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Misregulation of Trans	forming Growth Factor a	alpha (TGFalpha) and inc	creased Epidermal Grow	th Factor Receptor	(Eofr) activity has been associated with an
increased prognosis of	breast cancer. During	pogenesis in Drosophila	melanogaster, local Egfr	activation by the s	patially-restricted TGFalpha-like ligand,
Gurken (Grk), is requir	ed for axis formation in	the egg and future embry	o. Squid (Sqd), a hetero	geneous nuclear ri	bonucleoprotein (hnRNP) functions in the
localization and transla	tional regulation of grk	mRNA. The purpose of the	his project is to identify fa	actors that function	with Sqd to produce spatially-restricted Egfr
activation. We have inf	egrated genetic and bio	chemical methods to ide	ntify and characterize the	e following factors	that interact with Sqd: (1) an arginine
methyltransferase, CG	6554, which may serve	to methylate Sqd in vivo,	, (2) Hrb27C, an hnRNP	protein, and Ovaria	an tumor, which are required for proper grk
expression, and (3) Br	uno, Cup, and polyA-bir	iding protein, which funct	tion together to mediate	grk translational reg	gulation.
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Cover	1
SF 298	2
Introduction	4
Body	5-14
Key Research Accomplishments	15-17
Reportable Outcomes	
Conclusions	19
References	20-21
Appendix A	
Appendix B	

INTRODUCTION

Misregulation of Transforming Growth Factor alpha (TGFalpha) and increased Epidermal Growth Factor Receptor (EGFR) activity has been associated with an increased prognosis of breast cancer (LeMaistre et al., 1994). TGFalpha and EGFR are required for normal breast development, but deregulation of this signaling pathway can result in uncontrolled proliferation and transformation (Matsui et al., 1990). I am studying the mechanism of TGFalpha activation of EGFR in the model organism Drosophila melanogaster in order to understand how posttranscriptional regulators of TGFalpha function to establish proper ligand expression. The purpose of this project is to identify factors that establish and maintain the tight localization of Gurken (Grk; a Drosophila TGFalpha-like ligand) that is necessary to produce spatiallyrestricted activation of the EGFR during ovary development (Neuman-Silberberg and Schupbach, 1993; Neuman-Silberberg and Schupbach, 1994; Schupbach, 1987). These factors could define potential causes of EGFR misregulation, at the level of ligand production, that result in human breast cancer. We have integrated genetic and biochemical methods to study (1) the effects of a post-translational modification, methylation, on Squid (Sqd), a negative regulator of Grk (Kelley, 1993; Norvell et al., 1999), (2) the function of Hrb27C, an hnRNP protein, and Ovarian tumor for Gurken expression, and (3) the role of Bruno, Cup, polyA-binding protein, and Encore on the translational regulation of grk expression.

BODY

The role Squid (Sqd) methylation during oogenesis

The first task of the project was to determine the role of CG6554 in methylating Squid (Sqd). While we completed the initial proposed experiments, the interpretation has proven to be complicated. We found that CG6554 may function redundantly with another arginine methyltransferase to methylate Sqd. We generated multiple mutations in CG6554 and molecular characterization of these deletions suggests they are all null alleles as they remove the start codon and additional flanking sequence (Figure 1A). In addition, western blot analysis reveals the lack of a band corresponding to CG6554 in the mutants (Figure 1B). Interestingly, the mutants are homozygous viable and lay wild type eggs, suggesting *gurken* (*grk*) RNA and protein are properly localized. We have recombined SqdGFP onto the CG6554 mutant chromosomes and observed that SqdGFP is properly localized to the oocyte nucleus in the mutants. Immunoprecipitation of SqdGFP in the mutant backgrounds and subsequent western blot with antibodies that recognize di-methylarginine reveals that Sqd is still dimethylated in the mutant background (Figure 2). The lack of an eggshell defect in these mutants, in addition to proper localization of *grk* RNA, Grk protein, and SqdGFP makes the fact that Sqd is still methylated not surprising.

The lack of a phenotype in the mutants caused us to see if there were any other arginine methyltransferases in the *Drosophila* genome; six additional putative arginine methyltransferases were identified with homology to CG6554 (Table 1). For one of these genes, CG5358, there was an available ovarian EST (suggesting it was expressed in the ovary) and an available P-element insertion. We obtained this P-element and generated several mutations in CG5358 by P-element excision (Figure 3). As seen for CG6554, the mutants showed no eggshell phenotype, despite the molecular characterization of the mutants as null alleles. But, in these mutants, CG6554 is still present. It is possible that CG6554 and CG5358 function redundantly to methylate Sqd. To determine if CG6554 and CG5358 function redundantly to methylate Sqd, we generated flies doubly mutant for both genes and looked at their eggshell phenotype. While the females doubly mutant for these two arginine methyltransferases are rare, they do lay wild type eggs. As the eggs laid by these females are wild type, *grk* RNA and protein are most likely properly localized. This result suggests that either methylation is not important for egg formation during *Drosophila*

oogenesis or that there are other arginine methyltransferases that can function in the absence of CG6554 and CG5358 during oogenesis. Our current efforts are aimed at looking at the methylation status of Sqd in the mutant backgrounds. As there are several other methyltransferases for which there are no readily available tools to study, we have decided to take a different approach to investigating the function of Sqd methylation (described below). If Sqd methylation seems to be important for function, we will then generate tools to study the potential roles of other methyltransferases.

In order to study the SqdA and SqdS isoforms individually, we cloned HA-tagged genomic-cDNA transgenes and generated transgenic flies expressing each isoform. To determine if these transgenes functioned like wild-type, we expressed them in different sqd mutant backgrounds. Both SqdAHA and SqdSHA are able to rescue the sqd^1 mutant egg shell phenotype and females carrying either transgene lay mainly wild type eggs (72% for SqdSHA and 64% for SqdAHA, n> 900 for each genotype); whereas the sqd^1 mutants lay 100% dorsalized eggs. Both transgenes are also able to rescue the lethality of sqd null mutants as well. Using these transgenes, we were able to determine that both isoforms (SqdSHA and SqdAHA) are methylated in vivo (Figure 1). These results suggest that the HA tag does not interfere with the isoform function and the transgenes function like endogenous Sqd. The addition of the HA tag allowed me to observe the localization of each isoform during oogenesis by whole mount antibody staining with an HA antibody. This analysis confirmed previous results (obtained with tools that are no longer available) that SqdS is found in the nuclei of the oocyte and nurse cells while SqdA is found in the cytoplasm of the oocyte and nurse cells (Figure 2). We also looked at the localization of these transgenes in emrbryos and the localization was the same (data not shown). Using the HASqd transgenes, we were able to show that the SqdA and SqdS isoforms interact with endogenous Sqd (Figure 3), suggesting that the isoforms interact with each other. This data confirms a previous hypothesis that was also supported by data obtained in the initial yeast two hybrid screen with Sqd.

As there are so many arginine methyltransferases in the genome and we do not have mutants that eliminate Sqd methylation, we decided to alter the site of methylation to assay Sqd function in the absence of methylation. After determining that the HA tag did not disrupt Sqd function, we made HA tagged Sqd transgenes that should not be methylated. Methylation typically occurs on arginine residues that are found in an arginine-glycine-glycine (RGG) motifs. Sqd has several of these motifs and each isoform has a unique combination of them (Figure 4); SqdA contains 5 RGG motifs that are shared with SqdS, but Sqd S has three unique RGG motifs. To disrupt the methylation sites, we used site directed mutagenesis to change the arginines of the RGG motifs to lysines, as this was the most conservative amino acid change. It was the hope that this change would abolish methylation without disrupting other aspects of the protein. We generated an HA tagged SqdA transgene with all five RGG motifs mutated (AD5) as well as an HA tagged SqdS transgenes with all eight RGG motifs mutated (SD8) (Figure 4).

Methylation has been shown to be involved in mediating subcellular localization, proteinprotein interaction, and RNA-protein interaction (reviewed in McBride and Silver, 2001). To determine if the RGG motifs (and thus methylation) function in regulating the localization of Sqd, we performed whole mount antibody staining on ovaries from females carrying the mutant transgenes (SD8 and AD5). Each transgene was still properly localized: AD5 was found in the cytoplasm while SD8 was still found in the oocyte and nurse cell nuclei (Figure 5 compared to Figure 2). In addition, we assayed the requirement of the RGG motifs for the Sqd-Sqd interaction as seen in Figure 3. Figure 18 reveals that the RGG motifs and hence methylation is not required for the Sqd isoforms to interact with each other as the mutant transgenes still co-precipitate with endogenous Sqd, despite the mutated RGG motifs. It has been shown that Sqd binds directly to grk RNA (Norvel et al. 1999), to determine if interaction is isoform specific and if the RGG motifs mediate this interaction, we performed UV crosslinking analysis with the HA tagged wild type and RGG mutant transgenes. The results of this experiment reveal that both isoforms are capable of binding to grk RNA (Figure 7A) and that the RGG motifs do not mediate the protein-RNA interaction between Sqd and grk as the mutant transgenes can also bind to grk RNA (Figure 7B).

Since the RGG motifs in Sqd did not seem to be required for any of the previously described roles for arginine methylation, we wanted to establish if they were required for any function of Sqd during oogenesis. To determine if the RGG motifs are required for Sqd function, we assayed the ability of the SD8 and AD5 mutant transgenes (all RGG motifs disrupted) to rescue the sqd^1 mutant phenotypes compared to wild type transgene rescue. sqd^1 mutant females lay 100% doralized eggs. Any wild type eggs produced as a result of the presence of any transgene indicates that the transgene can rescue the mutant phenotype. In this experiment, females expressing two copies of the wild type SqdSHA transgene lay 72% wild type eggs, where

females expressing two copies of the SqdAHA transgene lay 64% wild type eggs. Using the same analysis, females expressing two copies of SD8 lay 5% wild type eggs and females expressing two copies of AD5 lay 68% wild type eggs (Figure 8). The inability of SD8 to rescue to the extent of SqdSHA indicates that SD8 has lost some function, but it is able to function partially to produce some wild type eggs; suggesting that the RGG motifs are not essential. It seems that the RGG motifs of SqdA are not required for function at all since the AD5 transgene can rescue as well as SqdAHA. To determine if the RGG altered transgenes are able to rescue the lethality of sqd mutants, we expressed two copies of each transgene in the mutant background and looked for adult flies to eclose. The lethal sqd mutants produce no adult flies, but when SD8 and AD5 are expressed in the mutant background, adult flies eclose suggesting that the RGG motifs are not required for the somatic function of Sqd either. To ensure that the mutant transgenes had lysines instead of arginines in RGG motifs and to determine if the lysines in the transgenes could still be methylated, we immunoprecipitated the SD8 transgene using HA antibodies from ovarian extract and sent this protein sample for mass spectrometromic analysis to identify any post-translational modifications. These results revealed that all arginines in the context of RGG motifs were changed to lysines and that there was no methylation of the SD8 protein. This result suggests that the changes to lysines did alter the methylation status of the protein as we expected and that the rescue ability of the mutant protein is due to residual function, not extraneous methylation. Knowing that there is no extraneous methylation occurring on the SD8 protein, which retains some function in the absence of the RGG motifs, we must conclude that methylation of Sqd does not seem to be required for function.

*The role of Hrb27C in Gurken expression and dorso-ventral patterning *this work is published in Goodrich et al., 2004 (see Appendix B)*

For the second task of determining the role of Hrb27C in Gurken (Grk) expression and dorsoventral (DV) patterning, we published an article (Goodrich et al., 2004, appendix) describing these results. Figure 2 describes the effects of *hrb27C* germline clone mutants on DV patterning. Hrb27C mutants lay eggs with a range of phenotypes that are consistent with dorsalization. These dorsalized eggs are caused by mislocalized *grk* RNA and Grk protein. Table 1 (Goodrich et al., 2004) demonstrates the in vivo genetic interaction between Hrb27C and Sqd; weak *hrb27C* mutants can strongly enhance the DV defects of weak *sqd* mutants. As Sqd binds to *grk* RNA, the interaction between Sqd and Hrb27C prompted us to ask if Hrb27C could bind directly to grk RNA by UV cross-linking analysis. Figure 3 (Goodrich et al. 2004) shows that Hrb27C does indeed bind to grk RNA. The Hrb27C antibodies do not function for whole mount staining, so we were not able to assay Hrb27C localization. However, we were able to observe that SqdGFP is properly localized in Hrb27C mutants. In addition, we observed another interesting phenotype in mutants of Sqd and Hrb27C: persistent polytene nurse cell chromosomes (Goodrich et al., 2004, Figure 4). We also found that Hrb27C interacts with another ovarian protein, Ovarian tumor (Otu). Otu seems to play a role in mediating proper Grk expression (Goodrich et al., 2004, Figure 5). Collectively, the data in this paper lead us to the model presented in Figure 6 (Goodrich et al., 2004): Sqd and Hrb27C associate with grk RNA in the oocyte nucleus and accompany it into the cytoplasm, where Otu then associates and other factors dissociate, to facilitate grk RNA localization, anchoring, and translational regulation in the dorsal anterior region of the oocyte. In the nurse cells, a distinct complex also containing Hrb27C, Sqd, and Otu in addition to other accessory factors functions in the cytoplasm to mediate the processing, localization, translational regulation, or stabilization of an unidentified RNA target, which can then regulate nurse cell chromosome dispersal at the appropriate time in oogenesis. In summary, this task was completed in full and all of the results were published.

*Identifying the mechanism of Bruno-dependent translational regulation of grk RNA *this work has been submitted to* Developmental Cell (*June 2006*)

The third task concerned the role of the translational regulator Bruno (Bru) in Gurken expression. We have observed that Grk RNA and protein seem to be properly localized in the flies expressing only the *grk* transgene lacking all Bru binding sites in an otherwise wild type background. We have obtained a new Bruno antibody that enhances our ability to assay which sites are important for Bru binding by UV cross-linking followed by immunoprecipitation. We assayed other regions of the *grk* transcript for Bru binding and confirmed our previous results that Bru only binds the *grk* 3' UTR.

We obtained additional information about the role of Bruno in *grk* expression by showing that Cup, a known Bruno-interacting protein (Chekulaeva et al., 2006; Nakamura et al., 2004; Wilhelm et al., 2003), also interacts with Sqd. We identified this interaction by preparing ovarian extracts and performing immunoprecipitations using either an α -Sqd antibody or a negative control antibody. Eleven bands were pulled down using the α -Sqd antibody but not by the negative control antibody (Figure 13A, arrows), and the identities of the bands were determined by mass spectrometry. One band of 150 kDa was identified as the translational repressor Cup, and a 65 kDa protein was identified as the translational activator polyA binding protein (pAbp55B, encoded by *CG5519*). The negative control antibody, α -Dorsal (α -Dl) was used because it is a monoclonal antibody that was generated in the same facility as α -Sqd, but *Dl* is not expressed during oogenesis and therefore should not specifically pull down any ovarian factors.

To verify the identity of the bands sequenced by mass spectrometry, we performed coimmunoprecipitations using antibodies that recognize Sqd (Goodrich et al., 2004), Cup (Keyes and Spradling, 1997; Nakamura et al., 2004; Nelson et al., 2004; Verrotti and Wharton, 2000), or pAbp55B (Matunis et al., 1992). In these assays, α -Sqd, but not α -Dl, immunoprecipitates Cup and pAbp55B in an RNA-dependent manner (Figure 13B and C), confirming the results of the mass spectrometry analysis. We also wanted to determine whether Cup or pAbp55B interacts specifically with one of the Sqd isoforms (Kelley, 1993), so we used an antibody that recognizes the HA epitope to perform immunoprecipitations out of ovarian extracts expressing either HA-SqdA or HA-SqdS. In these experiments, Cup and pAbp55B were able to interact with both Sqd isoforms, indicating that these interactions are not isoform-specific (data not shown).

To determine whether these biochemical interactions were relevant to *grk* function, we examined the eggs laid by *pAbp55B* or *cup* females and observed that these eggs display dorsoventral patterning defects. We obtained a P-element insertion in the 5' UTR of *pAbp55B* (*pAbp^{k10109}*), however this insertion is homozygous and hemizygous lethal, so we monitored the eggs that were laid by females heterozygous for the insertion. Significantly, these females lay $12.2 \pm 3.4\%$ (n=2006) ventralized eggs, characterized by the presence of a single, slender, fused dorsal appendage (Figure 14B) instead of two distinct appendages (Figure 14A). This phenotype is consistent with a reduction in Grk protein accumulation at the dorsal-anterior of the oocyte. Importantly, the biochemical and genetic tests described below suggest that the phenotype is not an artifact and is truly indicative of reduced Grk protein expression in the oocyte.

Since the $pAbp^{k10109}$ allele is lethal, we produced germline clones (Chou and Perrimon, 1996) to determine whether Grk protein expression is reduced in egg chambers with a germline homozygous for $pAbp^{k10109}$. Unfortunately, these germline clones were cell-lethal and did not permit the growth of egg chambers past stage 3 (data not shown). This suggests that there is an essential requirement for pAbp55B early in oogenesis that prevents the analysis of Grk protein or *grk* mRNA in mid-oogenesis in a germline that is homozygous for *pAbp55B*.

In contrast, homozygous cup^5 females lay 94.5 \pm 7.8% (n=135) dorsalized eggs, characterized by either one broad dorsal appendage or a crown of dorsal appendage material encircling the anterior of the egg (Figure 14C-E). $cup^{\Delta 212}$ seems to be a weaker allele, as homozygous females lay 95 \pm 4.9% (n=398) eggs with broad, fused appendages, a weaker phenotype than a crown of appendage material (data not shown). Both of these phenotypes are reminiscent of mutations in *sqd* (Norvell et al., 1999), and are consistent with ectopic expression of Grk protein in the oocyte. However, the phenotype also suggests that peak levels of *grk* signaling are not achieved in the mutant, since the dorsal midline fate is often missing.

To test whether the eggshell phenotypes seen in *cup* mutants were due to defective *grk* expression, we monitored Grk expression by indirect immunofluorescence. Consistent with the dorsalized eggshells, Grk protein accumulates improperly in *cup* mutants (Figure 15). The degree of severity of this defect was greater for cup^5 mutants than for $cup^{\Delta 212}$ mutants, which is also consistent with the degree of severity of the eggshell phenotypes.

In scoring, Grk localization was categorized as either localized properly to the future dorsalanterior of the oocyte (Figure 15A), dispersed throughout the oocyte, but somewhat accumulated at the future dorsal-anterior (Figure 15B), or evenly dispersed throughout the oocyte (Figure 15C). Grk protein was properly localized to the future dorsal-anterior of about 86% (n=548) of wild-type egg chambers, in contrast to 31% (n=196) of cup^5 and 61% (n=163) of $cup^{\Delta 212}$ egg chambers. The degree of severity of this defect was greater for cup⁵ mutants than for $cup^{\Delta 212}$ mutants, which is also consistent with the degree of severity of the eggshell phenotypes (Figure 15).

In order to determine whether the ectopic Grk protein expression is a result of improper translational regulation, we analyzed the localization of *grk* mRNA in *cup* mutants (Figure 16). In contrast to wild-type egg chambers, in which about 71% (n=349) of stage 8-9 egg chambers

display *grk* mRNA properly localized to the future dorsal-anterior of the oocyte, only 27% (n=271) of cup^5 and 54% (n=259) of $cup^{\Delta 212}$ egg chambers display this proper localization. Consistent with the eggshell phenotypes and Grk protein localization data, *grk* mRNA localization was less severely disrupted in $cup^{\Delta 212}$ than in cup^5 (Figure 16). This might suggest that Cup is required for *grk* mRNA localization, however, the effect of *cup* mutants may also be indirect. Perhaps removing Cup from the localization/repression complex built upon *grk* mRNA compromises the stable architecture of the complex, resulting in less efficient localization.

Importantly, the *grk* mRNA localization data show that Cup is required for the translational repression of unlocalized *grk* mRNA. This effect is also seen in *sqd* mutants (Norvell et al., 1999), and is in contrast to many ventralizing mutants in which *grk* mRNA is mislocalized but remains in a translationally repressed state, such as *encore* or the spindle class genes (Ghabrial et al., 1998; Gonzalez-Reyes et al., 1997; Hawkins et al., 1997).

Females transheterozygous for a weak allelic combination of $sqd (sqd^{l}/sqd^{kl2})$ lay only 13% wild-type eggs (n=355), and the remaining eggs display mild to strong dorsalized phenotypes. Females homozygous for the weak allele sqd^{kl2} lay only 5% weakly dorsalized eggs (n=160), but the frequency and severity of dorsalization in both sqd allelic combinations was dramatically enhanced by heterozygosity for cup^{20} (Table 2), a strong cup allele (Keyes and Spradling, 1997). This effect has been observed over multiple independent experiments, and representative data are shown (Table 2). This synergistic genetic interaction suggests that the Cup-Sqd biochemical interaction is functionally relevant for grk translational representation.

Females heterozygous for a null allele of *grk*, grk^{HF48} , lay 3% weakly ventralized eggs (n=1146), and reducing *pAbp55B* by one copy was able to greatly enhance this ventralization (n=1212, Table 2). This synergistic genetic interaction is consistent with pAbp55B functioning positively in *grk* translation.

Immunoprecipitation experiments show that α -pAbp55B is able to specifically pull down Cup protein in an RNA-dependent manner (Figure 13D), and α -Cup very weakly pulls down pAbp55B (data not shown). Considering that Cup and pAbp55B interact biochemically with each other and with Sqd, *cup* and *pAbp55B* females lay eggs with opposite phenotypes, and *cup-sqd* and *pAbp55B-grk* interact genetically, we propose that Cup and pAbp55B work antagonistically to regulate *grk* mRNA expression.

Encore (Enc) is a large, novel, cytoplasmic protein that co-localizes with *grk* mRNA (Van Buskirk et al., 2000) and is required for proper *grk* mRNA localization (Hawkins et al., 1997). In contrast to *sqd* and *K10* mutants, in which unlocalized *grk* mRNA is translated and dorsalized eggs are laid (Kelley, 1993; Norvell et al., 1999; Wieschaus et al., 1978), unlocalized *grk* mRNA in *enc* mutants is maintained in a translationally repressed state and ventralized eggs are laid (Hawkins et al., 1997). These data are consistent with a role for Enc in the translational activation of *grk* mRNA.

Enc has previously been shown to be a large cytoplasmic protein that interacts with subunits of the proteasome in early oogenesis (Ohlmeyer and Schupbach, 2003; Van Buskirk et al., 2000). Considering its function early in oogenesis, and given its cortical localization in the oocyte during mid-oogenesis, Enc may act as a scaffolding protein mediating the transition from translational repression to activation of *grk* mRNA. To test this hypothesis, we performed immunoprecipitations and showed that α -Enc specifically immunoprecipitates the translational activator pAbp55B. Furthermore, the data suggest that the interaction may be direct and not bridged by an RNA molecule, as α -Enc is able to immunoprecipitate pAbp55B even when RNase is added to the extract (Figure 17A).

In addition to the biochemical interaction, pAbp55B and *enc* also interact genetically. *enc* mutants are cold-sensitive for ventralization (Hawkins et al., 1997) and display much weaker ventralization at 25°C than at 18°C. However, heterozygosity for $pAbp^{k10109}$ enhances the both the frequency and severity of ventralized eggs laid by *enc* homozygotes at 25°C (Figure 17B). Although this enhancement is a subtle phenotype, the effect has been observed reproducibly over several independent experiments using two different *enc* alleles (*enc*^{Q4} and *enc*^{UU3}). This genetic interaction suggests that the pAbp55B-Enc biochemical interaction is functionally relevant in *grk* expression.

In addition to the enhanced ventralization, heterozygosity for $pAbp^{k10109}$ dramatically reduces the number of eggs that are laid by *enc* homozygotes and increases the percentage of collapsed eggs for which the eggshell phenotype cannot be determined. In fact, heterozygosity for $pAbp^{k10109}$ causes enc^{r17} and enc^{R1} homozygous females to lay no eggs. Taken together, the genetic interaction and RNA-independent physical association between Enc and pAbp55B suggest that these proteins may function together to activate translation of *grk* mRNA. The fact that Cup and pAbp55B interact with Sqd is important for elucidating the function of Bru in *grk* expression. Previous work has shown that Cup binds directly to Bru, which binds to well-defined sequences in the 3'UTR of specific RNA molecules. In addition, Cup binds directly to eIF4E, preventing eIF4G from binding to eIF4E. Because eIF4G binding to eIF4E is a prerequisite for translation initiation, Cup represses translation by blocking this interaction. Translation is activated when pAbp55B binds to the polyA tail of the same RNA. pAbp binds to eIF4G, and this interaction helps to increase the affinity of eIF4G for eIF4E. With this increased affinity, eIF4G is able to compete away Cup for binding to eIF4E, and translation is allowed to begin (reviewed in Richter and Sonenberg, 2005; Tarun and Sachs, 1996; Tarun et al., 1997). Our co-immunoprecipitation experiments suggest a specific mechanism by which Sqd, Bru, Cup, and pAbp function together to mediate *grk* translational regulation.

KEY RESEARCH ACCOMPLISHMENTS

Task 1: To determine the role Squid (Sqd) methylation during oogenesis

Production of 3 mutant alleles of CG6554 by imprecise P-element excision: 4D8-7 (403 bp deletion), 4D8-8 (609 bp deletion), and 4C16-2 (1700 bp deletion)

CG6554 mutants lay wild type eggs

Grk RNA and protein are properly localized in CG6554 mutants

SqdGFP is properly localized in CG6554 mutants

Sqd is still methylated in CG6554 mutants

CG5358 is another methyltransferase expressed in Drosophila ovaries

Generation of 3 mutant alleles of CG5358 by imprecise P-element excision: 2C (1718 bp deletion), 6B (845 bp deletion), and 14B (1091 bp deletion)

CG5358 mutants lay wild type eggs

Generation of CG6554 and CG5358 double mutants

CG6554 CG5358 double mutants lay wild type eggs

Grk RNA and protein are properly localized in CG6554 mutants

Generation of HASqdS transgenic flies

Generation of HASqdA transgenic flies

HASqdS is localized to the nuclei of the oocyte and the nurse cells

HASqdA is localized to the cytoplasm of the oocyte and the nurse cells

HASqdS and HASqdA are methylated in vivo

Sqd isoforms interact with each other

Generation of SqdS transgenic flies with eight arginines of RGG motifs changed to lysines

Generation of SqdA transgenic flies with five arginines of RGG motifs changed to lysines

Sqd isoforms interact with each other independent of RGG motifs

SqdA and SqdS both bind to grk RNA

SqdA and SqdS bind to grk RNA independent of RGG motifs

SD8 is not methylated on any residue

Sqd can function in the absence of the RGG motifs

Arginine methylation is not essential for Sqd function

Task 2: To determine the role of Hrb27C in Gurken expression and dorso-ventral patterning *this work is published in Goodrich et al., 2004

hrb27C mutants have dorsal-ventral defects

hrb27C mutants affect grk RNA localization and translational regulation

Hrb27C and Sqd interact genetically: weak transheterozygous alleles of Hrb27C strongly enhance the dorsal ventral defects of a weak *sqd* allele combination

Hrb27C binds to grk RNA

Hrb27C interacts with Ovarian tumor (Otu) by co-immunoprecipitation

otu mutants have dorsal-ventral eggshell defects

otu mutants have defects in grk RNA localization

Task 3: To identify the mechanism of Bruno-dependent translational regulation of grk RNA *this work has been submitted to Developmental Cell (June 2006)

grk RNA and protein are properly localized in the flies expressing a *grk* transgene lacking all Bru binding sites

Bru only binds to the grk 3'UTR, not other regions of the transcript

Cup, a Bruno-interacting protein, interacts with all Sqd isoforms in an RNA-dependent manner

Cup interacts with pAbp55B

pAbp55B interacts with Sqd in an RNA-dependent manner

cup mutant females lay dorsalized eggs

pAbp55B mutant females lay ventralized eggs

Grk protein is ectopically expressed in *cup* mutants

grk mRNA is less efficiently localized in cup mutants

cup and *sqd* interact genetically

- *pAbp55B* and *grk* interact genetically
- pAbp55B interacts biochemically with Enc, a known activator of *grk* translation, in an RNAindependent manner

pAbp55B and *enc* interact genetically

REPORTABLE OUTCOMES

- Goodrich, J.S. and Schüpbach, T. (in prep). Arginine methylation is not essential for Squid function during Drosophila oogenesis.
- Clouse, K.N. and Schüpbach, T. (submitted to *Dev Cell*). Cup and pAbp55B function with Sqd to mediate translational regulation of *grk* mRNA.
- Clouse, K.N. and Schüpbach, T. (2006). Dissertation: The role of Squid, an hnRNP protein, in RNA localization and translational control in *Drosophila melanogaster* oogenesis.
- Clouse, K.N. and Schüpbach, T. (2006). Squid, Cup, and pAbp cooperatively regulate *grk* mRNA expression. 47th Annual *Drosophila* Research Conference. Platform.
- Goodrich, J.S. and Schüpbach, T. (2005). EGFR activation of Spatially Restricted Ligands. Era of Hope, Department of Defense Breast Cancer Research Program Meeting. Poster.
- Goodrich, J.S. (2004). Dissertation: Regulation of Gurken expression by Squid-dependent RNA localization and translational control during *Drosophila melanogaster* oogenesis.
- Goodrich, J.S., Clouse, K. N., and Schüpbach, T. (2004). Hrb27C, Sqd, and Otu cooperatively regulate *gurken* RNA localization and mediate nurse cell chromosome dispersion in *Drosophila* oogenesis. Development 131 (9), 1949-58. ** paper attached in Appendix B
- Goodrich, J.S. and Schüpbach, T. (2004). The function of Squid methylation by CG6554 during *Drosophila* oogenesis. 45th Annual *Drosophila* Research Conference. Poster.

CONCLUSIONS

This work has allowed us to study the function of Sqd in more detail. First, it has shown us that arginine methylation of Sqd is not essential for function and that the arginine methyltransferases, CG6554 and CG5358, are not required for viability. The function of Sqd methylation is still unknown, but it does not seem to function in the regulation of Grk expression during oogenesis. Future efforts should be aimed at analyzing other potential post-translational modifications of Sqd that are required to regulate the different aspects of Sqd isoform function. This as of yet unidentified post-translational modification could be essential for proper regulation of Grk expression and the disruption of this modification could results in improper expression of the TGFalpha ligand and ultimately misexpression of EGFR that could lead to cancer. Second, these studies have shown us that Hrb27C and Otu function with Sqd to mediate proper Grk expression. Finally, our studies have identified a very important mechanism by which *grk* translation is regulated. Negative regulation is mediated by Sqd and Cup, and positive regulation is dependent on pAbp and Enc. Given that Sqd, Cup, and pAbp have human homologs, it will be interesting to test in the future whether these genes are also involved in the negative regulation of TGFalpha, and whether mutations in these genes could contribute to breast cancer development.

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APPENDIX A

Figures 1-18 Tables 1, 2





Figure 1. CG6554 mutants generated by excision analysis

(A) Schematic of the genomic region of CG6554 with a map and the names of the mutants generated by P-element excision. The deletion size is noted in parenthesis. (B) Western for CG6554 protein on ovarian extract from wild type and *CG6554* excision lines (4D8-7, 4D8-8, and 4C16-2) revealing the absence of the 46 kDa band, corresponding to CG6554, in the mutants. The generated mutants are null alleles.



Figure 2. Sqd is still methylated in CG6554 mutants

Western blot with an antibody to dimethylated arginines (Ab 413) recognizes Sqd after GFP immunoprecipitation (IP) from ovarian extract of flies expressing SqdGFP and SqdGFP in a *CG6554* mutant background. Multiple recombinants between *CG6554* and SqdGFP were made and tested in this assay. There is a background band smaller than SqdGFP in the GFP IP from HisGFP ovarian lysate, but the upper band is specific to SqdGFP as seen in all the lanes. #2 SqdGFP is the line that was used to make the recombinants.

Gene	Location	% identity to CG6554
CG6554	86C6	100%
CG3675	24E1	44%
CG6563	88E3	39%
CG9927	88A2	37%
CG5358	85F4	36%
CG16840	32D1	34%
CG9929	88A2	30%

Table 1. Seven arginine methyltransferases in the Drosophila genome

A list of the gene names, chromosomal location, and percent identity to *CG6554* of the at least six other arginine methyltransferases in the genome.

A.





Figure 3. CG5358 mutants generated by excision analysis

(A) Schematic of the genomic region of *CG5358* with a map and the names of the deletions generated by P-element excision. The deletion size is noted in parenthesis. (B) Western for CG5358 protein on ovarian extract from wild type and *CG5358* excision lines (2C, 6B, and 14B) revealing the absence of the 60 kDa band, corresponding to CG5358, in the mutants. The generated mutants are null alleles.

Figure 4. SqdS and SqdA are methylated in vivo

Western blot probed for methylated arginines after immunoprecipitation with HA antibodies from ovarian lysate of wild type flies or flies expressing either SqdSHA or SqdAHA. The methylarginine antibody recognizes bands specific to SqdSHA and SqdAHA suggesting that both isoforms are methylated in ovarian extract. Wild type lysate is the negative control since there is no HA tag to immunoprecipitate.

Figure 5. Localization of SqdS and SqdA

HA antibody staining of stage 9 egg chambers from females expressing SqdSHA (A) or SqdAHA (B) reveals that SqdS is localized to the nuclei of the oocyte and nurse cells(A). SqdA is localized to the cytoplasm (B).

Figure 6. Sqd isoforms interact with each other

Western blot probed for Sqd after immunoprecipitation with HA antibodies from ovarian lysate of wild type flies or flies expressing either SqdSHA or SqdAHA. Endogenous Sqd co-precipitates with both SqdSHA and SqdAHA. Wild type lysate is the negative control since there is no HA tag to immunoprecipitate.

Figure 7. Schematics of wild type and RGG mutant Sqd transgenes

The location of the HA tag is denoted by the green box. The striped and stippled boxes represent the RNA binding domains (RBD). The black boxes are the M9 nuclear transport motifs and the regions unique to each isoform appear after the alternative splice site. The RGG motifs are denoted by colored lines. Both SqdS and SqdA share 5 common RGG motifs (yellow line) and SqdS has three unique boxes (red and blue lines). The absence of the line represents the RGG motifs where the R was changed to K and hence should not be methylated.

Figure 8. RGG motifs are not required for proper Sqd localization

HA antibody staining of stage 9 egg chambers from females expressing SD8 (A) or AD5 (B) reveals that even without methylated arginines, SqdS is still localized to the nuclei of the oocyte and nurse cells (A). In the absence of arginine methylation, SqdA is still localized to the cytoplasm (B).

Figure 9. Sqd isoform interaction does not depend on RGG motifs

Western blot probed for Sqd after immunoprecipitation with HA antibodies from ovarian lysate of wild type flies or flies expressing either SD8 or AD5. Endogenous Sqd still co-precipitates even in the absence of the RGG motifs of SqdSHA and SqdAHA. Wild type lysate is the negative control as there is no HA tag to immunoprecipitate.

Figure 10. RGG motifs are not required for SqdS or SqdA binding to grk RNA

(A) Using ovarian lysate from flies expressing SqdSHA or SqdAHA, UV cross-linking analysis followed by immunoprecipitation (IP) with antibodies to Sqd or HA reveals that both SqdSHA and SqdAHA are able to bind to *grk* RNA. (B) Using ovarian lysate from flies expressing SD8 or AD5, UV cross-linking analysis followed by IP with antibodies to Sqd or HA reveals that the RGG motifs, and thus methylation, are not required for SqdS or SqdA to bind to *grk* RNA.

Figure 11. Rescue of sqd^1 eggshell phenotype by wild type and RGG mutant SqdS and SqdA transgenes

 sqd^{1} mutant females lay no wild type eggs. When two copies of the designated transgene are expressed in the sqd^{1} background, wild type eggs are produced indicating that both the wild type and mutant transgenes can rescue the sqd^{1} mutant eggshell phenotype (n> 900 for each genotype).

Figure 12. Bru binds only to the 3'UTR of grk RNA

(A) Schematic of the *grk* cDNA with the regions used for probes underlined. **denotes the region to which Bru binds. (B) UV cross-linking followed byimmunoprecipitation (IP) of Bru to identify the regions of *grk* to which Bru can bind.The region used for each probe is underlined in (A). In some experiments, IP with Sqd was used as a positive control.

Figure 13: Cup and pAbp55B interact with Sqd in ovarian extracts

(A) Immunoprecipitations were performed out of ovarian lysates using either α -Sqd or a negative control antibody, α -Dorsal. Specific Sqd interactors (marked by arrows) were excised from the gel and sequenced by mass spectrometry. Positively identified bands are labeled. (B-D) Immunoprecipitations were performed in the presence or absence of RNase (if indicated) using α -Sqd, α -Cup, α -pAbp55B, or α -Dorsal. Western blots were probed with α -pAbp55B (B) or α -Cup (C-D).

Figure 14. *cup* and *pAbp55B* females lay eggs with dorsoventral patterning defects

Most $pAbp^{k10109} / CyO$ eggs (n=2006) display wild-type morphology (A), but approximately 12% of eggs have a single, slender, fused dorsal appendage (B). A small percentage of cup^5 eggs (n=135) have wild-type morphology (A), but approximately 95% of eggs laid by cup^5 / cup^5 females have thick, fused dorsal appendages (C), a crown of dorsal appendage material (D), or open chorions (E).

	dorsal-anterior only	dispersed; dorsal-anterior bias	completely dispersed
OreR n=548	85.8 <u>+</u> 8.4%	13.0 <u>+</u> 4.8%	1.3 <u>+</u> 3.6%
<i>cup</i> ⁵ n=196	30.6 <u>+</u> 9.2%	46.9 <u>+</u> 4.1%	22.4 <u>+</u> 13.3%
<i>сир^{д212}</i> n=163	60.7 <u>+</u> 18.7%	35.6 <u>+</u> 21.3%	3.7 <u>+</u> 2.6%

Figure 15. Grk protein is not properly localized in *cup* mutants

OregonR, cup^5 , and $cup^{\Delta 212}$ egg chambers were stained for α -Grk (red), phalloidin (green), and Hoechst (blue). Grk protein distribution was categorized as either dorsal-anterior only (A), dispersed throughout the oocyte with a dorsal-anterior bias (B), or evenly dispersed throughout the ooplasm (C).

A	B	С
	2	2

	dorsal-anterior only	anterior ring; dorsal-anterior bias	anterior ring
OreR n=349	71.1 <u>+</u> 9.0%	23.5 <u>+</u> 8.4%	2.3 <u>+</u> 2.6%
<i>cup</i> ⁵ n=271	26.9 <u>+</u> 9.7%	54.2 <u>+</u> 12.2%	13.7 <u>+</u> 7.6%
<i>сир^{Д212}</i> n=259	53.7 <u>+</u> 5.2%	35.5 <u>+</u> 2.1%	2.7 <u>+</u> 3.6%

Figure 16. grk mRNA is localized less efficiently in cup mutants

In situ hybridization using a grk RNA probe was performed on OregonR, cup^5 , and $cup^{\Delta 212}$ egg chambers. grk mRNA distribution was categorized as either dorsal-anterior only (A), an anterior ring with a dorsal-anterior bias (B), or an anterior ring (C).

A.	wild-type like	weakly dorsalized	moderately dorsalized	strongly dorsalized
sqd^{k12} / sqd^1 (n=355)	13.2%	4.2%	54.1%	28.5%
$cup^{20} / +$; sqd^{k12} / sqd^{l} (n=443)	0%	6.8%	8.6%	84.7%
sqd^{k12} / sqd^{k12} (n=160)	94.4%	1.3%	4.4%	0%
$cup^{20} / +$; sqd^{k12} / sqd^{k12} (n=524)	25.2%	37.8%	28.8%	8.2%

B.	wild-type like	weakly ventralized	moderately ventralized	strongly ventralized
<i>grk</i> ^{HF48} / + (n=1146)	97.3 <u>+</u> 1.2%	2.7 <u>+</u> 1.2%	0%	0%
$grk^{HF48} / +$ $pAbp^{k10109} / +$ (n=1212)	45.1 <u>+</u> 5.0%	43.4 <u>+</u> 6.3%	10 <u>+</u> 2.0%	0%

Table 2. sqd interacts genetically with cup, and grk interacts genetically with pAbp

Heterozygosity for cup^{20} enhances the moderately dorsalized phenotype of sqd^{k12} / sqd^1 transheterozygotes and of sqd^{k12} / sqd^{k12} at 29°C (A). Eggs were characterized as either wild-type like, weakly dorsalized (single, broad fused appendage), moderately dorsalized (widely-spaced appendages), or strongly dorsalized (crown of appendage material). In addition, $grk^{HF48} / pAbp^{k10109}$ transheterozygotes lay an increased percentage of and more severely ventralized eggs at 25°C than does either heterozygote alone (B). Eggs were characterized as either wild-type like, weakly ventralized (appendages fused at the base), moderately ventralized (single, slender fused appendage), or strongly ventralized (no appendage material).

B.	wild-type like	weakly ventralized	moderately dorsalized	strongly ventralized	collapsed
enc^{Q4} / enc^{Q4} (n=305)	66.6%	4.3%	5.9%	8.2%	15.1%
enc^{Q4} / enc^{Q4} $pAbp^{k10109} / +$ (n=39)	5.1%	12.8%	20.5%	5.1%	51.3%
enc^{UU3} / enc^{UU3} (n=123)	52.8%	9.8%	5.7%	0.0%	31.7%
enc^{UU3} / enc^{UU3} $pAbp^{k10109} / +$ (n=48)	4.2%	4.2%	29.2%	10.4%	41.7%

Figure 17. pAbp55B interacts with Enc biochemically and genetically

(A) Immunoprecipitations were performed out of ovarian lysates in the presence or absence of RNase using α -Enc or α -Dorsal. Western blots were probed with α -pAbp55B. (B) Heterozygosity for $pAbp^{k10109}$ enhances the weakly ventralized phenotype of enc^{Q4} / enc^{Q4} and of enc^{UU3} / enc^{UU3} homozygotes at 25°C, increases the percentage of collapsed eggs, and decreases the overall number of eggs laid. Eggs were characterized as either wild-type like, weakly ventralized (appendages fused at the base), moderately ventralized (single, slender fused appendage), strongly ventralized (no appendage material), or collapsed.

APPENDIX B

Goodrich, J.S., Clouse, K. N., and Schüpbach, T. (2004). Hrb27C, Sqd, and Otu cooperatively regulate *gurken* RNA localization and mediate nurse cell chromosome dispersion in *Drosophila* oogenesis. Development 131 (9), 1949-58.

Hrb27C, Sqd and Otu cooperatively regulate *gurken* RNA localization and mediate nurse cell chromosome dispersion in *Drosophila* oogenesis

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Summary

Heterogeneous nuclear ribonucleoproteins, hnRNPs, are RNA-binding proteins that play crucial roles in controlling gene expression. In *Drosophila* oogenesis, the hnRNP Squid (Sqd) functions in the localization and translational regulation of *gurken* (*grk*) mRNA. We show that Sqd interacts with Hrb27C, an hnRNP previously implicated in splicing. Like *sqd*, *hrb27C* mutants lay eggs with dorsoventral defects and Hrb27C can directly bind to *grk* RNA. Our data demonstrate a novel role for Hrb27C in promoting *grk* localization. We also observe a direct physical interaction between Hrb27C and Ovarian tumor

Introduction

Heterogeneous nuclear ribonucleoproteins (hnRNPs) are RNA-binding proteins that function in a variety of cellular processes such as transcription, splicing, nuclear transport, localization, translation and protein stability (for a review, see Dreyfuss et al., 1993; Krecic and Swanson, 1999; Dreyfuss et al., 2002). HnRNP association is highly dynamic and can change with every step of RNA processing, as required by the specific RNA in the complex. Each mRNA is presumably bound by a unique combination of hnRNPs that ultimately specify its fate by influencing the structure of the RNA and modulating its interactions with trans-acting factors (Dreyfuss et al., 1993; Dreyfuss et al., 2002). Thus, hnRNPs play a crucial role in controlling gene expression.

Squid (sqd), also known as Drosophila hrp40 (Matunis et al., 1992a; Matunis et al., 1992b; Matunis et al., 1994), encodes an hnRNP that has been characterized mainly for its role in dorsoventral (DV) axis formation during oogenesis where it plays an important role in *gurken* (*grk*) mRNA localization and protein accumulation (Kelley, 1993; Norvell et al., 1999). All *Drosophila* hnRNPs identified so far have structural similarities to human hnRNP A/B proteins (Dreyfuss et al., 1993). Sqd contains the common hnRNP structural features (Krecic and Swanson, 1999; Dreyfuss et al., 2002): two RNA-binding domains, auxiliary glycine-rich and M9-like domains, and existence in multiple protein isoforms.

Grk is a TGF α -like ligand that is produced in the oocyte and signals to the EGFR (Epidermal Growth Factor Receptor) expressed in all follicle cells that comprise the epithelium surrounding the germline cells. During mid-oogenesis,

(Otu), a cytoplasmic protein implicated in RNA localization. We find that some *otu* alleles produce dorsalized eggs and it appears that Otu cooperates with Hrb27C and Sqd in the oocyte to mediate proper *grk* localization. All three mutants share another phenotype, persistent polytene nurse cell chromosomes. Our analyses support dual cooperative roles for Sqd, Hrb27C and Otu during *Drosophila* oogenesis.

Key words: *Drosophila*, Oogenesis, hnRNP, Gurken, mRNA localization, Nurse cell nuclear morphology, Hrp48

establishment of the DV axis of the egg and embryo depends on the precise spatial restriction of grk RNA and protein to the dorsal anterior region of the oocyte to produce localized activation of EGFR (Neuman-Silberberg and Schüpbach, 1993; Neuman-Silberberg and Schüpbach, 1994). In sqd mutants, grk RNA is mislocalized and translated around the entire anterior of the oocyte producing ectopic EGFR activation and induction of excess dorsal cell fates, which results in an expansion of dorsal appendage material around the anterior circumference of the egg (Kelley, 1993). The three Sqd isoforms (A, B and S) are generated by alternative splicing and differ only in their extreme C-terminal regions. They have different subcellular localization patterns and distinct roles in the regulated nuclear export and localization of grk mRNA, as well as in Grk protein accumulation (Norvell et al., 1999). A working model for the role of Sqd in the regulation of Grk expression as proposed by Norvell et al. (Norvell et al., 1999) suggests that SqdS associates with grk RNA in the oocyte nucleus to facilitate regulated nuclear export and RNA localization within the cytoplasm, where SqdA then associates with the grk transcript to facilitate translational regulation. SqdB does not appear to play a role in DV patterning.

To identify proteins that interact with Sqd to function in the regulation of Grk expression, we performed a yeast two-hybrid screen with Sqd and isolated the hnRNP, Hrb27C. We found that *hrb27C* mutants have DV defects as a result of mislocalized *grk* RNA. Thus, it appears that Hrb27C and Sqd function together to regulate Grk expression. We have also identified a physical interaction between Hrb27C and Ovarian tumor (Otu). Examination of *otu* mutants reveals that *otu* also

plays a role in regulating *grk* mRNA localization. *sqd*, *hrb27C* and *otu* mutants also share a nurse cell chromosome organization defect, indicating that these proteins function together in other processes during oogenesis. mRNA biogenesis is a multi-step process, presumably involving many trans-acting factors. We report the identification of several proteins that interact with each other and regulate both *grk* RNA localization and nurse cell chromosome dynamics during oogenesis.

Materials and methods

Yeast two-hybrid screen

The two-hybrid screen was performed using the Matchmaker LexA System (Clontech). The LexA-SqdA bait was constructed by PCR amplification of a fragment from pSC1.3 (Kelley, 1993). The 5' primer CCC CCC ATG GAA GCC GGA GAA CCA GAT G was used to add an *Nco*I site 5' to the third exon, and the 3' primer was T7. The resulting PCR product was cut using *Nco*I and *Xho*1 to generate a 566 bp fragment that was cloned into the pLexA vector (Clontech) to create a N-terminal fusion of LexA with amino acids 214-321of SqdA. The ovarian cDNA prey library, ovo1b, was provided by J. Großhans (Großhans et al., 1999) and 9.7×10^6 colonies were screened. 183 positive colonies were identified and four of these were Hrb27C.

Generation of Sqd antibodies, immunoprecipitations and western analysis

The full-length SqdA-GST fusion described in Norvell et al. (Norvell et al., 1999) was purified and used for mouse monoclonal antibody production. Immunoprecipitations were performed according to Van Buskirk et al. (Van Buskirk et al., 2000) with the following modifications: a complete mini protease inhibitor cocktail tablet (Roche) was used in lieu of other protease inhibitors in the lysis buffer, 1U/µl RNAse inhibitor (Roche) was added to the lysis buffer, lysates were not pre-cleared with pre-immune serum coated beads, and lysates were rotated with the antibody coated beads for 60 minutes at 4°C. The following antibodies were used: monoclonal anti-Sqd serum (8F3; 3:10 dilution), monoclonal anti-SpnF serum (10D8; 3:10 dilution) (U. Abdu, unpublished), polyclonal anti-Otu (guinea pig against amino acids 1-338; 1:10 dilution) (Glenn and Searles, 2001) or polyclonal anti-Odd skipped (1:10 dilution) (Kosman et al., 1998). For RNAse treated samples, 1 µg/µl RNAse A was added to the lysis buffer instead of RNAse inhibitor. NuPAGE Bis-Tris pre-cast gels (4-12%; Invitrogen) were used and the samples were transferred to nitrocellulose (Amersham) using the Xcell II blotting apparatus with standard protocol (Invitrogen), blocked in TBST (Tris-buffered saline + 0.1% Tween-20) + 5% milk + 1% BSA, incubated in anti-Hrp48 (Siebel et al., 1994) at 1:20,000 (or anti-Sqd at 1:100), washed and incubated in HRP-conjugated anti-rabbit antibody (Vector) at 1:7500 or HRP-conjugated anti-mouse antibody (Jackson ImmunoResearch) at 1:10,000. After washing, the bands were visualized by the ECL-Plus chemiluminescent system (Amersham). Germarial western analysis was performed according to Van Buskirk et al. (Van Buskirk et al., 2002) with 20 germaria in 50 µl of loading buffer (5 M Urea, 0.125 M Tris [pH 6.8], 4% SDS, 10% β-mercaptoethanol, 20% glycerol and 0.1% Bromophenol Blue). Samples were loaded on a 7% NuPAGE Tris-Acetate pre-cast gel (Invitrogen), transferred, blocked, probed and detected as described above using guinea pig anti-Otu (1:500 in TBST + 5% milk) or monoclonal anti-Tubulin (1:250 in TBST + 5% milk; Sigma T-9026). HRP-anti guinea pig or HRP antimouse (Jackson ImmunoResearch) was used at 1:2000 in TBST.

Fly stocks

The following *hrb27C* alleles were generously provided by Don Rio and the Bloomington Stock Center: *hrb27C* [10280, *rF680*, *k16303*,

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k10413, 02647, k02814]. The following alleles are lethal P-element insertions at various distances from the coding region: $hrb27C^{10280}$ (1.5 kb), hrb27C^{rF680} (1.8 kb), hrb27C^{k16303} (2.2 kb), hrb27C^{k10413} (2.2 kb) and *hrb27C*⁰²⁶⁴⁷ (3.3 kb) (Hammond et al., 1997). There are no molecular data on hrb27Ck02814; hrb27C377 is an EMS allele produced by Mary Lilly. hrb27C FRT alleles (FRT40A-377, -rF680, -02647, -10280, -k02814) alleles were generously provided by Mary Lilly. The FLP-DFS (yeast flipase recombination target-site specific recombinase-dominant female sterile) system described by Chou and Perrimon (Chou and Perrimon, 1992; Chou and Perrimon, 1996) was used to generate germline clones of hrb27C. Progeny from yw hsFLP; ovoD FRT40A/CyO × FRT40A-Hrb27C^X (x – one of the alleles listed above) were heat shocked at 37°C for 2 hours a day for 3 days during the second and third larval instar. The sqd^{1} allele is a P-element insertion that specifically disrupts germline expression during midoogenesis (Kelley, 1993; Matunis et al., 1994). The isoform specific transgenes of sqd in a sqd^1 mutant background were described in Norvell et al. (Norvell et al., 1999). The otu alleles and Df(1)RA2/FM7, which uncovers otu, were obtained from the Bloomington Stock Center. hfp9 (Van Buskirk and Schüpbach, 2002) was the allele used for RT-PCR and western analysis. Otu104 flies that carry a transgene expressing only the 104 kDa isoform of Otu under the control of the otu promoter (Sass et al., 1995) were provided by Lillie Searles.

In situ hybridization

Ovaries were dissected in PBS and fixed for 20 minutes in 4% paraformaldehyde in PBS with Heptane and DMSO. Subsequent steps were performed as previously described (Tautz and Pfeifle, 1989) using a grk RNA probe.

Immunohistochemistry

Ovaries were fixed for 20 minutes in 4% paraformaldehyde in PBS plus Heptane. After several rinses in PBST (PBS + 0.3% Triton), ovaries were blocked for 1 hour in 1% BSA + 1% Triton. After a 1 hour incubation in a 1:10 dilution of monoclonal anti-Grk sera (ID12) (Queenan et al., 1999) in PBST, the ovaries were washed in PBST overnight at 4°C. AlexaFluor 568-conjugated anti-mouse secondary (Molecular Probes) was used at 1:1000 in PBST. During secondary incubation, DNA was stained with 1:10,000 Hoechst (Molecular Probes). For DNA stain alone, ovaries were dissected and fixed as described above, incubated in 1:10,000 Hoechst for 1 hour, washed and mounted. Only stage 6 and older egg chambers were counted to assay the nurse cell phenotype.

UV cross-linking analysis

Fresh ovarian lysate was prepared according to Norvell et al. (Norvell et al., 1999) with the addition of a complete mini protease inhibitor tablet (Roche) to the lysis buffer. Radiolabeled RNA probes were made according to Norvell et al. (Norvell et al., 1999) using 1 µg of linearized (HindIII for grk and osk, XbaI for nos) DNA template. The templates for transcription include the 158 bp HincII-HindIII fragment from grk genomic DNA cloned into pBS, the 126 bp EcoRI-DraI fragment from osk genomic DNA cloned into pBS, and the nos+6 construct (Gavis et al., 1996). Unlabelled competitor RNAs were synthesized using Ampliscribe in vitro transcription system (Epicenter) and the final product was purified by phenol extraction and ethanol precipitation. For the binding reaction, lysate equivalent to approximately three ovaries was incubated with 1.5 μ l of 10× binding buffer [500 mM Tris-HCl (pH 8.3), 750 mM KCL, 30 mM MgCl₂], probe $(5 \times 10^5$ to 1×10^6 cpm), and water to a final volume of 15 µl. The reactions were incubated for 15 minutes on ice, crosslinked on ice at 999 mJ in a Stratalinker UV crosslinker. The probe was digested for 15 minutes at 37°C with 7 units of RNAse ONE (Promega) and 1 unit of RNAse H (Roche). The immunoprecipitations were performed after RNAse digestion, by incubation of the entire reaction with antibody-coated [anti-Hrp48 or anti-CycE (Santa Cruz#481)] protein A/G beads for 1 hour at 4°C. For the competition experiments, 200-fold excess competitor RNA was incubated with the binding reaction for 10 minutes on ice prior to addition of the probe. The concentration of competitor was determined by UV spectroscopy. After addition of loading buffer, the samples were boiled for 5 minutes, resolved on a 10% Tris-HCl Ready Gel (BioRad) and visualized by autoradiography.

Results

Hrb27C interacts with Sqd

Sqd has been shown to regulate Grk expression at the level of RNA localization and translational regulation (Kelley et al., 1993; Norvell et al., 1999). In an effort to understand the mechanism by which Sqd regulates Grk expression, a yeast two-hybrid screen with Sqd was initiated to identify interacting proteins. Using a bait containing the C-terminal region of SqdA extending from the M9-like domain to the end (Fig. 1A), we detected an interaction with four independent clones of Hrb27C. This interaction is specific (Fig. 1B) and has been confirmed by co-immunoprecipitation. Surprisingly, the association of Hrb27C and Sqd is RNA dependent; it can be disrupted by the addition of RNAse to the lysate prior to immunoprecipitation (Fig. 1C). The in vivo RNA dependence of this interaction is unexpected because the Sqd bait and at least one of the Hrb27C clones lack part of the RRMs (Fig. 1A) and are presumably not able to bind to RNA, yet the proteins are able to interact in the yeast two hybrid assay (see Discussion). Hrb27C is an hnRNP that maps to 27C and was also previously referred to as Hrp48 (Matunis et al., 1992a; Matunis et al., 1992b). Hrb27C contains two RNA recognition motifs (RRMs) (Matunis et al., 1992a) (Fig. 1A) and has been previously studied for its role in the regulation of the tissuespecific inhibition of splicing of the P-element third intron (IVS3) (Siebel et al., 1994; Hammond et al., 1997). As Sqd affects grk RNA localization and translational control, our findings raise the possibility that the splicing of grk is directly linked to localization, or, the model we favor, that Hrb27C has functions in oogenesis other than splicing.

hrb27C mutants affect DV axis formation

To assess the effects of hrb27C mutations during oogenesis, we analyzed seven alleles of hrb27C. As hrb27C is an essential gene (Hammond et al., 1997), we made germline clones using the FLP-DFS system (Chou and Perrimon, 1992; Chou and Perrimon, 1996). Eggs laid by females that have Hrb27C disrupted in their germline display a variety of phenotypes, ranging from wild-type morphology in the weaker alleles to completely dorsalized eggs in the stronger alleles (Fig. 2A-D). The different classes of abnormal eggshell phenotypes are consistent with dorsalization. For two of the stronger alleles, hrb27C^{k02814} and hrb27C^{rF680}, the observed DV defects range from being slightly dorsalized, with an expansion of the operculum (Fig. 2B), to a crown of appendage material (Fig. 2D), similar to eggs laid by strong sqd mutants (sqd^1) (Kelley, 1993). We also observed intermediate phenotypes with a broad/fused appendage that spans at least the width of the eggshell occupied by wild-type appendages (Fig. 2C). As observed in sqd^1 mutants, most of the eggs were short, which is probably because of a failure to transfer all the nurse cell cytoplasmic contents to the oocyte prior to egg deposition.

Fig. 1. Sqd and Hrb27C interact. (A) A schematic of the SqdA protein [321 amino acids (aa)] containing two RNA-binding domains (RRMs) (striped boxes), an M9-like nuclear transport motif (black box) and a glycine rich C-terminal region (hatched box). The underlined region represents the region used as bait in the yeast twohybrid screen. The Hrb27C protein (422 aa) also contains two RNAbinding motifs (striped boxes). We isolated four different clones of Hrb27C: two contain all but the first 11 amino acids, one contains all but the first 28 amino acids and one is truncated to exclude the first 109 amino acids (underlined). (B) The Hrb27C-activation domain (AD) fusion interacts with the LexA-SqdA fusion but not with LexA alone or a LexA-p53 fusion. The AD alone does not interact with any LexA fusion proteins. (C) Western blot probed for Hrb27C and Sqd after immunoprecipitation (IP) from ovarian lysate with either Sqd or SpnF antibody in the absence (-) or presence (+) of RNAse A. Hrb27C co-purifies in an RNA-dependent manner with Sqd but not SpnF. WCL, whole cell lysate (one-tenth volume).

To determine whether the observed dorsalized eggshell phenotypes of hrb27C are a result of defects in grk expression, we analyzed grk mRNA localization by in situ hybridization in ovaries from females whose germline was mutant for hrb27C. In wild-type stage 9 egg chambers, grk mRNA is localized to the region overlying the oocyte nucleus. Grk protein is also restricted to this same region and dorsal cell fates are induced by a local activation of EGFR in the overlying follicle cells (Neuman-Silberberg and Schüpbach, 1993; Neuman-Silberberg and Schüpbach, 1994) (Fig. 2E,G). In hrb27C mutants, we observed defects in grk RNA localization that varied depending on the strength of the allele. In the weaker mutants, all of the RNA was properly localized at stage 9, while in two of the stronger mutants, $hrb27C^{rF680}$ and $hrb27C^{377}$, 26% (n=23) and 12% (n=67), respectively, of stage 9 egg chambers showed grk mRNA mislocalized in an anterior ring (Fig. 2F). Thus, it seems that Hrb27C is involved in the proper localization of grk mRNA to the dorsal anterior region of the oocyte.

As Sqd plays a role in both RNA localization and translation

1952 Development 131 (9)

Fig. 2. hrb27C mutant germline clones display a dorsalized phenotype because of defects in Grk expression. (A) Wild-type egg with two dorsal appendages that mark the dorsal anterior surface. (B-D) hrb27C mutants lay a range of dorsalized eggs. For the two strongest alleles $hrb27C^{k02814}$ and $hrb27C^{rF680}$, the appendage defects observed range from being (B) widely spaced, with an expansion of the operculum in 28-62% of the eggs, to (D) a crown of appendage material that surrounds the anterior circumference of the egg in 5-6% of the eggs. More intermediate phenotypes were also observed (C) with a broad appendage that spans at least the width of the normal appendages in 31-60% of the eggs $(n=86 \text{ for } hrb27C^{k02814} \text{ and } n=262 \text{ for } hrb27C^{rF680}).$ (E,F) grk in situ hybridizations of stage 9 egg chambers. In wild-type (E) grk is tightly localized to the dorsal anterior region of the oocyte, but is detected in a ring around the anterior circumference of a fraction of the egg chambers in hrb27C mutants (F). (G-I) Grk antibody staining of stage 9 egg chambers from hrb27C mutants reveals that Grk protein has a variable distribution that includes (G) tight localization to the dorsal anterior region as in wild-type, (H) diffuse localization throughout the oocyte with an enrichment in the dorsal anterior region, and (I) an anterior ring around the oocyte.

regulation of grk RNA (Norvell et al., 1999), we wanted to know if the same was true for Hrb27C. To determine if the mislocalized RNA in hrb27C mutants is translated, we analyzed Grk protein expression by whole-mount antibody staining. We did observe egg chambers where Grk was mislocalized throughout the oocyte cytoplasm and in a ring around the entire anterior of the oocyte (Fig. 2I), indicating that at least in some cases, mislocalized grk RNA is translated in the absence of Hrb27C. In many egg chambers, Grk was diffuse throughout the oocyte with an enrichment in the dorsal anterior region, but we also observed some egg chambers where Grk protein was localized normally (Fig. 2G,H). However, the occasional observation of Grk protein on the ventral side of the egg chamber clearly indicates a lack of translational repression in hrb27C mutants. Presumably, the egg chambers in which Grk is mislocalized in a ring would give rise to the completely dorsalized eggs laid by hrb27C mutant females, as this is the cause of the dorsalized eggs laid by females mutant for sqd.

To test whether Sqd and Hrb27C cooperate during oogenesis, we tested genetic interactions between the two genes. For this experiment, we used the few viable transheterozygous combinations of hrb27C alleles that produce eggs. These mutant combinations produce mildly abnormal eggshell phenotypes, which are less severe than those produced by germline clones. The hypomorphic allele sqd^{k12} also shows mild phenotypes (Kelley, 1993). sqd^{1}/sqd^{k12} transheterozygotes produce a range of mutant eggshell phenotypes, but the majority of these eggs are wild type. Mutations in *hrb27C* can enhance the eggshell phenotype of sqd^{1}/sqd^{k12} ; the eggs laid by females doubly mutant for hrb27C and sqd are much more dorsalized, 18-37% of the eggs have a crown of dorsal appendage material (Table 1). This nonadditive enhancement of the dorsalized phenotype is consistent with Sqd and Hrb27C cooperating in the regulation of Grk expression.

Hrb27C binds to grk RNA

The genetic and physical interactions of Sqd and Hrb27C suggest that these two proteins function together to regulate grk mRNA localization. As Sqd can bind to grk RNA (Norvell et al., 1999) and Hrb27C interacts with Sqd, we wanted to determine whether Hrb27C is able to bind to grk RNA. By performing UV crosslinking analysis in ovarian lysate using a small proportion of the grk 3'UTR (Fig. 3A) as a probe, we observed an interacting protein with a molecular weight (approximately 48 kDa) that is consistent with that of Hrb27C. We confirmed the identity of this protein by specifically immunoprecipitating it with Hrb27C antibodies and not with control antibodies (Fig. 3B). As a positive control, we performed the same experiments with the AB region of the osk 3'UTR (Fig. 3A) (Kim-Ha et al., 1995) which has previously been shown by Gunkel et al. (Gunkel et al., 1998) to bind p50, recently identified as Hrb27C (T. Yano, S. Lopez de Ouinto, A. Shevenchenko, A. Shevenchenko, T. Matsui and A. Ephrussi, personal communication). Furthermore, Hrb27C

 Table 1. hrb27C enhances the dorsalized phenotype of sqd

 mutants

	% Eggshell phenotype				
Genotype	Wild type	Fused	Wide space	Crown	п
sqd ¹ /sqd ^{k12}	54.7	18.3	22.4	4.6	856
hrb27C ¹⁰²⁸⁰ /hrb27C ¹⁰⁴¹³	97.7	1.1	1.3	0.0	946
$hrb27C^{10280}/hrb27C^{10413}; sqd^{l}/sqd^{k12}$	1.4	4.4	76.7	17.6	734
hrb27C ¹⁰²⁸⁰ /hrb27C ¹⁶²⁰³	97.6	0.0	2.4	0.0	742
$hrb27C^{10280}/hrb27C^{16203}; sqd^{1}/sqd^{k12}$	0.0	10.0	71.4	18.6	140
hrb27C ³⁷⁷ /hrb27C ⁰²⁶⁴⁷	1.1	0.8	98.1	0.0	795
hrb27C ³⁷⁷ /hrb27C ⁰²⁶⁴⁷ ; sqd ¹ /sqd ^{k12}	0.0	3.7	59.3	37.0	246

The percentage of dorsalized eggshell phenotypes observed from females transheterozygous for *sqd*, transheterozygous for *hrb27C* or transheterozygous for both.

Research article

Fig. 3. Hrb27C can bind to the 3'UTR of *grk*. (A) Schematics of the entire 3'UTR of *grk*, *osk* and *nos* with the regions used as probes or competitors underlined. (B) UV crosslinking analysis of ovarian proteins that can interact with *grk*, *osk* or *nos*. The banding profiles reveal that the same proteins bind to *grk* and *osk* RNAs, but these proteins do not bind to *nos* RNA. The identity of the protein that binds to *grk* and *osk* is Hrb27C because it can be immunoprecipitated with Hrb27C antibodies, but not with CycE antibodies. (C) UV crosslinking analysis of *osk* (labeled probe) in the presence of *grk*, *osk* (positive control) or *nos* (negative control) as unlabelled competitor. A 200-fold excess *grk* or *osk* is able to compete binding of Hrb27C (arrow) from *osk*, while excess *nos* is not.

does not interact with the 3'UTR of nanos (nos) and no proteins are precipitated by the Hrb27C antibody after crosslinking with nos probe (Fig. 3B). Purified GST-Hrb27C can be crosslinked to the same region of the grk 3'UTR, while other GST fusions are not able to bind to grk RNA (data not shown). Finally, to demonstrate the specificity of the Hrb27C interaction with grk RNA and to confirm that the same protein binds to both grk and osk UTRs, we performed UV crosslinking in the presence of cold competitor RNAs. For these experiments, the 3'UTR regions of grk, osk or nos that were previously used as probes were added as unlabeled competitors prior to addition of the labeled osk 3'UTR probe. Hrb27C is completely competed away from osk RNA by addition of 200-fold excess grk 3'UTR but not by the nos 3'UTR (Fig. 3C). The same results were obtained using grk as the probe and osk as the competitor (data not shown). Together, these data indicate that Hrb27C can specifically bind to grk RNA and suggest that a complex containing Sqd and Hrb27C binds to grk RNA to promote RNA localization and translational regulation of Grk.

Nurse cell chromosomes of *sqd* and *hrb27C* mutants fail to disperse

We have also detected a phenotype earlier in oogenesis that is shared by both *sqd* and *hrb27C* mutants, raising the possibility that the two proteins participate in multiple processes during oogenesis. In wild-type oogenesis, the nurse cell chromosomes undergo several cycles of endoreplication that result in chromatids that are in tight association, otherwise known as polytene. Prior to their dispersal, the chromosomes take on a characteristic blob-like appearance and by stage 6, they are completely dispersed (King, 1970; Dej and Spradling, 1999) (Fig. 4A,B). In both sqd and hrb27C mutants, the nurse cell chromosomes fail to completely disperse and remain in the blob-like polytene conformation (Fig. 4C,D). Strong alleles of sqd (sqd¹ and germline clones of a null allele, sqd^{ix77}) have nurse cell chromosomes that are arrested in the blob-like conformation, whereas the nurse cell chromosomes of the hypomorphic allele, sqd^{k12} , disperse properly. Germline clones of all lethal hrb27C alleles and some of the viable transheterozygous allele combinations display some degree of polytene chromosome morphology (Fig. 4D). The sqd^{1}/sqd^{K12} allele combination and other viable transheterozygous hrb27C allele combinations (hrb27C¹⁰²⁸⁰/hrb27C^{k10413}, hrb27C¹⁰²⁸⁰/ hrb27Ck16303) have nurse cell chromosomes that disperse properly. Interestingly, when sqd^{1}/sqd^{K12} is combined with the transheterozygous *hrb27C* allele combinations that disperse normally, the nurse cell chromosomes of the double mutants become arrested in the blob-like conformation and fail to disperse.

To determine the role of the Sqd isoforms in the dispersion of the nurse cell chromosomes, we assayed the ability of genomic cDNA transgenes that express one particular Sqd isoform (Norvell et al., 1999) to rescue the nurse cell phenotype of sqd^1 mutants. Expressing either two copies of SqdA, SqdB or SqdS in a *sqd¹* background partially rescues the nuclear morphology from only 3% wild type to 14%, 54% or 61% wild type, respectively (Fig. 4E). These data suggest that each isoform is able to participate in regulating nurse cell chromosome morphology. Whereas SqdS and SqdA play the major roles in establishing the DV axis (Norvell et al., 1999), SqdS and SqdB, the two isoforms found in nuclei, have the largest effects in regulating nurse cell chromosome morphology. Consistent with the distinct yet overlapping function of the isoforms described by Norvell et al. (Norvell et al., 1999), expressing one copy of each of two different isoforms is able to rescue the nurse cell nuclear morphology better than expressing two copies of the same isoform. SqdA/SqdB rescues 96%, SqdB/SqdS rescues 96% and SqdS/SqdA rescues 92% of the nurse cell nuclear morphology to wild type (Fig. 4E).

The *ovarian tumor* gene can rescue the nurse cell nuclear morphology of *sqd* mutants

Nurse cell chromosomes that fail to disperse are also observed in certain alleles of ovarian tumor (otu) (King et al., 1981; King and Storto, 1988; Heino, 1989; Mal'ceva and Zhimulev, 1993; Heino, 1994; Mal'ceva et al., 1995). otu produces two protein isoforms, Otu98 and Otu104, by alternative splicing of a 126 bp exon. Genetic and molecular analyses reveal distinct requirements for each isoform during oogenesis (Storto and King, 1988; Steinhauer and Kalfayan, 1992; Sass et al., 1995; Tirronen et al., 1995). In particular, a mutant that specifically disrupts the Otu104 product has persistent polytene nurse cell chromosomes, suggesting that the 98 kDa Otu isoform is not capable of mediating wild-type chromosome dispersion (Steinhauer and Kalfayan, 1992). This phenotype was also described for mutants in half pint (hfp; pUf68 – FlyBase). Hfp encodes a polyU-binding factor and plays an important role in the alternative splicing of otu. Fig. 4. sqd and hrb27C mutants have nurse cell chromosomes that fail to disperse and this phenotype is rescued by isoform-specific transgenes of Sqd and Otu. (A) Wild-type ovariole stained with Hoechst to show the polytene (arrowhead) nurse cell chromosomes that have a blob-like appearance (double arrowhead) at stage 5 and are fully dispersed (arrow) by stage 6. (B) Wild-type stage 8 egg chamber stained with Hoechst with properly dispersed nurse cell chromosomes. (C,D) sqd (C) and hrb27C (D) mutant egg chambers at stage 8 stained with Hoechst reveal that the nurse cell chromosomes fail to disperse throughout oogenesis. (E) The percentage of polytene nurse cell nuclei in stage 6 and later egg chambers from sqd^1 mutants and from sqd¹ mutants carrying transgenes expressing the specified Sqd isoforms (A, B or S) (n>400 for each genotype). (F) The percentage of polytene nurse cell nuclei in stage 6 and later egg chambers from sqd^1 mutants and from sqd^{1} mutants carrying one (n=339) or two copies (n=818) of the Otu104 transgene. (G) RT-PCR analysis of otu transcripts in previtellarial egg chambers. Although the larger otu transcript is absent from hfp mutants, it is present in sqd mutants. (H) Western analysis using an Otu antibody of previtellarial egg chambers from wild type (WT), sqd¹ and hfp mutants. sqd mutants show a reduction in the amount of the 104 kDa Otu isoform. Western analysis of the samples using a Tubulin antibody reveals equal loading of the sqd and wild-type samples.

Е F chromos Gell nurse 40 % polytene 20 B/A sad1 2x A S/A: S/B: 1x Otu104; sqd1 2x Otu104; sqd1 sqd1 sad1 sqd1 sad1 sad1 sqd1 sadi н WT sqd1 hfp9 wт hfp9 sad 104 otu 98

Research article

tub

In *hfp* mutants, there is a dramatic decrease in the levels of the Otu104-encoding transcript as seen by RT-PCR analysis of germarial RNA, and Otu104 is not detectable on a western blot. The nurse cell phenotype of hfp mutants can be rescued by a transgene that expresses the 104 kDa Otu isoform under the control of the otu promoter (Van Buskirk and Schüpbach, 2002). To determine whether defects in Otu104 cause the polytene phenotype of sqd mutants, we assayed the ability of the Otu104 transgene to rescue this defect in sqd mutants. In sqd^1 mutants, 98% of the egg chambers (stage 6 and older) have nurse cells chromosomes that are not dispersed. When one copy of Otu104 is expressed in a sqd^{1} background, only 17% of the egg chambers have persistent polytene nurse cell chromosomes, and expressing two copies of Otu104 leaves only 8% of the egg chambers with polytene nurse cell chromosomes (Fig. 4F).

% polytene nurse cell chromosomes

G

As Otu104 can rescue the nurse cell phenotype of both hfp and sqd, we asked whether sqd affects the alternative splicing of otu, as is the case for hfp mutants. RT-PCR analysis of RNA isolated from sqd^{1} germaria reveals that *otu* is properly spliced in sqd mutants (Fig. 4G). To determine if Otu104 protein accumulates properly in sqd^1 mutants, we performed a western blot on extracts prepared from germaria of wild-type and sqd^1 mutant females. Western analysis reveals that although both Otu isoforms are present in sqd^1 ; the level of the 104 kDa isoform appears reduced in sqd^1 mutants compared with wild type (Fig. 4H). Though the decrease in the levels of Otu104 observed in sqd^1 mutants is not as striking as that seen in hfpmutants, the decrease is consistent over many experiments. As a single copy of Otu104 can rescue the nurse cell phenotype of sqd mutants (Fig. 4F), and the polytene nurse cell phenotype is observed in females heterozygous for a deficiency that removes otu and even in females heterozygous for the Otu104specific mutants, otu^{11} or otu^{13} (data not shown), it seems that the level of Otu104 is crucial for proper nurse cell chromosome dispersion.

Given that *hrb27C* mutants share the polytene nurse cell chromosome phenotype of sqd mutants, we assayed the ability of two copies of Otu104 to rescue the phenotype of a viable transheterozygous combination of hrb27C ($hrb27C^{377/}$ $hrb27C^{02647}$). While the Otu104 rescue of the hrb27C phenotype was not as complete as that of sqd^1 mutants, we consistently observed a qualitatively improved (less blob-like) nuclear morphology. The Otu104-partial rescue of the nurse cell dispersion defect of hrb27C suggests that both Sqd and Hrb27C may act with or through Otu104 to regulate chromosome dispersal.

Otu interacts with Hrb27C and affects DV patterning

As *otu* mutants share a similar nurse cell nuclear morphology defect with sqd and hrb27C, we tested whether Otu physically interacts with these proteins. Although we have not been able to detect a physical interaction between Sqd and Otu, we do detect a physical interaction between Otu and Hrb27C by co-immunoprecipitation (Fig. 5A). This interaction is RNA independent as RNAse treatment of the lysate prior to immunoprecipitation does not disrupt it (data not shown). It is therefore possible that Otu could be part of the same complex with Sqd and Hrb27C through its interaction with Hrb27C. To evaluate a potential role for Otu in grk regulation, we analyzed the eggshell phenotype of eggs laid by otu^7 and otu^{11} mutant females. Indeed, although only a few mature eggs are produced, some of these eggs are clearly dorsalized with a crown of dorsal appendage material (Fig. 5B). otu mutant eggs show a range of dorsalization similar to the phenotypes of hrb27C mutants (see Fig. 2). We also assessed grk RNA localization in otu⁷ mutants by in situ hybridization and found that grk RNA is mislocalized in 34% (n=92) of the stage 9 egg chambers (Fig. 5C). grk RNA is also mislocalized in egg chambers from otu¹¹ females. Additional support for the role of Otu cooperating with Sqd in regulating Grk expression derives from the fact that eggs laid by females expressing two to four additional copies of Otu104 in a sqd mutant background have extra appendage material, but not a complete crown (data not shown). These results suggest that Otu could be in a complex with Hrb27C and Sqd that regulates grk RNA localization and potentially translational regulation.

Collectively, the data presented here suggest that Otu, Hrb27C and Sqd function in two different cell types in at least two processes during oogenesis. Interestingly, there seem to be differential requirements for Sqd and Otu depending on the cell type. In the nurse cells, Otu seems to be essential as expressing additional copies of Otu104 rescues the Otu104 reduction seen in *sqd* mutants (data not shown) and rescues the nurse cell chromosome morphology defect. In the oocyte, Sqd is essential for proper *grk* RNA localization. Here, extra copies of Otu104 only partially alleviate the DV defects of the *sqd*¹ mutation. Thus, it seems that specific interacting factors make differential contributions to the coordinated function of these proteins in various cell types.

Discussion

The important roles of nuclear RNA-binding proteins in the regulation of gene expression (for a review, see Dreyfuss et al., 2002) are becoming increasingly evident as more protein complexes and their RNA targets are identified. Some hnRNPs can play multiple roles in the processing of a single RNA target; for example, in vertebrates, hnRNP A2 has been

Fig. 5. Otu interacts with Hrb27C and plays a role in regulating *grk* localization. (A) Western blot probed for Hrb27C after immunoprecipitation (IP) of ovarian lysate using either Otu or Odd skipped (Odd) antibody. Only the Otu-IP shows a specific interaction. WCL, whole cell lysate (one-tenth volume). (B) A representative dorsalized egg laid by an otu^7 mutant. (C) *grk* in situ hybridization of a late stage 9 egg chamber from an otu^7 mutant. In wild type, *grk* is tightly localized to the dorsal anterior region of the oocyte (see Fig. 2E), but is mislocalized along the anterior circumference of the egg chamber in otu^7 and otu^{11} mutants.

implicated in the nuclear export, cytoplasmic localization and translational regulation of myelin basic protein (for a review, see Carson et al., 2001). In *Drosophila*, the hnRNP Sqd has been shown to regulate one of its target RNAs, *grk*, at the level of localization as well as translational regulation (Norvell et al., 1999). Identification of the various partners that cooperate with Sqd should allow us to understand how one particular hnRNP can play such diverse roles in various cellular environments.

Hrb27C and Sqd interact and regulate the localization of *grk* mRNA

We have identified a physical and genetic interaction between Hrb27C and Sqd. The two proteins were previously biochemically purified as part of an hnRNP complex from Drosophila cells (Matunis et al., 1992a; Matunis et al., 1992b), but it was not known if they interact directly nor had their RNA targets been isolated. We originally identified the interaction in a two-hybrid screen and we have confirmed it biochemically by co-immunoprecipitation. In vivo, this interaction requires the presence of RNA; this result was unexpected because the yeast two-hybrid constructs that originally revealed the interaction did not contain the RRMs and presumably cannot bind RNA. The interaction may require the full-length proteins to be in unique conformations that are achieved only when they are bound to RNA. The truncated proteins used in the yeast twohybrid screen may be folded in such a manner that the proteinprotein interaction domains are exposed even in the absence of RNA binding, making it possible for the interaction to occur in yeast.

We also observed a striking genetic interaction between Sqd and Hrb27C; weak *hrb27C* mutants can strongly enhance the DV defects of weak *sqd* mutants. This suggests that the physical interaction has in vivo significance. The phenotypes of sqd and hrb27C place both proteins in a pathway required for proper grk localization and suggest a previously undetected role for Hrb27C. hrb27C mutants lay variably dorsalized eggs, and in situ hybridization analysis reveals that grk RNA is mislocalized. As the mislocalized RNA in hrb27C mutants is translated, causing a dorsalized egg phenotype, it is likely that Hrb27C also functions with Sqd in regulating Grk protein accumulation. A role in the regulation of RNA localization and translation is novel for Hrb27C/Hrp48; it has been previously described as functioning in the inhibition of IVS3 splicing of P-element encoded transposase (Siebel et al., 1994; Hammond et al., 1997). Hrb27C has nuclear functions and has been observed in the nucleus and cytoplasm of somatic and germline cells in embryos (Siebel et al., 1995). As Sqd is localized to the oocyte nucleus (Matunis et al., 1994) where it is thought to bind grk RNA (Norvell et al., 1999), we favor a model where Hrb27C and Sqd bind grk RNA together and are exported possibly as a complex into the cytoplasm.

Two unpublished studies have implicated Hrb27C in osk RNA localization (J. Huynh, T. Munro, K. Litière-Smith and D. St Johnston, personal communication) and translational regulation (T. Yano, S. Lopez de Quinto, A. Shevenchenko, A. Shevenchenko, T. Matsui and A. Ephrussi, personal communication). Translational repression of osk RNA until it is properly localized is essential to prevent disruptions in the posterior patterning of the embryo (Gavis and Lehmann, 1992; Gavis and Lehmann, 1994; Kim-Ha et al., 1995; Webster et al., 1997). An analogous mechanism of co-regulation of mRNA localization and translation appears to control Grk expression. Certain parallels between osk and grk regulation are very striking. Both RNAs are tightly localized and subject to complex translational regulation; they also share certain factors that mediate this regulation. In addition to Hrb27C, the translational repressor Bruno appears to be a part of the regulatory complexes that are required for the proper expression of both RNAs (Kim-Ha et al., 1995; Webster et al., 1997; Norvell et al., 1999; Filardo and Ephrussi, 2003). It will be interesting to determine if there are other shared partners that function in the regulation of both RNAs, as well as identify the factors that give each complex its localization specificity.

Otu plays a role in *grk* RNA localization and interacts with Hrb27C

Otu is also involved in the localization of grk mRNA and interacts physically with Hrb27C. Though a definitive role of Otu in regulating Grk expression has not been previously described, Van Buskirk et al. (Van Buskirk et al., 2002) found that four copies of Otu104 are able to rescue the grk RNA mislocalization defect of *hfp* mutants. Our analysis of the hypomorphic alleles, otu^7 and otu¹¹, reveals a requirement for Otu in localizing grk RNA for proper DV patterning. Alternative splicing of the otu transcript produces two protein isoforms: a 98 kDa isoform and a 104 kDa isoform that differ by the inclusion of a 126 bp alternatively spliced exon (6a) in the 104 kDa isoform (Steinhauer and Kalfayan, 1992). This alternatively spliced exon encodes a tudor domain, a sequence element present in proteins with putative RNA-binding abilities (Ponting, 1997). Interestingly, the otu¹¹ allele, which contains a missense mutation in exon 6a (Steinhauer and Kalfayan, 1992), shows the grk localization and dorsalization defect, strongly suggesting that the tudor domain of Otu plays an important function in *grk* localization. As *otu* mutants also show defects in *osk* localization (Tirronen et al., 1995), it appears that like several other factors, Otu is required for both *grk* and *osk* localization. Additionally, Otu has been isolated from cytoplasmic mRNP complexes (Glenn and Searles, 2001).

Hrb27C, Sqd and Otu function in nurse cell chromosome regulation

Surprisingly, we found an additional shared phenotype of hrb27C, sqd and otu mutants. During early oogenesis, the endoreplicated nurse cell chromosomes are polytene. As they begin to disperse, the chromosomes are visible as distinct masses of blob-like chromatin that completely disperse by stage 6 (King, 1970; Dej and Spradling, 1999). The formation of polytene nurse cell chromosomes is due to the endocycling that occurs during early oogenesis (Dej and Spradling, 1999) (for a review, see Edgar and Orr-Weaver, 2001). The mechanism of chromosome dispersal is hypothesized to involve the degradation of securin by the anaphase-promoting complex/cyclosome as a result of separase activity that cleaves cohesin (Kashevsky et al., 2002) (for a review, see Nasmyth et al., 2000). The significance of chromosome dispersion is not clear, but Dej and Spradling (Dej and Spradling, 1999) suggest that it could facilitate rapid ribosome synthesis. A defect in the dispersal of polytene chromosomes has been previously described for otu mutants (King et al., 1981; King and Storto, 1988), and we have found that both sqd and hrb27C mutants also have nurse cell chromosomes that fail to disperse. Weak double transheterozygous allele combinations of sqd and *hrb27C* produce the persistent polytene nurse cell chromosome phenotype that is not observed in either of the weak mutants alone. Thus, genetic and physical interactions between these gene products suggest that they function together in regulation of this process.

The failure to disperse nurse cell chromosomes has also been observed in mutants of hfp where the nurse cell nuclear morphology defect is due to improper splicing of Otu104 (Van Buskirk and Schüpbach, 2002). As was shown for hfp, expressing Otu104 in a sqd mutant background is able to rescue the polytene nurse cell chromosome phenotype. Although Hfp affects otu splicing, it is not clear how Sqd functions to mediate this phenotype, but evidence supports a role in Otu104 protein accumulation. The transcript that encodes Otu104 is clearly present in sqd mutants, as assayed by RT-PCR, but the level of Otu104 protein is decreased. Our results suggest that the level of Otu104 is crucial to maintaining proper nurse cell chromosome morphology as the polytene phenotype of sqd mutants can be rescued by expressing only one extra copy of Otu104 and the majority of egg chambers from females heterozygous for otu^{13} , otu^{11} , or a deficiency lacking otu, have nurse cell chromosomes that fail to disperse.

The reduced level of Otu104 in sqd mutants raises the possibility that Sqd could translationally regulate *otu* RNA and that the polytene nurse cell phenotype may be a result of the decreased level of Otu104. Alternatively, Sqd and Hrb27C could form a complex that recruits and stabilizes Otu104 protein, and this complex in turn functions directly or indirectly to regulate chromosome dispersion. An indirect role seems more likely, given that Otu has never been observed in the nucleus (Steinhauer and Kalfayan, 1992). As Otu104 can rescue the phenotype of sqd mutants and we can co-precipitate

Fig. 6. Model for the dual roles of a RNP complex containing Hrb27C, Sqd and Otu in oogenesis. In the oocyte nucleus, Sqd, Hrb27C and additional factors (rectangle and oval) form a complex that binds to *grk* mRNA prior to export. Once in the cytoplasm, Sqd and Hrb27C remain part of the complex, some factors dissociate and Otu, along with different accessory factors (rounded rectangle), associate to form a distinct complex to facilitate *grk* RNA localization, anchoring and translational regulation in the dorsal anterior region of the oocyte. In the nurse cells, a distinct complex also containing Hrb27C, Sqd and Otu in addition to other accessory factors (trapezoid) function in the cytoplasm to mediate the processing, localization, translational regulation or stabilization of an unidentified RNA target (X), which can then regulate nurse cell chromosome dispersal at the appropriate time in oogenesis.

Otu and Hrb27C, it seems plausible that Sqd and Hrb27C interact with Otu in the nurse cell cytoplasm to affect an RNA target that could then mediate chromosome dispersion.

Model for a RNP complex containing Sqd, Hrb27C, and Otu

Our genetic data support a model in which Sqd, Hrb27C, and Otu function together in a complex that affects at least two processes during oogenesis: DV patterning within the oocyte and mediation of nurse cell chromosome dispersion. Although our biochemical data are consistent with this model, it is also possible that Hrb27C and Sqd could form a complex that is distinct from a complex containing Hrb27C and Otu. However, the in vivo genetic interactions and mutant phenotypes reveal that all three proteins affect both processes and lead us to favor a model in which all three proteins function in one complex.

Our results allow us to expand upon the model proposed by Norvell et al. (Norvell et al., 1999) for the regulation of grkRNA expression (Fig. 6). Sqd and Hrb27C associate with grkRNA in the oocyte nucleus. Hrb27C and Sqd remain associated with grk in the cytoplasm where Otu and possibly other unidentified proteins associate with the complex as necessary to properly localize, anchor and translationally regulate grkRNA. A likely candidate to be recruited to the complex is Bruno, which interacts with Sqd (Norvell et al., 1999). Although we do not know the RNA target of Sqd, Hrb27C and Otu in the nurse cells, the proteins could form a complex composed of different accessory factors to regulate localization and/or translation of RNAs encoding proteins that affect chromosome morphology.

Although Hrb27C, Sqd and Otu function together to affect different processes in the oocyte and the nurse cells, they probably function to mediate the precise spatial and temporal regulation of a target RNA that is unique to each cell type where the presence of additional factors would provide specificity to each complex. The importance of the precise localization and translational regulation of *grk* is well defined, and we have now identified Hrb27C and Otu as additional proteins that facilitate these processes. The transition from polytene to dispersed chromosomes in the nurse cells is less well characterized, but most probably requires precise spatial and temporal regulation as well. A complex containing Sqd, Hrb27C and Otu could function to ensure that the target RNA is properly localized and functional in the nurse cells only at the proper stage of development to promote chromosome dispersal.

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