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Role of the insulin/Tor signaling network in starvation-induced programmed cell death in *Drosophila* oogenesis

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Amino-acid starvation leads to an inhibition of cellular proliferation and the induction of programmed cell death (PCD) in the *Drosophila* ovary. Disruption of insulin signaling has been shown to inhibit the progression of oogenesis, but it is unclear whether this phenotype mimics starvation. Here, we investigate whether the insulin-mediated phosphoinositide kinase-3 pathway regulates PCD in mid oogenesis. We reasoned that under well-fed conditions, disruption of positive components of the insulin signaling pathway within the germline would mimic starvation and produce degenerating egg chambers. Surprisingly, mutants did not mimic starvation but instead produced many abnormal egg chambers in which the somatic follicle cells disappeared and the germline persisted. These abnormal egg chambers did not show an induction of caspases and lysosomes like that observed in wild-type (WT) degenerating egg chambers. Egg chambers from insulin signaling mutants were resistant to starvation-induced PCD, indicating that a complete block in insulin-signaling prevents the proper response to starvation. However, *target of rapamycin* (*Tor*) mutants did show a phenotype that mimicked WT starvation-induced PCD, indicating an insulin independent regulation of PCD via Tor signaling. These results suggest that inhibition of the insulin signaling pathway is not sufficient to regulate starvation-induced PCD in mid oogenesis. Furthermore, starvation-induced PCD is regulated by Tor signaling converging with the canonical insulin signaling pathway.
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The insulin-mediated class I phosphoinositide kinase-3 (PI3K) pathway is a highly conserved nutrient-sensing pathway in diverse organisms. In mammals, insulin signaling affects oocyte maturation and fertilization.[1,2] Mutations in the insulin signaling pathway in *C. elegans* can lengthen life span but reduce fertility.[3,4] Improper insulin signaling in *Drosophila* leads to reduced body size and female sterility.[5] Thus, insulin signaling is essential for reproduction in diverse organisms, but how this pathway regulates fertility is not fully understood.

Proper nutrition during *Drosophila* oogenesis is critical for egg chamber production.[6–8] Depriving flies of yeast as a protein source leads to programmed cell death (PCD) at two stages: early oogenesis in the germarium and mid oogenesis during stages 7–9.[7] Egg chambers undergoing PCD during mid oogenesis are characterized by nuclear condensation and fragmentation of germline-derived nurse cells (NCs), engulfment by somatic follicle cells (FCs), and ultimately FC death.[5]

During PCD in mid oogenesis, dying NCs show characteristics of both apoptotic and autophagic PCD.[5] The effector caspase Death caspase-1 (Dcp-1) is essential for germline PCD in mid oogenesis. *dcp-1* mutants that have been starved display mid-stage egg chambers that have a persistence of uncondensed NC nuclei but an absence of FCs.[5] This phenotype indicates that the *dcp-1* mutant germline is unable to die in response to starvation, although the FCs respond and undergo PCD. *dcp-1* mutants show decreased autophagy,[5] but autophagy appears to have a minor role during PCD in mid oogenesis. Autophagy-deficient egg chambers show normal chromatin condensation, but reduced levels of DNA fragmentation.[5]

The cell death pathway upstream of *dcp-1* is unclear. Double mutants lacking the initiator caspases Strica and Dronc only partially disrupt PCD in mid oogenesis.[5] Levels of the caspase inhibitor *Drosophila* inhibitor of apoptosis protein 1 (DIAP1) decline during mid oogenesis,[5] which may be the mechanism that makes this stage of oogenesis more susceptible to PCD. However, mid oogenesis PCD is not regulated by the IAP-binding proteins Rpr, Hid, Grim and Skl,[9] so the mechanism controlling DIAP1 levels is unknown. Recently, we have determined that mitochondrial events and the Bcl-2 family partially control PCD in mid oogenesis.[10] However, how nutritional cues link to Dcp-1, DIAP1, or mitochondria has not been determined. One candidate for the regulation of starvation-induced PCD in the ovary is the insulin signaling pathway. The insulin signaling pathway is involved in the regulation of apoptosis and autophagic PCD at several points during *Drosophila* development.[11,12]

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In the *Drosophila* ovary, insulin signaling is important for germline stem cell division,[7,13] but evidence that it is involved in the regulation of PCD in mid oogenesis is limited. The insulin receptor (InR) and the InR substrate (Chico) are required for egg chamber progression and proliferation of FCs;[5,7] however, the phenotype of the terminal egg chambers has not been closely analyzed. The terminal egg chambers of *target of rapamycin* (*Tor*) mutant germline clones (GLCs) show an induction in apoptosis as visualized by acridine orange staining.[14] Recently, rapamycin treatment has been shown to induce autophagy in the fly ovary.[15] These studies suggest that the insulin signaling pathway may be involved in the regulation of starvation-induced PCD in mid oogenesis; however, detailed analysis of mid-stage egg chambers has not been reported.

In this study, we analyze multiple components of the insulin signaling pathway within the germline to determine whether their disruption mimics the effects of starvation in the ovary. Interestingly, we find that these mutants show an arrest early in oogenesis but most of these egg chambers do not mimic starvation-induced PCD. We provide evidence that caspases and lysosome formation are blocked in abnormal egg chambers produced by GLCs of *InR*, *chico*, and ribosomal protein *S6 kinase* (*S6k*). However, *Tor* GLCs do produce degenerating mid-stage egg chambers, mimicking starvation-induced PCD. These findings suggest that Tor signaling acts independently of the insulin pathway during mid oogenesis. To determine whether another pathway acts in parallel to insulin signaling during starvation, we starved the mutants but found that only *Tor* mutants resembled wild-type (WT)-starved flies. These findings indicate that the insulin and Tor signaling network are required in the ovary for proper progression of oogenesis and prevention of PCD.

Results

Insulin pathway mutants do not mimic starvation-induced cell death in the ovary. Given the known role of insulin signaling in controlling the response to nutrition, we reasoned that in the presence of nutrients, mutants of positive components of the insulin signaling pathway would mimic the PCD seen in starved WT flies. We chose to analyze two upstream genes, *InR* and *chico*, and two downstream genes, *Tor* and *S6k* (Figure 1a). All four alleles that were selected have been described as likely null alleles.[16–19] Because most of these mutants are homozygous lethal, we generated GLCs. Flies were well-fed, but mutant GLCs did not progress into mid oogenesis (Supplementary Figure 1),
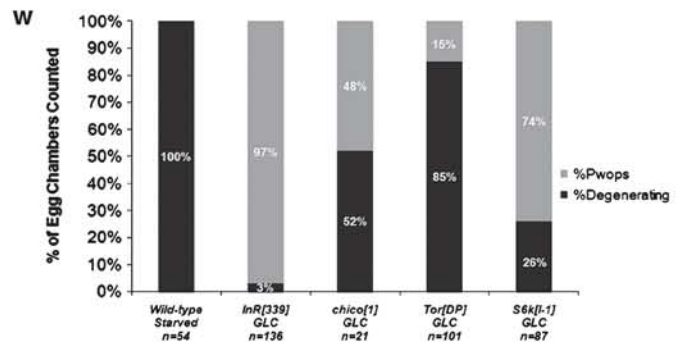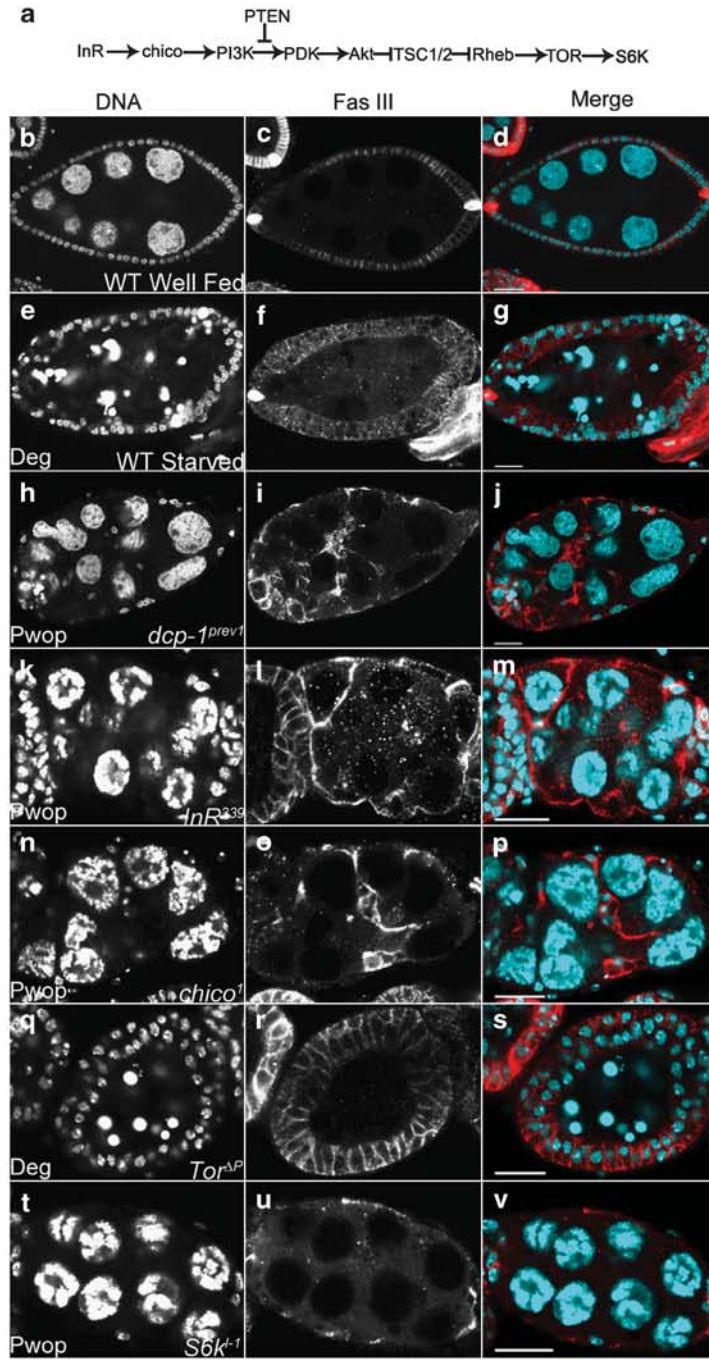
consistent with previous studies.[7,14,20] To determine the stage of oogenesis in which growth arrested, the last healthy egg chamber at the end of an ovariole was scored (Supplementary Figure 1m). Stage 4 of oogenesis was the predominant stage at which oogenesis arrested for InR^{339} (38%), $chico^1$ (50%), $Tor^{\Delta P}$ (59%), and $S6k^{l-1}$ (54%) GLCs.

Surprisingly, we found that many of the mutant egg chambers did not mimic starvation-induced PCD (Figure 1e). All four mutants produced egg chambers that failed to degenerate, showing an aberrant morphology where the germline persisted and FCs disappeared (Figures 1k, n and t). This phenotype was reminiscent of that seen in *dcp-1* mutants Figure 1h), where the germline is unable to die despite receiving a death signal. We refer to this phenotype as 'peas without pods' (Pwop), where the peas represent the uncondensed NCs and the pod represents the FC layer.[5] Fas III labeling showed that most of the FCs were lost from the pwops, and some of the remaining FC membranes became stretched over the NCs (Figures 1i, l, o and u).

Some egg chambers from the insulin pathway mutants did appear to degenerate normally (Figure 1q), so the occurrence of Pwops and degenerating egg chambers was quantified (Figure 1w, Supplementary Figure 1n). Well-fed or starved WT flies produced degenerating egg chambers (100%) and did not produce any Pwops. As previously reported, starved WT flies produced more degenerating egg chambers than well-fed flies (data not shown).[5] In contrast to WT, InR^{339} GLCs produced a large number of Pwops (97%) as did $Akt1^q$ (71%) and $S6k^{l-1}$ (74%). In GLCs of the hypomorphic allele InR^{E19} (Supplementary Figure 1d), later stages of oogenesis were present but the major phenotype was Pwop (98%). Hemizygotes of the hypomorphic allele $S6K^{07084}$ (Supplementary Figure 1j) produced mid-stage egg chambers that degenerated (54%) and almost an equal number of Pwops (46%). Similarly, $chico^1$ GLCs produced an almost equal distribution of degenerating (48%) and Pwops (52%). To further analyze $chico^1$, we looked at a potentially stronger allelic combination of $chico^1$ in trans to a deficiency (Supplementary Figure 1f). This combination produced more Pwops (75%), suggesting that $chico^1$ is not a null allele, or that the FC genotype influences germline survival in the $chico^1$ mutant. $Tor^{\Delta P}$ GLCs largely resembled WT starvation (85%) but still produced some Pwops (15%). However, *Tor* mutant egg chambers degenerated earlier in oogenesis than that normally occurs in WT (Figures 2e and q).

To further investigate how insulin signaling is involved in starvation-induced PCD, we compared the progression of apoptotic and autophagic events in WT flies and insulin signaling mutants. One of the hallmarks of PCD in the ovary is

Figure 1 Insulin signaling pathway mutants have two distinct phenotypes in the *Drosophila* ovary. All egg chambers were stained with DAPI to label DNA and Fas III to label the FC membranes. (**a**) The simplified *Drosophila* insulin signaling cascade. (**b–d**) WT well-fed stage 8 egg chambers display no NC nuclear fragmentation and FCs are still present. (**e–g**) WT starved stage 8 egg chamber undergoing degeneration (Deg), in which NC nuclei fragment and FCs are still present. (**h–j**) Starved $dcp-1^{prev}$ mutants display the Pwop phenotype in a stage 8 egg chamber with uncondensed NC nuclei but a disappearance of FCs. (**k–m**) Stage 4 egg chamber from InR^{339} GLCs shows Pwop phenotype like *dcp-1*. (**n–p**) Stage 5 Pwop from $chico^1$ GLCs. (**q–s**) Stage 4 degenerating egg chamber from $Tor^{\Delta P}$ GLCs. (**t–v**) Stage 4 Pwop from $S6k^{l-1}$ GLCs. Scale bar: 20 μm. (**w**) Quantification of insulin signaling pathway mutant ovary phenotypes. Egg chambers classified as degenerating displayed fragmented NC nuclei (Black). Pwop egg chambers displayed uncondensed persisting NC nuclei and a premature disappearance of the FCs (Grey). Healthy egg chambers were not included because we wished to compare the frequency of degenerating vs. Pwop egg chambers. Degenerating egg chambers in WT ranged from stages 7 to 9 (mid oogenesis). Degenerating and Pwop egg chambers from insulin signaling mutants ranged from stages 2 to 6 (early oogenesis)
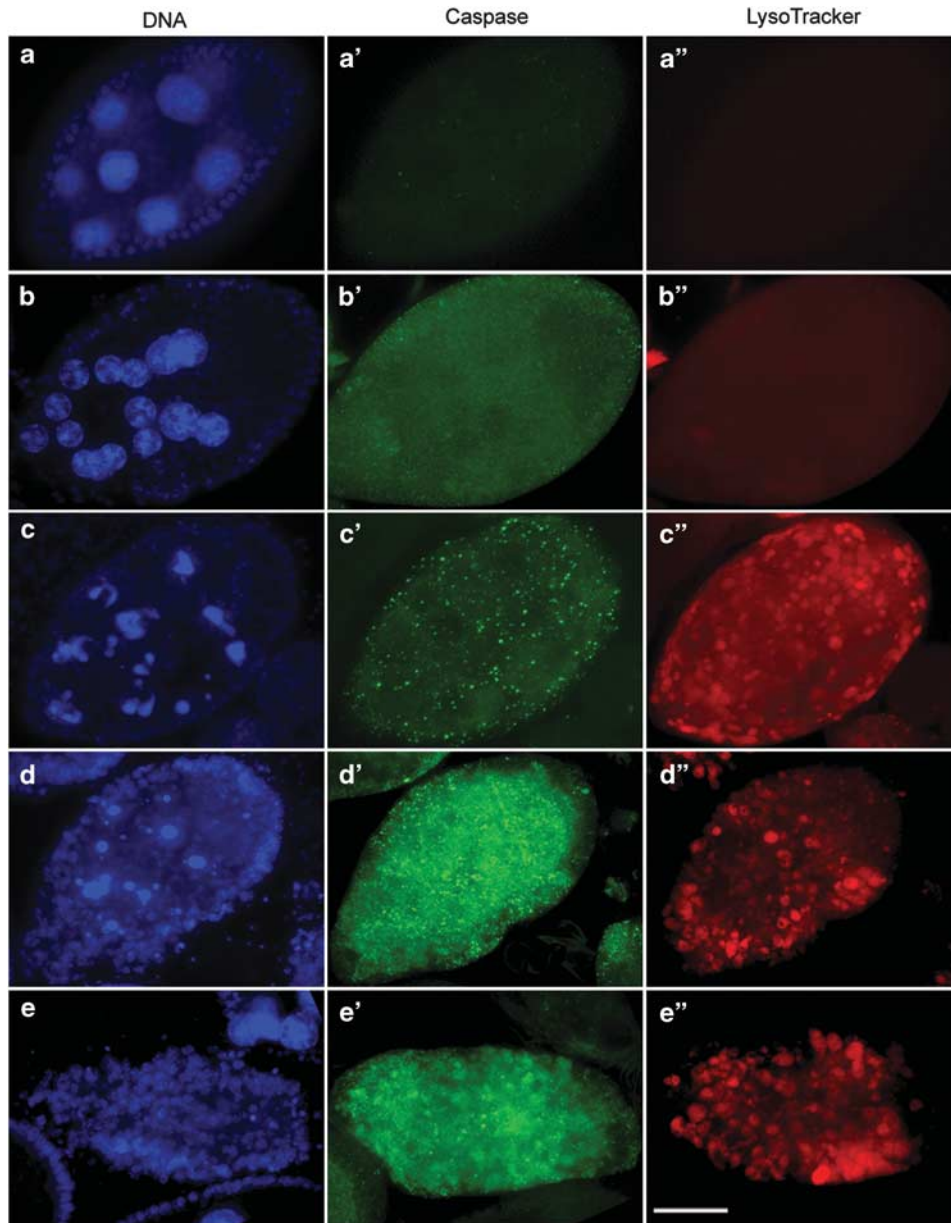
Figure panels (a–w): signaling pathway diagram and fluorescence micrographs with bar graph.

DNA	Caspase	LysoTracker

Figure 2 WT progression of starvation-induced PCD at mid oogenesis. All egg chambers were stained with DAPI (blue) to label DNA, anti-cleaved caspase-3 (green) and LT (red) to label lysosomes. (**a–a″**) A healthy egg chamber from a well-fed fly. (**b–e″**) Egg chambers from starved flies in the process of degeneration. (**b–e**) In degenerating egg chambers, NC nuclei become condensed and fragment into smaller pieces that are engulfed by the surrounding FCs. (**a′–e′**) As the NC nuclei fragment, caspase activity increases. (**a″–e″**) LT positive spots increase, indicating an increase in lysosomes and/or autolysosomes. All egg chambers are at stage 8. All images were taken at the same magnification and are projections of 15–25 images. Scale bar is 15 μm

nuclear condensation and fragmentation, so we used the extent of nuclear breakdown to stage degenerating WT egg chambers (Figures 2a–e).[5] Apoptotic events were marked with anti-cleaved caspase-3 to label active *Drosophila* caspases[21] (Figures 2a′–e′). Active caspases were first detected when NC nuclei began to fragment, and caspase activity increased as NC nuclei fragmented into smaller pieces. Lysosome formation was visualized with LysoTracker (LT) (Figures 2a″–e″). LT positive spots appeared at the start of NC nuclear fragmentation and increased as nurse nuclei fragmented into smaller pieces, similar to anti-cleaved caspase-3 staining.

However, Pwops produced by *InR^339*, *chico^1* and *S6k^l−1* GLCs displayed reduced punctate caspase-3 staining (Figure 3a, b and e) compared with WT degenerating egg chambers (Figure 2d′). The caspase-3 staining in the Pwop egg chambers may be associated with the dying FCs (Supplementary Figure 2). In *InR^339* and *chico^1* GLC Pwops, there were few LT positive spots, and there were larger domains, suggesting acidification of the NC cytoplasm (Figures 3a and b). In *S6k^l−1* GLCs, LT staining was not detectable (Figure 3e′), suggesting that S6k has a positive role in autophagy induction. This is similar to observations in the fat body of starved *S6K^l−1* homozygotes in which LT staining is not present.[11] Degenerating
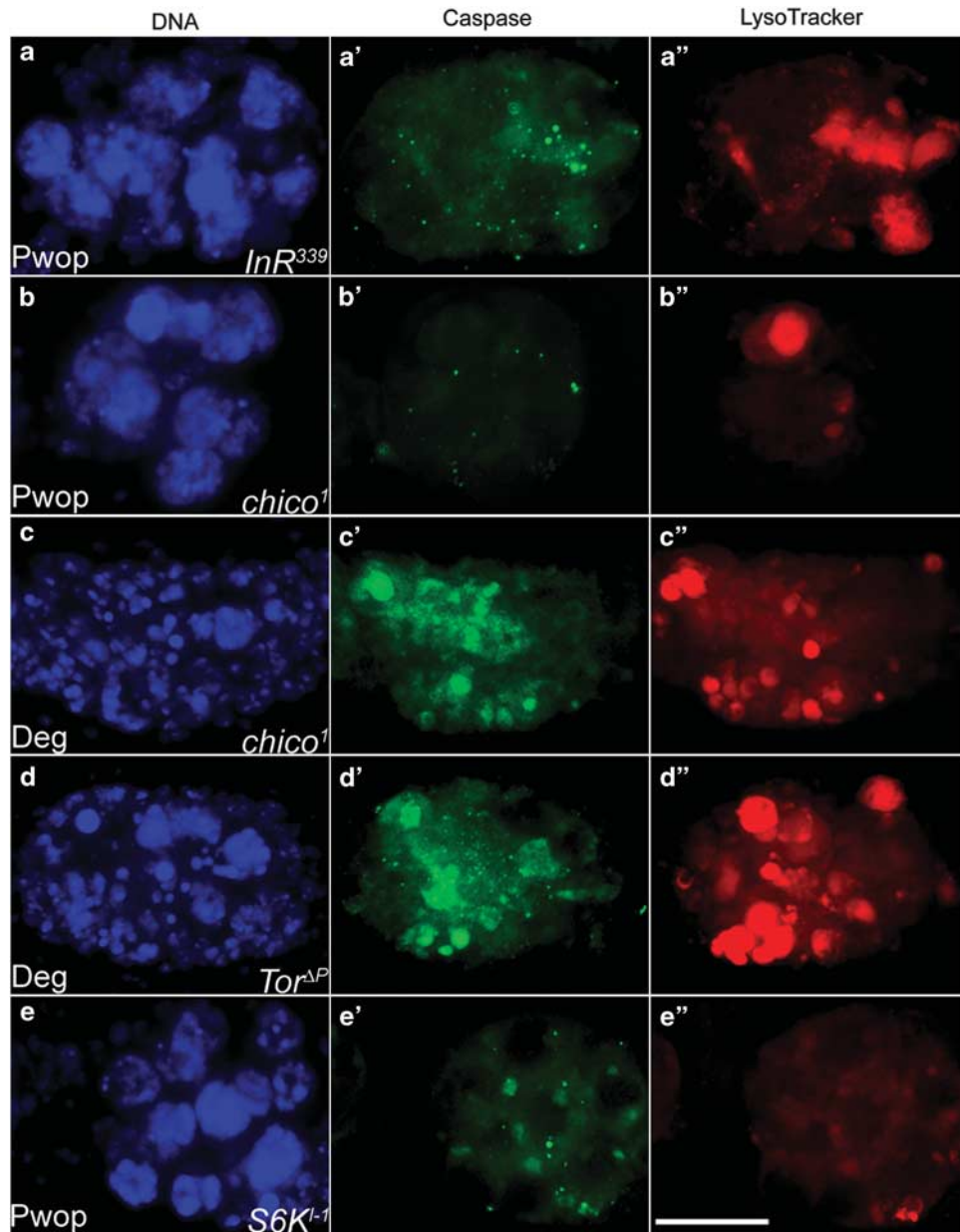
Figure 3 Insulin pathway mutant degenerating egg chambers have caspase and lysosome activity, whereas Pwops show weak caspase activation and acidification. All egg chambers were stained with DAPI (blue), anti-cleaved caspase-3 (green) and LT (red). (**a–a″**) InR^{339} GLC Pwop stage 6 egg chamber. (**b–b″**) A stage 5 Pwop from $chico^1$ GLCs. (**c–c″**) A degenerating stage 4 egg chamber from $chico^1$ GLCs. (**d–d″**) A degenerating stage 5 egg chamber from $Tor^{\Delta P}$ GLCs. (**e–e″**) Pwop stage 4 egg chamber from $S6k^{l-1}$ GLCs. Pwop and degenerating egg chamber stages are approximate due to difficulties in staging abnormal egg chambers. All images were taken at the same magnification and are projections of 15–20 images. Scale bar is 15 μm

egg chambers from $chico^1$ and $Tor^{\Delta P}$ GLCs (Figures 3c and d) mimicked the caspase-3 staining seen in degenerating WT egg chambers. The LT staining of $chico^1$ and $Tor^{\Delta P}$ GLC degenerating egg chambers showed a mix of spots similar to WT starved and large domains similar to InR^{339}. We were unable to visualize autophagy more directly using the auto-phagosome marker GFP-LC3[22] in early stage (1–6) egg chambers of WT or $chico^1$ mutants. We found that chico mutant egg chambers showed elevated levels of the autophagic adaptor protein, p62 (Supplementary Figure 3). Elevated levels of p62 have previously been reported in autophagy-deficient egg chambers.[23] Taken together, these data indicate that mutant egg chambers that degenerate like WT display a similar induction in apoptosis and autophagy, whereas Pwop egg chambers are defective in caspase activation and lysosome formation. Furthermore, Tor mutants more closely resemble WT starved flies than the other components of the pathway.

DNA fragmentation is blocked in Pwop egg chambers from insulin signaling pathway mutants. To further inve-stigate a disruption in apoptotic events, we used TUNEL to see whether we could detect DNA fragmentation in Pwops.
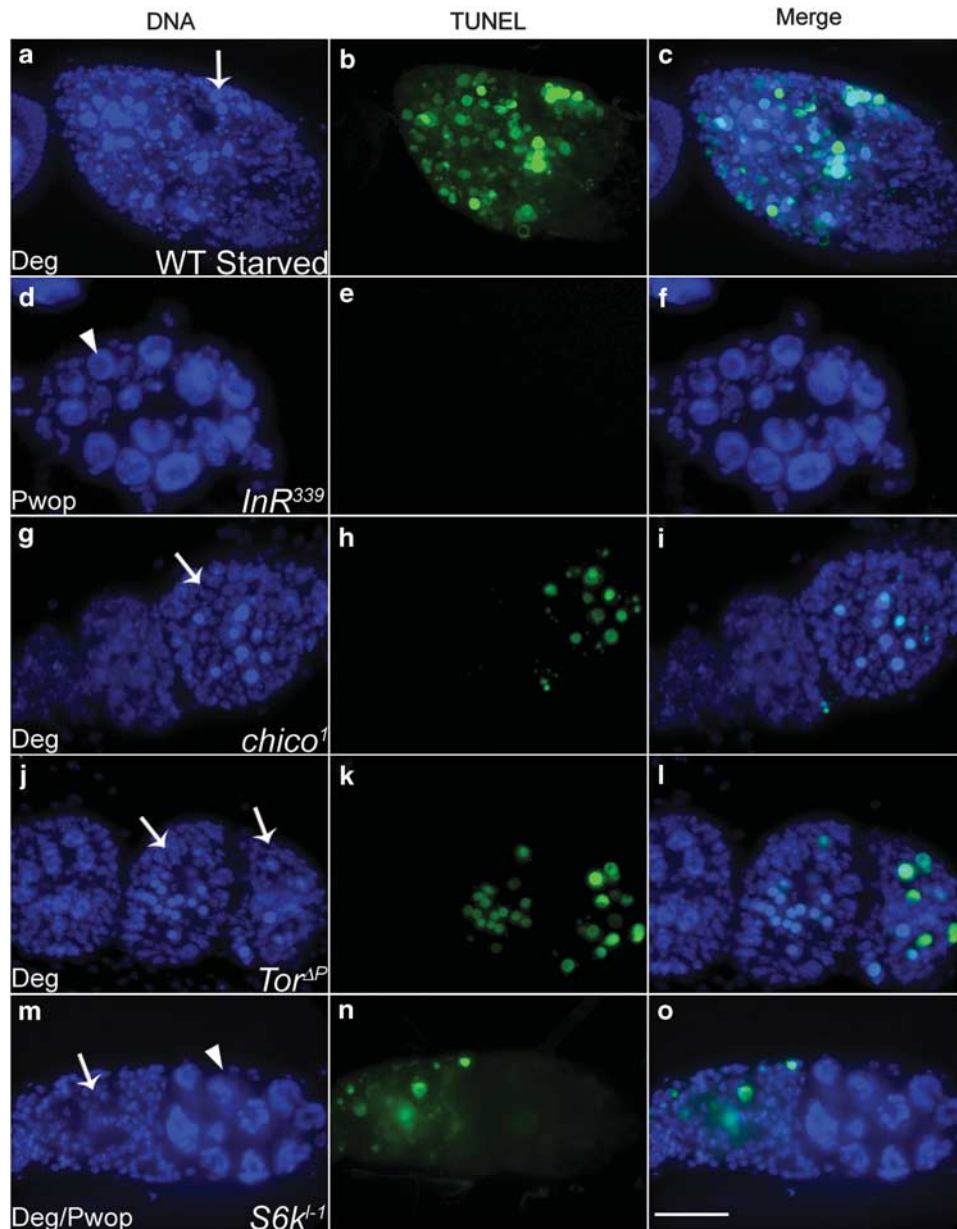
Figure 4 DNA fragmentation is blocked in insulin signaling mutant Pwop egg chambers. All egg chambers were assayed for fragmented DNA using the TUNEL ApopTag Plus Fluorescein *In Situ* Detection Kit (green), and stained with DAPI (blue). (**a–c**) WT degenerating egg chamber, in which NC nuclear fragmentation has occurred, shows many TUNEL-positive spots. (**d–f**) InR^{339} GLC stage 5 Pwop egg chamber. (**g–i**) $chico^1$ GLC healthy early stage egg chambers and a degenerating stage 2 egg chamber. (**j–l**) $Tor^{\Delta P}$ GLC healthy (stage 4) and degenerating (stages 4 and 5) egg chambers. (**m–o**) $S6k^{l-1}$ GLC degenerating (stage 1) and Pwop stage 4 egg chambers. Arrows indicate degenerating egg chambers and arrowheads indicate Pwops. All images were taken at the same magnification and are projections of 15–20 images. Scale bar is 15 μm

WT starved degenerating egg chambers produced numerous TUNEL-positive spots indicating that DNA fragmentation had occurred (Figures 4a–c). Likewise, mutant degenerating egg chambers displayed TUNEL-positive spots similar to starved WT egg chambers (Figures 4g–l). However, Pwops had no TUNEL-positive spots (Figures 4d–f). This trend was most apparent in mutants with mixed phenotypes like $S6k^{l-1}$ GLCs: degenerating egg chambers were TUNEL-positive and Pwops were TUNEL-negative (Figures 4m–o). The lack of DNA fragmentation in Pwops provides further evidence that apoptotic events are blocked.

DIAP1 levels remain moderate in Pwop egg chambers of insulin signaling mutants. The insulin pathway mutant Pwop egg chambers resembled egg chambers of *dcp-1* mutants, suggesting that PCD had initiated, but was unable to progress. DIAP1 is a potent caspase inhibitor, and its expression level has been shown to decrease in mid-stage egg chambers,[5] perhaps to lower the threshold for caspase activation and make this stage more sensitive to starvation-induced PCD. To determine whether DIAP1 could be protecting the germline of Pwops in the insulin pathway mutants, we examined DIAP1 protein levels with immunocytochemistry.
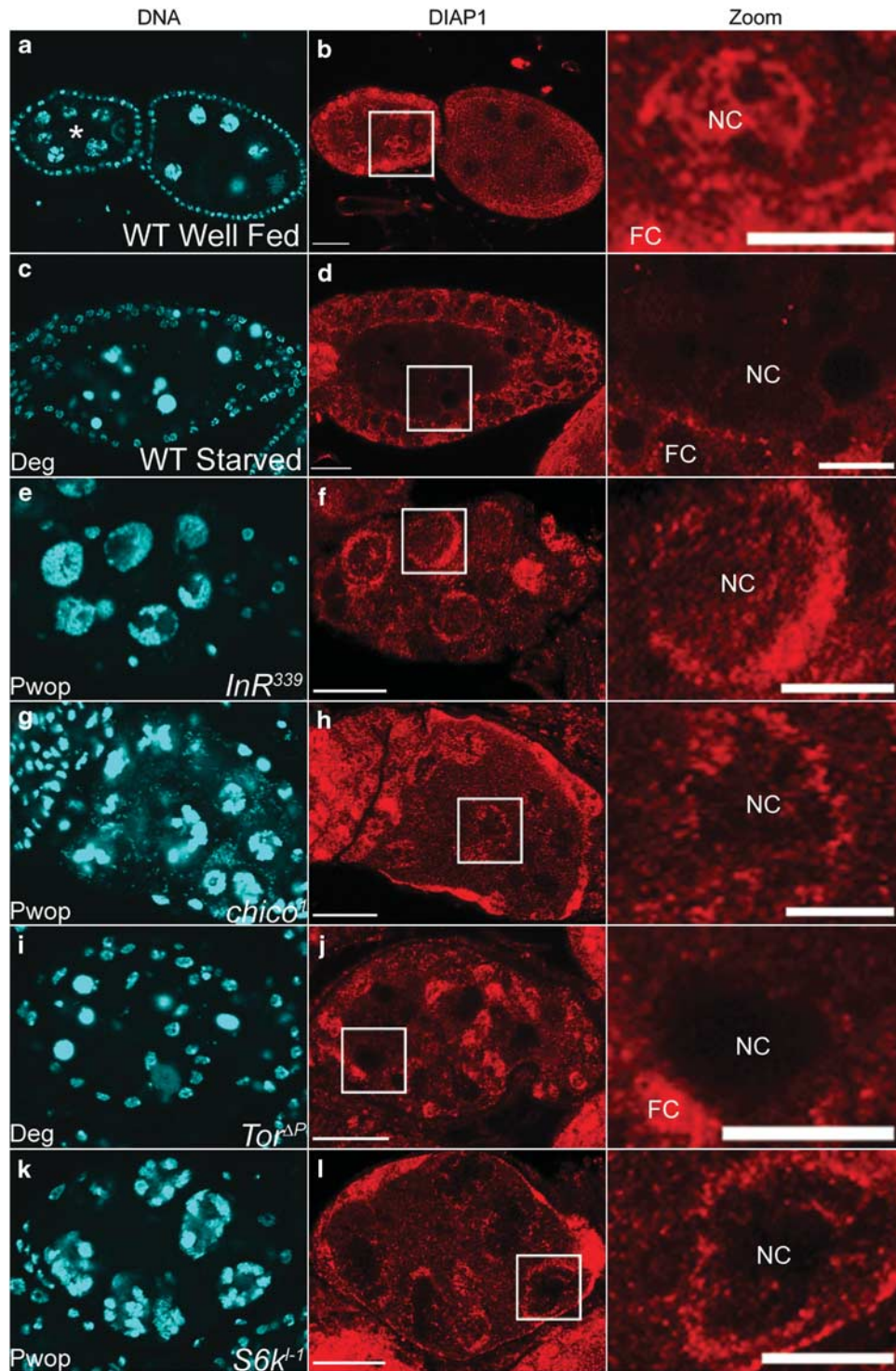
Figure 5 DIAP1 levels are maintained in insulin signaling mutant Pwop egg chambers. All egg chambers were stained with DAPI (Blue) and Anti-DIAP1 (Red). (**a** and **b**) WT healthy stage 5 egg chamber (*) and healthy stage 8 egg chamber. Boxed region from b shows DIAP1 in the NC and FC of an early stage egg chamber. (**c** and **d**) WT starved degenerating stage 9 egg chamber. Boxed region from d shows that DIAP1 is depleted in the NC. (**e** and **f**) InR^{339} GLC Pwop stage 5 egg chamber. Boxed region in **f** shows that the NC retains DIAP1 surrounding the nucleus. (**g** and **h**) $chico^1$ GLC Pwop stage 4 egg chamber. Similar to InR mutants, the boxed region from **k** displays DIAP1 surrounding a NC nucleus. (**i** and **j**) $Tor^{\Delta P}$ GLC-degenerating stage 4 egg chamber. Similar to the degenerating WT egg chamber, the boxed region from **j** displays reduced DIAP1 staining in the NC. (**k** and **l**) $S6k^{l-1}$ GLC Pwop stage 4 egg chamber. A NC from the boxed region in **l** displays DIAP1 surrounding a NC nucleus. Boxed regions are cropped zooms. Scale bar is 20 μm
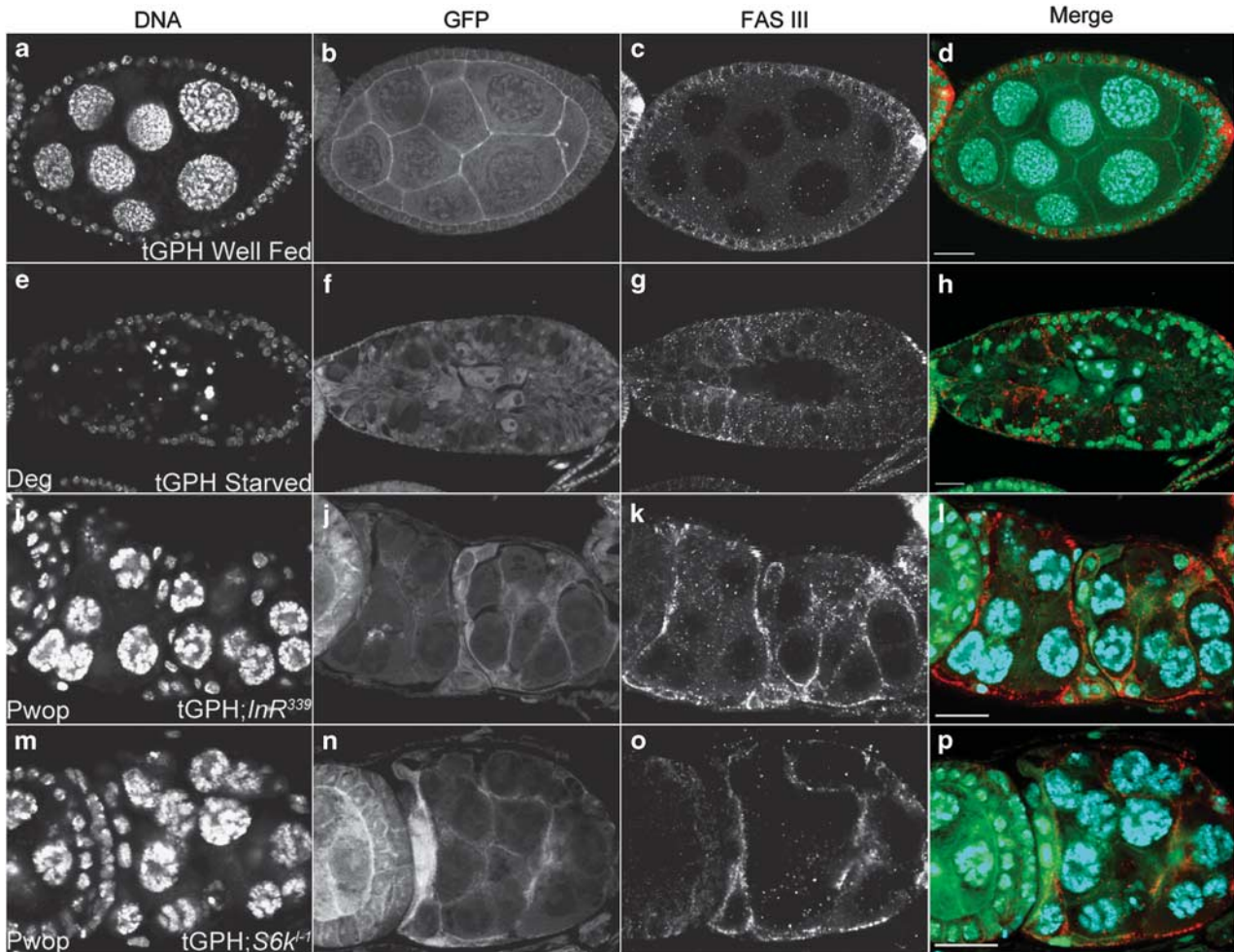
Figure 6 PI3K activity is lost in *InR* and *S6k* Pwops. All egg chambers expressing the tGPH reporter (green) were stained with DAPI (blue) and Fas III (red) to label FCs. FasIII is enriched in the polar cells in mid-stage egg chambers. (**a**) *tGPH* mid-stage egg chamber from well-fed fly displayed (**b**) PI3K activity at the plasma membranes of FCs and NCs. (**c**) Fas III and (**d**) Merge indicates co-localization at the FC plasma membrane of tGPH reporter and Fas III. (**e**) *tGPH*-degenerating mid-stage egg chamber from starved fly displayed (**f**) no PI3K activity at the plasma membranes of the FCs or NCs. (**g**) Fas III staining shows that FCs increased in size during engulfment of NC remnants. (**h**) Merge. (**i–k**) *tGPH;InR*[339] GLC Pwop stage 4 egg chambers. (**l**) Merge indicates co-localization at the FC plasma membrane of tGPH reporter and Fas III. (**m–o**) *tGPH;S6k*[l−1] GLC Pwop stage 3 egg chamber. (**p**) The merge displays co-localization at the FC plasma membrane of tGPH reporter and Fas III. Scale bar is 20 μm

In healthy WT egg chambers, DIAP1 staining was seen in the NCs of early stage egg chambers but levels decreased in the NCs of mid-stage egg chambers (Figures 5a and b), as previously shown.[5] Degenerating WT egg chambers had greatly reduced levels of DIAP1 in the NCs (Figures 5c and d). Pwops retained moderate levels of DIAP1 in and surrounding the persisting NC nuclei (Figures 5e, f and k). However, in *Tor*[4P] GLC-degenerating egg chambers, DIAP1 levels were depleted similar to WT (Figures 5i and j). The moderate levels of DIAP1 that remained in the Pwops of insulin signaling mutants suggests that DIAP1 could indeed be protecting the germline.

PI3K activity is lost in *InR* and *S6k* Pwops. PI3K is a target of InR that phosphorylates inositol lipids at the plasma membrane, which in turn recruit proteins with phosphoinositide-interacting domains called Pleckstrin Homology (PH) domains.[24] We used a tubulin-green fluorescent protein PH reporter (tGPH)[25] to visualize PI3K activity in egg chambers of WT and insulin pathway mutants. In well-fed control flies, egg chambers displayed the tGPH reporter at the plasma membrane of both the NCs and the FCs, demonstrating PI3K activity (Figures 6a–d). Degenerating egg chambers from starved control flies lost tGPH from the plasma membrane of the FCs and the NCs (Figures 6e–h). Thus, our results indicate that PI3K activity is lost in degenerating egg chambers.

To determine whether PI3K activity was reduced in the mutant egg chambers, flies were generated to carry the tGPH reporter in *InR*[339] or *S6k*[l−1] GLCs. GLCs of *S6k*, which acts downstream of PI3K, would be expected to display normal recruitment of tGPH. *S6k*[l−1] GLCs displayed membrane localization of the tGPH reporter in 67% ($n=15$) of Pwop egg chambers (Figures 6m and n). Most of the tGPH labeling in Pwop egg chambers of *S6k*[l−1] GLCs localized with the FC marker Fas III, indicating that the remaining PI3K activity was not in the NCs (Figures 6n–p). Similarly, *InR*[339] GLCs showed the tGPH reporter at the plasma membrane in 40% ($n=25$) of Pwops (Figures 6i and j), but the reporter was not localized in the NCs of anterior healthy egg chambers (data not shown).
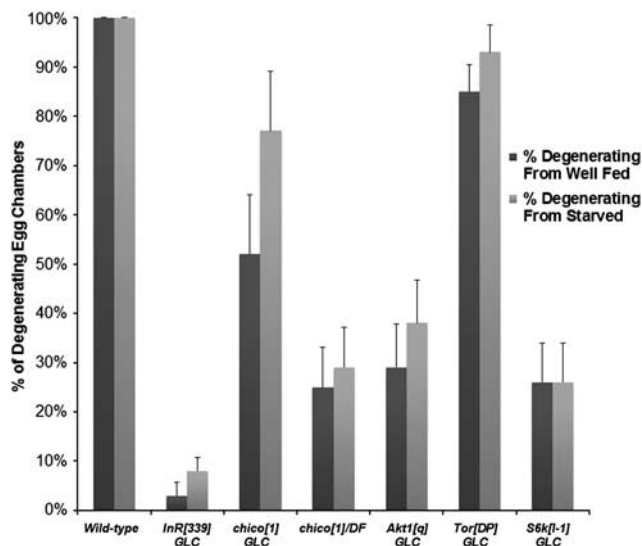
Figure 7 Quantification of starved insulin signaling pathway mutant ovary phenotypes. Insulin mutants were deprived of amino acids similar to WT starved flies. Bar graphs shows the percentage of degenerating egg chambers compared with Pwops in well-fed *versus* starved flies. WT, InR^{339} GLC, $chico^1$/Df, Akt^q GLC, Tor^{1P} GLC, and $S6k^{l-1}$ GLC comparison of well fed *versus* starved had a *P*-value > 0.05 indicating there was no significant difference, whereas $chico^1$ GLCs had a *P*-value < 0.05. (WT *P*-value = 1, InR^{339} GLCs *P*-value = 0.064, $chico^1$ GLCs *P*-value = 0.039, $chico^1$/Df *P*-value = 0.6234, Akt^q GLCs *P*-value = 0.308, Tor^{1P} GLCs *P*-value = 0.1471, and $S6k^{l-1}$ GLCs *P*-value = 1). The total numbers of egg chambers (degenerating + Pwop) for starved flies were: 54 (WT), 192 (InR^{339} GLCs), 61 (Akt^q GLCs), 47 ($chico^1$ GLCs), 49 ($chico^1$ /Df), 55 (Tor^{1P} GLCs), 46 ($S6k^{l-1}$ GLCs). Data for the well-fed flies were the same as in Figure 1 or Supplementary Figure 1. Error bars represent S.E. calculated using a standard two-tailed *t*-test

These data indicate that the insulin signaling pathway is not intact in *InR* or *S6k* GLCs, because we do not see PI3K activity in healthy NCs. Furthermore, PI3K activity is observed in many of the Pwops; however, this activity localized to the membranes of the remaining FCs that have stretched to surround the NCs.

Pwop egg chambers cannot undergo germline PCD in response to starvation. The block in egg chamber degeneration in insulin pathway mutants could be because another pathway acts in parallel to insulin signaling to control egg chamber survival. Therefore, we reasoned that starvation of the insulin signaling mutants would affect such a parallel pathway and the mutants would show an increase in degenerating egg chambers when they were starved. Surprisingly, GLCs from starved insulin pathway mutants still showed the Pwop phenotype (Figure 7). InR^{339} and Akt^q GLCs, and $chico^1$ hemizygotes showed slight increases in the frequency of degenerating egg chambers, but the majority were still Pwops and there was no statistically significant change. $chico^1$ GLCs did show a significant change between well-fed and starved, suggesting either that this is not a null allele or the surrounding WT tissue in the GLC-carrying flies could be contributing to the degeneration of the NCs. Starved Tor^{1P} and $S6k^{l-1}$ GLCs also showed no significant difference between well-fed and starved conditions. These data indicate that the abnormal Pwop phenotype seen in insulin signaling

mutants cannot be explained simply by a parallel pathway acting during starvation.

Discussion

Following starvation, mid-stage egg chambers degenerate via PCD that has hallmarks of both apoptosis and autophagic PCD. In the *Drosophila* salivary gland and midgut, autophagic PCD is regulated by the insulin signaling pathway.[11,12] Results from our study indicate that the insulin signaling pathway may regulate PCD in the ovary similar to other *Drosophila* tissues, but additionally there is cross-talk from other pathways. This cross-talk could come from Tor signaling that is independent of InR. Interestingly, Tor has recently been shown to also have insulin-independent roles in germline stem cell proliferation in the *Drosophila* ovary.[13]

We expected that disruption of the insulin signaling pathway would lead to degenerating egg chambers, resembling those seen in starved WT flies. Surprisingly, we found many abnormal Pwop egg chambers in which the NCs persisted and the FCs disappeared, indicating that PCD was initiated but incomplete. These Pwop egg chambers exhibited a block in DNA fragmentation, weak to no caspase activation, moderate levels of DIAP1 protein, and large domains or a lack of LT staining. Some of the mutant egg chambers did appear to be degenerating normally and these displayed PCD hallmarks such as DNA fragmentation, active caspases, reduced DIAP1 protein, and LT staining. It is also possible that the Pwop phenotype arises because insulin signaling in the NCs is required for FC survival, and viable FCs are needed for germline death.[26]

WT egg chambers that degenerate due to starvation occur specifically during mid oogenesis.[7] *InR, chico, Akt, Tor*, and *S6k* GLCs have a growth delay in which they produce only early stage egg chambers, indicating that insulin signaling is required to progress into mid oogenesis.[7,13] Because insulin signaling mutant egg chambers arrest before entering mid oogenesis, the early stages may be incompatible for the induction of PCD. DIAP1 levels remain high in early stage WT egg chambers but are reduced in mid-stage egg chambers, likely making them more vulnerable to starvation-induced PCD.[5] Perhaps insulin signaling mutant egg chambers at early stages are still protected by DIAP1 and do not reach the susceptible mid-stage. Pwops from *InR, chico*, and *S6k* GLCs did retain moderate levels of DIAP1, suggesting that DIAP1 could be protecting these abnormal egg chambers from PCD. However, early stage degenerating egg chambers with a depletion of DIAP1 were observed in *Tor* GLCs, suggesting that *Tor* mutants can overcome this block, and a pathway in addition to insulin signaling acts to regulate Tor. Consistent with this model, we found that *chico Tor* double GLCs mimicked *Tor* single GLCs (Supplementary Figure 1).

To determine whether an alternative pathway acts in parallel to insulin signaling, we starved the mutant GLCs. If a parallel survival pathway was acting, we expected that egg chambers from starved insulin pathway mutants would degenerate in a manner similar to egg chambers of starved WT flies. Interestingly, *InR, chico, Akt*, and *S6k* mutants still produced the Pwop phenotype. This could be because early stage egg chambers are resistant to PCD. An alternative

possibility is that complete loss of the insulin pathway leads to an aberrant state. Perhaps, dying egg chambers require a certain threshold of insulin signaling to enter into starvation-induced PCD. This hypothesis is supported by our observations of InR and S6K hypomorphic alleles (InR[05545] and S6K[07084]), which produce mid-stage egg chambers that degenerate in a manner similar to egg chambers of starved WT flies (Supplementary Figure 1). These findings support the notion that a small amount of InR activity permits progression into mid oogenesis but also promotes PCD.

Tor can be regulated by nutrients through multiple pathways. The presence of amino acids promotes Tor activity via Rag small guanosine triphosphatases (Rag GTPases) and MAP4K3.[27–30] In Drosophila, MAP4K3 and Rag GTPases are required for growth in response to amino acids and overexpression of Rag GTPases suppresses starvation-induced autophagic PCD in the fat body.[29,30] Furthermore, MAP4K3 has been shown to physically interact with Rag GTPases and amino acids regulate their association.[30] Thus, Tor is an integration point for multiple nutrient sensing pathways, which may explain why Tor mutant egg chambers resemble degenerating egg chambers seen in WT starved flies.

Tor is found in two distinct complexes, TORC1 and TORC2,[31,32] which leads to further complexity in the Tor signaling network. Rag GTPases, MAP4K3 and the insulin signaling pathway converge on TORC1, which then phosphorylates downstream targets such as S6K.[33] The regulation and activity of TORC2 is less well understood. However, the Tor mutants we analyzed would be disrupted for both TORC1 and TORC2, which could explain why Tor mutants showed a different phenotype than the other mutants. In one possible scenario, complete removal of Tor leads to PCD in the germline, whereas disruption of insulin signaling or S6K is not sufficient. Because S6K is regulated only by TORC1,[33] this model explains why the phenotype of S6K more closely resembles chico and InR mutants than Tor mutants.

Using a reporter for PI3K activity, we determined that PI3K activity was associated with healthy egg chambers and lost from degenerating egg chambers in WT flies. Healthy egg chambers from S6k or InR GLCS did not display the tGPH reporter in NCs, indicating that both InR and S6K are required to maintain PI3K activity. Surprisingly, PI3K activity appeared to be restored in many of the Pwop egg chambers from both S6k and InR GLCs; however, this staining was predominantly associated with FC membranes that had stretched over the NCs. One possible explanation for residual PI3K activity is that PI3K is activated by an alternative mechanism in Pwop egg chambers. The regulatory subunit of PI3K binds to a SRC homology-2 (SH2) domain on Chico, but recently the adaptor protein Lnk has been shown to have an SH2 domain that may interact with PI3K.[34] Mutants of Lnk are reduced in size and produce abnormal egg chambers remarkably similar to those seen in InR, chico, Akt, and S6k GLCs.[34]

The pattern of LT suggested that the insulin signaling mutant egg chambers cannot induce autophagy properly. WT starved mid-stage egg chambers that are dying display LT positive spots, whereas InR and chico Pwop egg chambers display LT staining as large domains that partially overlap with NC nuclei. Interestingly, similar large domains of LT staining occur during developmental PCD of NCs, and may represent large lysosomes, or an acidification of NC remnants.[35] Disruption of insulin signaling may not be sufficient to induce apoptosis and autophagic PCD, but the presence of acidified persisting NCs may indicate necrosis.[36] It may be that these abnormal egg chambers progress to a necrotic state because they cannot induce apoptosis.

Our results indicate that the insulin signaling pathway is an important factor for cell survival in the Drosophila ovary, similar to its role in mammals. Mammalian ovaries cultured in serum-free media show an induction in apoptotic and autophagic PCD.[37] Survival of mouse primordial follicles requires PI3K, phosphoinositide-dependent protein kinase-1 (PDK1), and S6k1, which are members of the insulin signaling cascade.[38] Treatment with rapamycin, a drug known to block Tor activity, inhibits oocyte growth in cultured Drosophila and mammalian ovaries, and leads to apoptosis and autophagy.[37,39,40,26] Taken together, these findings suggest an evolutionarily conserved role for insulin and Tor signaling in promoting survival in the ovary. Furthermore, characterization of starvation-induced PCD in the Drosophila ovary may give insight into the mechanisms of degeneration of defective oocytes in mammalian systems during reproductive aging and fertility disorders.

Materials and Methods

Drosophila stocks. *Drosophila* stocks were maintained at 25 °C. All stocks were obtained from the Bloomington *Drosophila* Stock Center unless noted. *tubulin-GPH* was a gift from Bruce Edgar.[25] This reporter for PI3K activity has the PH domain of the *Drosophila* homolog of the general receptor for phosphoinositides-1 (GRP1) fused to GFP and placed under the control of the β-tubulin promoter (*tGPH*).[25] GRP1 binds to phosphatidylinositol-3,4,5-P3 (PIP$_3$) generated by PI3K activity on phospholipids at the plasma membrane; thus, this GFP fusion construct is localized to the plasma membrane when PI3K is active. *Df(2L)J2* is a deficiency that removes *chico*. *chico[116]* was recombined onto the *FRT40A* chromosome. *InR[339]FRT82B* was a gift from Daniela Drummond-Barbosa.[17] *S6k[l−1 41]* was a gift from Tom Neufeld and recombined onto the *FRT82B* chromosome. GLCs were generated with these *FRT* recombinants as well as *Tor[1P]FRT40*[18] using the *FLP/FRT/ovo[D]* system as described.[9] To induce clones, larvae were heat shocked for 2 days at 37 °C for 1 h. Under these conditions, <20% of egg chambers have large FC clones (data not shown).

Ovary staining. Flies were conditioned on yeast paste for 2–3 days. For starvation experiments, flies were conditioned 2–3 days and then moved to apple juice agar for 1–2 days and dissected. Ovaries were dissected in *Drosophila* Ringers (130 mM NaCl, 4.7 mM KCl, 1.9 mM CaCl$_2$, 10 mM Hepes, pH 6.9). Well-conditioned WT ovaries were included with mutant ovaries as carrier to retain mutant ovaries that were greatly reduced in size. Ovaries were fixed for 10 min in one part Buffer B (100 mM KH$_2$PO$_4$/K$_2$HPO$_4$ (pH 6.8), 450 mM KCl, 150 mM NaCl, 20 mM MgCl$_2$ 6H$_2$O), 1 part 36% formaldehyde, 4 parts dH$_2$O, and an equal volume heptane, then washed in PBS plus 0.1% TritonX (PBT). LysoTracker Red (Invitrogen, Grand Island, NY, USA) was used at 1:50 for 3 min and washed with PBS before fixation. Anti-cleaved caspase-3 (Cell Signaling, Danvers, MA, USA) was used at 1:100. Anti-DIAP1 was a gift from Bruce Hay and used at 1:200.[42] Anti-Ref(2)P was a gift from Ioannis Nezis and used at 1:1000.[23] FC-specific antibody anti-Fas III[43] (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) was used at 1:50. Secondary antibodies Goat-anti-rabbit Alexa Fluor 488 (Invitrogen), Goat-anti-rat DyLight 488 and Goat anti-mouse Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were all used at 1:200 for 2 h. TUNEL was performed using the ApopTag Plus Fluorescein *In Situ* Detection Kit (Millipore, Billerica, MA, USA).[44] For the TUNEL assay, ovaries were treated with 20 μg/ml of Proteinase K for 20 min, then post fixed for 5 min. Ovaries were washed after staining three times for 10 min each in PBT to reduce background, and mounted onto microscope slides in Vectashield mountant with DAPI (Vector Labs, Burlingame, CA, USA). Images were taken using an Olympus BX51 microscope with DSU spinning disc attachment, except anti-DIAP1, tGPH,

anti-Ref(2)P, and anti-Fas III images, which were taken on an Olympus FluoView 10i Confocal microscope. All images were prepared in Adobe Photoshop.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)