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PRINCIPAL INVESTIGATOR: Jennifer S. Goodrich

CONTRACTING ORGANIZATION: Princeton University
Princeton, NJ 08544

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Jennifer S. Goodrich

E-Mail: goodrich@molbio.princeton.edu

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ABSTRACT

Misregulation of Transforming Growth Factor alpha (TGFalpha) and increased Epidermal Growth Factor Receptor (EGRF) activity has been associated with an increased prognosis of breast cancer. During oogenesis in *Drosophila melanogaster*, local EGFR activation by the spatially restricted TGFalpha–like ligand, Gurken (Grk), is required for axis formation in the egg and future embryo. Squid, a heterogeneous nuclear ribo-nucleoprotein (hnRNP) functions in the localization and translational regulation of *grk* mRNA. The purpose of this project is to identify factors that function with Squid to produce spatially-restricted EGFR activation. I have identified an arginine methyltransferase (CG6554) that interacts with Squid, but mutations in this enzyme do not produce a phenotype. I have gathered evidence that Squid is methylated on arginine residues. This methylation could function to mediate Squid's different roles in regulating Grk expression. I generated several Squid transgenes in which the potential methylation sites have been mutated. Characterization of these transgenes should allow us to elucidate the function of Squid methylation and its role in the regulation of Grk expression. These factors, which interact with Squid to control Grk expression, could define potential causes of EGFR misregulation that result in human breast cancer, and they may provide possible targets for drug development.

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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, 1994). TGFalpha and EGFR are required for normal breast development, but deregulation of this signaling pathway can result in uncontrolled proliferation and transformation (Matsui, 1990). I am studying the mechanism of TGFalpha activation of EGFR in the model organism *Drosophila melanogaster* in order to understand how post-transcriptional 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 spatially-restricted activation of the EGFR during ovary development (Schüpbach, 1987; Neuman-Silberberg & Schüpbach, 1993; 1994). These factors could define potential causes of EGFR misregulation, at the level of ligand production, that result in human breast cancer. I am integrating genetic and biochemical methods to study the effects of a post-translational modification, methylation, on Squid (Sqd), a negative regulator of Grk (Kelly, 1993; Norvell et al. 1999).

BODY

The role of Squid (Sqd) methylation during oogenesis

In my first year, I determined that Sqd was methylated and that CG6554 was able to methylate Sqd, but null mutants in CG6554 are homozygous viable and lay wild type eggs, suggesting *gurken* (*grk*) RNA and protein are properly localized. It seemed likely that CG6554 was functioning redundantly with another arginine methyltransferase to methylate Sqd. This possibility was not surprising as there are six additional putative arginine methyltransferases in the *Drosophila* genome. CG5358 was a potential candidate because there was an available ovarian EST (Expressed Squence Tag--suggesting it was expressed in the ovary) and an available P-element insertion. I generated null mutants in CG5358 and these were also homozygous viable and showed no eggshell phenotype. To determine if CG6554 and CG5358 function redundantly to methylate Sqd, I 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. My 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, I have decided to take a different approach to investigating the function of Sqd methylation (see task 2). If Sqd methylation seems to be important for function, I will then generate tools to study the potential roles of other methyltransferases.

The function of the RGG motifs in the SqdA and SqdS protein isoforms

In order to study the SqdA and SqdS isoforms individually, I cloned HA-tagged genomic-cDNA transgenes and generated transgenic flies expressing each isoform. To determine if these transgenes functioned like wild-type, I 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 (Fig. 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 (Fig. 2). I also looked at the localization of these transgenes in emrbryos and the localization was the same (data not shown). Using the HASqd transgenes, I was able to show that the SqdA and SqdS isoforms interact with endogenous Sqd (Fig. 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, I 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, I 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 (Fig. 4); SqdA contains 5 RGG motifs that are shared with SqdS, but Sqd S has three unique RGG motifs. To disrupt the methylation sites, I 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. I 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) (Fig. 4).

Methylation has been shown to be involved in mediating subcellular localization, protein-protein interaction, and RNA-protein interaction (for review, see McBride and Silver, 2001). To determine if the RGG motifs (and thus methylation) function in regulating the localization of Sqd, I 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 (Fig. 5 compared to Fig. 2). In addition, I assayed the requirement of the RGG motifs for the Sqd-Sqd interaction as seen

in Fig. 3. Figure 6 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, I 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 (Fig. 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 (Fig. 7B).

Since the RGG motifs in Sqd did not seem to be required for any of the previously described roles for arginine methylation, I 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, I 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 (Fig. 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, I 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, I 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 the translational regulator Bruno in Gurken expression

The first two tasks as outlined above have encompassed all of my time and I have been unable to concentrate on this task.

KEY RESEARCH ACCOMPLISHMENTS

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

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

Task 2: To determine the function of the RGG motifs in the SqdA and SqdS protein isoforms

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 ransgenic 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

REPORTABLE OUTCOMES

Goodrich, J.S. and Schüpbach, T. (in prep). Arginine methylation is not essential for Squid function during Drosophila oogenesis.

CONCLUSIONS

This work has allowed us to study the individual functions of the Sqd isoforms in more detail. It has also 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.

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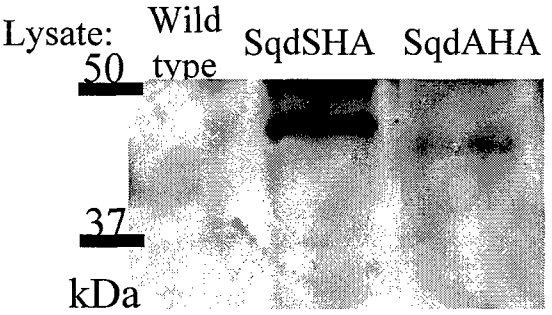

Figure 1. SqdS and SqdA are methylated in vivo.
Western blot probed for methylated arginines after immunoprecipitation with HA antibodies from ovarian lysate of wild type flies or flies expressing either SqdSHA or SqdAHA.The methylarginine antibody recognizes bands specific to SqdSHA and SqdAHA suggesting that both isoforms are methylated in ovarian extract. Wild type lysate is the negative control since there is no HA tag to immunoprecipitate.

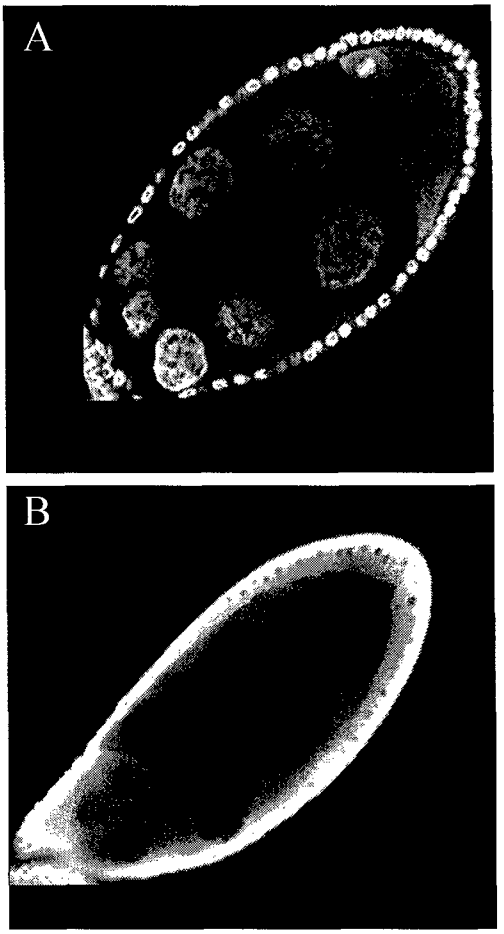

Figure 2. Localization of SqdS and SqdA.
HA antibody staining of stage 9 egg chambers from females expressing SqdSHA (A) or SqdAHA (B) reveals that SqdS is localized to the nuclei of the oocyte and nurse cells.(A). SqdA is localized to the cytoplasm (B).

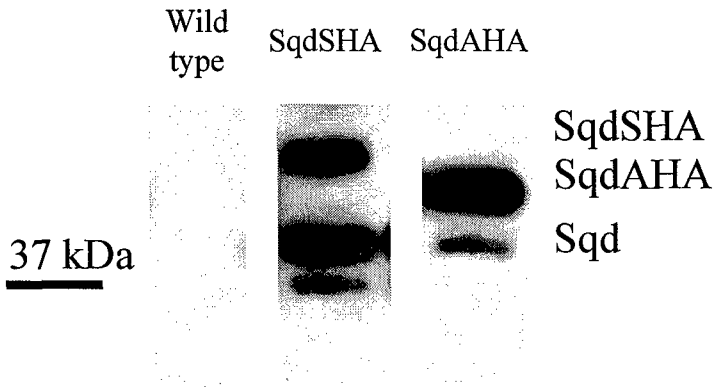

Figure 3. Sqd isoforms interact with each other.
Western blot probed for Sqd after immunoprecipitation with HA antibodies from ovarian lysate of wild type flies or flies expressing either SqdSHA or SqdAHA. Endogenous Sqd co-precipitates with both SqdSHA and SqdAHA. Wild type lysate is the negative control since there is no HA tag to immunoprecipitate.

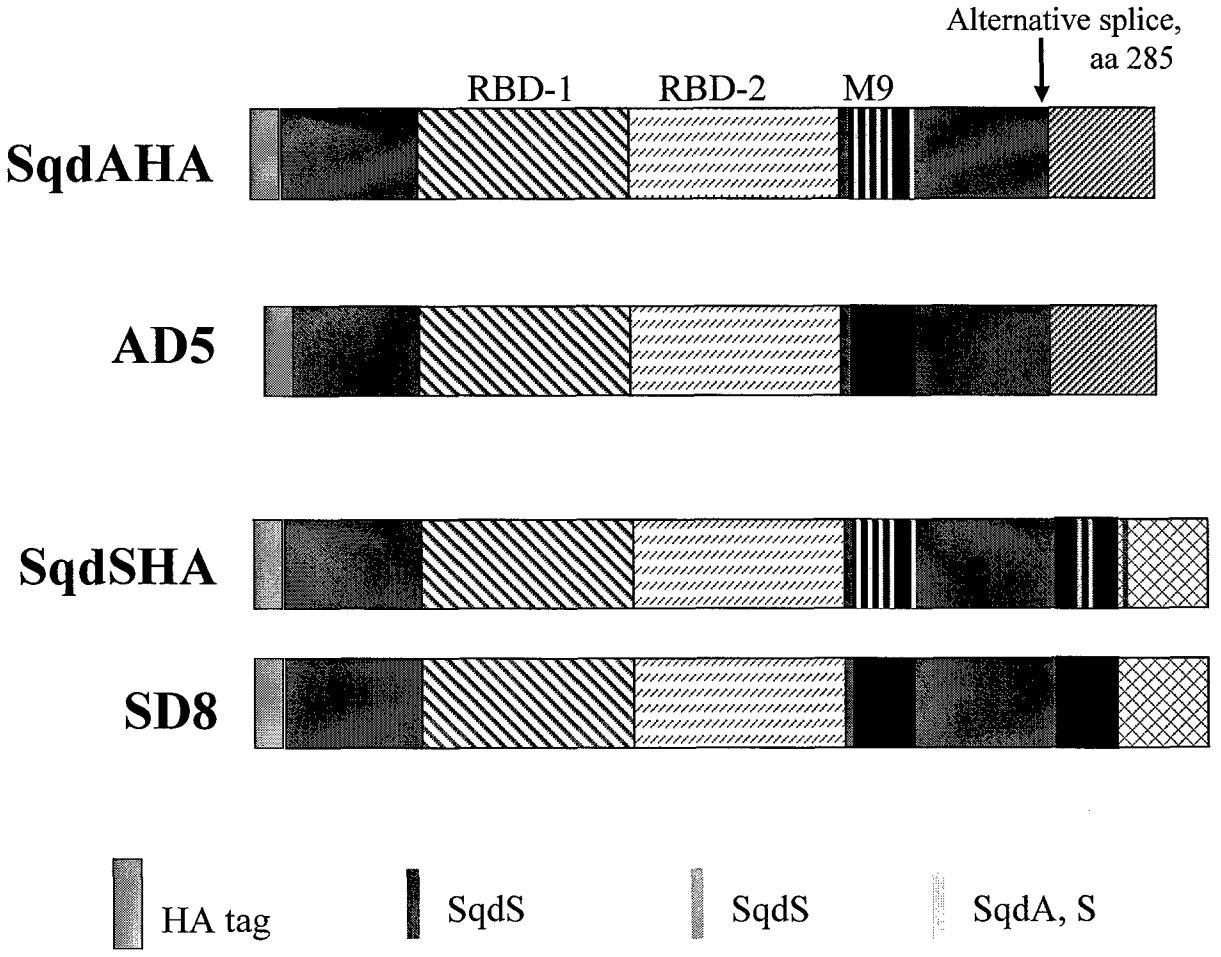

Figure 4. Schematics of wild type and RGG mutant Sqd transgenes.
The location of the HA tag is denoted by the green box. The striped and stippled boxes represent the RNA binding domains (RBD). The black boxes are the M9 nuclear transport motifs and the regions unique to each isoform appear after the 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.

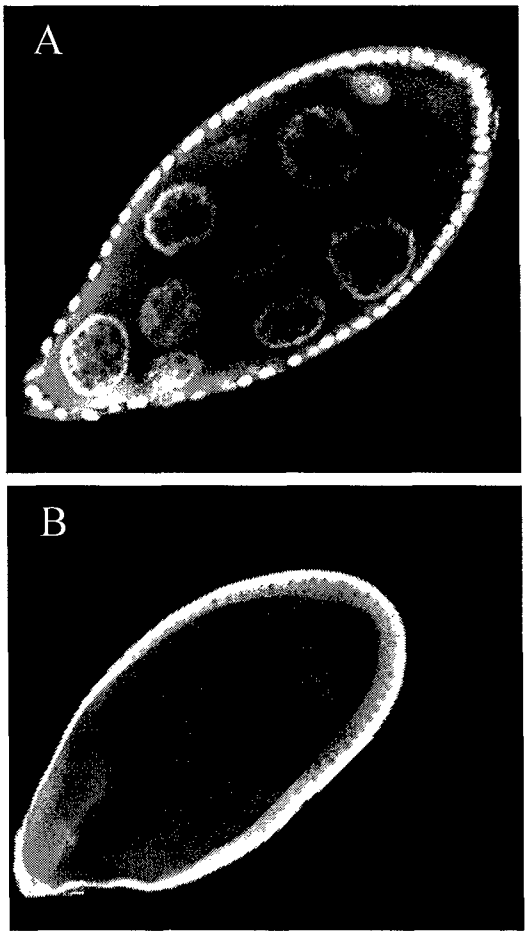

Figure 5. RGG motifs are not required for proper Sqd localization. HA antibody staining of stage 9 egg chambers from females expressing SD8 (A) or AD5 (B) reveals that even without methylated arginines, SqdS is still localized to the nuclei of the oocyte and nurse cells.(A). In the absence of arginine methylation, SqdA is still localized to the cytoplasm (B).

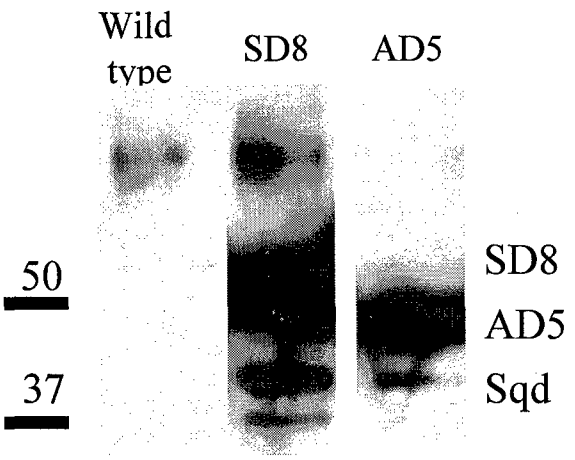

Figure 6. Sqd isoform interaction does not depend on RGG motifs. Western blot probed for Sqd after immunoprecipitation with HA antibodies from ovarian lysate of wild type flies or flies expressing either SD8 or AD5. Endogenous Sqd still co-precipitates even in the absence of the RGG motifs of SqdSHA and SqdAHA. Wild type lysate is the negative control as there is no HA tag to immunoprecipitate.

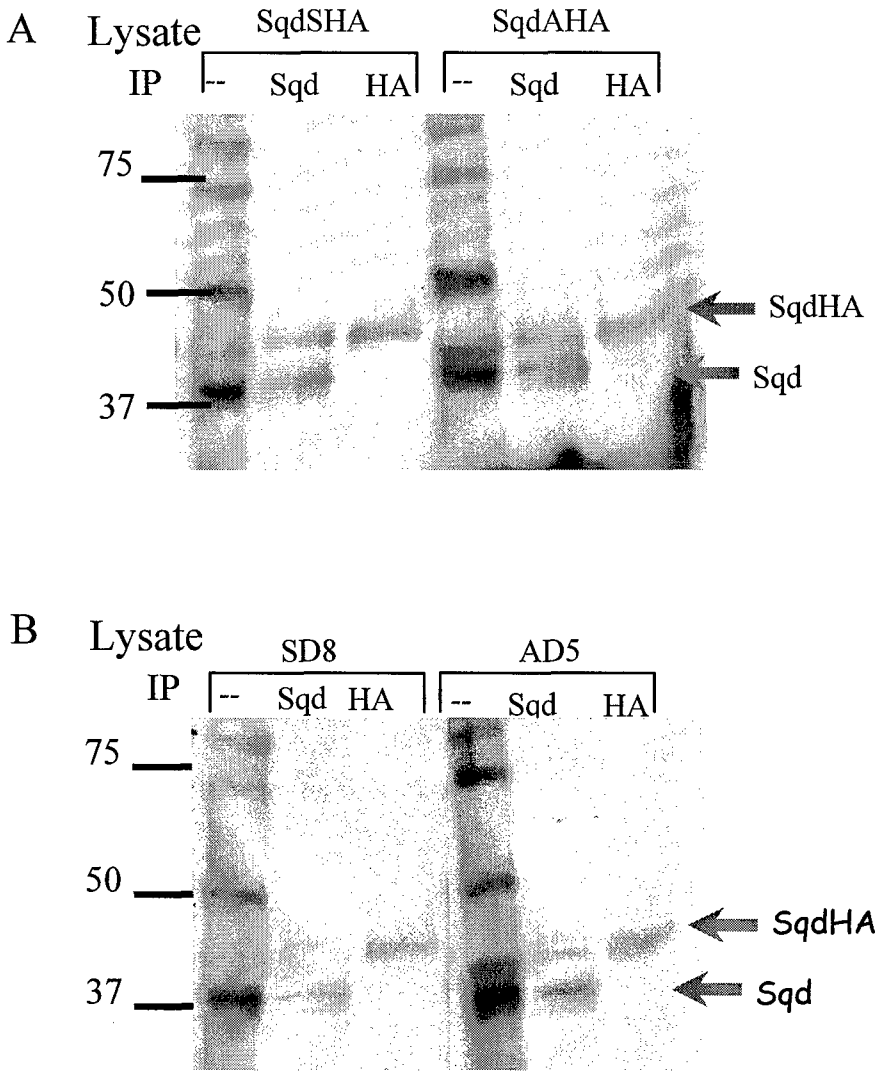

Figure 7. RGG motifs are not required for SqdS or SqdA binding to *grk* RNA.

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 immunoprecipitation (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.

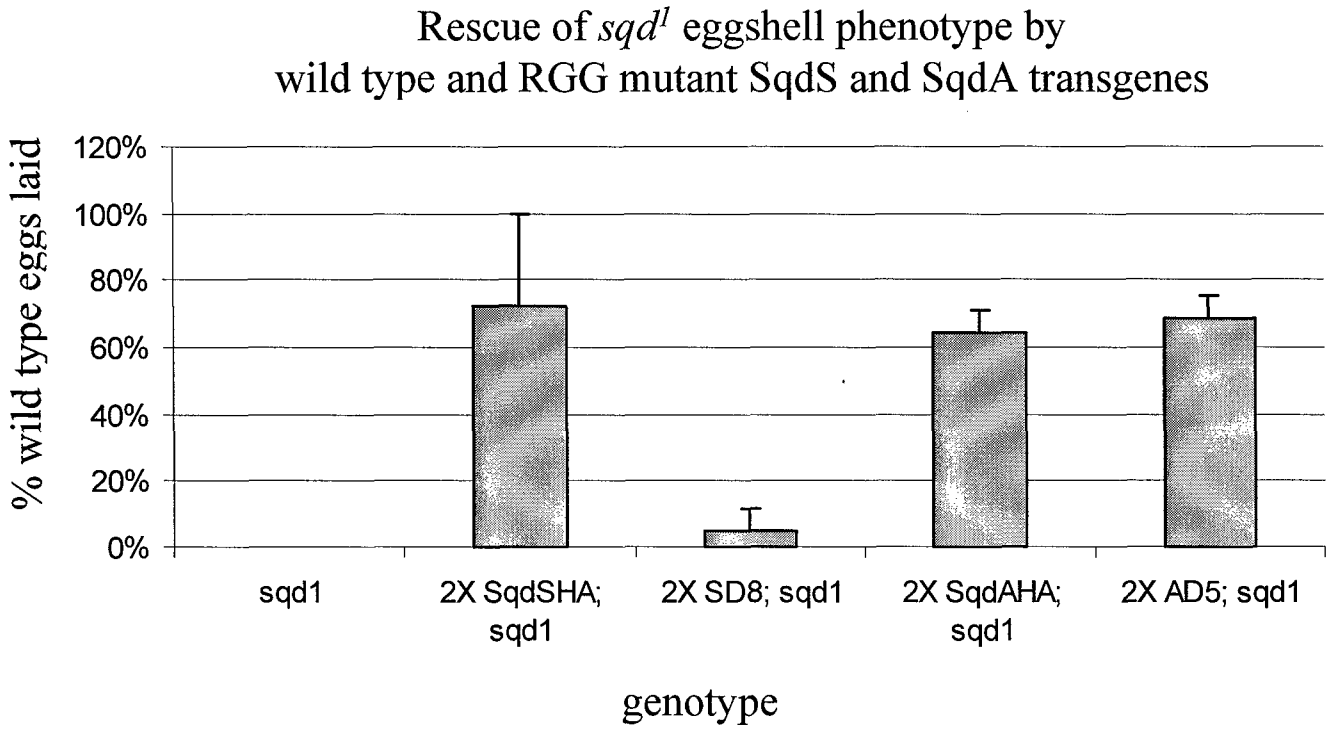

Figure 8. Rescue of *sqd*^{1} eggshell phenotype by wild type and RGG mutant SqdS and SqdA transgenes.

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).