Repression of Gurken translation by a meiotic checkpoint in *Drosophila* oogenesis is suppressed by a reduction in the dose of eIF1A

Wei Li, Martha Klovstad and Trudi Schüpbach*

**ABSTRACT**

In *Drosophila melanogaster*, the anteroposterior (AP) and dorsoventral (DV) axes of the oocyte and future embryo are established through the localization and translational regulation of *gurken* (*grk*) mRNA. This process involves binding of specific factors to the RNA during transport and a dynamic remodeling of the *grk*-containing ribonucleoprotein (RNP) complexes once they have reached their destination within the oocyte. In ovaries of *spindle*-class females, an activated DNA damage checkpoint causes inefficient Grk translation and ventralization of the oocyte. In a screen for modifiers of the oocyte DV patterning defects, we identified a mutation in the *eIF1A* gene as a dominant suppressor. We show that reducing the function of eIF1A in *spnB* ovaries suppresses the ventralized eggshell phenotype by restoring Grk expression. This suppression is not the result of more efficient DNA damage repair or of disrupted checkpoint activation, but is coupled to an increase in the amount of *grk* mRNA associated with polysomes. In *spnB* ovaries, the activated meiotic checkpoint blocks Grk translation by disrupting the accumulation of *grk* mRNA in a translationally competent RNP complex that contains the translational activator Oo18 RNA-binding protein (Orb); this regulation involves the translational repressor Squid (Sqd). We further propose that reduction of eIF1A allows more efficient Grk translation possibly because of the presence of specific structural features in the grk 5′ UTR.

**KEY WORDS:** Gurken translation, EIF1A, Meiotic checkpoint, RNP complex, *Drosophila*

**INTRODUCTION**

In order to maintain genome stability, eukaryotes have evolved a signaling network called the DNA damage response (DDR) to sense and correct various forms of lesions in their DNA. In addition to their roles in activating cell cycle checkpoints, coordinating DNA damage repair and regulating apoptosis, many of the DDR proteins also function to ensure the progression of important developmental processes such as homologous recombination (HR) at meiosis (reviewed by Su, 2006). Studies of *Drosophila* oogenesis have demonstrated that a prolonged meiotic checkpoint, caused by the defective repair of DNA double-strand breaks (DSBs) that form during meiotic HR, leads to inefficient accumulation of a *Drosophila* TGF-α-like protein Gurken (Grk) and results in an improperly specified DV axis in *Drosophila* oocytes (Ghabrial and Schüpbach, 1999; Ghabrial et al., 1998). During *Drosophila* meiosis, HR is initiated in the early meiotic prophase by the Spo11 homolog Mei-W68 and by Mei-P22, both of which are essential for DSB formation. Proper progression of HR depends on efficient DSB repair, a process that requires a conserved set of DNA repair enzymes, such as SpnB, a RecA/Rad51 family member, and Okr, a helicase homologous to Rad54 (reviewed by Lake and Hawley, 2012). In *Drosophila* females that carry mutations in genes encoding these repair enzymes, a meiotic checkpoint involving the ATR homolog Mei-41 and its downstream kinase Chk2 is activated in the ovary and leads to decreased levels of Grk protein (Abdu et al., 2002; Ghabrial and Schüpbach, 1999). Although many of the key factors of this meiotic checkpoint have been identified, the molecular mechanisms underlying the checkpoint-mediated repression of Grk translation is still elusive.

During *Drosophila* oogenesis, spatial and temporal regulation of the localization and translation of *grk* mRNA underpins the establishment of both the AP and DV axes of the oocyte (reviewed by Lasko, 2012). During the early stages of oogenesis, *grk* mRNA is localized to the posterior of the oocyte where it provides a local source of Grk protein that signals through the EGF receptor (EGFR) to the follicle cells to establish posterior follicle cell fate and to ultimately determine the AP axis (González-Reyes et al., 1995; Roth et al., 1995). During stage 7 of oogenesis, after the posterior follicle cells have signaled back to the oocyte, the oocyte nucleus is relocated to the anterior cortex. Subsequently, *grk* mRNA moves toward the region surrounding the oocyte nucleus and eventually accumulates in a crescent between the nucleus and the neighboring cortex by becoming tightly associated with electron-dense structures called sponge bodies. This localization and anchoring process requires a heterogeneous nuclear ribonucleoprotein (hnRNP) Sqd, and promotes the local activation of Grk translation (Delanoue et al., 2007; Jaramillo et al., 2008; Weil et al., 2012). The localized Grk protein is secreted and activates the EGFR in lateral follicle cells to establish the DV axis of the egg and future embryo (Neuman-Silberberg and Schüpbach, 1993). During transport, *grk* mRNA remains translationally silent and this repression requires a complex of proteins, including Sqd and another translational repressor Cup (Clouse et al., 2008; Norvell et al., 1999). At the dorsoanterior corner, the localized *grk* mRNA was found to be anchored near the translational activator Orb (Weil et al., 2012). As a member of the cytoplasmic polyadenylation element-binding protein (CPEB) family, Orb is presumed to activate Grk translation by regulating polyA addition (Chang et al., 2001). Another polyA-binding translational regulator protein PABP55B has also been shown to mediate the translational activation of the localized *grk* mRNA together with a large cytoplasmic protein Encore (Enc) that colocalizes with *grk* mRNA at the dorsoanterior corner (Clouse et al., 2008; Van Buskirk et al., 2000). Therefore, activation of Grk translation at the dorsoanterior corner may be facilitated by the association between the anchored *grk* mRNA and a protein complex containing Orb, PABP55B and Enc. It is not clear whether the
activated meiotic checkpoint represses Grk translation by interfering with interactions between grk mRNA and the proteins that activate its translation or by some other mechanism.

In order to gain insight into the translational regulation of grk mRNA by the meiotic checkpoint, we have conducted a genetic screen for modifiers of the DV patterning defects observed in eggs laid by spnB mutant females. We identified a recessive lethal mutation in the gene encoding the eukaryotic translation initiation factor 1A (eIF1A) as a dominant suppressor of the spnB mutations. eIF1A promotes the initiation of protein translation by mediating the 43S pre-initiation complex (PIC) assembly, assisting the scanning of the 5'UTR of mRNA by PIC, ensuring correct AUG start codon selection and facilitating formation of the 80S initiation complex (IC) (reviewed by Hinnebusch, 2014). Here, we show that reducing the function of eIF1A in spnB ovaries restores Grk protein accumulation and suppresses the ventralized eggshell phenotype. This suppression is not the result of restored DSB repair or of disrupted checkpoint activation, but is coupled to an increase in Grk translation. We provide genetic and biochemical evidence indicating that the negative regulation of Grk translation in spnB mutant ovaries involves Sqd. We propose a model in which a Sqd-dependent translational repressive complex inhibits the translation of grk mRNA in spnB mutants and that reduction of eIF1A allows more efficient grk mRNA translation, possibly because of the presence of specific structural features in the grk 5'UTR.

RESULTS
Mutations in eIF1A suppress the DV patterning defects of spnB mutants
To study the molecular mechanism underlying the downregulation of Grk translation caused by the activated meiotic DNA damage checkpoint, we conducted a screen for mutations on the third chromosome that could suppress the ventralization of the eggshell in spnB females. BH167 was isolated as a homozygous lethal mutation that dominantly and strongly suppressed the eggshell patterning defects of spnB mutants (Fig. 1B). We mapped BH167 to the locus of eIF1A, which encodes a protein that is crucial for eukaryotic translational initiation (reviewed by Hinnebusch, 2014), and found that eIF1A<sup>BH167</sup> carries a nonsense mutation that truncates the Drosophila eIF1A protein by converting glutamine 37 into a stop codon (Fig. 1C). Meanwhile, we sequenced two previously isolated eIF1A homozygous lethal alleles, eIF1A<sup>645</sup> and eIF1A<sup>2232</sup> (Collins and Cohen, 2005). The eIF1A<sup>645</sup> allele carries a nonsense mutation that converts tryptophan 70 into a stop codon, whereas the eIF1A<sup>2232</sup> allele has a missense mutation that converts the highly conserved arginine 82 into a cysteine (Fig. 1C). We found that both
Importantly, as heterozygosity of a chromosomal deletion type or mildly ventralized (category V2) eggshell patterns. Scale bars: 50 egg chambers. of egg chambers examined per genotype. Numbers in parentheses indicate the total number staining in the oocyte. Error bars represent the standard error between five (D) Percentage of stage 9 and 10 egg chambers showing different levels of Grk accumulation (arrowhead) at the dorsoanterior corner of the oocyte (oo). By contrast, 37% of spnB<sup>BU</sup> eIF1<sup>ABH167</sup> egg chambers contained oocytes in which Grk protein was undetectable (supplementary material Fig. S2B,D). Therefore, only a small proportion (12%) of spnB<sup>BU</sup> eIF1<sup>ABH167</sup> egg chambers at these stages exhibited complete absence of Grk in the oocytes, whereas 35% of spnB<sup>BU</sup> egg chambers had no visible Grk protein (Fig. 2D). Therefore, a notable recovery of Grk levels is apparent at all mid-oogenesis stages, resulting in a strong suppression of the <em>spnB</em> eggshell phenotype.

It has previously been shown that grk mRNA localization is somewhat delayed at mid-oogenesis stages when the meiotic DNA damage checkpoint is activated (Ghabrial et al., 1998). As Grk translation occurs after the phosphorylated form of a histone H2A variant Chα7, which eliminates the eIF1A locus, also strongly suppressed the DV patterning defects in <em>spnB</em> mutants (supplementary material Fig. S1), we conclude that it is the reduction in functional eIF1A protein that accounts for the observed suppression. We further analyzed the effects of the eIF1A<sup>ABH167</sup> mutation and found that the eIF1A<sup>ABH167</sup> mutation also suppressed the eggshell patterning defects caused by mutations of <em>spnA</em> (Fig. 1B), indicating that its effect in DV patterning is not specific to <em>spnB</em>.

**eIF1A<sup>ABH167</sup> affects Grk protein accumulation but not grk mRNA localization**

We examined whether eIF1A<sup>ABH167</sup> suppresses the DV patterning defects of <em>spnB</em> mutants by restoring Grk protein levels. By immunostaining, we found that apparently normal Grk protein levels were restored in <em>spnB<sup>BU</sup>/eIF1A<sup>ABH167</sup>/spnB<sup>BU</sup></em> (hereafter <em>spnB<sup>BU</sup>eIF1<sup>ABH167</sup></em>) egg chambers. At early mid-oogenesis stages (stages 7 and 8), only 4% of <em>spnB<sup>BU</sup></em> egg chambers contained oocytes with wild-type level of Grk accumulation, whereas 69% contained oocytes in which Grk protein was undetectable (supplementary material Fig. S2B,D). By contrast, 37% of <em>spnB<sup>BU</sup> eIF1<sup>ABH167</sup></em> egg chambers contained oocytes in which Grk was absent (supplementary material Fig. S2C,D). The restoration of Grk protein accumulation in <em>spnB<sup>BU</sup> eIF1<sup>ABH167</sup></em> egg chambers is more striking during late mid-oogenesis stages (stages 9 and 10). At these stages, about 61% of <em>spnB<sup>BU</sup> eIF1<sup>ABH167</sup></em> egg chambers had a level of Grk accumulation in the oocytes that was comparable with wild type, whereas only 23% of <em>spnB<sup>BU</sup></em> egg chambers showed a similarly robust level of Grk in their oocytes (Fig. 2C,D). Moreover, only a small proportion (12%) of <em>spnB<sup>BU</sup> eIF1<sup>ABH167</sup></em> egg chambers at these stages exhibited complete absence of Grk in the oocytes, whereas 35% of <em>spnB<sup>BU</sup></em> egg chambers had no visible Grk protein (Fig. 2D). Therefore, a notable recovery of Grk levels is apparent at all mid-oogenesis stages, resulting in a strong suppression of the <em>spnB</em> eggshell phenotype.

**eIF1A<sup>ABH167</sup> does not affect DSBs repair or activation of Chk2 in <em>spnB</em> mutant ovaries**

In <em>Drosophila</em> ovaries, programmed DSBs that facilitate normal progression of meiosis are generated and repaired in the region called the gerarium. In region 2a of the gerarium, two cells in each 16-cell germline cyst become pro-oocytes and enter the early pachytene stage of meiosis by forming a full-length synaptonemal complex (SC) that can be detected using an anti-C(3)G antibody (Page and Hawley, 2001). Meanwhile, DSBs are generated to initiate HR in pro-oocytes and can be detected with an antibody recognizing the phosphorylated form of a histone H2A variant.
As the cyst progresses through meiosis, DSBs are gradually repaired and the number of γH2Av foci declines. In region 3 of the germarium, where the cyst reaches mid-pachytene (stage 1 of oogenesis), one of the two pro-oocytes retains the SC and differentiates into the oocyte, while the other pro-oocyte disassembles the SC and becomes a nurse cell. At the same stage, γH2Av foci disappear from the oocyte nucleus, indicating the effectiveness of DSB repair (Fig. 3A) (reviewed by Lake and Hawley, 2012).

Owing to delayed DSB repair, persistent accumulation of DSBs becomes apparent in spnB oocytes at mid-pachytene and later stages of oogenesis (Jang et al., 2003). We observed perduring large γH2Av foci in the C(3)G-positive nuclei in all stage 1 spnB cysts (Fig. 3B, n = 176). In addition, owing to the activation of a pachytene checkpoint that delays oocyte selection (Joyce and McKim, 2009), we found that these stage 1 spnB cysts often contained two cells that still showed robust nuclear C(3)G staining (Fig. 3B,G). We tested whether the elf1A mutation would suppress these phenotypes. We found that all stage 1 spnB eIF1ABH167 cysts still exhibited strong accumulation of γH2Av foci in their C(3)G-positive nuclei (Fig. 3C, n = 182), and we frequently saw stage 1 spnB eIF1ABH167 cysts with two cells exhibiting robust nuclear C(3)G accumulation (Fig. 3C,G). These results show that eIF1ABH167 heterozygosity does not enhance DSB repair, nor does it interfere with the pachytene checkpoint in a spnB mutant.

Repression of Grk translation in spnB mutants requires the activation of Chk2 (Abdu et al., 2002). As Chk2 activation also leads to defects in oocyte chromosomal organization by suppressing a histone kinase NHK-1 (Lancaster et al., 2010), we reasoned that if eIF1A heterozygosity suppresses the DV patterning defects in spnB mutant oocytes, it should also suppress the chromosomal organization defects seen in spnB mutant oocytes. We

**Fig. 3.** eIF1A<sub>bH167</sub> does not affect DNA damage repair or checkpoint activation. (A-C) Germaria stained for γH2Av foci (red), the synaptonemal complex component C(3)G (green) and DNA (blue). Regions of the germarium are indicated. (A) A spn<sup>B</sup>TM6 germarium contains a stage 1 cyst with only one C(3)G-positive pro-oocyte (arrowhead) and no γH2Av foci in its nucleus (arrow). (B,C) The stage 1 cyst in the spn<sup>B</sup> (B) or spn<sup>B</sup>eIF1<sub>A<sup>bH167</sup></sub> (C) germarium contains two C(3)G-positive pro-oocytes (arrowheads) with strong nuclear accumulation of γH2Av foci (arrows). (D-F) DNA staining (cyan) showing the morphology of the oocyte karyosome during mid-oogenesis. The nuclear envelope is stained for the nuclear pore complex marker WGA in red. (D) In spn<sup>B</sup>/TM6 oocytes, the chromatin is condensed into a sphere, called karyosome (arrowhead). In spn<sup>B</sup> (E) and spn<sup>B</sup>eIF1<sub>A<sup>bH167</sub></sub> (F) oocytes, the chromatin is dispersed (arrows). (G) eIF1A<sub>bH167</sub> heterozygosity causes no significant change in the percentage of cysts containing two C(3)G-positive pro-oocytes. Error bars represent the standard error between three independent experiments. The numbers in parentheses indicate the total number of stage 1 cysts examined per genotype. (H) eIF1A<sub>bH167</sub> heterozygosity causes no significant change in the percentage of egg chambers showing dispersed chromatin. Error bars represent the standard error between four independent experiments. The numbers in parentheses indicate the total number of egg chambers examined per genotype. P values were generated by two-sample two-tail heteroscedastic t-tests. Scale bars: 20 µm.
examined the morphology of the oocyte karyosome, which normally contains the condensed oocyte chromosomes and appears spherical (Fig. 3D), in both spnBBU and spnBBU eIF1ABH167 egg chambers at mid-oogenesis (stages 7 to 10). As expected, the chromatin inside the oocyte nucleus in spnBBU egg chambers was dispersed and remained in separate fragments (Fig. 3E). Heterozygosity of the eIF1ABH167 mutation did not rescue this defect (Fig. 3F,H). Therefore, we conclude that loss of one copy of eIF1A does not significantly affect Chk2 activation.

**Association of grk mRNA with polysomes and RNP complexes of large molecular size**

In order to understand the translational regulation of grk mRNA, we performed sucrose gradient fractionation using ovary extracts and analyzed the distribution profile of grk mRNA in each gradient by qRT-PCR. Based on the UV absorbance profile and the distribution pattern of ribosomal RNAs, we concluded that the vast majority of polysomes was present in the fast-sedimenting fractions of the gradients not being treated with EDTA (fractions 10 to 14; Fig. 4; supplementary material Figs S4 and S5A), because the EDTA treatment, which disrupts the magnesium-dependent interaction between the large (60S) and small (40S) ribosomal subunits (Fleischer et al., 2006), almost completely eliminated rRNAs from these fractions (Fig. 4; supplementary material Figs S4 and S5A’).

We first examined grk mRNA distribution in the gradient prepared from wild-type ovary extracts. Two reference mRNAs, aTub67C and RpL32, were used to test the efficiency of our fractionation. The aTub67C mRNA, which remains translationally active throughout oogenesis, was highly enriched in the polysome-containing fractions (fractions 10 to 14; Figs 4D and 5D). By contrast, fewer than 40% of RpL32 transcripts were found in these polysome-containing fractions and a significant amount of RpL32 transcripts were present in the fractions that were devoid of ribosomes (fractions 1 to 3; Figs 4D and 5F; supplementary material Fig. S4A), which is consistent with the previous observation that RpL32 mRNA is only actively translated during the early stages of oogenesis and its association with polysomes declines considerably after mid-oogenesis (Al-Atia et al., 1985).

grk mRNA also appeared enriched in the polysome-containing fractions and this distribution was significantly altered by EDTA (fractions 10 to 14; Fig. 4D,D’), suggesting that a significant number of grk transcripts found in the fast-sedimenting fractions were associated with polysomes and actively translated.

In Drosophila egg chambers, grk mRNA remains translationally silent until it is properly localized (reviewed by Lasko, 2012). We expected that, in our gradients, those unlocalized translationally silent grk transcripts would be present in fractions 1 to 3 where there were no ribosomes. However, unlike RpL32, most of the grk transcripts that were not present in the polysome-containing fractions were actually found in the intermediate fractions...
containing the 40/60S ribosomal subunits and 80S monosomes (fractions 4 to 9; Fig. 4D; supplementary material Fig. S4A). This observation indicates that in wild-type ovaries a substantial fraction of untranslated grk mRNA is associated with particles of large molecular size, possibly the RNP complexes involved in RNA localization and translational regulation (Delanoue et al., 2007; Weil et al., 2012). In contrast to the reference mRNAs, this accumulation of grk mRNA in large RNP complexes became even more pronounced after EDTA treatment because a significant amount of grk transcripts were found in the heavier intermediate fractions sedimenting at faster speeds (fractions 7 to 10; Fig. 4D′).

The activated meiotic checkpoint reduces the amount of polysome-associated grk transcripts

Next, we compared the polysome profile between the wild-type and spnBBU gradients. The UV absorbance profiles of the wild-type and spnBBU gradients were similar (Fig. 4A,B), which suggests that the meiotic checkpoint activated in spnB ovaries does not cause a significant global change in protein translation. In addition, EDTA treatment also caused a loss of polysomes in the fast-sedimenting fractions of the spnBBU gradient (Fig. 4B′; supplementary material Fig. S4B′). When we assessed mRNA distribution in the spnBBU gradient, we found that compared with the wild-type gradient, there was a significant reduction in the amount of grk mRNA present in the polysome-containing fractions, while no significant difference in the distribution profiles of αTub67C or RpL32 was observed (fractions 10 to 14; Fig. 5). These results indicate that the meiotic checkpoint in spnB mutant ovaries downregulates Grk protein synthesis at least partly by reducing the amount of polysome-associated grk transcripts.

We then examined the effects of the eIF1ABH167 mutation on the polysome association of grk, αTub67C and RpL32 in spnB mutants. We found that for all three transcripts there was a consistent increase in the amount of transcripts detected in the polysome-containing fractions when one copy of the eIF1A<sup>BH167</sup> mutation was present, with the increase of RpL32 polysome association being the most significant (Fig. 5). The modest increase in the amount of grk mRNA present in the polysome-containing fractions of the spnBBU eIF1ABH167/spnBBU gradient is consistent with the partial suppression of the eggshell patterning defects by the eIF1A mutation. Moreover, these results also indicate that the eIF1A mutation has a modest global effect on protein synthesis in developing egg chambers by promoting the association of mRNAs with polysomes.

Regulation on Grk translation caused by the activated meiotic checkpoint involves Squid (Sqd)

Previous studies have suggested that a dynamic change in protein components occurs in grk-containing RNP complexes as the RNA is being localized at the dorsoanterior corner of the developing oocyte. During transport, grk mRNA is associated with a repressive
RNP complex containing Sqd (Goodrich et al., 2004; Norvell et al., 1999). When grk mRNA is properly localized, the initiation of Grk translation is triggered by grk mRNA becoming part of a RNP complex that likely contains the translational activator Orb (Weil et al., 2012). Therefore, we tested whether the meiotic DNA damage checkpoint represses Grk translation by affecting the grk-containing RNP complexes.

In sucrose gradients made from wild-type ovary extracts, we observed a substantial amount of grk mRNA in the intermediate fractions (fractions 4 to 9; Fig. 4D) that might represent a mixed population of grk transcripts that are associated with either a translationally repressive RNP or a translationally competent RNP complex. In order to examine the composition of these intermediate fractions, we compared the grk mRNA distribution between the EDTA-treated wild-type (blue circles, wild type), spnB\textsuperscript{nu} (red squares, spnB), spnB\textsuperscript{nu} elf1A\textsuperscript{Bireli} (green triangles, spnB elf1A) and sqd\textsuperscript{I} (purple diamonds, sqd) gradients. (D) Comparison of the amount of grk (D) and Rpl32 (D') mRNA in the heavier intermediate fractions (fractions 7 to 10) of the EDTA-treated gradients. Error bars in the figure represent standard error from three independent experiments. P values were generated by two-sample one-tail heteroscedastic t-tests. (E) Distribution of Orb, Sqd and Me31B proteins in the EDTA-treated gradients.

Fig. 6. Reduced association of grk mRNA with a translationally competent RNP complex accounts for the translational repression caused by the meiotic DNA damage checkpoint. (A–C) Distribution of grk (A,B,C) and Rpl32 (A',B',C') mRNA in the EDTA-treated wild-type (blue circles, wild type), spnB\textsuperscript{nu} (red squares, spnB), spnB\textsuperscript{nu} elf1A\textsuperscript{Bireli} (green triangles, spnB elf1A) and sqd\textsuperscript{I} (purple diamonds, sqd) gradients. (D) Comparison of the amount of grk (D) and Rpl32 (D') mRNA in the heavier intermediate fractions (fractions 7 to 10) of the EDTA-treated gradients. Error bars in the figure represent standard error from three independent experiments. P values were generated by two-sample one-tail heteroscedastic t-tests. (E) Distribution of Orb, Sqd and Me31B proteins in the EDTA-treated gradients.
In the EDTA-treated spnB¹⁰⁴ gradient, the amount of grk transcripts detected in the heavier intermediate fractions was much lower than that detected in the wild-type gradient (fractions 7 to 10; Fig. 6B,D). Moreover, compared with the EDTA-treated spnB¹⁰⁴ gradient, we were able to detect a recovery of grk transcripts in these fractions of the EDTA-treated spnB¹⁰⁴ eIF1A¹⁰⁴ gradient (Fig. 6C,D), which would be consistent with the partial derepression of Grk translation and suppression of the spnB¹⁰⁴ phenotype. No such differences were observed for the reference mRNAs (Fig. 6B,C,D; supplementary material Fig. S5B,C). These results suggest that the meiotic checkpoint represses Grk translation at least partly by preventing grk mRNA from associating with the translationally competent RNP complexes.

We further tested whether Sqd could mediate the suppression of Grk translation by the meiotic checkpoint. First, we observed that introducing one copy of the sqd¹ mutation into spnB¹⁰⁴ females partially suppressed the eggshell ventralization defects (Fig. 7A). We then compared the amount of Sqd-bound grk transcripts in control ovary lysates with that in the spnB¹⁰⁴ ovary lysates, and found that in the latter there were more grk transcripts associated with Sqd (Fig. 7B). No such difference was observed for the RpL32 mRNA. In addition, the amount of grk transcripts that were pulled down using an anti-Cyclin B antibody was comparable between the control and the spnB mutant. Therefore, we conclude that the translational repression of grk mRNA by the meiotic checkpoint also involves Sqd.

In summary, we have shown that lowering the dosage of eIF1A restores Grk translation in ovaries in which the meiotic checkpoint is active. Our results further suggest that spnB¹⁰⁴ ovaries contain fewer grk transcripts that are associated with either polysomes or with translationally competent RNP complexes that sediment to these heavier intermediate fractions after EDTA treatment, and that the loss of one copy of eIF1A partially restores that population. Furthermore, it appears that repression of Grk protein synthesis by the meiotic checkpoint is partly due to the retention of grk transcripts in translationally repressive RNPs, which are found in the lighter intermediate fractions, a process that involves Sqd and prevents the grk mRNA from being recruited to translationally competent RNP complexes.

**DISCUSSION**

In this study, we provide both biochemical and genetic insights into the molecular mechanism underlying the translational control of grk mRNA in Drosophila oogenesis, focusing on the downregulation of Grk translation caused by the meiotic DNA damage checkpoint. Mutations in DNA repair enzyme genes, such as spnB and spnA, cause prolonged persistence of DSBs in the germline, which activates an ATR-Chk2-dependent checkpoint. In these mutants, checkpoint activation results in low levels of Grk protein accumulation in developing oocytes, which leads to ventralized eggshell phenotypes. Using forward genetics, we isolated an allele of the eukaryotic translation initiation factor eIF1A as a suppressor of this phenotype. A single copy of this mutation in mutant females with activated meiotic checkpoint restores Grk protein levels largely back to normal. Our results show that neither DSB repair nor checkpoint activity is affected by the eIF1A mutation. Therefore, the eIF1A mutation has a rather specific effect on the regulation of Grk translation caused by the meiotic checkpoint. We also present biochemical evidence that the activated meiotic checkpoint downregulates Grk translation by reducing the amount of polysome-associated grk transcripts. Furthermore, our data suggest that the reduction of Grk translation in spnB mutants also involves the hnRNP factor Sqd, which blocks
Grk translation by holding grk transcripts in translationally repressive RNP complexes.

**Effects of meiotic checkpoint activity on Grk translation**

Sucrose density gradient assays using *Drosophila* ovary extracts have yielded valuable insights into the translational control of other maternally expressed transcripts, including nanos and oskar (Andrews et al., 2011; Braat et al., 2004; Chekulaeva et al., 2006; Clark et al., 2000). By combining this assay with qRT-PCR, we were able to compare the translational profile of grk mRNA of the wild-type with *spnB* ovary extracts. We found that in the *spnB* mutant gradient the change in the amount of grk transcripts present in the polysome-containing fractions was statistically significant, whereas the changes we observed for *αTub67C* and *Rpl32* were not. This indicates that effects of meiotic checkpoint activity on oocyte protein translation are relatively specific for grk mRNA. Previous work has shown that Grk protein accumulation is strongly affected in *spnB* mutant egg chambers at the mid-oogenesis stages, and less so at earlier stages (Ghabrial et al., 1998). Our sucrose gradient analyses were performed using total ovary lysates containing egg chambers at all stages of oogenesis; therefore, it is possible that an even more pronounced loss of grk mRNA from the polysome fraction would be apparent if only egg chambers at mid-oogenesis stages were isolated.

In support of our interpretation that the activated meiotic checkpoint more specifically affects the translation of grk mRNA, we did not observe any clear changes in the general polysome profiles of the wild-type compared with the *spnB* gradients, which further indicates that meiotic checkpoint activity does not block global protein synthesis. However, it has previously been shown that UV irradiation-induced DNA damage can cause an overall inhibition of translation in mammalian cells (Powley et al., 2009; Wu et al., 2002). It is possible that an increased sample complexity in *Drosophila* ovary extracts, such as the presence of large amount of RNP complexes and yolk, could limit the resolution of sucrose fractionation and make it difficult to visualize a more general change in protein synthesis. Alternatively, as some of the key regulators that mediate the inhibitory effect of DNA damage on translation in mammalian cells (such as p53) (Budanov and Karin, 2008; Cam et al., 2014) are not required by the *Drosophila* meiotic DNA damage checkpoint to repress Grk translation (Abdu et al., 2002), it is likely that the meiotic checkpoint specifically regulates the translation of grk and some other mRNAs that may share unique features in their sequence and/or structure that account for this specificity. Future biochemical experiments will be needed to address this possibility.

**Involvement of Sqd and the RNP complex in Grk translational control by meiotic checkpoint**

Our genetic and biochemical analyses suggest that Grk translational control by the meiotic checkpoint involves a dynamic change in the protein composition of grk RNP complexes. The distribution pattern of grk mRNA in the EDTA-treated wild-type gradient was rather different from the EDTA-treated *spnB* gradient, which we interpret to indicate that fewer grk transcripts in *spnB* oaries are associated with the translationally competent RNP complexes that sediment to the heavier intermediate fractions following EDTA treatment. The further increase in the amount of grk transcripts present in the same fractions of the EDTA-treated sqd gradient and the apparent accumulation of Orb, an activator of Grk translation, in these fractions supports this interpretation. Moreover, reducing the dose of Sqd in *spnB* oaries suppresses the eggshell DV patterning defects, suggesting that Sqd is involved in repressing Grk translation in *spnB* oaries.

Sqd is required to regulate the nuclear export, dorsoanterior localization and translational control of grk mRNA (Caceras and Nilson, 2009; Goodrich et al., 2004; Norvell et al., 1999). An earlier study from our laboratory showed that Sqd functions together with Cup and Hrb27C to mediate the translational repression of grk (Clouse et al., 2008). As we found that *spnB* oaries contain more Sqd-bound grk transcripts, it is possible that the meiotic checkpoint activity inhibits the machinery that frees grk transcripts from the Sqd-containing translationally repressive RNP complex, leaving more grk transcripts translationally repressed.

An ultrastructural study showed that at the dorsoanterior corner of the oocyte localized grk transcripts are enriched at the edge of sponge bodies where Orb is also present (Weil et al., 2012). Here, we found that Orb and grk RNA accumulate in the heavier intermediate fractions of the EDTA-treated gradients. Therefore, we propose that the observed RNAs and proteins present at the edge of P bodies correspond to the translationally competent RNP complexes described in our study.

In light of these results, we propose the following model: in wild-type oaries, unlocalized grk transcripts are associated with repressive RNP particles containing Sqd, which sediment to the light intermediate fractions of the gradient. Once localized, grk transcripts become associated with translationally competent RNP complexes containing Orb and are ready to become actively translated. In *spnB* mutant oaries, more grk transcripts are excluded from the translationally competent RNP complexes and remain translationally repressed; this repression involves Sqd protein as it does in wild type.

**Mutations in eIF1A leads to derepression of Grk translation**

We found that mutations in eIF1A suppress the eggshell patterning defects in *spnB* mutants by derepressing Grk translation. This finding was initially very surprising, given that eIF1A encodes a general translation factor whose loss one might expect to enhance the defects in Grk translation. The eIF1A<sup>ΔH1657</sup> allele discovered in our screen encodes a short truncated form of eIF1A missing most of its functional domains, including the OB (oligonucleotide binding) fold that is crucial for its ribosome-binding activity (Battiste et al., 2000; Fekete et al., 2005). Two other previously isolated eIF1A alleles (Collins and Cohen, 2005), eIF1A<sup>e645</sup> and eIF1A<sup>2322</sup>, showed a similar suppressing effect when introduced into the *spnB* mutant background. The eIF1A<sup>e645</sup> allele encodes a truncated protein missing half of the OB fold, whereas the eIF1A<sup>2322</sup> allele encodes a mutant protein with a key RNA-binding residue in the OB fold, Arg82, changed to a cysteine (Battiste et al., 2000). In addition, a chromosomal deletion that eliminates the eIF1A locus showed a strong suppressing effect, which strongly suggests that the suppression by all the eIF1A alleles used in this study is the result of a loss of protein function and not because of some gain-of-function effect. Based on the well-characterized functions of eIF1A during translation initiation, we propose that the effect on grk translation may reflect some special features of the grk transcript.

During translation initiation, eIF1A facilitates the assembly of the 43S pre-initiation complex (PIC) by promoting the binding of the Met-tRNA<sub>i</sub>-eIF2-GTP ternary complex (TC) to the 40S ribosomal subunit (reviewed in Hinnebusch, 2014). A mutation in the ribosome-binding OB fold of eIF1A impairs TC recruitment and reduces general translation (Fekete et al., 2005). Interestingly, the same mutation increases the translation of *GCN4* mRNA and the overexpression of TC specifically abolishes this increase (Fekete
et al., 2005). In yeast, the translation of GCN4 is regulated by four upstream open reading frames (uORFs) located in its 5′-UTR (reviewed by Hinnebusch, 2014). When TC levels are reduced due to amino acid starvation or, in this case, due to impaired TC recruitment because of the eIF1A mutation, a fraction of ribosomes fails to rebind TC until they scan past the last uORF and reinitiate translation at the AUG preceding the open reading frame of GCN4 instead, resulting in increased GCN4 translation. Like yeast GCN4, Drosophila grk contains three uORFs in its 5′-UTR. It is therefore possible that these uORFs also regulate Grk translation. Analogous to the yeast model, mutations in eIF1A would diminish the amount of 43S PIC and allow more ribosomes to reinitiate translation at the start codon of grk. In support of this hypothesis, a protein-rich diet (yeast paste) often exacerbates the DV patterning defects in eggs laid by the spnB females (Ferguson et al., 2012). It would be interesting to test whether eliminating these uORFs from the grk 5′-UTR has an effect on Grk translation and whether such deletions would upregulate Grk translation in the spnB mutant.

An alternative explanation could be proposed based on the notion that toward the end of translation initiation eIF1A needs to vacate the newly formed 80S initiation complex (IC) to free the A site of the 80S ribosome for the subsequent translational elongation to start (Jackson et al., 2010). There is strong evidence that efficient dissociation of eIF1A from 80S IC depends on GTP hydrolysis by the initiation factor eIF5B (Acker et al., 2006, 2009; Fringer et al., 2007). Interestingly, the Drosophila eIF5B homolog has been shown to interact with Vasa, a key regulator of Grk translation, and this interaction is indispensable for Vasa to promote Grk protein synthesis in the ovary (Carrera et al., 2000; Johnstone and Lasko, 2004). Earlier results from our laboratory showed that activation of the meiotic checkpoint in the germ line results in a posttranslational modification of Vasa (Abdu et al., 2002; Ghabrial and Schüpbach, 1999). It is therefore possible that this modification of Vasa by the meiotic checkpoint may inhibit the GTP hydrolysis activity of eIF5B, thus impeding eIF1A dissociation and delaying the progression of Grk translation into the elongation phase. When an eIF1A mutation is present in those mutants, there might be less eIF1A protein retained in the 80S IC, which may offset the inhibitory effect of the Vasa modification on the hydrolysis activity of eIF5B and expedite the start of the elongation phase of Grk translation.

In mammalian cell culture it has been shown that the DNA damage response deregulates the global rate of protein synthesis by modulating the core factors of the eukaryotic translation initiation machinery ( Braunstein et al., 2009; Hayman et al., 2012; Powley et al., 2009). Studies have also started highlighting the surprising specificity in the classes of mRNAs that are affected at the translational level during the cellular responses to DNA damage and other stresses (reviewed by Spriggs et al., 2010). Here, we have shown that the meiotic DNA damage checkpoint that operates during Drosophila oogenesis affects grk mRNA specifically and prevents a significant fraction of the mRNA from associating with polysomes. However, reduction of functional eIF1A results in a more general upregulation of translation and promotes various mRNAs to enter the polysome fraction. Although the regulation of Grk translation in oogenesis may be an insect-specific process, it will be interesting in the future to explore whether checkpoint activities in other developmental contexts also impede translation of specific mRNAs in order to coordinate repair processes in the nucleus with developmental processes in the cytoplasm, and to test whether altering the amount of functional eIF1A in other cell types can affect the regulation of protein synthesis by the DNA damage checkpoint.

 MATERIALS AND METHODS

Eggs shell phenotype scoring

Eggs were collected by placing 1-day-old flies of the appropriate genotypes into egg laying blocks (Wieschaus and Nüsslein-Volhard, 1998). The eggs shell phenotype was scored as previously described on days 3-7 (Klovstad et al., 2008).

Drosophila strains

The Oregon-R strain was used as wild-type control for sucrose gradient analysis. eIF1A(BH167) was identified in this work and carries a C to T transition at nucleotide 109 of the eIF1A open reading frame (ORF). eIF1A(2232) and eIF1A(232) are two previously reported EMS-generated alleles provided by S. Cohen (Collins and Cohen, 2005). eIF1A(E935) and Df(3R) Cha7 were acquired from the Bloomington Stock Center. Other mutant alleles used in this study were: spnB97, spnB153, spnA7, spnA93 and sqd' (Ghabrial et al., 1998; Kelley, 1993; Staeava-Vieira et al., 2003). Standard genetic procedures were used to generate double mutant combinations.

Generation and mapping of BH167

Males of the genotype st spnB97 sr eTM6,Hu were treated with EMS according to standard procedures and mated to appropriate females to establish stocks (Ferguson et al., 2012). To find dominant suppressor mutations, males from these lines were crossed to st spnB153 TM3,St females and the eggs of the resulting st spnB97 sr e spnB153 females were scored. Line BH167 showed a strong dominant suppressing phenotype. Meiotic recombination mapping combined with deficiency complementation tests narrowed down the candidate region containing the BH167 mutation to the chromosomal region between 87C3 and 87C7. One P-element insertion in this region, P(EP);eIF1A(E935), failed to complement the lethality of BH167. To confirm that BH167 is an allele of eIF1A, we sequenced the coding region of eIF1A using genomic DNA extracted from BH167 homozygous embryos.

Immunostaining

Ovary fixation and whole-mount antibody labeling were performed as previously described (Klovstad et al., 2008). Primary antibodies were used at the following concentrations: 1:10, mouse anti-Grk clone 1D12; 1:500, rabbit anti-γH2Av (Jang et al., 2003); and 1:500, mouse anti-C(3)G clone 1A8-1G2 (Anderson et al., 2005). Secondary antibodies (Molecular Probes) were used at 1:1000 in PBST. Hoechst 33342 (Molecular Probes) and wheat-germ agglutinin (Alexa Fluor 633 Conjugate, Molecular Probes) were used at 1:1000 in PBST. Hoechst 33342 (Molecular Probes) and wheat-germ agglutinin (Alexa Fluor 633 Conjugate, Molecular Probes) were used at 1 μg/ml. Samples were mounted in Aqua-Poly/Mount (Polysciences) and visualized using a Nikon A1 laser scanning confocal microscope.

Ovary extract preparation, sucrose gradient fractionation, RNA and protein isolation

Ovary extract preparation and sucrose gradient fractionation were performed as previously described (Clark et al., 2000) with small changes in protocol (see methods in the supplementary material). To analyze the polysome profile, after centrifugation, gradients were pumped through a Bio-Rad Mini Exclusion column and visualized using a Nikon A1 laser scanning confocal microscope.

RNA was purified from fraction samples by a 30 min incubation at 37°C in a solution containing 150 μg/ml proteinase K, 1% SDS and 10 mM EDTA, followed by extraction with TRI Reagent LS (Sigma-Aldrich). To ensure efficient cDNA synthesis, purified RNA was cleaned by lithium chloride precipitation (Del Prete et al., 2007). Protein isolation was performed by TCA precipitation.

Immunoprecipitations and RNA isolation

Immunoprecipitations using monoclonal anti-Sqd serum or monoclonal anti-CyclinB serum were performed as described previously (Clouse et al., 2008) with small modifications (see methods in the supplementary material). RNA was purified from the IP and input samples with TRI Reagent LS (Sigma-Aldrich) and RNeasy Mini Kit (Qiagen).
qRT-PCR and western blot
cDNA was synthesized using the Transcripter First Strand cDNA Synthesis Kit (Roche). qRT-PCR was conducted on the Mastercycler realeplex® 2 real-time PCR system (Eppendorf) using the FastStart TaqMan Probe Master (Roche) and probes from the Universal Probe Library (Roche).

Western blotting was performed using standard protocols with the following antibodies: mouse anti-Orb monoclonal antibodies (clone 4H8, 1:30; clone 6H4, 1:30) (Lantz et al., 1994), mouse anti-Sqd monoclonal antibody (clone 8F3, 1:200) (Goodrich et al., 2004) and mouse anti-Mc31B monoclonal antibody (clone 2M, 1:100) (Nakamura et al., 2001). HRP-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratory) was used at 1:10,000 dilution. Data were collected using the FluorChem HD2 system (ProteinSimple).

Acknowledgements
We thank Elizabeth Gavis and Kim McKim for providing antibodies and reagents. We are grateful to Elizabeth Gavis, Danielle Snowflack and Nicolette Belleliet for assistance with sucrose gradient analysis, to Joe Goodhouse and Gary Laevesky for help with confocal microscopy, and to Gail Barcelo for technical assistance. We also thank members of the Ščupbach and Wieschaus laboratories for insightful discussions, and Elizabeth Gavis, Nicolette Belleliet, Julie Merkle, Bing He, Blythe Shelby, Amanda Norvell and Attlee Plane for their critical reading of the manuscript.

Funding
This work was supported by the Howard Hughes Medical Institute and by the National Institutes of Health [NIH-R01 GM076260]. Deposited in PMC for release after 6 months.

Supplementary material
Supplementary material available online at http://dev.biologists.org/lookup/suppl?doi=10.1242/dev.109306/-/DC1

References


Supplementary Methods

Ovary extract preparation and sucrose gradient fractionation

Ovaries from 100 females, fed on yeast for 4 days, were dissected in ice-cold PBS. Extra PBS was removed from the tube and ovaries were snap frozen in liquid nitrogen and stored at -80°C. To prepare ovary extracts, approximately 400 µl of frozen ovaries were homogenized on ice in 1 ml of polysome buffer (PB) containing 250 mM NaCl, 25 mM magnesium acetate, 50 mM Tris HCl, pH 7.5, 2.5 mg/ml heparin, 2 mM DTT, 0.2% Triton, 1 mg/ml cycloheximide, 1x EDTA-free Complete protease inhibitor (Roche) and 50 U/ml RNasin (Promega). Homogenates were cleared by centrifugation at 4°C for 10 minutes at 16000 x g and the supernatant was collected for density centrifugation. For EDTA treatment, the supernatant was treated with 50 mM EDTA on ice for 5 minutes before it was layered onto the sucrose gradient.

After measuring the A_{254}, ultraviolet (u.v.) absorbance of the cleared supernatant, 700 µl of ovary extracts containing 25 OD units was layered onto an 11 ml 10-50% sucrose gradient containing 250 mM NaCl, 25 mM magnesium acetate and 50 mM Tris HCl, pH 7.5. The EDTA-treated supernatant was layered onto a gradient containing 10 mM EDTA in place of magnesium acetate. Gradients were centrifuged in a Beckman SW41 Ti rotor at 36000 rpm for 3.5 hours at 4°C. After centrifugation, thirteen equivalent volume fractions (900 µl each, fractions 1 to 13) were manually collected from each gradient. Since the gradient pellet has been shown to contain very large polysomes (Clark et al., 2000), we solubilized it with 900 µl PB and treated it as an equivalent fraction (fraction 14). In total, 14 fractions were collected per gradient. In addition, 850 µl PB was added into 50 µl of the previously saved supernatant to make the lysate sample (L).
**Ovary lysate preparation and immunoprecipitation**

Ovaries from 50 females, fed on yeast for 4 days, were dissected in ice-cold PBS. Extra PBS was removed from the tube and ovaries were homogenized on ice in 150 μl of immunoprecipitation buffer (IB) containing 150 mM NaCl, 50 mM Tris HCl, pH 7.5, 1 mM EDTA, pH8.0, 0.4% NP-40, 0.1% Triton, 1x EDTA-free Complete protease inhibitor (Roche), 1x PhosSTOP phosphatase inhibitor (Roche) and 40 U/ml SUPERase• In™ RNase Inhibitor (Ambion). Homogenates were cleared by centrifugation at 4°C for 10 minutes at 16000 x g and after three repeats 350 μl of supernatant was collected for IP. 100 μl of ovary lysate was added onto 200 μl of IP mix containing pre-cleared antibody-bound Protein G Sepharose 4 Fast Flow beads (GE Healthcare) and incubated for 8 hours at 4°C. Beads were rinsed 4x in cold IB before RNA extraction. RNA extracted from 10 μl of ovary lysate was used as input.

**List of PCR primers**

The following PCR primers were used to amplify the genomic DNA at the eIF1A locus and sequence the eIF1A coding sequence: 5’ PCR primer for eIF1A genomic region 1, ACATGTTTATAATTTAAGATTCAATCGCT; 3’ PCR primer for eIF1A genomic region 1, AGCCACCTTAGAGAGGGGC; 5’ PCR primer for eIF1A genomic region 2, CAGAATTACTCACAACACAAAAAGAAAC; 3’ PCR primer for eIF1A genomic region 2, CGCTAGCTAACCTCACATCTCG; 2nd forward sequencing primer for eIF1A genomic region 2, GCAGATAACAATGTTCATTTACGGATT; 2nd reverse sequencing primer for eIF1A genomic region 2, TTATTAGTTATGTTCGCAATGTCC.

The following PCR primers and UPL probes were used to perform the qPCR analysis: grk 5’ primer, CCAATGATGCAATCCCCAT; grk 3’ primer, GCGTATGCTCTCGGAGAAGT; UPL probe ID, #152 (cat. no. 04694384001, Roche). αTub67C 5’ primer, CACCCCAATTTTAGTCCAGCAA; αTub67C 3’ primer, CAGGTACAGCTCCAGCGCAG;
UPL probe ID, #58 (cat. no. 04688554001, Roche). \(RpL32\) 5’ primer,
CGGATCGATATGCTAAGCTGT; \(RpL32\) 3’ primer, CGACGCACTCTTTGTCG; UPL
probe ID, #117 (cat. no. 04693515001, Roche).
Fig. S1. Additional eggshell phenotype analyses. (A) Suppression of the DV patterning defects by other eIF1A alleles in spnB^{Blu} mutants. Percentages of eggs showing different categories of the eggshell phenotype were plotted for each genotype. Error bars represent the standard error between the indicated numbers (n) of independent experiments. Numbers in the parentheses indicate the total number of eggs counted per genotype.
**Fig. S2.** The eIF1A<sup>BH167</sup> mutation restores the level of Grk protein accumulation in the <i>spnB</i> mutant egg chambers during the early stages of mid-oogenesis. (A-C) Stage 7/8 egg chambers with Grk stained in red and DNA in blue. Scale bars: 50 µm. (D) Percentage of stage 7 and 8 egg chambers showing different levels of Grk staining in the oocyte. Error bars represent the standard error between the indicated numbers (n) of independent experiments. Numbers in parentheses indicate the total number of egg chambers examined per genotype.
Fig. S3. \( eIF1A^{BH167} \) heterozygosity does not suppress the \( grk \) mRNA localization defects in \( spnB \) mutants. (A) RNA in situ images showing either dorsoanterior concentration (D.A. Conc., arrow) or anterior ring (arrowheads) localization of \( grk \) mRNA in stage 9 \( spnB^{BU} eIF1A^{BH167} \) egg chambers. (B) Percentage of stage 9 egg chambers with \( grk \) mRNA either concentrated at the DA corner (D.A. Conc.) or localized in a ring on the anterior margin of the oocyte (Anterior Ring). Error bars represent the standard error between three independent experiments. The numbers in parentheses indicate the total number of egg chambers examined per genotype.
**Fig. S4. Distribution of rRNA in the fraction samples.** Ethidium bromide staining of agarose gels containing RNA extracted from individual fractions of the wild-type (A), *spnB<sup>BU</sup>* (B), *spnB<sup>BU</sup>* *eIF1A<sup>BU167</sup>* (C), and *sqd<sup>i</sup>* (D) gradients without (A, B, C, D) or with (A’, B’, C’, D’) EDTA treatment. Representative gel images are shown. Fractions containing 18S rRNA and 28S rRNA are indicated. Polysomes were completely disrupted by EDTA, as shown by the prominent reduction of 18S and 28S rRNA staining in the heavy fractions (fractions 10 to 14) of the EDTA-treated gradients.
Fig. S5. (A) Representative UV absorbance profiles of the sqd' gradients without (A) or with (A') EDTA treatment. (B) The distribution profile of αTub67C mRNA in EDTA-treated wild-type (blue circles, *wild type*), *spnB* (red squares, *spnB*), *spnB* eIF1A (green triangles, *spnB eIF1A*), and *sqd* (purple diamonds, *sqd*) gradients. Data are plotted as the percentage of total mRNA in the gradient detected in each fraction. (C) Comparison of the amount of αTub67C mRNA in the heavier intermediate fractions (fractions 7 to 10) of the EDTA-treated gradients. Error bars represent standard error from three independent experiments. P values in the figure were generated by two-sample one-tail heteroscedastic t-tests.