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Sqd interacts with the *Drosophila* retinoblastoma tumor suppressor Rbf

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Abstract

RB serves as a scaffold to coordinate binding of numerous proteins, including E2F and histone deacetylases, through its C-terminal domain. The amino-terminal half of RB has few known binding partners and its function is not well understood. We used the amino-terminal domain of the *Drosophila* retinoblastoma tumor suppressor Rbf (RbfN) to identify novel binding partners by immunoprecipitation coupled with mass spectrometry. Our experiment reveals that the RNA-binding protein Squid (Sq) is a putative interacting partner of RbfN. Western blot confirmed that Sq interacts with the amino-terminal domain of Rbf. We observed that Sq colocalizes with RbfN in *Drosophila* salivary gland cells. We also show that double RNAi knockdown of Rbf and Sq in the eye results in an extensive loss of eye bristles, suggesting that Rbf and Sq function in a common pathway. We conclude from our studies that Rbf physically and genetically interacts with Sq. We propose that the retinoblastoma tumor suppressor may play a novel role in RNA processing through interaction with RNA binding proteins.

Keywords

RB; Rbf; Rbf1; retinoblastoma; tumor suppressor; Sq; hnRNP; RNA binding; RNA processing

Introduction

The retinoblastoma tumor suppressor (RB) is a conserved protein that plays an important role in development and cell cycle regulation in all multicellular organisms. The major function of RB is best characterized by its repression of the E2F family of transcription factors which serves to arrest cells in G1. RB also has an influence over other cellular processes, including apoptosis and tissue differentiation [1]. RB restricts cell cycle progression by serving as a scaffold to coordinate binding of numerous proteins, including E2F and histone deacetylases, through its C-terminal domain. As such, most studies have focused on elucidating the function of RB through analysis of its C-terminal half [2]. On the other hand, the amino-terminal half of RB has few known binding partners and its function is not well understood [3].

The goal of the present study is to infer new functions for the retinoblastoma protein by using the *Drosophila melanogaster* retinoblastoma homologue, Rbf, to identify novel

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binding partners of its amino-terminal domain. We immunoprecipitated the Rbf N-terminal domain (RbfN) expressed in *Drosophila* S2 cells and identified binding partners by mass spectrometry. We identified the RNA-binding protein Squid as a novel binding partner of Rbf and discuss the possible implications of this protein interaction in development and cancer.

Materials and Methods

S2 cell culture and protein expression

Drosophila Schneider S2 cells were grown under standard conditions in M3 medium (Sigma) supplemented with antibiotics and 12% fetal bovine serum. V5 epitope-tagged RbfN protein was expressed in S2 cells, as described previously [4].

Antibodies, immunoprecipitations, and immunoblots

Immunoprecipitations were performed as described previously [4]. Protein-A beads (Sigma) were used for all reactions. For immunoprecipitations: Anti-Sqd [5] and anti-V5 (AbD Serotec) antibodies were used at 1:50 dilutions. Anti-Rbf and its pre-immune serum were used at 1:20 [6]. Immunoblotting was done by standard techniques as described previously [4]. For immunoblots: Anti-Sqd was used at 1:200. HRP-conjugated anti-mouse was used as a secondary antibody (Jackson ImmunoResearch). Chemiluminescence was used to visualize the immunoblots (Pierce). Ethidium bromide was included in the immunoprecipitations to eliminate DNA-mediated interactions. Sqd and Rbf antibodies were kind gifts from Trudi Schupbach and David Arnosti, respectively.

Microscopy

Transgenic UAS>RbfN-RFP virgins were collected and crossed to males containing salivary gland specific Sgs3>GAL4 driver (Bloomington). UAS>RbfN-RFP; Sgs3>GAL4 flies were then crossed to GFP trap lines for *sqd* stock #YB0291DE or *betaTub56D* stock #YC0063 [7, 8]. Salivary glands were dissected from wandering third instar larvae in Grace's medium and fixed with 8% formaldehyde in Buffer B [9]. Images were obtained using a Zeiss LSM 510 Meta microscope.

Proteomics

Proteins from V5 antibody immunoprecipitations either from S2 cells expressing RbfN-V5 or from control S2 cells were eluted with SDS-PAGE sample buffer by boiling at 95 degrees for 10 minutes. Eluates were resolved by 12.5% SDS-PAGE (BioRad). The protein gels were stained with comassie and nonspecific bands found in both the experimental and control samples, including the V5 antibody, were cut out and discarded. The proteins remaining in the rest of the gel were subjected to in gel digestion using trypsin and then extracted from the gel. The extracted peptides were then submitted for mass spectrometry analysis, as previously described [10].

All MS/MS samples were analyzed using Sequest (ThermoFinnigan, San Jose, CA; version 27, rev. 12) and X! Tandem (www.thegpm.org; version 2007.01.01.1). X! Tandem was set up to search a subset of the drosomay05 database also assuming trypsin. Sequest was set up to search the drosophila database (downloaded from NCBI on August 12, 2008 with 87625 entries) assuming the digestion enzyme trypsin. Scaffold (version Scaffold_2_01_01, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they exceeded specific database search engine thresholds. Proteins were considered as strong candidates if they contained at least 2 unique peptide identifications.

Results

Proteomic identification of Sqd as a novel binding partner of Rbf

To gain more insight into the function of the amino-terminal domain of Rbf (RbfN) we wished to identify novel binding partners by immunoprecipitation coupled with mass spectrometry. We performed an immunoprecipitation using a V5 antibody with *Drosophila* S2 cell lysates expressing RbfN-V5, as described previously [4]. Lysates from untransfected cells were used for a parallel immunoprecipitation as a control for nonspecific binding to the antibody or beads. After extensive washing, the immunoprecipitated proteins were eluted and then subjected to SDS-PAGE followed by LC/LC-MS/MS (MudPIT). MudPIT spectra were manually validated for those proteins that had more than one unique peptide and that were not also identified in the control immunoprecipitation MudPIT (Table 1).

Our MudPIT experiment reveals that the heat shock cognate protein Hsc70-4 is a putative binding partner of RbfN. In addition, the RNA-binding protein Squid (Sqd) was identified as a putative interacting partner of RbfN. We also identified Sqd as a putative partner of RbfN in an independent nano-LC-MS/MS experiment (data not shown). To confirm our MudPIT data we probed a western blot of an RbfN immunoprecipitation with a Sqd monoclonal antibody [5]. The western blot showed a band of approximately 40 kDa in the cell extract that corresponds to Sqd (Figure 1A, lane 1). A control monoclonal antibody fails to pull down Sqd in extracts containing RbfN-V5 (lane 2). Immunoprecipitation with V5 antibody from cells not expressing RbfN also failed to pull down Sqd (lane 3). However, immunoprecipitation with V5 or Sqd monoclonal antibodies from RbfN lysates resulted in an enrichment of Sqd protein (lanes 4 and 5). These results confirm that Sqd interacts with the amino-terminal domain of Rbf in *Drosophila* S2 cells.

We next determined whether Sqd can interact with full-length endogenous Rbf. We homogenized ovaries from wild-type female flies and performed an immunoprecipitation using a polyclonal Rbf antibody and its pre-immune serum as a control antibody [6]. We performed a western blot and probed with Sqd antibody, as before. We observe a band of approximately 40 kDa in the ovarian lysate indicating the Sqd protein (Figure 1B, lane 1). The pre-immune serum antibodies were unable to pull down Sqd (lane 2), whereas Rbf and Sqd antibodies show a single protein band corresponding to Sqd (lanes 3 and 4). We conclude that Rbf physically interacts with Sqd *in vivo* and that this interaction is mediated by the Rbf N-terminal domain.

RbfN colocalizes with Sqd

We next wished to determine if RbfN exhibited an overlapping pattern of cellular localization with Sqd. We chose to use larval salivary glands that contain highly secretory cells with large subcellular structures that are good for colocalization studies. We expressed RbfN labeled with red fluorescent protein in salivary glands that also express a GFP-tagged Sqd protein [4, 11]. We dissected salivary glands from third instar larvae, fixed them in formaldehyde, and stained them with DAPI. Confocal imaging of these glands demonstrate nuclear and cytoplasmic localization of RbfN-RFP (Figure 2A), as shown previously [4]. Sqd-GFP shows a cytoplasmic and nuclear localization that overlaps significantly with RbfN-RFP (Figure 2D). However, Sqd-GFP appears in several bright bands on the polytene chromosomes that do not show a correlated brightness in RbfN-RFP signal, which indicates that Sqd may localize to chromatin independently of Rbf. On the other hand, the RNA binding protein Rm62 did not show any cytoplasmic localization and did not colocalize with nuclear RbfN-RFP, which demonstrates the specificity of the RbfN interaction with Sqd (supplementary data). We conclude that Sqd colocalizes extensively with RbfN in the cytoplasm and throughout the nucleus.

We were intrigued by the distinct fishnet pattern shown by both RbfN and Sqd in the cytoplasm of salivary gland cells, so we investigated this further. It has been shown in the ovary that Sqd localizes gurken mRNA to the cytoplasm around the oocyte nucleus by means of microtubule-mediated transport [12]. We hypothesized that the localization pattern of Sqd and RbfN may correspond to the highly active microtubule networks of salivary glands. We expressed RbfN-RFP in salivary glands of larvae expressing a GFP-tagged Beta-tubulin and prepared the salivary glands as before. Cytoplasmic Beta-Tubulin is organized in salivary gland cells as a network that is fishnet in appearance (Figure 2B). As we predicted, RbfN-RFP colocalizes with cytoplasmic Beta-tubulin. It is interesting to note that Beta-Tubulin also localizes to the nucleus in salivary gland cells, although not as strongly as RbfN. Others have reported nuclear Beta-tubulin localization in mammalian cells, although the role of nuclear tubulin is unclear [13-15]. We also observe a cytoplasmic colocalization of RbfN-RFP with Rab11-GFP, a marker of microtubule-associated transport vesicles, which appears similar to Beta-tubulin and Sqd cytoplasmic localization (supplementary data). We believe this cytoplasmic localization is specific, since we do not see cytoplasmic localization of the RNA binding proteins Rm62-GFP or pUf68-GFP in the presence of RbfN-RFP (supplementary data). Interestingly, RB has also been shown to associate with microtubules in human cells [16, 17]. Taken together, our data suggests that Rbf can associate with cytoplasmic microtubule networks in *Drosophila* salivary gland cells.

Rbf genetically interacts with Sqd

We have shown that Rbf and Sqd physically interact, and we next tested whether these proteins work together in proliferating tissues. Using the GAL4-UAS system, we expressed UAS-RNAi against *rbf* and/or *sqd* in the developing eye disc under the influence of GMR-GAL4. Scanning electron microscopy shows a wild-type adult eye in the absence of GMR-GAL4 (Figure 3A). Lowered expression by RNAi of either *Rbf* (Figure 3B) or *Sqd* (Figure 3C) results in a modest reduction of eye bristles. However, a simultaneous RNAi knockdown of *Rbf* and *Sqd* results in an extensive loss of eye bristles (Figure 3D). In addition, these eye ommatidia have lost underlying structural integrity and have a flattened appearance. These results suggest that Rbf and Sqd function in a common pathway, where reduction of both gene products results in an enhanced mutant phenotype. We conclude from our studies that Rbf physically and genetically interacts with Sqd.

Discussion

The retinoblastoma tumor suppressor (RB) is an important regulator of the cell proliferation during development. Despite the volumes of research literature dedicated to RB function since its molecular identification in the 1980s [18], much remains to be learned about the scope of its function. In particular, very little is known about the function of the RB amino-terminal domain [3]. To gain further insight into the function of RB, we used the *Drosophila* retinoblastoma homologue Rbf to identify novel binding partners of its amino-terminal domain (RbfN) by immunoprecipitation followed by mass spectrometry.

Our mass spectrometry data indicate that Hsc70-4 interacts with RbfN (Table 1). Several lines of evidence support the validity of this finding. Hsc70-4 was found in a complex with the chromatin protein Brahma that physically interacts with Rbf [19]. Hsc70-4 deficiency dominantly suppresses a cyclin E hypomorphic phenotype [19]. Since cyclin E is an upstream suppressor of Rbf, one interpretation of this data would suggest that Hsc70-4 may act as an effector of Rbf function. Finally, a protein interaction has been shown between the N-terminal domain of the human retinoblastoma protein RB and hsc73, a homologue of Hsc70-4 [20]. Thus, our data confirms that this interaction is conserved between flies and

humans, although the significance of this interaction remains undefined. Additional studies will be necessary to test the functional significance of this interaction.

We also find through our proteomic analysis that the RNA binding protein Squid (Sqd) associates with RbfN (Table 1). Sqd is an RNA binding protein which has homology to human hnRNP A proteins that are involved in many processes relative to RNA metabolism and gene expression, including regulation of transcription, RNA splicing, and transport [21, 22]. We show that Sqd interacts with Rbf in *Drosophila* S2 cells and ovaries (Figure 1). Sqd also colocalizes with RbfN in *Drosophila* salivary gland cells (Figure 2). Although this colocalization is extensive, we note that several chromosomal locations are enriched with Sqd that do not correspond to an enrichment of RbfN. These genomic locations may correspond to highly transcribed regions where Sqd is recruited independently of Rbf. We also investigated whether Rbf and Sqd might genetically interact *in vivo*. We demonstrated that double RNAi knockdown of *Sqd* and *Rbf* in the *Drosophila* eye results in an enhanced phenotype (Figure 3). We therefore infer that Sqd and Rbf function together during development, which may constitute a novel mode of regulation by the retinoblastoma tumor suppressor.

Although the role of Sqd during *Drosophila* eye development has not been studied, the function of Sqd is best defined by its role in EGFR signaling during oocyte development where it participates in dorsoventral patterning by localizing *gurken* mRNA [23]. Gurken is a ligand for EGFR that locally acts as a signal from the oocyte to instruct a subpopulation of the dorsal epithelium to adopt a dorsal cell fate. *sqd* mutants do not properly localize *gurken* mRNA, resulting in broad *gurken* signaling that produces a dorsalized egg [24]. On the other hand, mutations that reduce *gurken*-EGFR signaling result in reduced dorsal cell fates that give a ventralized phenotype [25].

The RB/E2F pathway may have a role in EGFR signaling during *Drosophila* oogenesis. Loss of the E2F binding partner DP during oogenesis results in a ventralized egg, which appears to be due to the reduced ability to transport *gurken* mRNA into the oocyte from the nurse cells [26]. Another study found that reduction of *E2F* or *cyclin E* enhances a dorsalization phenotype caused by overexpression of *Imp*, an RNA binding protein that functions in *gurken* mRNA localization [27]. In light of this data, our finding that the Rbf protein associates with Sqd may indicate a direct role for the retinoblastoma protein in regulation EGFR signaling. Further studies will reveal to what degree Rbf influences dorsoventral patterning during oogenesis.

The nematode *C. elegans* also has a system of EGFR signaling, where EGF signals locally from the adjacent gonad to induce only one of six vulval precursor cells to adopt a vulval cell fate [28]. Analogous to what is observed during *Drosophila* oogenesis, broad misexpression of EGF from neighboring cells induces all of the vulval precursor cells to adopt a vulval fate, resulting in a worm with multiple vulvae. RB normally suppresses EGF signaling in this context, since mutations in the *C. elegans* orthologues of RB/E2F/DP result in a multivulval phenotype [29]. In *Drosophila*, *rbf* mutation sensitizes cells to E2F-induced apoptosis that can be modulated by EGFR activity [30]. Studies in mammalian cell culture also demonstrate functional interplay between the EGFR/Ras and Rb/E2F signaling pathways [31-33]. Thus, it appears that the interaction of these two pathways may be conserved among multicellular organisms, and we suggest that the physical interaction of Rbf with Sqd may constitute one method by which these pathways converge. Our findings also raise the interesting possibility that the retinoblastoma tumor suppressor proteins may have a role in RNA processing, since Sqd is an RNA binding protein that functions in other pathways of RNA processing, such as alternative splicing of transcripts [34]. A role in

EGFR signaling and influence on RNA processing through interaction with RNA binding proteins may be novel mechanisms for tumor suppression by RB.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Rbf interacts with Sqd

(A) RbfN-V5 transfected cell extracts were immunoprecipitated with anti-V5 and subjected to SDS-PAGE followed by western blotting using monoclonal Sqd antibody. Sqd appears as a band of approximately 40 kDa. V5 or Sqd antibodies from RbfN lysates pulled down Sqd protein. Note that extracts from untransfected cells treated identically with monoclonal V5 antibody failed to immunoprecipitate Sqd. A control monoclonal antibody also fails to pull down Sqd from cell extracts containing RbfN-V5, demonstrating that the interaction between Sqd and RbfN is specific. (B) Endogenous Rbf or Sqd was immunoprecipitated from wild-type ovarian extracts. Western immunoblotting with monoclonal Sqd antibody shows that Rbf antibody serum can pull down Sqd protein, whereas a pre-immune control serum did not pull down Sqd.

Figure 2. RbfN colocalizes with Sqd

RbfN-RFP localizes to the nucleus and cytoplasm in *Drosophila* larval salivary gland cells. (A) Sqd-GFP shows a pattern of cytoplasmic localization that overlaps extensively with RbfN-RFP. Nucleoplasmic RbfN and Sqd also significantly colocalize. However, nuclear localized Sqd-GFP shows enrichment in some chromosomal regions that appear to be independent of RbfN-RFP localization. (B) Beta-Tubulin-GFP localizes to discrete cytoplasmic structures in *Drosophila* larval salivary gland cells that overlaps with RbfN-RFP, indicating the RbfN potentially associates with microtubule networks.

Figure 3. Rbf and Sqd in genetically interact in *Drosophila* eyes

We expressed RNAi against *rbf* and/or *sqd* in the developing eye disc using the GAL4-UAS system. (A) A wild-type adult eye shows a normal arrangement of ommatidia and eye bristles. (B) RNAi of *rbf* or (C) RNAi of *sqd* produces a modest reduction of eye bristles. (D) Double RNAi knockdown of *rbf* and *sqd* results in an extensive loss of eye bristles. Also note that these eye ommatidia have a flattened appearance, which may indicate a loss of underlying cells.

Table 1

Identified Proteins	Accession Number	Molecular Weight (kDa)	Unique Peptides	Coverage (%)
RbfN	gi 24638969	42 ^a	10	26.38 ^a
Hsc70-4	gi 103190	71	4	6.91
Squid	gi 476947	35	2	7.17

^aRbfN amino acids 1-345.