Modulation of *gurken* Translation by Insulin/TOR Signaling in *Drosophila*

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Summary:

Localized Gurken translation specifies the anterior/posterior and dorsal/ventral axes of the developing Drosophila oocyte. *spindle*-class females lay ventralized eggs resulting from inefficient *grk* translation. This phenotype is thought to result from inhibition of the Vasa RNA helicase. In a screen for modifiers of the eggshell phenotype in *spn-B* flies, we identified a mutation in the *lnk* gene. We show that *lnk* mutations restore Grk expression, but do not suppress the persistence of double strand breaks nor other *spn-B* phenotypes. This suppression does not affect Egfr directly, but rather overcomes the translational block of *grk* messages seen in *spindle* mutants. Lnk was recently identified as a component of the insulin/insulin-like growth factor signaling (IIS) / TOR pathway. Interestingly, direct inhibition of TOR with rapamycin can also suppress the ventralized eggshell phenotype in *spn-B* or *vasa* mutant mothers. When dietary protein is inadequate, reduced IIS/TOR activity inhibits cap-dependent translation by promoting the activity of the translation inhibitor eIF4E binding protein. We hypothesize that reduced TOR activity promotes *grk* translation independent of the canonical Vasa/cap-dependent mechanism. This model suggests a means by which flies can maintain the translation of developmentally important transcripts during periods of nutrient limitation when bulk cap-dependent translation is repressed.

Introduction

Reproduction represents a substantial energy investment for an organism. Many studies have shown that ovarian physiology is exquisitely sensitive to nutritional status. Limitation of dietary protein intake results in a dramatic slowing of egg chamber maturation via developmental arrest, programmed cell death, or loss of germline stem cells (Drummond-Barbosa and Spradling, 2001; Hsu et al., 2008; LaFever and Drummond-Barbosa, 2005). Several signaling pathways are integrated to bring about this response including 20-hydroxyecdysone, Juvenile Hormone (JH), and insulin/insulin-like signaling (IIS). IIS is stimulated by protein feeding and is required for oogenesis to progress. The IIS pathway integrates nutritional signals at two distinct points during oogenesis. The first is in region 2A of the germarium where developing germline cysts undergo apoptosis in the absence of a source of maternal dietary protein. The second point of nutritional control is at stage 8 of oogenesis during the onset of vitellogenesis. In the absence of food, egg chambers develop to stage 8, where they are arrested until a favorable food source is located. These two checkpoints represent points at which the energetically expensive process of oogenesis can be halted if insufficient resources are available (Hsu et al., 2008; LaFever and Drummond-Barbosa, 2005).
The IIS pathway elicits its effect on Drosophila physiology through several effector pathways, namely the dFOXO transcription factor and the Target of Rapamycin kinase (TOR) (Grewal, 2009; Teleman, 2010). IIS inhibits dFOXO activity by promoting its phosphorylation by PKB/Akt and subsequent exclusion from the nucleus. Starvation or mutations in the insulin pathway allow dFOXO to translocate to the nucleus where it directs the transcription of genes that promote longevity, stress resistance, fat storage, and growth attenuation (Hwangbo et al., 2004; Giannakou et al., 2004; Junger et al., 2003). TOR activity is stimulated by both IIS through the dRheb GTPase and by amino acids via Rag GTPases (Grewal, 2009; Gao and Pan, 2001; Kim et al., 2008; Sancak et al., 2008). When nutrients are plentiful, high TOR activity stimulates the translation of mRNA by phosphorylating S6K which in turn phosphorylates eIF4B and promotes its interaction with eIF3 (Holz et al., 2005). These steps are critical for recruiting the translation preinitiation complex (PIC) to the m7G cap at the 5' end of the mRNA. Once bound, the PIC recruits the small ribosomal subunit and proceeds to scan the transcript for an initiating AUG codon. This process requires the activity of the eIF4A RNA helicase (Sonenberg and Hinnebusch, 2009). TOR also phosphorylates and inactivates the inhibitory eIF4E binding protein, 4EBP. Starvation inhibits cap-dependent translation through reduced TOR activity. When nutrients are limiting and TOR activity is low, eIF4B is not phosphorylated and can no longer participate in PIC assembly, furthermore 4EBP inhibition is lifted and it proceeds to inhibit cap-recognition by eIF4E (Richter and Sonenberg, 2005). Both activities have the effect of strongly blocking cap-dependent translation initiation when nutrients are scarce. A select few transcripts escape this translational block by upregulating the utilization of an alternative mechanism that relies on an Internal Ribosomal Entry Site (IRES) that obviates the requirement for cap recognition and start codon scanning. The list of transcripts that contain IRES sequences is growing (Mokrejs et al., 2009) and includes numerous growth factors such as VEGF-A (Huez et al., 2001), FGF2 (Arnaud et al., 1999), PDGF2 (Bernstein et al., 1997), and IGF-II (Pedersen et al., 2002). A prominent example of IRES-mediated nutritional adaptation is the Drosophila insulin receptor dInR, the translation of which is upregulated in response to starvation as a way to sensitize the cell to insulin when nutrients become available (Marr et al., 2007).

Control of translation is vitally important to developmental patterning. The transcripts of many morphogens, including nanos, oskar, and gurken, are co-transcriptionally packaged into silencing particles and transported in a translationally quiescent form (Tomancak et al., 1998; Besse and Ephrussi, 2008; Chekulaeva et al., 2006; Martin and Ephrussi, 2009; Delanoue et al., 2007; Norvell et al., 1999). Once localized, this repression is alleviated and translation proceeds in the developmentally appropriate locale. Gurken (Grk) is a TGF-α related ligand for the Drosophila Egfr. Localized translation of the spatially restricted grk transcript results in signaling by germline-derived Grk to the Egfr in the overlying
follicle cells. This signal is required to specify the posterior fate in early oogenesis and the dorsal fate during mid oogenesis (Gonzalez-Reyes et al., 1995; Roth and Lynch, 2009). Mutations that reduce grk translation are female sterile due to an inability to correctly pattern the developing oocyte and result in concomitant patterning defects in the embryo. grk translation requires the eIF4A-related DEAD-box helicase Vasa (Vas). Mutations in vas are female sterile owing to a failure to specify dorsal structures in the egg shell or posterior structures in the embryo (Tomancak et al., 1998; Styhler et al., 1998; Lasko and Ashburner, 1988; Tinker et al., 1998; Schüpbach and Wieschaus, 1986).

Spindle class genes are responsible for repairing DNA double strand breaks (DSBs) that are induced during homologous recombination in Drosophila oogenesis (Jang et al., 2003; McKim et al., 2002; Ghabrial et al., 1998; Staeva-Vieira et al., 2003). In wild type females, DSBs are induced in germ line cells entering pachytene in region 2A of the germarium. This process is initiated by the Spo11 homologue Mei-W68 and Mei-P22, a protein that aids in break site selection (Liu et al., 2002; McKim and Hayashi-Hagihara, 1998). These breaks are then repaired by homologous recombination, a process that requires the RAD-51 homologue spindle-B (spn-B). Mutations in spn-B result in an accumulation of unrepaired DSBs that lead to activation of a meiotic checkpoint (Ghabrial et al., 1998; Ghabrial and Schüpbach, 1999; Jang et al., 2003). The checkpoint is comprised of the ATR homologue mei-41 and the downstream kinase chk-2 (Ghabrial et al., 1998; Ghabrial and Schüpbach, 1999; Abdu et al., 2002; Jang et al., 2003). Persistent DSBs in spn-B<sup>BU</sup> females activate the checkpoint that requires the Mei-41 and Chk2 kinases and leads to inefficient grk translation and ventralized eggshell phenotypes. Checkpoint activation also results in phosphorylation of Vasa, a modification that is thought to inhibit its function (Ghabrial and Schüpbach, 1999; Abdu et al., 2002). Early in oogenesis, the oocyte nucleus becomes arrested in pachytene and forms a compact structure called the karyosome. The formation of the karyosome is disrupted in spindle-class mutants where the chromatin appears fractured or ellipsoid (Ghabrial et al., 1998; Abdu et al., 2002). Weak grk translation and an inability to properly form the karyosome are both spindle phenotypes that are consistent with reduced Vasa activity (Tomancak et al., 1998).

In the current study, we identified the SH2B family adaptor gene lnk in a genetic screen for modifiers of the ventralized eggshell phenotype seen in spn-B<sup>BU</sup> mutant flies. SH2B proteins are known to regulate intracellular signaling by membrane bound receptor tyrosine kinases (RTKs). SH2Bs can promote signaling by scaffolding downstream effectors to the RTK or mediate proteosomal receptor destruction by recruiting the Cbl ubiquitin ligase. Lnk was recently identified as a positive regulator of the Insulin/Insulin-like Signaling (IIS) pathway that functions at the level of the insulin receptor substrate Chico (Slack et al., 2010; Werz et al., 2009). Here we show that lnk mutations can promote grk translation and suppress the ventralized eggshell phenotype in a spn-B<sup>BU</sup> mutant background. This suppression
occurs independent of Vasa activity and does not suppress the karyosome phenotype. We did not find any genetic interactions with a weak grk allele nor downstream targets of Egfr suggesting that lnk-mediated suppression of spindle phenotypes does not occur by directly modulating Egfr activity. Our data suggest that lnk mutations promote grk translation by inhibiting TOR activity as Rapamycin feeding experiments can also suppress the eggshell phenotype of spn-B and vas mutant flies. We propose a model in which reduced IIS/TOR signaling inhibits cap-dependent translation and promotes utilization of an alternative translation initiation mechanism of the grk mRNA. This mechanism enables flies to faithfully pattern their oocytes when nutrients are scarce.

**Results**

*lnk* Mutations Suppress Dorsal / Ventral Patterning Defects in *spindle-B* Mutants

To identify factors that mediate the interface between the meiotic checkpoint and grk translation, we conducted an EMS screen on the third chromosome for mutations that could suppress the ventralized eggshell phenotype seen in *spn-B^{BU}* females (Figure 1A). Two lines, CA1215 and CR642, yielded strong suppression and produced a majority of wild type eggs despite being mutant for *spn-B^{BU}* (Figure 1B). The mutations were positionally cloned and the molecular lesions were identified. CA1215 is an allele of the mei-P22 gene. mei-P22 is required to initiate DSB formation in pachytene and mutations in mei-P22 suppress ovarian phenotypes associated with *spn-B^{BU}* mutations (Ghabrial and Schüpbach, 1999; Liu et al., 2002). Similarly mei-P22^{CA1215}, *spn-B^{BU}* mutants lay eggs with wild type D/V polarity, normal Grk expression, and no indication of DSB formation in the germarium (Figures 1B and 5D). This result validated our experimental approach and illustrated that we were able to isolate alleles of genes known to be involved in the meiotic recombination process.

CR642, a second strong suppressor mutation, was mapped to the lnk locus and found to be a G to A transition mutation at nucleotide 894 of the lnk ORF. (Figure 1C). Lnk is a member of the SH2B adapter family that is known to modulate receptor tyrosine kinase (RTK) signaling. Three functional domains have been described including a phenylalanine zipper dimerization domain, a plextrin homology domain, and an SH2 domain. There is also a conserved C-terminal tyrosine (Y720) that is required for binding to the Cbl ubiquitin ligase in mammalian homologues (Hu and Hubbard, 2005). The lnk^{CR642} allele results in a nonsense codon after serine 297 that truncates the protein leaving the dimerization domain and most of the PH domain intact. The lnk^{CR642} allele was tested in trans to the Df(3R)ExpL3 and Df(3R)BSC140 deficiencies as well as with transposon integration alleles. The lnk^{f02642} and lnk^{f05062} alleles are PBac[WH] insertions in the first intron of the coding sequence, while the lnk^{d07478} allele is a P[XP] insertion in the
5′UTR (Thibault et al., 2004). Homozygous and hemizygous allelic combinations yielded similar levels of suppression with greater than 80% wild type eggs being laid by spn-B, lnk double mutants. Notably, heteroallelic spn-B<sup>BU</sup>, lnk<sup>CR642</sup>/lnk<sup>d07478</sup> females show particularly strong suppression and lay 96% wild type eggs in contrast to spn-B<sup>BU</sup> flies that only lay 28% wild type eggs (Figure 1B). lnk<sup>CR642</sup>/lnk<sup>d07478</sup> also suppressed the spindle-A mutation, indicating that this effect is not specific to spn-B. The suppression of spn-B<sup>BU</sup> reflects restored Grk protein levels in mid-oogenesis (Figure 1D-F). Genomic lnk rescue constructs are able to revert the suppression phenotype and produce a ventralization profile similar to that seen in spn-B<sup>BU</sup> flies (Figure 2E). While loss of lnk activity is able to suppress the ventralized eggshell phenotype, eggs laid by spn-B<sup>BU</sup>, lnk<sup>CR642</sup> females do not hatch. We have also observed that eggs laid by females homozygous mutant for lnk<sup>CR642</sup> but wildtype for spn-B are patterned correctly, however 18% do not hatch.

**Lnk is Expressed in both Germline and Follicle Cells**

To determine where Lnk protein is expressed, we generated a rabbit polyclonal antibody against amino acids 56-542 of Lnk. Whole mount immunostaining of lnk<sup>CR642</sup> follicle cell clones revealed a membrane localization in heterozygous and twin spot follicle cells but a nearly undetectable level of expression in lnk<sup>CR642</sup> clones (Figure 2A and A′). Lnk protein is also strongly expressed in the nurse cells and accumulates at the membrane. Genomic EGFP-lnk fusion constructs recapitulated the localization seen by immunofluorescence (Figure 2B - D). The level of expression in the germline was somewhat more robust than in follicle cells, especially in regions 1 and 2A of the germarium where nutritional control of germline cells is most pronounced.

The localization of Lnk throughout the ovary prompted us to assess the functional requirements for Lnk expression. We generated a UAS-lnk expression construct and crossed it with the follicle cell driver CY2-Gal4 or the germline driver nos-Gal4 to determine which tissue mediated the spn-B<sup>BU</sup> suppression. Consistent with a role in the regulation of the germline morphogen Grk, nos-Gal4 driven UAS-lnk rescued the suppression seen in spn-B<sup>BU</sup>, lnk<sup>CR642</sup> flies while expression in the somatic follicle cells did not (Figure 2F).

The rate of division of developing cysts in the germarium drops rapidly in flies with a protein poor diet (Drummond-Barbosa and Spradling, 2001). These results are consistent with the role of SH2B proteins as signal transduction scaffolds that interface with membrane bound RTKs. If Lnk attenuates Egfr signaling, loss of this activity could potentiate signaling and counteract the inefficient grk translation seen in spn-B<sup>BU</sup> flies. Alternatively, the suppression phenotype may be related to the ability of lnk to regulate
signaling by the Drosophila Insulin Receptor (dInR) which modulates ovarian development in response to nutritional availability (Slack et al., 2010; Werz et al., 2009).

**lnk Does Not Directly Modulate Egfr Signaling in the Ovary**

In mid oogenesis Grk is translated in the future dorsal anterior of the oocyte and interacts with the EGF receptor to specify the dorsal fate in the follicle cell epithelium whose default fate is ventral (see Roth and Lynch, 2009 for a recent review). It has been shown that there is crosstalk between the IIS pathway and the Egfr in the developing eye (McNeill et al., 2008). Furthermore, mammalian SH2B proteins have been shown to recruit the Cbl ubiquitin ligase and promote clathrin-mediated endocytosis and recycling of ligand-bound receptors as a negative feedback mechanism (Yokouchi et al., 1999; Ahmed and Pillay, 2001; Thien and Langdon, 2005). Mutations in Drosophila *cbl* lead to activation of the Egfr even in lateral and ventral follicle cells that are exposed to the lowest levels of the Grk gradient (Pai et al., 2000, 2006; Chang et al., 2008). Therefore we tested the hypothesis that Lnk can directly regulate the activity of the Egfr in ovarian follicle cells.

If *lnk* mutations were to sensitize the Egfr to Grk signaling, reduced Grk levels caused by *spn-B<sup>BU</sup>* mutations should have the same effect as a hypomorphic allele of the *grk* gene from the perspective of the follicle cells. To test this hypothesis, we made flies that were double mutant for *lnk*<sup>CRI642</sup> and *grk*<sup>ED22</sup>. The *grk*<sup>ED22</sup> allele is a missense mutation in the EGF domain that affects RNA levels and may also potentially reduce Egfr binding. *grk*<sup>ED22</sup> females lay eggs with a single appendage due to inefficient activation of the Egfr (Clifford and Schüpbach, 1989; Neuman-Silberberg and Schüpbach, 1993; Thio et al., 2000). Surprisingly, the eggs laid by *grk*<sup>ED22</sup>; *lnk*<sup>CRI642</sup> flies are indistinguishable from those produced by *grk*<sup>ED22</sup> single mutants and all have a single appendage (data not shown).

Mutations that promote Egfr signaling result in ectopic dorsal fates in follicle cell clones. For example, follicle cell clones of *cbl* cause dorsalization of lateral domains of the eggshell due to hyper-activation of the Egfr (Pai et al., 2000). If Lnk attenuates Egfr signaling by promoting Cbl-dependent Egfr endocytosis, mutations in *lnk* should also result in ectopic Egfr activation. Unlike *cbl* clones, eggs laid by *lnk*<sup>CRI642</sup> mutant flies are indistinguishable from wild type (data not shown). This result was supported by looking directly at a LacZ enhancer trap for the Egfr transcriptional target *kekkon* (*kek-LacZ*) in *lnk*<sup>CRI642</sup> follicle cell clones. The intensity of *kek-LacZ* expression is proportional to the level of Egfr activity. In contrast to *cbl* clones (Pai et al; 2000) no difference in *kek-LacZ* activity was observed across the border of *lnk* homozygous mutant, heterozygous or wild type twin spot clones (Figure 3A and B).
To further rule out that the eggshell suppression was due to effects on Cbl, we generated \textit{lnk} mutants that block interactions with Cbl in mammalian Lnk orthologues. Phosphorylation of the intracellular domain of tyrosine kinase receptors induces the association of APS family proteins. The human insulin receptor recruits APS following insulin binding and subsequently phosphorylates APS at a C-terminal tyrosine. Phosphorylation of this tyrosine in APS is strictly required to recruit the TKB domain of Cbl to the activated insulin receptor (Liu et al., 2002). The context of a conserved tyrosine residue at position 720 in Drosophila Lnk conforms to the consensus binding site for Cbl and is identical (RAVxNQYS) to human APS (Hu and Hubbard, 2005). To determine if this tyrosine is required to rescue \textit{lnk} suppression of \textit{spn-B}^{RU}, we performed a rescue experiment with a wild type \textit{lnk} transgene or a \textit{lnkY720F} allele driven by the genomic promoter. These rescue constructs were integrated into the \textit{attP40} docking site at 25C7 using the \textit{ΦC31} site-specific integrase system to control for position effects on transgene expression (Markstein et al., 2008; Venken and Bellen, 2007). Both alleles were able to rescue the suppression of \textit{spn-B}^{RU} / \textit{spn-B}^{Δ57C}, \textit{lnkCR642} / \textit{lnkY720F} to a similar extent, indicating that phosphorylation of this residue is not crucial for recruiting Cbl activity and thus downregulation of Egfr in the follicle cells (Figure 3C). Taken together, these data suggest that Lnk does not directly shape the gradient of Egfr activity in the follicle cells.

\textit{lnkCR642} Reduces Insulin / Insulin-Like Signaling and Slows the Rate of Oogenesis

Lnk is a positive regulator of insulin/insulin like signaling in Drosophila (Werz et al., 2009; Slack et al., 2010). Based on epistatic analysis, Lnk was shown to function upstream of PI3K at the level of the Chico insulin receptor substrate. \textit{chico}^{1} / \textit{chico}^{2}, \textit{lnk}^{Q3}/\textit{lnk}^{SS2} double mutants exhibit synthetic lethality that can be suppressed by heterozygosity of the \textit{pten} phosphatidylinositol-(3,4,5)-trisphosphate (PIP3) phosphatase (Werz et al., 2009). Reduction of IIS either by mutation or dietary restriction (DR) results in atrophic ovaries with a dearth of egg chambers older than stage 8. This nutritional control point coincides with the onset of the energetically expensive process of vitellogenesis and arrests development until adequate resources are available. We find that while the P-element insertion \textit{lnk}^{07478} allele produces a classical IIS ovarian atrophy with minimal yolk production (Figures 4B and E), \textit{lnkCR642} ovaries are highly vitellogenic and morphologically indistinguishable from wild type (Figures 4C and F).

In light of the vitellogenic phenotype of \textit{lnkCR642} ovaries, we wanted to determine the effect of \textit{lnk} mutations on PI3K signaling in the ovary. The tGPH reporter is sensitive to the levels of PIP3 in the cell and exhibits strong membrane localization when PI3K activity high (Britton et al., 2002). Follicle cell clones of the strong \textit{lnk}^{02642} allele reduced the membrane localization of the tGPH reporter (Figure 4G). This is consistent with results obtained by Wertz et al. in the larval fat body (Werz et al., 2009).
To determine if \( \text{lnk}^{\text{CR642}} \) causes a developmental delay in oogenesis similar to that seen under conditions of moderate nutritional deprivation we monitored the rate of oocyte maturation by lineage tracing (Drummond-Barbosa and Spradling, 2001). The X-15 marker system was used to induce \( \text{lacZ}^+ \) clones in dividing germline cells in the germarium (Harrison and Perrimon, 1993). Four days following heat shock Flp induction, the extent of egg chamber maturation was determined by counting the most mature cyst in each ovariole with multiple \( \beta \)-galactosidase positive nuclei. The data show distinctly different rates of development with wild type ovaries typically supporting development to stage 10B, while \( \text{lnk}^{\text{CR642}} \) egg chambers were most frequently observed at developmental stage 6 (Figure 4G). These data suggest that \( \text{lnk}^{\text{CR642}} \) brings about a modest reduction in IIS that slows oogenesis but is not severe enough to completely arrest development like the more severe \( \text{lnk}^{\text{d07478}} \) allele.

**\( \text{lnk} \) Does Not Affect DNA Repair or Vasa Phenotypes**

In region 2A of the germarium Mei-W68 and Mei-P22 generate double strand breaks as an obligate step in meiotic recombination that can be detected by immunostaining of phosphorylated His2Av (\( \gamma \)-H2Av) (Mehrotra and McKim, 2006). In wild type flies, these breaks are initiated in both nurse cells and pro-oocytes, however they are repaired in region 2B and by the time cysts reach region 3 and bud off into the vitellarium no \( \gamma \)-H2Av foci are evident (McKim et al., 2002). Mutations in the spindle class genes delay the formation of breaks until region three of the germarium and preclude the repair of these DSBs resulting in \( \gamma \)-H2Av foci that are evident until stage 4 in the vitellarium (Staeva-Vieira et al., 2003; Jang et al., 2003). It is unclear if the disappearance of \( \gamma \)-H2Av in spindle mutants indicates that other repair pathways such as non-homologous end joining (NHEJ) are compensating for a lack of homologous recombination or if \( \gamma \)-H2Av is simply dissipating while the DSBs remain. Because \( \text{lnk}^{\text{CR642}} \) slows the rate of oogenesis, it is possible that this delay provides additional time to repair the breaks that persist in \( \text{spn-}B^{\text{BU}} \) mutants. If compensatory repair is occurring, one would predict that all of the ovarian phenotypes seen in \( \text{spn-}B^{\text{BU}} \) flies such as karyosome malformation and Vasa phosphorylation would also be suppressed.

To determine the prevalence of DSBs, we stained ovaries for \( \gamma \)-H2Av. Consistent with previous results, \( \text{spn-}B^{\text{BU}} \) ovaries had breaks in region 3 while \( \text{spn-}B^{\text{BU}}, \text{mei-P22}^{\text{CA1215}} \) did not exhibit any staining due to a defect in the generation of DSBs (Figure 5B and D). Despite the rate of oogenesis being slower in \( \text{spn-}B^{\text{BU}}, \text{lnk}^{\text{CR642}} \) mutants, \( \gamma \)-H2Av foci were still prevalent in region 3, indicating that DNA repair activity is not enhanced in \( \text{lnk} \) mutants (Figure 5C). Consistent with previous findings for \( \text{spn-}B^{\text{BU}} \) (Jang et al., 2003), the \( \gamma \)-H2Av foci in \( \text{spn-}B^{\text{BU}}, \text{lnk}^{\text{CR642}} \) ovaries persisted until stage 4 after which they disappeared (data not shown). We also examined the morphology of the karyosome in mid-oogenesis in these mutant...
backgrounds. In a \textit{spn-B}\textsuperscript{Blu} background, mutation of \textit{mei-P22} was able to suppress the fragmented karyosome (Figure 5G), however similar to the \(\gamma\)-H2Av result, \textit{lnk}\textsuperscript{CR642} was not able to rescue the karyosome malformation (Figure 5H).

The fragmented karyosome phenotype seen in \textit{spindle} mutant flies is shared with flies carrying alleles of the \textit{vasa} RNA helicase. Though this has yet to be shown directly, Vasa phosphorylation resulting from Mei-41/Chk2 checkpoint activation is believed to inhibit Vasa activity and lead to both karyosome malformation and reduced Grk translation. We examined the phosphorylation state of Vasa in \textit{spn-B}\textsuperscript{Blu}, \textit{lnk}\textsuperscript{CR642} ovaries by Western blotting. The mobility of ovarian Vasa from \textit{spn-B}\textsuperscript{Blu}, \textit{lnk}\textsuperscript{CR642} was slower than wild type and identical to Vasa from \textit{spn-B}\textsuperscript{Blu} ovaries indicating that Vasa is still phosphorylated in this background (Figure 5I). These results were somewhat surprising given that the primary etiology of the ventralized eggshell phenotype in \textit{spindle} mutants is inefficient \textit{grk} translation, a process that is thought to require the activity of Vasa.

\textit{spn-B} and \textit{vas} Mutants Can be Suppressed by Rapamycin

The observation that robust \textit{grk} translation can be supported despite continued phosphorylation of Vasa in \textit{spn-B}\textsuperscript{Blu}, \textit{lnk}\textsuperscript{CR642} mutants suggests that the cap-dependent mode of translation initiation that typically governs \textit{grk} translation is being bypassed (Clouse et al., 2008). IIS is known to regulate cap-dependent translation by controlling the activity of the eIF4E binding protein (4EBP). Low levels of IIS and TOR activity, such as occurs during starvation or rapamycin feeding, allow 4EBP to interact with eIF4E and inhibit its cap-binding activity and subsequent translation initiation. When IIS activity is high, it promotes the activity of the TOR kinase which in turn phosphorylates 4EBP. This inhibitory phosphorylation blocks the interaction between 4EBP and eIF4E and permits eIF4E to participate in cap-dependent translation initiation. (Ruggero and Sonenberg, 2005). Recently it was shown that the Drosophila insulin receptor (InR) transcript uses an alternative translational initiation mechanism to respond to nutrient availability. In times of nutrient limitation when IIS signaling is low and 4EBP activity is high, the InR is robustly translated at an internal ribosomal entry site (IRES). The IRES in the 5’ UTR of the \textit{InR} mRNA allows translation to bypass the 4EBP block of bulk cap-dependent translation (Marr et al., 2007).

To determine if \textit{grk} translation can overcome a block in the cap-dependent pathway induced by \textit{spn-B}\textsuperscript{Blu} or \textit{vasa}\textsuperscript{PH165} / \textit{vasa}\textsuperscript{RG53} mutations, we attempted to suppress the ventralized eggshell phenotype of these flies by promoting 4EBP activity with rapamycin. Through interactions with FK506 binding proteins, rapamycin inhibits TOR activity thereby alleviating the repression of 4EBP. Rapamycin was added to yeast paste and grape juice agar and fed to the flies for 7 days. The eggshell phenotypes were scored on days 4 - 6. Surprisingly, rapamycin promoted a greater proportion of wild type eggs in \textit{spn-B}\textsuperscript{Blu} flies up to
a concentration of 5 μM while 10 μM completely arrested oogenesis (Figure 6A). This indicates that moderate inhibition of TOR activity can phenocopy the lnk<sup>CR642</sup> mutation and promote grk translation. This result suggests that even in situations where the translation of most cellular mRNA is repressed, grk translation can persist.

Finally, we looked directly at the phenotype of vasa mutants that are unable to translate grk (Figure 6B). Strong vasa mutants still accumulate grk mRNA in the oocyte, however Grk protein is not translated and strongly ventralized eggs are laid (Styhler et al., 1998). We hypothesize that in these flies, cap-dependent translation of grk is blocked due to an inability to navigate secondary structure that is encountered during scanning of the 5’ UTR and/or to promote joining of the 60S ribosomal subunit via interactions with eIF5B (Johnstone and Lasko, 2004). While the null vasa<sup>PH165</sup> allele did not produce many eggs, the weaker heteroallelic vasa<sup>PH165</sup> / vasa<sup>RG53</sup> combination resulted in increased fecundity and an eggshell distribution similar to spn-B<sup>BU</sup> flies. When these flies were fed rapamycin, strong suppression of the ventralized eggshell phenotype was evident and greater than 90% of the eggs laid by the 10 μM rapamycin cohort were wild type (Figure 6B).

**Discussion**

In the current study we demonstrate a novel interaction between a meiotic checkpoint, the insulin/insulin-like signaling pathway, and translation of gurken mRNA in Drosophila oogenesis. Mutations in meiotic DNA repair enzymes such as spn-B result in persistent DSBs in early oogenesis that activate an ATR-Chk2-dependent meiotic checkpoint. Checkpoint activation results in phosphorylation of the eIF4A-like RNA helicase Vasa, the activity of which is important for grk translation. In these mutants, low levels of Grk protein are synthesized which is insufficient to pattern the eggshell correctly and results in ventralized eggs. Using forward genetics, we isolated an allele of the insulin receptor adapter, lnk. This mutation can suppress the weak grk translation phenotype and restore normal patterning to eggs laid by spn-B<sup>BU</sup> flies. We have shown through clonal analysis that lnk mutations reduce IIS in a cell-autonomous manner in the ovary. As in mammals, Drosophila IIS controls the rate of cap-dependent translation initiation in the cell by regulating the activity of the TOR kinase. Rapamycin inhibits TOR activity and we have shown that feeding rapamycin can suppress the ventralized eggshell phenotype not only in spn-B<sup>BU</sup> females, but also in vasa<sup>PH165</sup> / vasa<sup>RG53</sup> flies. These data suggest an alternative translation initiation mechanism for the grk mRNA by which flies can maintain D/V axis patterning in times of moderate nutrient limitation.

*Inhibition of TOR activity leads to activation of grk translation*
The discovery that mutations in \textit{lnk}, a positive regulator of IIS, can suppress the patterning defects in \textit{spn-B} flies was initially surprising. The eggshell phenotypes of the different genotypes were assessed after keeping the flies on apple or grape juice agar plates on which abundant amounts of yeast paste had been added thus allowing the females to eat a very protein rich diet (Wieschaus and Nusslein-Volhard, 1998). A protein rich diet stimulates the activity of the TOR kinase via two mechanisms. Insulin-like peptides (dilps) are secreted into the hemolymph by neuroendocrine cells in response to nutrient availability (Ikeya et al., 2002). This in turn activates the IIS cascade comprised of Chico/Lnk, PI3K, Akt, Tsc1/2, and Rheb which promotes TOR-C1 activity (Grewal, 2009). The second mechanism acts more directly through the levels of intracellular amino acids that are imported in part by the \textit{slimfast} and \textit{pathetic} transporters. Both of these mechanisms stimulate TOR-C1 activity which has been shown to promote cap-dependent translation by inhibiting 4EGBP sequestration of eIF4E (Holz et al., 2005; Sonenberg and Hinnenbusch, 2009; Teleman, 2010). Therefore, reducing TOR activity either by a mutation in \textit{lnk} or by addition of rapamycin, would be expected to interfere with cap-dependent translation and therefore further enhance the mutant phenotype. However, in \textit{spn-B} mutant flies, cap-dependent translation is already inhibited by the activity of the checkpoint, presumably acting via Vasa modification. The fact that we observed a suppression of the ventralized phenotype in \textit{lnk} mutants indicates that reduction in TOR signaling must activate a second mode of translation that allows Gurken protein to be produced independently of the block in cap-dependent translation.

\textbf{A Model of Vasa Independent gurken Translation}

Several ovarian phenotypes are shared between mutations in \textit{spindle} genes and \textit{vas} mutants, including failure to form a compact karyosome, very weak \textit{grk} translation, and ventralized eggs. Combined with the reproducible phosphorylation of Vas protein in \textit{spindle}-class mutants, these phenotypes are consistent with a defect in Vas activity. While the specific effect of this phosphorylation is unknown, Vas serves several functions in cap-dependent translation initiation of \textit{grk} mRNA. Vasa has been shown to interact with eIF5B and mutations that interfere with this interaction inhibit \textit{grk} translation (Johnstone and Lasko, 2004). This interaction is thought to facilitate assembly of the 60S ribosomal subunit at the AUG start codon. Furthermore, as a DEAD-box RNA helicase, Vasa may permit the pre-initiation complex to scan the 5’ UTR of \textit{grk} and negotiate secondary structures that may impede the progress of this complex (Liang et al., 1994). IRES sequences adopt strong secondary structures in the 5’ UTR of RNAs that they regulate (Kanamori and Nakashima, 2001). If it can be demonstrated in the future that \textit{grk} possess an IRES sequence, this may explain the requirement for Vasa helicase activity to unwind this structure when translation is initiated from the 5’ cap during conditions of adequate nutrient availability. Whether the checkpoint dependent phosphorylation of Vas affects its stability, RNA helicase activity, or its eIF5B
interaction, the expected result is a block in cap-dependent translation initiation of \textit{grk} mRNA and concomitant D/V patterning defects. Our observation that \textit{grk} translation can be induced to occur in \textit{spn-B}^{BU} and in \textit{vas}^{PH165} / \textit{vas}^{RG53} flies indicates that an alternative mechanism for supporting translation initiation is taking place. Because reduced IIS and TOR activity both block bulk cap-dependent translation initiation through sequestration of eIF4E by 4EBP, yet stimulate IRES activity we propose that the latter may provide an explanation for our results.

\textit{Proposed Mechanism of Spindle Phenotype Suppression by IIS/TOR}

Grk plays a central role in shaping the development of the egg and subsequent embryo. Mutations that disrupt Grk / Egfr signaling during oogenesis result in female sterility (Schüpbach, 1987). Blocking the translation of this essential morphogen in \textit{spindle} class mutants that are unable to repair DNA damage is an effective mechanism to prevent the transmission of mutations to the progeny (Abdu et al., 2002; Ghabrial and Schüpbach, 1999). This reproductive checkpoint is effective when nutrients are abundant, however as we have demonstrated, the strategy breaks down when IIS/TOR activity is low. Under these conditions, \textit{grk} can be translated and result in eggs that are patterned correctly (Figure 1), even though the DNA damage and karyosome malformation phenotypes persist (Figure 5). We propose that this difference occurs because the DNA-damage checkpoint can only impinge on one of the two mechanisms by which \textit{grk} translation can be initiated.

One mechanism by which suppression of the D/V patterning defects of \textit{spn-B}^{BU} may occur is through the effects of the additional time that \textit{lnk}^{CR642} egg chambers spend completing oogenesis. While Grk production is reduced in \textit{spn-B}^{BU} flies, it is not completely blocked and some Grk protein is made. If the reduced rate of Grk production is integrated over the extended time spent during mid oogenesis, sufficient Grk levels could accumulate and support normal D/V patterning. However, this model is inconsistent with the inability of \textit{lnk}^{CR642} to suppress the ventralized eggs laid by \textit{grk}^{ED22} females. These flies do retain some Grk activity as is evident by the single appendage that is specified, however if the mechanism of suppression were via accumulation, then \textit{grk}^{ED22} should be suppressed by \textit{lnk} mutations. Therefore, we favor the IRES-dependent model proposed herein.

The selective pressure that may have driven the evolution of this bi-modal translation mechanism for \textit{grk} can be best understood by considering that in wild populations of \textit{Drosophila}, females feed and oviposit at locations where yeast is abundant (Good and Tatar, 2001). This behavior ensures adequate nutrition to support oogenesis in the female as well as for the developing larvae (Drummond-Barbosa and Spradling, 2001; LaFever and Drummond-Barbosa, 2005). If however nutrients become scarce, females adjust the rate of oogenesis to match nutrient availability. In response to complete starvation, egg chambers undergo
apoptosis and are reabsorbed, however moderate reductions in IIS slow the rate of oogenesis until an abundant protein source is found. The conserved response to dietary restriction is to repress cap-dependent translation of most cellular transcripts while a select population of RNAs that are essential for survival escape this repression by utilizing a cap-independent IRES mechanism. We posit that grk may be one such transcript. Oocytes that are in mid development when nutrients are scarce must still be patterned appropriately so that the resulting eggs are fertile. IRES activity may facilitate Grk expression to maintain normal D/V patterning in times of lean whereas when nutrients are abundant, cap-dependent translation predominates.

**Materials and Methods**

**Eggshell Phenotype Scoring**

Five female flies of the indicated genotypes were placed in egg laying blocks with an equal number of sibling males of any genotype (Wieschaus and Nusslein-Volhard, 1998). The blocks were inverted on apple juice or grape juice agar plates onto which active yeast paste had been spotted and kept at 25°C. The plates were changed daily before the flies had consumed the available yeast to guard against dietary restriction. Eggshell phenotypes were scored on days 4-7 as previously described (Ghabrial et al., 1998). When indicated, rapamycin (LC Laboratories) dissolved in DMSO was added to both the agar substrate as well as the yeast paste. The final concentration of DMSO in the media was 0.1%.

**EMS Mutagenesis Screen**

75-100 st, spn-B<sup>BU</sup>, sr, e / TM6B, Hu, e adult males were starved for 2 hours, then fed a 1% sucrose solution containing 0.25% ethylmethanesulfonylate for 24 hours, and cleaned by transferring to fresh food four times. The mutagenized males were crossed to ras, st, e / TM8, DTS, Sb, st, e, th virgin females at 18°C. The males were discarded after 5-7 days. In the F<sub>1</sub> generation single st, spn-B<sup>BU</sup>, sr, e * / TM8, DTS, Sb, st, e, th (where * indicates that the chromosome was present during the mutagenesis) virgin females were crossed to P[w<sup>+</sup>, ovoD] / TM8, DTS, Sb, st, e, th males at 18°C. The F<sub>2</sub> generation was crossed inter se at 29°C for 7 days and the cross was moved to 25°C for the remainder of development. In the F<sub>3</sub> generation, 5 homozygous st, spn-B<sup>BU</sup>, sr, e * females and 3-5 males of any genotype were placed in laying chambers on yeasted apple juice plates at room temperature. Fresh yeasted plates were applied approximately every 24 hours. Eggs laid on days 5-7 were examined. Lines that showed a noticeable decrease in the percentage of ventralized eggs laid relative to spn-B<sup>BU</sup> were selected for further analysis.

**Fly Stocks**

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The \textit{spn-B}^{BU} allele is a G107E missense mutation and has been previously described (Ghabrial et al., 1998). The \textit{spn-B}^{A17C} allele was generated by imprecise excision of the \textit{P(GSV6)}^{GS15042} P-element resulting in deletion of the entire \textit{spn-B} open reading frame and 9 bp of the 3’ UTR. 633 bp of the P-element remained in the deficiency. \textit{mei-P22}^{CA1215} was identified in this work and consists of an EMS generated missense mutation at codon R31C and a 36 bp deletion that removes amino acids 67 through 78. \textit{lnk}^{CR642} was identified in this work and consists of an EMS generated nonsense mutation at codon 298. \textit{lnk}^{A07478} is a \textit{P}[XP] insertion in the 5’ UTR of the \textit{lnk} locus 701 bp upstream of the start codon while the \textit{lnk}^{F02642} and \textit{lnk}^{G3062} alleles are \textit{PBac[WH]} insertions in the second intron (Thibault et al., 2004). The \textit{vasPH165} allele is a deletion generated by P-element excision (Styhler et al., 1998) while the \textit{vasP}^{RG3} allele is an undefined EMS-generated allele (Schüpbach and Wieschaus, 1991). \textit{P[w+ lac-Z]BB142} is an enhancer trap of the \textit{kekkon} gene (Schüpbach and Roth, 1994). We generated follicle cell clones in \textit{e22c-Gal4, UAS-FLP/+; FRT82, lnk}^{CR642}/\textit{FRT82, ubi-GFP} flies (Xu and Rubin, 1993; Duffy et al., 1998). To assess Egfr activity we generated clones in flies with the genotype \textit{e22c-Gal4, UAS-FLP/P[w+ lac-Z]BB142; FRT82, lnk}^{CR642}/\textit{FRT82, ubi-GFP}. PI3K activity was compared in clones generated in ovaries with the genotype \textit{e22c-Gal4, UAS-FLP/tGPH; FRT82, lnk}^{F02642}/\textit{FRT82, ubi-GFP} (Britton et al., 2002). UAS constructs were expressed using the \textit{CY2-Gal4} or \textit{nos-Gal4} drivers (Queenan et al., 1997; Van Doren et al., 1998). Lineage tracing experiments were conducted as previously described with flies of the genotypes \textit{hs-FLP ; X15-29 / X15-33 ; lnk}^{CR642} (Harrison and Perrimon, 1993; Drummond-Barbosa and Spradling, 2001).

\textbf{Mapping of CR642}

The \textit{lnk}^{CR642} suppressor mutation was positionally cloned by meiotic recombination. Rough meiotic mapping with visible markers placed the suppressor between \textit{ebony} and \textit{claret}. Fine scale SNP mapping was conducted by recombination with the \textit{P[EPgy2]EY09907} insertion at 98B1 (Venken and Bellen, 2005). It was necessary to select \textit{sr, e, w+} recombinants to maintain \textit{spn-B}^{BU} in the background as no phenotype was evident in flies carrying only the \textit{CR642} mutation. Intergenic SNPs were identified that allowed us to define the region between 96F3 and 96F9. \textit{lnk} was identified as one of four genes with ovarian ESTs and the \textit{CR642} allele was confirmed by sequence comparison with the \textit{spn-B}^{BU} parental line.

\textbf{Transgenic Constructs}

The \textit{lnk} genomic rescue construct (\textit{pSF10}) was amplified from BAC22N13 with primers 5’-
\text{ATGCAGGTACCGAATTGCTTTAATGTCTGATCGGACGTTGG-3’} and 5’-
\text{ATGCAGGCGCGCTGAAAGAAGACCGACGTAG-3’} and cloned into \textit{P[ACMAN]-attB-Amp} as an
EcoRI/NotI fragment (Venken et al., 2006). This construct contains 903 bp of sequence upstream of the start of transcription and the entire 3’ UTR. The *lnkY720F* rescue construct (*pSF11*) was generated by site-directed mutagenesis with primers 5’-

GACGGCCGTCGATAATCAGTTCAGCTTCACCTAAGTCCG-3’ and 5’-

CGGACTTAGGTGAAGCTGAACTGATTATCGACGGCCCGTC-3’. The EGFP-tagged Lnk construct (*pSF12*) contains all of the sequence elements in *pSF10*, however the EGFP sequence is cloned in frame with the 5’ end of the *lnk* ORF. These constructs were integrated into the *attP16* and *attP40* docking sites using the ΦC31 system (Markstein et al., 2008). A UAS-*lnk* expression construct (*pSF44*) was amplified from cDNA clone LD10453 with primers 5’-ACTGGGTACCATGGGTGGCAATAGCACAGG-3’ and 5’-ACTGGAATTCTTAGGTGAAGCTGTACTGATTATCG-3’ and cloned as a KpnI/EcoRI fragment into a derivative of pUASP called pTIGER. Transgenic flies were generated by standard P-element transgenesis (Genetic Services, Inc).

**Antibodies and Immunohistochemistry**

Grk staining was performed with monoclonal mouse α-Grk ID12 at 1:10 as previously described (Queenan et al., 1999). Mouse α-βGal was used at 1:1000 (Promega). Rabbit α-γ-H2Av (a gift of Kim McKim) was used at 1:500 (McKim et al., 2009; Mehrotra and McKim, 2006). Rabbit α-Vas was generated by immunization with a KLH-conjugated peptide CGDGVGGSGGEGGGY and used at 1:3000 (Epitomics, Inc). Rabbit α-Lnk was used at 1:500. The antigen was expressed as a recombinant GST-Lnk 56-542 fusion protein and was purified on a GSTrap FF column (GE Healthcare) according to the manufacturer. This antigen contains sequences that are predicted to remain in protein made by the *lnk*<sup>CR642</sup> (This work), *lnk*<sup>02642</sup> and *lnk*<sup>035062</sup> alleles (Thibault et al., 2004), however the existence / stability of these truncations could not be determined. Attempts to detect Lnk protein by western blotting were unsuccessful. Secondary Alexa Fluor 488 or 546 α-mouse and/or α-rabbit antibodies and Alexa Fluor 546 phalloidin were used at 1:1000 (Invitrogen). Hoechst 33342 was used at 1 μg/mL. HRP-conjugated donkey α-rabbit was used at 1:5000 (Jackson ImmunoResearch).

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References


**Figure Legends**

**Figure 1:** *lnk* Mutations Suppress D/V Patterning Defects in Eggs Laid by *spn-B* Flies. Mutations in *spindle* class genes result in female sterility and variable ventralization of the eggs laid by homozygous females. (A) SEM images of eggs laid by *spn-B<sup>BU</sup>* flies demonstrating a range of ventralization phenotypes. D/V polarity was classified as wild type if two distinct dorsal appendages were evident. Fusion at the base of the appendages was classified as mild class 2 ventralization (V2), whereas fusion past the anterior aspect of the egg when viewed dorsally was classified as moderate class 3 ventralization (V3). Severely ventralized eggs lacking all appendage material were designated as V4. The scale bar in A is 100 μm. (B) Genetic suppression of the *spn-B<sup>BU</sup>* ventralized eggshell phenotype by mutations in *mei-P22<sup>CA1215</sup>* and several allelic combinations with *lnk<sup>CR642</sup>*. Mutations in *lnk* are also able to suppress D/V patterning defects in eggs laid by *spn-A<sup>093/003</sup>* mutant females. (C) A domain map of the Lnk protein illustrating the location of the *CR642* mutation and conserved tyrosine phosphorylation site. (D-F) The eggshell suppression reflects a rescue of Grk protein expression in the ovary. Grk is stained green while F-actin is shown in red. The scale bar in D-F is 25 μm.

**Figure 2:** Lnk Functions in Both the Germline and Follicle Cells. (A) Immunostaining of Lnk protein (red in A and white in A’) in follicle cell mosaics generated in *e22c-FLP/+; FRT82, lnk<sup>CR642</sup> / FRT82, ubi-GFP* flies. Clones are marked by the absence of EGFP and DNA was stained blue with Hoechst. Images are a maximum intensity projection of multiple confocal sections. (B-D) Localization of an N-terminal EGFP-Lnk fusion construct expressed from the genomic promoter. B is a single confocal section through the germarium and early stage cysts while C is a section through a stage 10A cyst. Panel D shows a maximum intensity projection of multiple sections through the nurse cell cluster of a stage 10B egg chamber. Scale bars are 50 μm in A, C and D and 25 μm in B. (E) Expression of a genomic *lnk* rescue construct can restore a ventralized eggshell phenotype to *spn-B<sup>BU</sup>, lnk<sup>CR642</sup>* flies. (F) *nos-Gal4* or *CY2-Gal4* were used to drive expression of a UAS-*lnk* construct in the germline or follicle cells respectively of *spn-B<sup>BU</sup>, lnk<sup>CR642</sup>* flies. *Act 5C-Gal4* was used to drive expression in both tissues. Germline
expression of \( \text{lnk} \) resulted in a significant increase in the number of ventralized eggs whereas follicle cell expression did not. Eggshell classes are as described in Figure 1.

**Figure 3:** \( \text{lnk} \) Mutations Do Not Affect Egfr Activity in the Follicle Cells. (A and B) Immunostaining of follicle cell clones in stage 10 e22c-FLP/\( \text{kek-LacZ} \); \( \text{FRT82, lnk}^{\text{CR642}} / \text{FRT82, ubi-GFP}} \) ovaries. \( \beta \)-galactosidase (red) expression from the \( \text{kek-LacZ} \) enhancer trap reflects the gradient of Egfr activity in the follicle cells. Homozygous \( \text{lnk}^{\text{CR642}} \) cells are marked by the absence of EGFP. A is a maximum intensity projection of multiple confocal sections encompassing the peak of Egfr activity at the future dorsal anterior that declines towards the ventral and posterior axes. B is a single confocal section with the dorsal anterior on the top left. \( \text{kek-LacZ} \) expression is unaffected by the \( \text{lnk} \) genotype. Scale bars are 50 \( \mu \text{m} \) in both A and B. (C) Cbl binds to the conserved tyrosine at 720 in SH2-B family members. Mutation of Lnk tyrosine 720 to a non-phosphorylatable phenylalanine in a genomic rescue construct does not affect the ability of the transgene to restore a ventralized eggshell phenotype in \( \text{spn-B}^{\text{BU}} / \text{spn-B}^{\text{A77C}}, \text{lnk}^{\text{CR642}} / \text{lnkf}^{\text{05062}} \) flies. Eggshell classes are as described in Figure 1.

**Figure 4:** \( \text{lnk} \) Mutations Affect IIS and Slow the Rate of Oogenesis. Whole ovaries were imaged from Oregon R (A & D), \( \text{lnkd}^{\text{07478}} \) (B & E), and \( \text{lnk}^{\text{CR642}} \) (C & F) flies. Ovaries in D-F were stained with Draq5 and imaged with UV epi-illumination. Following the onset of vitellogenesis, the oocyte autofluoresces blue. Note the strong arrest in early vitellogenesis in \( \text{lnkd}^{\text{07478}} \) ovaries while many late stage egg chambers are evident in \( \text{lnk}^{\text{CR642}} \) ovaries. The scale bar is 500 \( \mu \text{m} \) in A-F. (G) Membrane localization of the \( \text{tGPH} \) reporter is sensitive to phosphatidylinositol-(3,4,5)-trisphosphate (PIP3) levels. A follicle cell clone from \( \text{tGPH}^{+/+} / \text{FRT82, lnkf}^{\text{02642}} / \text{FRT82} \) ovaries stained with \( \alpha \)-Lnk antibodies (Red and G’) illustrating the reduced membrane localization of \( \text{tGPH} \) (Green and G”) in cells lacking Lnk activity. The clone border is illustrated by the dotted line with heterozygous tissue on the left and \( \text{lnk}^{\text{J02642}} \) homozygous cells on the right. The scale bar in G is 10 \( \mu \text{M} \). (H) Linage tracing in wild type and \( \text{lnk}^{\text{CR642}} \) ovaries using the \( \text{hsFLP X15} \) system. Clones were induced in the germarium by transient heat shock and allowed to develop on plates with live yeast paste for four days. The latest stage egg chamber with multiply marked nurse cell nuclei in each ovariole was recorded for each genotype. \( \text{lnk}^{\text{CR642}} \) egg chambers developed more slowly than wild type.

**Figure 5:** \( \text{lnk} \) Suppression Does Not Affect DNA Repair or Vasa Phenotypes. (A–D) Immunostaining of \( \gamma \)-H2Av foci (Green) in germaria. Orb (Red) stains individual cysts in the germarium and accumulates in the oocyte in region 2B. DNA is shown in blue. \( \gamma \)-H2Av foci appear in region 2A of wild type germaria and are resolved in oocytes in region 2B / 3 (arrowheads) whereas DSBs are evident in region 2B through stage 4 in \( \text{spn-B}^{\text{BU}} \) ovarioles. While \( \text{spn-B}^{\text{BU}}, \text{mei-P22}^{\text{CA1215}} \) and \( \text{spn-B}^{\text{BU}}, \text{lnk}^{\text{CR642}} \) flies both lay wild type...
eggs, only mei-P22^{CA1215} does so by blocking the formation of DSBs. spn-B^{BU}, lnk^{CR642} germaria have persistent DSBs in region 3 to a similar extent as spn-B^{BU}. The scale bar in A-D is 5 μm. (E-H) A single confocal section of the karyosome from mid-stage egg chambers stained with hoechst reveals that the karyosome fails to properly condense in spn-B^{BU}, lnk^{CR642} oocytes and resembles the phenotype seen in spn-B^{BU}. The scale bar in E-H is 1 μm. (I) Western blot of Vasa protein from ovarian extracts reveals that the electrophoretic mobility of Vasa from spn-B^{BU}, lnk^{CR642} retains the modification seen in spn-B^{BU}.

**Figure 6: Suppression of spn-B and vasa Eggshell Phenotypes by Rapamycin.** (A) spn-B^{BU} and (B) vas^{PH165/RG53} flies were fed yeast paste on grape juice agar containing the indicated concentration of rapamycin. Eggs from days 4-7 were scored for eggshell phenotypes. In both cases, TOR inhibition results in strong suppression of eggshell ventralization. At 10 μM rapamycin completely inhibited egg deposition in spn-B^{BU} flies. Eggshell classes are as described in Figure 1.

**Figure 7: A model of Vasa-independent gurken translation.** (A) When nutrients are abundant, cap-dependent translation predominates. Vasa helicase activity facilitates 43S Pre-Initiation Complex (PIC) scanning of the 5’ UTR, allowing it to navigate secondary structures. Once an AUG codon is identified, Vasa-eIF5B interactions promote joining of the 60S subunit to form the 80S ribosome. (B) When nutrients are low, Insulin / Insulin-like Signaling (IIS) is compromised, or flies are fed the TOR-C1 inhibitor rapamycin TOR activity falls and eIF4E Binding Protein (4EBP) is free to inhibit the cap-binding protein eIF4E. The resulting increase in free ribosomes favors IRES translation initiation of the localized grk transcript allowing oocytes to be patterned correctly, thereby preserving their viability for when nutrients are again available. (C) spindle-class mutants such as spn-B^{BU} inhibit grk translation by phosphorylating Vasa in a checkpoint-dependent manner. This disrupts scanning of the 43S PIC and/or interactions with eIF5B (5B) and therefore subunit joining at the initiation AUG codon. In these conditions, grk cannot be translated and ventralized eggs result. (D) Reduced TOR activity in a spn-B mutant background permits grk translation independent of Vasa activity. Despite the inhibition of cap-dependent translation initiation resulting from Vasa phosphorylation and increased 4EBP activity, grk translation persists. We hypothesize that this is due to alternative translation initiation at an Internal Ribosomal Entry Site (IRES) in the grk 5’ UTR.
**A** Wild Type - Rich Diet
Normal D/V Patterning

*Cap-dependent Translation Initiation*

**B** Wild Type - Nutrient Limitation
Normal D/V Patterning

*IRES-Mediated Translation Initiation*

**C** *spindle* Mutant - Rich Diet
Ventralized Eggs

*Vasa Phosphorylation
Blocks Scanning or Subunit Joining*

**D** *spindle* Mutant - Nutrient Limitation
Normal D/V Patterning

*IRES-Mediated Translation Initiation*