(3L)armi-J*/*Df(3L)Exel6095* transheterozygote, which have a phenotype identical to *Df(3L)armi-J*. (D) A higher magnification of the terminal filament region in C. (E) Ovaries from 3-day old *Df(3L)armi-J* females contain abnormal egg chambers with more than the normal number of endoreplicating nuclei. Some cells appear to be undergoing cell death as indicated by the characteristic pycnotic nurse cell nuclei (open arrows), which is evident at higher magnification in (F). (G) Ovaries from 8-day old *Df(3L)armi-J* females contain empty ovarioles (bracket) with disorganized germaria (open arrowheads). Anterior is generally toward the top of each figure; in (G) the anterior of the lower ovariole is toward the right. Size bar is 50 μm in all panels except E and G, in which it is 20 μm.

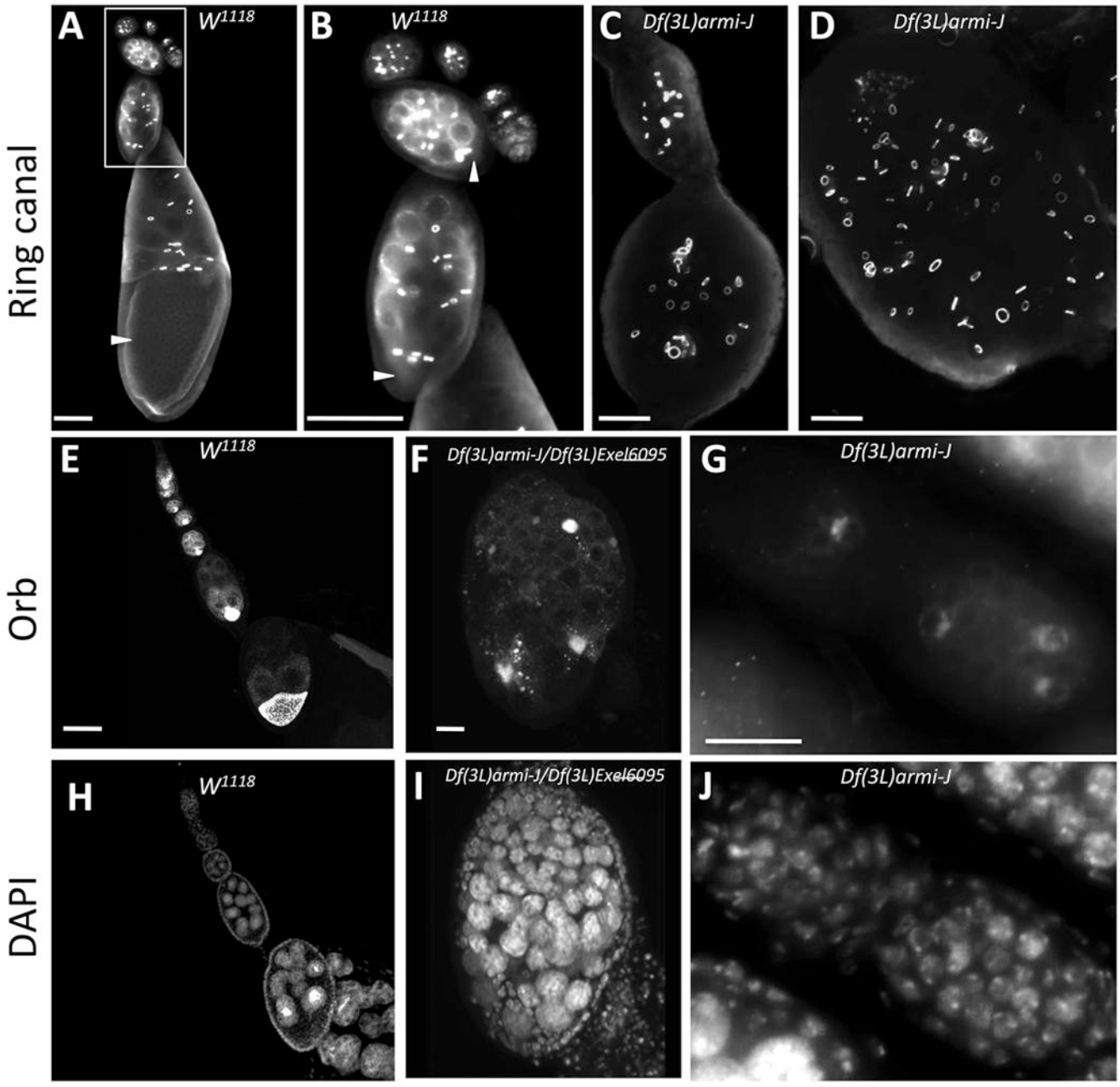

Figure 3.

Multiple cystoblasts divide and differentiate within a single egg chamber in *armi-CycJ* mutants. Ovaries from wild type (w^{1118}) (A, B) and *Df(3L)armi-J* (C,D) adults stained with anti-hts-RC antibody to visualize ring canals. In wild-type ovarioles each egg chamber contains a single posteriorly located oocyte (arrowheads) with four associated ring canals. (B) shows the boxed region in A at higher magnification. The *Df(3L)armi-J* mutant ovarioles show multiple abnormalities (C and D). Germarial patterning is disrupted and each egg chamber carries a giant cyst with multiple clusters of 16 cells interconnected by ring canals. (E-J) Ovarioles from wild-type (w^{1118}) (E, H), *Df(3L)armi-J*/*Df(3L)Exel6095* (F, I),

and *Df(3L)armi-J* (G, J) stained with DAPI (H-J) and Orb (E-G) to visualize oocytes. In wild-type ovarioles each egg chamber contains one Orb-staining oocyte. In the mutant, each giant cyst contains multiple Orb-staining nuclei adjacent to endoreplicating nurse cells. Anterior is towards the top in A and upper left in the rest of the images. Size bars are 50 μm in A-E and H, and 20 μm in F, G, I, and J.

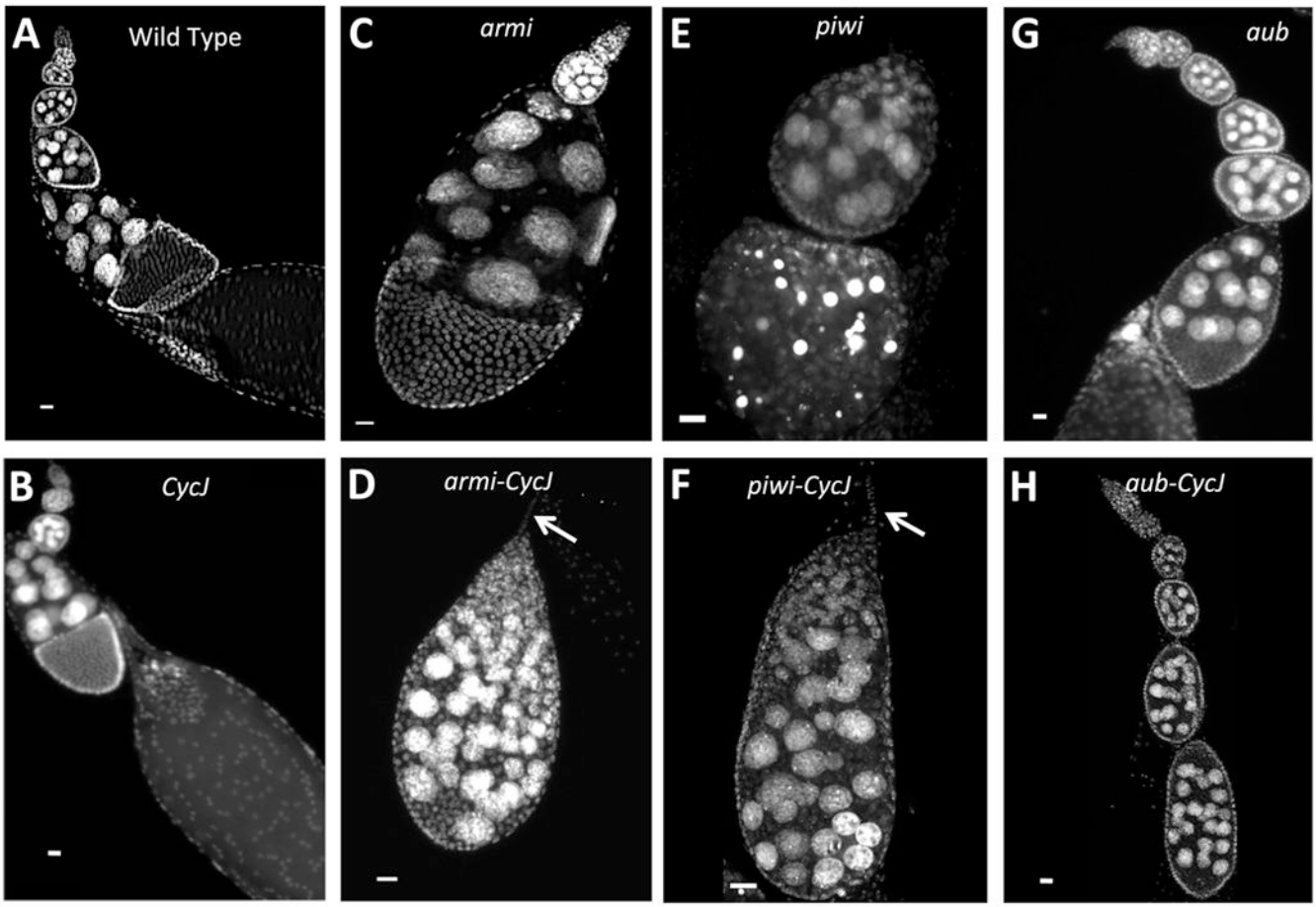

Figure 4.

CycJ genetically interacts with *armi*, *piwi*, and *aub*. As in wild type (A), ovarioles from the *CycJ* null (*Df(3L)armi-J* with the P{*armi*} transgene) appear normal (B), with long chains of egg chambers each containing 15 nurse cell nuclei. Ovarioles from the *armi* null (C) (*Df(3L)armi-J* with a P{*CycJ*} transgene) frequently have just two egg chambers per ovariole, and egg chambers have 15 nurse cell nuclei. The *armi-CycJ* null (D) exhibits drastic oogenesis defects epitomized by production of ovarioles with one egg chamber that often has many more than 15 nurse cells. Like the *armi* null, *piwi* mutants (E) (*piwi[06843]/Df(2L)BSC145)* exhibit a decreased number of egg chambers per ovariole. *piwi-CycJ* double mutants (F) phenocopy the *armi-CycJ*, producing compound egg chambers with more than 15 nurse cells. The *aub* mutant (G) (aub^{HN}/aub^{QC42}) showed all stages of egg chamber development each with the normal number (15) of nurse cell nuclei. In *aub-CycJ* double mutants (H), oogenesis arrested prior to stage 8 with some egg chambers harboring many more than 15 nurse cells. Arrows in D and F point to the terminal filament. Anterior is towards the top and size bars are 20 μm in all panels.

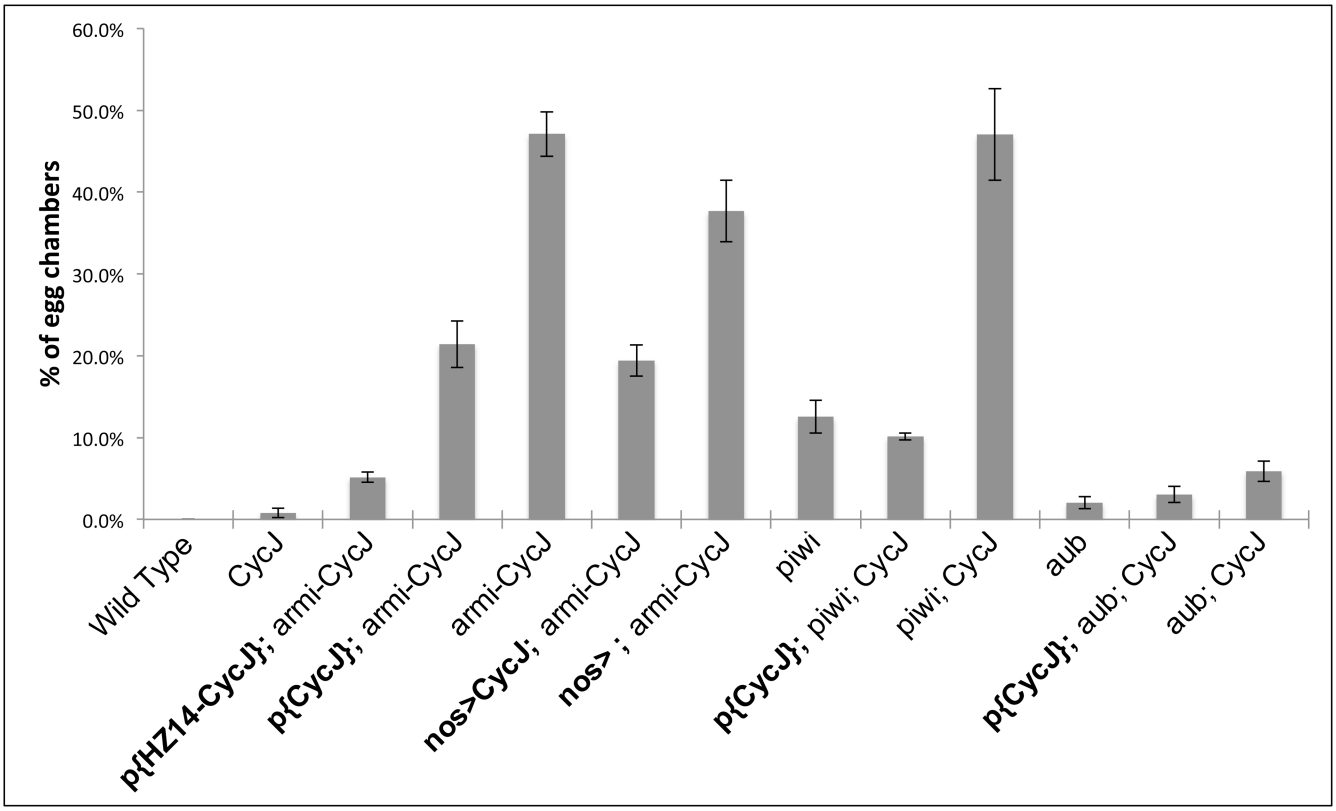

Figure 5.

CycJ expression suppresses compound egg chamber production in *armi-CycJ*, *piwi-CycJ*, and *aub-CycJ*. The percent of egg chambers with more than 15 nurse cell nuclei is shown, error bars = SEM (0.00% to 5.58%). Transgenes are in bold. *CycJ* null egg chambers are the same as wild type with respect to the number of nurse cell nuclei. In *armi-CycJ* null ovaries, 47.1% +/-2.7% of egg chambers have an excess of germline cells, which is indicative of a compound egg chamber (see examples in Fig. 2). *CycJ* genomic transgenes (P{*CycJ*} or P{*HZ14_CycJ*}) added to the *armi-CycJ* null significantly decreased the frequency of compound egg chambers. Germline specific expression of *UAS-Myc-CycJ* with the *VP16::nos-Gal4* driver in *armi-CycJ* null also decreased compound egg chamber production. P{*CycJ*} added back to both *piwi-CycJ* and *aub-CycJ* was able to rescue compound egg chamber production back to the level of the *piwi* and *aub* single mutants, respectively. Note that *armi-CycJ* and *piwi-CycJ* exhibit the same high level of compound egg chamber production. The *CycJ* null is *P{armi}, Df(3L)armi-J*/*Df(3L)Exel6095*. The *armi-CycJ* double null in each case is *Df(3L)armi-J*/*Df(3L)Exel6095*. The *piwi* mutant is *piwi[06843]/Df(2L)BSC145*. The *aub* mutant is aub^{HN}/aub^{QC42}. P-values are shown in Figure S4. All ovaries are from females 2-4 days post eclosion except *piwi* mutants, which are 0-2 days.

Table 1

Egg laying, morphology, and hatching rate defects in *armi* and *CycJ* mutants

Maternal Genotype	Genotype abbreviation	Eggs laid[a]	Egg morphology[b] (dorsal appendages) Two	One	None	Hatching Rate[c]
Df(3L)armi-J /+		100%	100%	0%	0%	96%
Df(3L)armi-J or Df(3L)armi-J/Df(3L)Exel6095	$armi^{null}$ $CycJ^{null}$ ($CG14971^{null}$)	NONE	NA	NA	NA	NA
P{CG14971}; Df(3L)armi-J	$armi^{null}$ $CycJ^{null}$	NONE	NA	NA	NA	NA
P{CycJ}; Df(3L)armi-J	$armi^{null}$ ($CG14971^{null}$)	<1%	0%	0%	100%	0%
P{CG14971}; P{CycJ} ; Df(3L)armi-J	$armi^{null}$	<1%	0%	0%	100%	0%
P{armi}; Df(3L)armi-J	$CycJ^{null}$ ($CG14971^{null}$)	65.3%	92%	7%	1%	30%
P{armi} / P{CG14971}; Df(3L)armi-J	$CycJ^{null}$	65.1%	91%	8%	1%	30%
P{armi}; P{CycJ}; Df(3L)armi-J	$CG14971^{null}$	66.7%	94%	6%	0%	73%
P{armi}; P{CycJ}; Df(3L)armi-J/Df(3L)Exel6095	$CG14971^{null}$	79.2%	90%	7%	3%	76%
P{armi} / P{CG14971}; P{CycJ};Df(3L)armi-J		67.4%	93%	7%	0%	75%
Df(3L)armi-J / $armi^{72.1}$	$armi^{72.1}$ (hypomorph)	35.3%	0%	28%	72%	0%
aub^{HN} / aub^{QC}	*aub*	75.0%	8%	68%	24%	0%
P{armi}; aub^{HN} / aub^{QC}*; Df(3L)armi-J*	$CycJ^{null}$, *aub*	NONE	NA	NA	NA	NA
mnk^{P6}*; Df(3L)armi-J /* $armi^{72.1}$	$armi^{72.1}$, *mnk*	89.4%	85%	8%	7%	0%
mnk^{P6}*; Df(3L)armi-J*	$armi^{null}$ $CycJ^{null}$, *mnk*	NONE	NA	NA	NA	NA
P{CycJ}; mnk^{P6}*;Df(3L)armi-J*	$armi^{null}$, *mnk*	16.8%	77%	12%	9%	0%

[a] Percent of the number eggs laid by *Df(3L)armi-J*/+ (n=1118) over the same period of time. For the two *armi* null genotypes, repeated collections yielded only 4 or 5 eggs per collection. *armi-CycJ* null and *CycJ* null *aub* double mutants laid no eggs.

[b] Number of dorsal appendages per egg (wild-type is two). Axis establishment defects lead to ventralized eggs, which have fewer than two dorsal appendages. NA, not applicable (no eggs laid).

[c] Embryo hatching rates relative to wild-type (Materials and Methods).