Drosophila Syncrip binds the *gurken* mRNA localisation signal and regulates localised transcripts during axis specification

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Summary

In the *Drosophila* oocyte, mRNA transport and localised translation play a fundamental role in axis determination and germline formation of the future embryo. *gurken* mRNA encodes a secreted TGF-α signal that specifies dorsal structures, and is localised to the dorso-anterior corner of the oocyte via a cis-acting 64 nucleotide *gurken* localisation signal. Using GRNA chromatography, we characterised the biochemical composition of the ribonucleoprotein complexes that form around the *gurken* mRNA localisation signal in the oocyte. We identified a number of the factors already known to be involved in *gurken* localisation and translational regulation, such as Squid and Imp, in addition to a number of factors with known links to mRNA localisation, such as Me31B and Exu. We also identified previously uncharacterised *Drosophila* proteins, including the fly homologue of mammalian SYNCRIP/hnRNPQ, a component of RNA transport granules in the dendrites of mammalian hippocampal neurons. We show that *Drosophila* Syncrip binds specifically to *gurken* and *oskar,* but not *bicoid* transcripts. The loss-of-function and overexpression phenotypes of *syncrip* in *Drosophila* egg chambers show that the protein is required for correct *grk* and *osk* mRNA localisation and translational regulation. We conclude that *Drosophila* Syncrip is a new factor required for localisation and translational regulation of *oskar* and *gurken* mRNA in the oocyte. We propose that Syncrip/SYNCRIP is part of a conserved complex associated with localised transcripts and required for their correct translational regulation in flies and mammals.

Key words: *Drosophila*, Syncrip, localised translation, mRNA localization, oogenesis

Introduction

The intracellular localisation and localised translation of messenger RNA (mRNA) is a widespread and evolutionarily ancient strategy used to temporally and spatially restrict specific proteins to their site of function (Holt and Bullock, 2009; Martin and Ephrussi, 2009; Meignin and Davis, 2010). High-throughput studies have shown that a large proportion of mRNAs are localised in many cell types, indicating that it is a highly prevalent mechanism for the local regulation of gene expression (Blower et al., 2007; Lécuyer et al., 2007; Mili et al., 2008). The best-studied examples of localised mRNAs include transcripts whose protein products play roles in the regulation of cell polarity (Condeelis and Singer, 2005; Horne-Badovinac and Bilder, 2008; Li et al., 2008), the segregation of cell fate and embryonic polarity determinants (Broadus et al., 1998; King et al., 2005; Kugler and Lasko, 2009) and in the plastic changes at neuronal synapses that underlie memory and learning (Guzowski et al., 2000; Miller et al., 2002; Ashraf et al., 2006; Sánchez-Carbente and Desgroseillers, 2008; Wang et al., 2009).

The transport of mRNA cargoes to their cellular destinations occurs in large ribonucleoprotein (RNP) complexes termed transport particles or granules. Visualisation of these particles using light and electron microscopy (MacDougall et al., 2003; Weil et al., 2006; Delanoue et al., 2007; Jaramillo et al., 2008), and purification using biochemical sedimentation techniques (Krichevsky and Kosik, 2001) have shown that they consist of mRNA molecules complexed with their associated proteins. Such factors can play various roles including regulation of translation, and cytoskeletal dependent molecular motor activities. However, many questions remain unanswered about the composition, organisation and regulation of the transport RNPs and their association with cis-acting localisation signals.

In the developing *Drosophila* oocyte, the localisation of *gurken* (*grk*), *bicoid* (*bcd*), *nanos* (*nos*) and *oskar* (*osk*) mRNAs plays a fundamental role in the determination of the axes of the oocyte and future embryo. *grk* encodes a secreted TGF-α signal, and is localised to the dorso-anterior of the oocyte where it is required for dorso-ventral axis determination. The majority of the known proteins that function in *grk* mRNA localisation and translational regulation have been identified through genetic studies. In *squid* (*sqd*) mutants, *grk* mRNA is mislocalised and translated along the entire anterior cortex of the oocyte (Kelley,

1993; Neuman-Silberberg and Schüpbach, 1993). Sqd is a heterogeneous nuclear ribonucleoprotein (hnRNP), a family of proteins that has been implicated in all steps of RNA processing and that are thought to shuttle between the nucleus and cytoplasm. Sqd is present in *grk* transport particles and anchoring structures (Delanoue et al., 2007), where it is required for localisation, translational repression and anchoring of the mRNA (Norvell et al., 1999; Delanoue et al., 2007; Cáceres and Nilson, 2009). Sqd associates with a number of other factors that are also required for *grk* localisation and translational control including Hrb27C and Imp (Goodrich et al., 2004; Geng and Macdonald, 2006). Several of the proteins that regulate *grk* are also required for the localisation and translation of *osk* mRNA (Huynh et al., 2004; Norvell et al., 2005), suggesting that these mRNAs may be regulated by shared core components.

We previously defined the *grk* localisation signal (GLS), a 64-nucleotide stem loop structure in the coding region of the *grk* transcript, as necessary and sufficient for dorso-anterior localisation (Van De Bor et al., 2005). The GLS is thought to specify the destination of *grk* mRNA through its recognition by trans-acting protein factors. However, it has been unclear how many more important factors are present that have been missed in genetic screens, nor how the trans-acting factors are related to the GLS cis-acting stem loop. Here, we have used a biochemical approach to identify the proteins that specifically associate with the GLS. We identified known factors previously shown to be required for *grk* mRNA localisation and translational regulation, including Sqd and Imp. We also identified a number of previously uncharacterized RNA binding proteins, most notably CG17838, the *Drosophila* homologue of mammalian SYNCRIP/hnRNPQ, a component of RNA granules in the dendrites of hippocampal neurons (Bannai et al., 2004). We therefore named CG17838 as Syncrip (Syp). We show that *Drosophila* Syp associates specifically with *grk* and *osk* mRNAs together with Sqd and Hrb27C. The loss-of-function and overexpression phenotypes of *syp* in *Drosophila* egg chambers show that the protein is required for correct *grk* and *osk* mRNA localisation and translational regulation, processes known to require Sqd and Hrb27C. We propose that Syp is a novel conserved component of localised RNPs, regulating translation of localised transcripts in flies and mammals.

Results

Drosophila Syp associates with the *gurken* RNA localisation signal

To study the biochemical composition of *grk* RNP particles we used GST-RNA (GRNA) affinity chromatography (Czaplinski et al., 2005). GRNA resins were prepared using a region of *grk* containing the GLS (5′ORF), the same region with the GLS deleted (5′ORFΔGLS), as well as *hunchback* (*hb*), a non-localising negative control mRNA (Fig. 1A). The resin was incubated with *Drosophila* ovary lysate, and eluted proteins analysed in bulk by mass spectrometry. A total of 16 candidate GLS specific proteins were identified by subtracting the proteins able to associate specifically with negative controls from those able to associate with 5′ORF RNA containing the GLS (Fig. 1B; Table 1; supplementary material Table S1). We found a number of known proteins required for *grk* mRNA localisation and translational regulation, including Sqd and Imp, thus validating the biochemical approach (Fig. 1B; Table 1; supplementary material Tables S1, S2). We also found a number of uncharacterised RNA binding proteins, most notably CG17838, an hnRNP protein that shares 47% sequence identity and 60% similarity to mammalian SYNaptotagmin-binding Cytoplasmic RNA-Interacting Protein (SYNCRIP)/hnRNPQ and R (Fig. 1C). Western blot analysis of GRNA chromatography samples confirmed the enrichment of this protein in 5′ORF eluates (supplementary material Fig. S1). Mammalian SYNCRIP is a component of neuronal RNA granules associated with localised dendritic mRNAs (Bannai et al., 2004; Kanai et al., 2004; Elvira et al., 2006), and is thought to regulate translation via an interaction with the non-coding RNA BC200/BC1, itself a translational repressor (Duning et al., 2008). *Drosophila syp* has a similar structure to the mammalian homologues, sharing RRM RNA binding domains and nuclear localisation signal(s), as well as encoding a number of isoforms (A to H) (Fig. 2A). We have found that although Syp protein and *syp* transcripts are absent throughout embryogenesis, they are present in third instar larvae, enriched in larval brains, and are also expressed in ovaries (Fig. 2B–D; supplementary material Fig. S2). Tissue-specific expression is also observed for a number of isoforms. Isoforms A, D, E, and H are restricted to male testis whilst isoforms C, F and G are found in larvae, pupae, and in all adult tissues tested, including ovaries (Fig. 2B; supplementary material Fig. S2).

Syp is a component of *grk* and *osk* RNP complexes and is required for axis specification and germline formation

To test whether Syp associates *in vivo* with *grk* mRNA and its known trans-acting factors we immunoprecipitated native RNP complexes from ovarian lysates, followed by detection of proteins and of oocyte mRNAs by reverse transcription and quantitative PCR (qRT-PCR). Immunoprecipitation was carried out using an anti-Syp antibody which we raised against recombinant Syp protein. We found that Syp co-immunoprecipitates with Sqd and with Hrb27C (Fig. 3A). The interaction with Sqd, but not Hrb27C, is RNA-dependent as it is disrupted by RNase treatment, suggesting that Syp and Sqd interact via RNA targets and that Hrb27C interacts with Syp through a protein-protein interaction. *grk* and *osk* are both enriched in the fraction precipitated from wild-type ovaries using anti-Syp antibody (Fig. 3B). In contrast, we found no specific enrichment of *bcd*, *nos*, *hb* or of an abundant transcript encoding a ribosomal protein (*rp49*). Immunoprecipitation of SqdGFP and Syp from SqdGFP protein trap ovaries, that contain a viable replacement of endogenous *sqd,* confirmed the association of both Sqd and Syp with *grk* and *osk* mRNAs, whilst also indicating that Sqd can associate with *bcd* mRNA (Fig. 3B). Our results are consistent with, and extend, previous studies showing that Sqd and Hrb27C are present in *grk* mRNA transport particles and anchoring structures (Delanoue et al., 2007) and that they interact with both *grk* and *osk* mRNAs (Norvell et al., 1999; Goodrich et al., 2004; Norvell et al., 2005). We conclude that Syp is present in *grk* and *osk* mRNPs together with other known trans-acting factors. The GRNA and immunoprecipitation experiments do not distinguish whether Syp interacts directly with *grk* and *osk* mRNAs, or indirectly through these other mRNP components. Although we cannot exclude indirect interactions between Syp and its RNA targets, we believe that the simplest interpretation of our data, given that Syp contains three highly conserved RNA-binding RRM domains, is a direct interaction between Syp, and *grk* and *osk* mRNAs.

To investigate the function of Syp during oogenesis, we characterised the *syp* loss of function phenotypes associated with

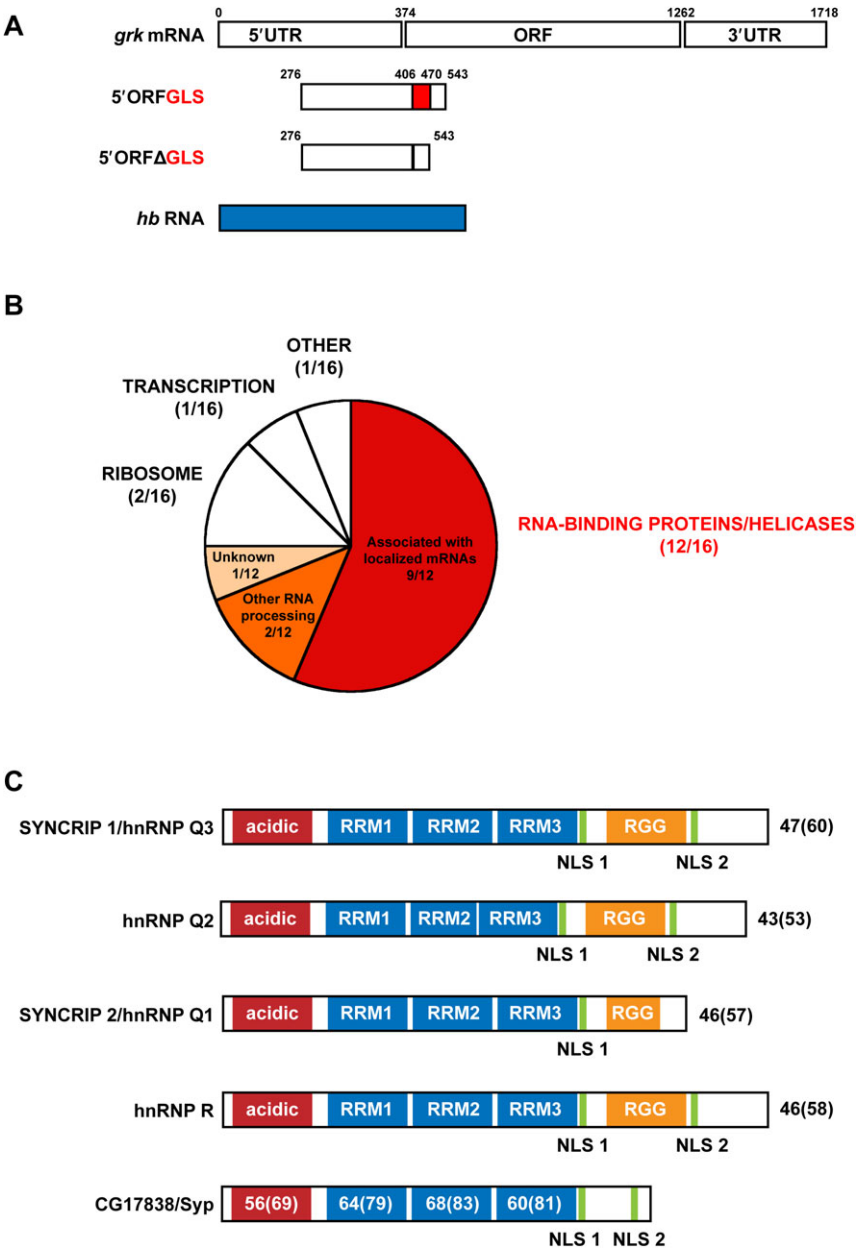

Fig. 1. GRNA affinity chromatography identifies the previously uncharacterized *Drosophila* homologue of mammalian SYNCRIP. (**A**) Illustration of the RNA fragments used to prepare GRNA resins. 5′ORF RNA consists of the GLS surrounded by 211 nucleotides spanning the 3′ of the 5′UTR into the 5′ of the *grk* open reading frame. 5′ORFΔGLS is the same sequence, but lacks the GLS. (**B**) Classification of the proteins able to specifically associate with 5′ORF RNA by protein type and function. 16 proteins were found to specifically associate with 5′ORF RNA, 12 of which were RNA binding proteins or RNA helicases. Nine of these proteins, or their orthologues, have also been shown by previous studies to be associated with localised mRNAs in a variety of model organisms. (**C**) Schematic representation of the mouse and human SYNCRIP/hnRNP Q isoforms, hnRNP R and *Drosophila* CG17838 (isoform F)/Syp. Acidic, protein domain rich in acidic amino acids; RRM, RNA Recognition Motif; NLS, Nuclear Localisation Signal; RGG, arginine-glycine-glycine-rich domain. hnRNP Q2 has a deletion within RRM 2, whilst hnRNP Q1 lacks the second putative nuclear localisation signal and has a unique C-terminus. hnRNP R differs only in the identity of a number of amino acids at the very N-terminus of the protein. The N-terminal acidic domain and the three RRMs are highly conserved in *Drosophila* CG17838, which lacks the C-terminal RGG boxes. Percent amino acid identity and similarity shared by the *Drosophila* protein is indicated for each mammalian protein and for individual domains. Percent similarity is given in brackets.

two insertions in the *syp* gene, syp^{e00286} and syp^{f03775}, which are predicted to affect all isoforms (Fig. 2A). Homozygotes and trans-heterozygotes of these alleles in combination with the deficiency *Df(3R)BSC124*, show highly reduced adult viability, *syp* mRNA and Syp protein levels (Fig. 2E,F; Table 2). Mutants survive until late pupal stages, but mostly fail to eclose. It was therefore necessary to generate germline clones of the syp^{e00286} allele (Chou and Perrimon, 1992) in order to study the oogenesis phenotype. The eggs laid by females with a germline homozygous for syp^{e00286} display a range of dorso-ventral patterning defects including ectopic, fused and missing dorsal appendages, or defects in the spacing and position of dorsal appendages (Fig. 4A; Table 3). Eggs laid by these females also had a range of morphological defects including short, round eggs and a ‘moose antler’ dorsal appendage phenotype, resembling that seen in *bullwinkle* mutants (Fig. 4B; Table 3). The resulting embryos also have a reduced number of Vasa-positive pole cells at the cellular blastoderm stage, indicative of a reduction in Osk protein level or function (Lehmann and Nüsslein-Volhard, 1986) (Fig. 4D,E; Table 4).

Overexpression of UAS-SypGFP in the germline, driven by tubulinGal4 (Fig. 2B), also resulted in dorso-ventral axis specification and pole cell number defects that were more severe and penetrant than those observed in the syp^{e00286} germline clones (Fig. 4C,F; Tables 3, 4). Therefore, altering the level of Syp in the germline results in *grk* and *osk* phenotypes, which is consistent with Syp being a functional component of *grk* and *osk* RNP complexes and being required to modulate *grk* and *osk* function.

Syp modulates *grk* and *osk* mRNA localisation and translation

To determine the basis of the dorso-ventral patterning and pole cell defects in *syp* mutants, we carried out *in situ* hybridization and immunostaining for *grk*, *osk* and *bcd* mRNAs and Grk and

Table 1. 16 trans-acting factors identified by GRNA affinity chromatography to associate with 5′ORF RNA. CG17838, now named *Drosophila* Syp, is highlighted in bold.

Function	Type	Protein name
mRNA localisation	RNA-binding protein	IGF-II mRNA-binding protein (Imp), Exuperentia (Exu)
mRNA localisation and translational regulation	RNA-binding protein	Squid (Sqd), Polypyrimidine tract binding protein (PTB)/Hephaestus (Heph)
Translational regulation	RNA-binding protein	Dodeca satellite binding protein 1 (Dp1), Bicaudal C (BicC), Polyadenylate-binding protein (PABP)
	RNA helicase	Maternal expression at 31B (Me31B)
Other RNA processing	RNA-binding protein	Fibrillarin (Fib), CG6745
Unknown function	RNA-binding protein	**CG17838 *Drosophila* Syp**
	RNA helicase	CG5205
Structural constituent of the ribosome	Ribosome	Ribosomal protein L14 (RpL14), Ribosomal protein S19 (RpS19)
Regulation of transcription	Transcription	Rigor mortis (Rig)
Other	Other	Deadhead (Dhd)

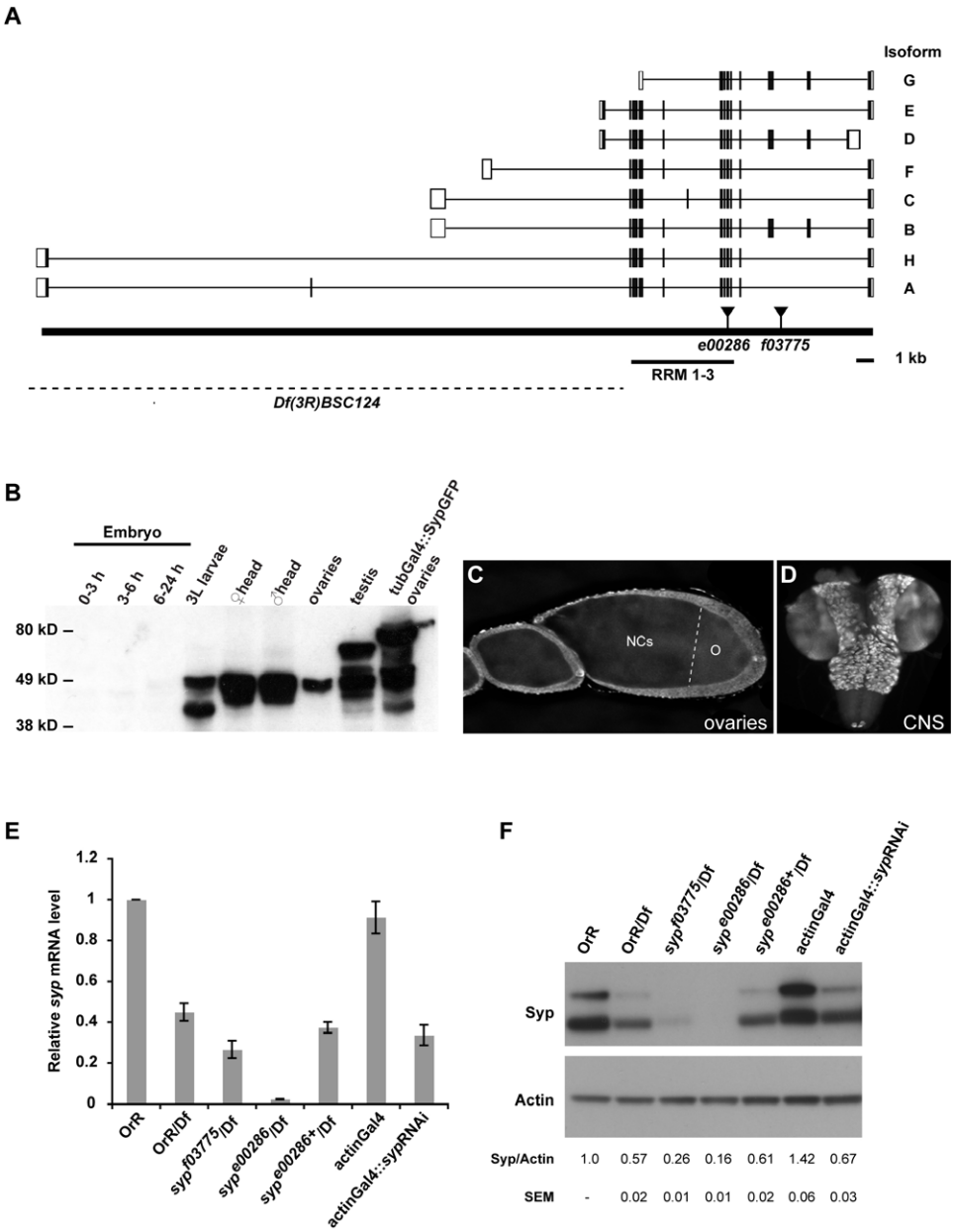

Fig. 2. Syp isoforms are expressed during oogenesis and in the larval nervous system. (**A**) The genomic structure of the *syp* gene and associated alleles. Black bars represent coding sequence exons and white boxes represent untranslated sequence exons. Alternative splicing is predicted to produce a number of transcripts corresponding to isoforms A to H. The syp^{e00286} allele is an insertion within an exon coding for RRM 3, whilst syp^{f03775} lies within a C-terminal intron. The deficiency *Df(3R)BSC124* uncovers a large portion of the *syp* gene. (**B**) Western blot of extracts from a range of tissues and developmental stages, probed with guinea-pig anti-Syp antibodies. Extracts are OrR, unless otherwise stated. (**C**) Wild-type (OrR) egg chambers stained with guinea-pig anti-Syp antibodies. Syp is expressed both in the germline and in the somatic follicle cells. The dashed line delineates the approximate boundary between the oocyte (O) and nurse cells (NCs). (**D**) Wild-type (OrR) third instar larval brain stained with guinea-pig anti-Syp antibodies. (**E**) The levels of *syp* mRNA in third instar larvae of the indicated genotypes were quantified by qRT-PCR and normalized to *rp49* mRNA. Error bars indicate the mean ± SEM from three independent experiments. qPCR primers were designed to amplify all *syp* isoforms. The *syp* RNAi line is 33012 from VDRC. (**F**) Syp protein levels in larvae of the same genotypes as in (E) were assessed by western blot analysis and quantified by densitometry using actin for normalization. The upper Syp band was used for quantification. Data are shown as means ± SEM from three independent experiments. The reduction in Syp protein levels in syp^{e00286}/*Df(3R)BSC124*, syp^{f03775}/*Df(3R)BSC124*, and actinGal4::*syp*RNAi larvae shows that the anti-Syp antibody is highly specific.

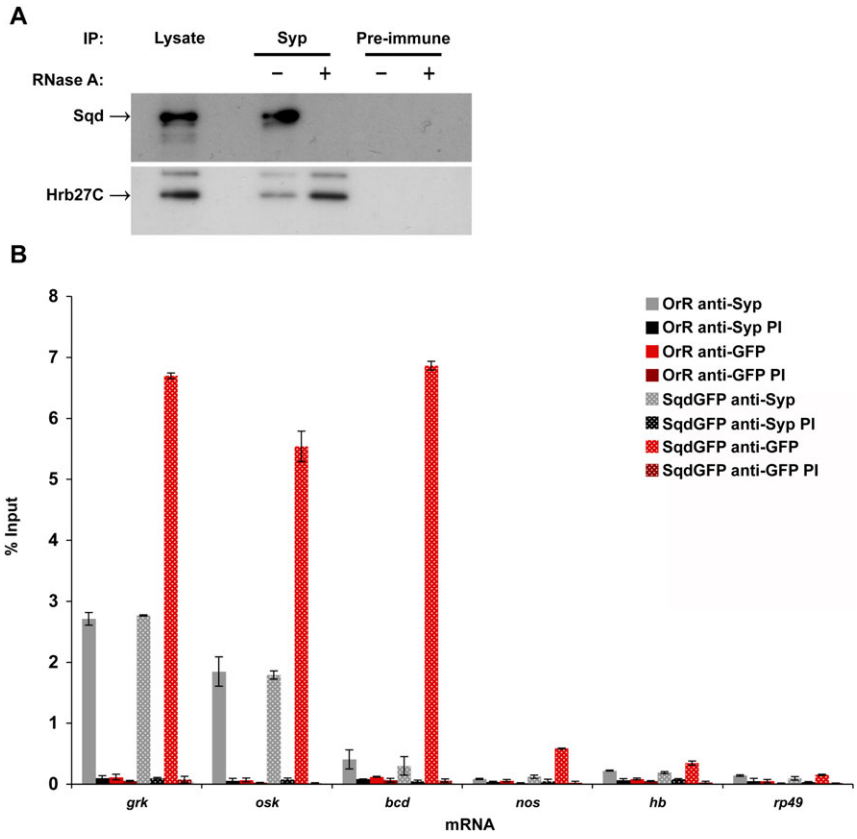

Fig. 3. Syp is a component of *grk* and *osk* RNP complexes. (**A**) Western blot of immunoprecipitation from wild-type ovarian lysate with anti-Syp antibodies or pre-immune serum with (+) or without (−) RNase A treatment. An amount of lysate equal to 0.5% of that used for immunoprecipitation was also loaded. (**B**) RNA immunoprecipitation from wild-type (OrR) or SqdGFP ovarian lysate with anti-Syp or anti-GFP antibodies, or pre-immune sera (PI). The indicated mRNAs were detected by RT-qPCR analysis. Results are expressed as the percentage of input mRNA. Data are shown as means ± SEM from three independent experiments.

Osk proteins. We found that *bcd* mRNA localisation was unaffected in *syp* mutant germline clones and in Syp overexpression oocytes (Fig. 5A,B; data not shown), consistent with our RNA immunoprecipitation data and also indicating that the overall polarity of the egg chamber is unaffected. In a small percentage of syp^{e00286} oocytes (9%; n=5/55), *grk* mRNA is mislocalised at the anterior cortex (Fig. 5D). However the majority of syp^{e00286} oocytes had wild-type grk localisation (data not shown). 26% (n=12/47) syp^{e00286} oocytes displayed defects in the distribution of Grk protein (Fig. 5F–H). In these oocytes, Grk is more spread out along the anterior cortex of the oocyte (11%; n=5/47), or is entirely absent (15%; n=7/47). These results correspond with the range of observed eggshell phenotypes, and we interpret them as indicating that the presence of Syp is required to regulate *grk* translation, repressing the translation of unlocalised *grk* mRNA and promoting the translation of *grk* that is correctly localised. Overexpression of Syp results in a much more penetrant *grk* mislocalisation phenotype than syp^{e00286} germline clones. In SypGFP egg chambers 94% (n=31/33) of the oocytes have *grk* mRNA mislocalised at the anterior cortex (Fig. 5E) and 98% (n=39/40) have Grk protein dispersed throughout the oocyte cytoplasm (Fig. 5I). Grk protein is also observed in rings around the nurse cell nuclei in these egg chambers, indicating that overexpression of Syp leads to abnormal translation of unlocalised *grk*. We interpret these results as indicating that the overexpression of Syp produces a neomorphic phenotype, for example by displacement of essential translational repressors (see discussion). Western blots of wild-type, *syp* mutant and Syp overexpressing whole ovary lysates, probed with anti-Gurken antibody revealed only subtle and differences in the level of Gurken protein in these lysates (data not shown). *gurken* mRNA is also localised to the posterior of the oocyte and translated in earlier stage egg chambers than those shown, and this does not seem to be affected by reducing or increasing the levels of Syp (data not shown). We believe that Syp affects Gurken protein levels only at later stages and such changes, while easily visible by immunostaining, are very difficult to detect when using whole ovary lysates (with all stages averaged), especially if the phenotype is not completely penetrant.

In wild-type oocytes, localised *osk* mRNA and Osk protein form a tight crescent at the posterior cortex at stage 10 of oogenesis (Fig. 5J). In 45% (n=10/22) syp^{e00286} oocytes *osk* mRNA and Osk protein colocalise in a highly diffuse cytoplasmic distribution at the posterior, and in some cases are found in a dot near the posterior pole (Fig. 5K). Through stage 9, the distribution of *osk* mRNA in syp^{e00286} oocytes was comparable to that in wild-type oocytes (100%; n=14/14) (data

Table 2. Homozygotes and transheterozygotes of syp^{f03775} and syp^{e00286} alleles show highly reduced adult viability in combination with each other and with the deficiency (Df) mutation *Df(3R)BSC124*. Viability is restored by precise excision of the syp^{e00286} insertion ($syp^{e00286+}$).

Genotype	n	% Viability
$syp^{f03775}/syp^{f03775}$	703	10
syp^{f03775}/Df(3R)BSC124	429	2
$syp^{f03775}/syp^{e00286}$	195	3
$syp^{e00286}/syp^{e00286}$	730	12
syp^{e00286}/Df(3R)BSC124	400	2
$syp^{e00286+}/syp^{e00286}$	332	100
$syp^{e00286+}$/Df(3R)BSC124	356	100

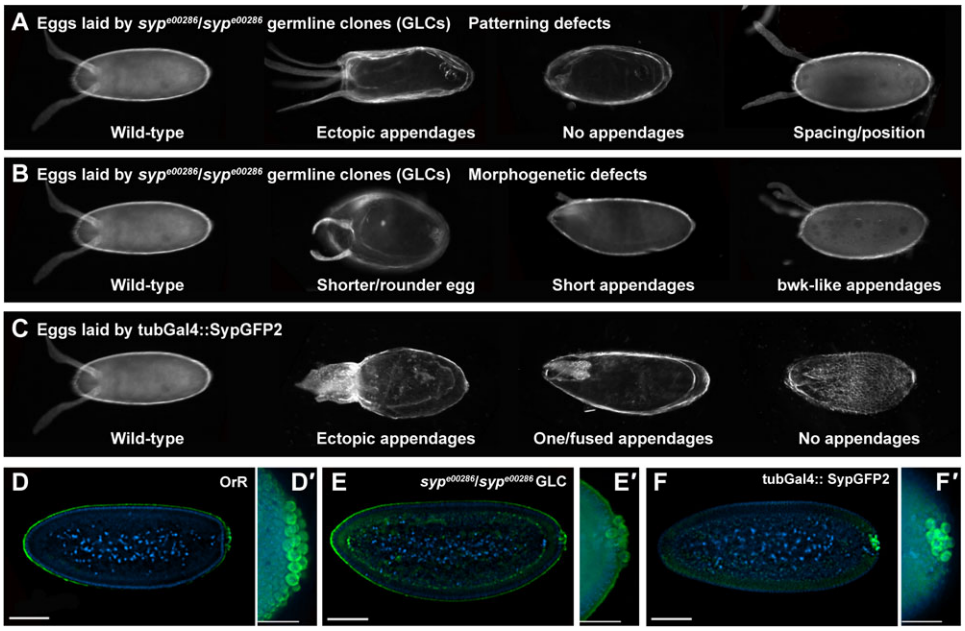

Fig. 4. Altering the level of Syp in the germline results in *grk* and *osk* phenotypes. (**A**–**C**) The range of dorso-ventral patterning and morphogenetic phenotypes of eggs laid by females with a syp^{e00286} mutant or SypGFP2 overexpressing germline. 'bwk-like appendages' refers to a dorsal appendage phenotype resembling that observed in *bullwinkle* mutants. See also Table 3. (**D**–**F**) Cellular blastoderm embryos collected from wild-type (OrR) females and females with a syp^{e00286} mutant or SypGFP2 overexpressing germline were stained with anti-Vasa antibody (green) to visualize pole cells. Many embryos from mutant germline and overexpressing females have a reduced number of Vasa-positive pole cells. Scale bars 40 µm. See also Table 4.

not shown), indicating that Syp is not necessary for the initial transport of the mRNA to the posterior. This suggests that the diffuse distribution in stage 10 syp^{e00286} oocytes is due to defective posterior anchoring of *osk* mRNA and Osk protein. Overexpression of Syp results in weak *osk* mRNA localisation in 43% (n=6/14) oocytes, with a corresponding decrease in localised Osk protein (Fig. 5L). In a further 43% of these oocytes, *osk* mRNA and Osk protein are not detectable by *in situ* hybridization and immunostaining. We conclude that the precise level of Syp in the germline is important for the correct localisation and anchoring of *grk* and *osk* mRNAs respectively, and for the translation of Grk and Osk proteins. We interpret the effect of *syp* mutations and Syp overexpression on *osk* mRNA anchoring as being due to an indirect effect of reducing Osk protein levels, as Osk is known to be required for anchoring of *osk* mRNA, and that the primary requirement for Syp in *osk* mRNA function is in its translational regulation (Vanzo and Ephrussi, 2002).

Discussion

Using GRNA chromatography we have identified *Drosophila* Syp as a novel conserved component of localised RNP granules (Fig. 6). We show that Syp associates specifically with *grk* and *osk* and is required for their localisation and translational

Table 3. Quantitation of *syp* germline clone and SypGFP overexpression eggshell phenotypes. The range of phenotypes observed suggests that Syp plays a number of roles during oogenesis. '*bwk*-like' refers to a 'moose-antler' dorsal appendage phenotype resembling that observed in *bullwinkle* mutants, indicating that Syp may have a number of mRNA targets that regulate follicle cell migration.

% eggshell phenotype									
Maternal genotype	WT	Ectopic appendages	One/fused appendages	No appendages	Spacing/ position	Shorter/ rounder egg	Short appendages	*bwk*-like	cup-like eggs
OrR n=600	100	0	0	0	0	0	0	0	0
syp^{e00286}/TM3 n=152	90	0	0	3	0	0	4	3	0
$syp^{e00286}/syp^{e00286}$ germline clones n=474	57.2	2.5	3.6	7.4	7.4	4	8	9.7	0.2
tubGAL4/TM3 n=598	99.7	0	0.3	0	0	0	0	0	0
tubGAL4::SypGFP n=279	4.3	69.6	16.8	9.3	0	0	0	0	0
tubGAL4::SypGFP2 n=123	15.4	81.3	3.3	0	0	0	0	0	0

Table 4. Effects of *syp* mutation and SypGFP overexpression on Vasa-positive pole cell numbers in progeny embryos. The counts are reported as mean ± standard deviation. Statistical significance was calculated using Welch's t test.

Maternal genotype	Number of embryos examined	Vasa-positive pole cell number	*P*
OrR	42	33.0±6.6	
$syp^{e00286}/syp^{e00286}$ germline clones	40	25.8±9.1	<0.0001
tubGAL4::SypGFP	14	15.0±5.3	<0.0001
tubGAL4::SypGFP2	20	17.2±7.0	<0.0001

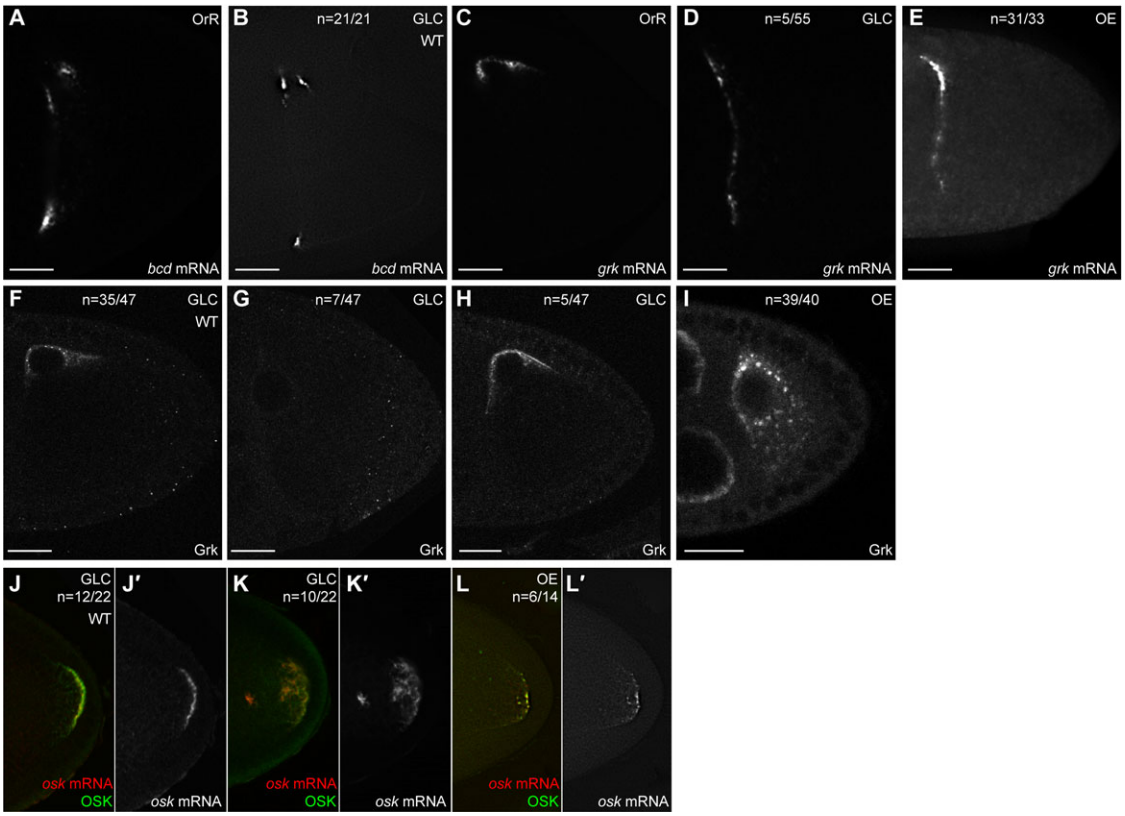

Fig. 5. Syp modulates *grk* and *osk* mRNA localisation and translation. (**A**–**E**) *In situ* hybridization using a *grk* or *bcd* probe was carried out on wild-type (OrR), *syp*^{e00286} germline clone (GLC) or SypGFP2 overexpressing (OE) egg chambers. WT indicates a wild-type pattern of mRNA localisation. Scale bar 20 µm. (**F**–**I**) *syp*^{e00286} germline clone (GLC) or SypGFP2 overexpressing (OE) egg chambers were stained with anti-Grk antibodies. WT indicates a wild-type distribution of Grk protein. Scale bar 20 µm. (**J**–**L**) *In situ* hybridization using an *osk* probe was carried out on *syp*^{e00286} germline clone (GLC) and SypGFP2 overexpressing (OE) egg chambers that were also stained with anti-Osk antibodies. WT indicates a wild-type distribution of *osk* mRNA and Osk protein.

regulation in the *Drosophila* germline. Although SYNCRIP has been studied in mammalian cells using biochemical approaches, our study is the first to address the function of Syp *in vivo*, and particularly in generating cellular asymmetry in the germline. Despite a number of genetic screens that have been carried out to identify the genes required for axis specification in flies, Syp was not previously identified as being required for axis determination. Our work together with a number of other biochemical based studies (Arn et al., 2003) illustrates that there are many other essential factors that are still to be identified as having a role in axis specification.

We present evidence in this study that Syp is required for axis specification and germline formation by affecting the localisation and translation of *grk* and *osk* mRNAs. The phenotypes of loss of function mutations in the gene, and overexpression of the protein support an interesting role for Syp in regulating *grk* and *osk* mRNAs. We note that the loss of function phenotypes are of low penetrance and are such that further studies are required to uncover the precise mechanism of Syp function. However, Syp is not the only component of *grk* and *osk* mRNPs that has a partially penetrant loss of function phenotype. Indeed, others such as Imp give a stronger phenotype only when in combination with other mutations (Geng and Macdonald, 2006). Unexpectedly, overexpression of Syp gives the same phenotype as its loss of function, but at a higher penetrance. These results are potentially interesting, but difficult to interpret with certainty. We favour the interpretation that a certain stoichiometry is necessary within the RNP complexes in which Syp is found. Overexpression of Syp may cause the displacement of certain translational repressors, allowing *grk* mRNA to be prematurely translated in the nurse

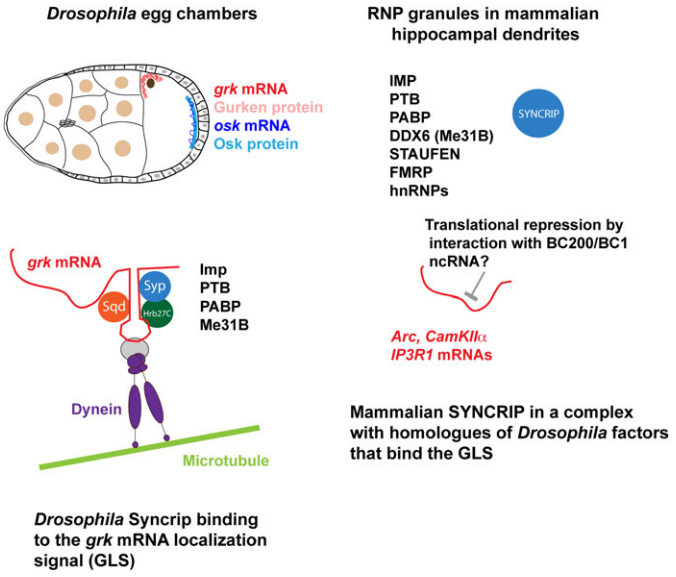

Fig. 6. Syncrip is a conserved regulator of mRNA localisation and translational repression in *Drosophila* oocytes, as well as mammalian hippocampal dendrites.

cells. In contrast, with loss of function of Syp, *grk* mRNA localisation and translational regulation may be disrupted in different ways because loss of Syp could lead to decreased stability of the RNP complex. This could in turn lead to the loss of certain components necessary for localization and translational regulation. The eggshell phenotypes we observe in the *syp* germline clones also include some defects that are not typical of a disruption of *grk* mRNA localisation and translation. We interpret these results as indicating that Syp has additional target mRNAs in the germline whose localisation and/or translation are affected in the *syp* mutant. On the basis of the morphological defects we observe in a number of eggs these targets may include mRNAs involved in follicle cell migration or actin organisation during oogenesis, such as *bullwinkle* (*bwk*) or *chickadee* (*chic*). Mislocalisation and/or altered translation of these mRNAs could result in the shorter eggs and the *bwk*-like dorsal appendage defects that are observed (Cooley et al., 1992; Rittenhouse and Berg, 1995; Dorman et al., 2004).

The association of Syp with these mRNAs in GRNA and RNA immunoprecipitation experiments further supports a function for Syp in the regulation of *grk* and *osk* mRNAs, although it is unclear whether this is through a direct or indirect interaction. Syp does not appear to colocalise with localised *grk* or *osk* in the oocyte, and so Syp may function before the mRNAs reach their final destination in the oocyte in order to influence localisation and translation. Syp was also identified biochemically with a number of the factors already known to be required for *grk* and *osk* mRNA localisation and translational regulation (Table 1; supplementary material Table S2). These include Sqd (Kelley, 1993; Norvell et al., 1999; Norvell et al., 2005; Delanoue et al., 2007; Cáceres and Nilson, 2009), Imp (Geng and Macdonald, 2006), PTB (Besse et al., 2009), PABP (Clouse et al., 2008), Me31B (Nakamura et al., 2001) and BicC (Saffman et al., 1998). The homologues of these factors were also identified in biochemical studies of SYNCRIP interactors in mammalian cells (Bannai et al., 2004; Kanai et al., 2004; Elvira et al., 2006). Therefore, we propose that our results have uncovered a conserved module of RNA binding proteins that are required for both mRNA transport and translational regulation. The Syp associated complex that we have uncovered binds to *grk* via a relatively small stem loop sequence, the GLS. While Syp also associates with *osk* mRNA, the minimal region necessary and sufficient for *osk* mRNA localisation has not yet been defined. Therefore, it is unknown whether a small region is required in this case, and if so whether it is in any way similar to the GLS, structurally or in primary sequence.

Taking the results of this study in the context of our other data and the previous publications on SYNCRIP and its associated proteins in mammalian hippocampal neurons, we propose that Syp may be present at neuronal synapses in a complex with at least some of the same proteins that are required for *grk* mRNA localisation. This idea is supported by the fact that at least some of these factors, namely Sqd (Flyprot annotation), Imp (Boylan et al., 2008; Adolph et al., 2009), PTB (Davis et al., 2002), PABP (Sigrist et al., 2000) and Me31B (Barbee et al., 2006) are also present in the *Drosophila* nervous system. Our expression studies show that Syp is absent from embryos but is highly expressed in the larval nervous system. Given the role of Syp in mRNA localization and translational regulation in the oocyte, and its presence in larval brains, we propose that Syp could have a similar function in the nervous system. In comparison with the oocyte, much less is known about localised transcripts and their translational regulation in the nervous system, and it remains to be determined to what extent this proposal is valid and in which neuronal tissues Syp is required in larvae. Nevertheless, our work is the first demonstration that Syp functions in mRNA localisation and translational control in the oocyte and coupled with the work on mammalian SYNCRIP showing association with RNP granules in the dendrites of hippocampal neurons (Bannai et al., 2004; Kanai et al., 2004; Elvira et al., 2006) it is attractive to propose that Syp protein also has a conserved function in regulation of neuronal mRNAs.

Materials and Methods

Genetics

Stocks were raised on standard cornmeal-agar medium at 25℃. The wild-type control strain was Oregon R (OrR). The allele syp^{e00286} is the PBac{RB}CG17838^{e00286} insertion line and syp^{f03775} is the PBac{WH}f03775 insertion line (Exelixis Collection at Harvard). Germline clones of syp^{e00286} were generated using the FLP/FRT dominant female sterile technique (Chou and Perrimon, 1992). syp^{e00286} was recombined with P{ry$^+$, FRT}82B (Bloomington Stock Center) and yw P{w=hs-FLP}122; P{ry$^+$, FRT}82B syp^{e00286}/TM3 sb was generated. Females were mated to P{ry$^+$, FRT}82B P{w$^+$ovoD1}/TM3 sb males and the progeny third instar larvae were heat shocked at 37℃ for 2 h each day for 3 days. syp^{e00286}/ovo^{D1} females were collected and mated to OrR males. A precise excision of syp^{e00286} was generated and called $syp^{e00286+}$. *syp* mutant third instar larvae were $syp^{e00286}/Df(3R)BSC124$ (*Df(3R)BSC124;* Bloomington Deletion Project, Bloomington Stock Centre). Gal4 drivers were actinGal4/TM6B, Tb[1] and tubulinGal4 (tubGal4; germline driver). *syp*RNAi lines were stocks 33011 and 33012 (Vienna Drosophila RNAi Center). SquidGFP was a GFP protein trap line (A. Debec, unpublished).

The *syp-F* coding region was amplified by PCR (Expand High Fidelity PCR system, Roche) from cDNA prepared using total OrR ovarian mRNA, Superscript III Reverse Transcriptase (RT) and oligo(dT)20 primers. Syp cDNA was tagged C-terminally with GFP (from vector KS-GFP from A. Vincent). This construct was cloned into pUASp for germline expression. Several independent transgenic lines were obtained following transformation with this plasmid. UASp-SypGFP (chromosome II) and UASp-SypGFP2 (chromosome III) were used in this study, driven by tubGal4. UASp-SypGFP2 was more highly expressed and gave more severe phenotypes in the germline, and so was used for immunostaining and *in situ* hybridization in ovaries.

Eggshell preparation

Axis specification defects were studied in eggs from *syp* mutant germline clones and flies overexpressing tubGal4::SypGFP. For eggshell preparation, freshly laid eggs were mounted in a 1:1 mixture of lactic acid:Hoyer's medium and incubated overnight at 65℃.

Antibodies

Polyclonal anti-Hrb27C rabbit peptide antibody that was affinity purified by Eurogentec using two peptides; 1: N-CRTGPGNSASKSGSEY-C and 2: N-EGASNYGAGPRSAYGNC-C, which correspond to nonconserved regions in the C-terminal Glycine-rich domain (Huynh et al., 2004). Polyclonal guinea-pig anti-Syp antibodies (Eurogentec) were raised against GST-Syp (isoform F) that had been expressed in *E. coli* and purified with Glutathione Sepharose (GE Healthcare Life Sciences). This anti-Syp antibody gives particularly good results in western blotting and immunoprecipitation experiments. However, it gives a poor signal-to-noise ratio in immunostaining experiments. All antibodies used in this study are listed in supplementary material Table S3.

GRNA affinity chromatography and mass spectrometry

Complexes formed on GLS-containing RNA were purified according to a protocol developed by Czaplinski et al. (Czaplinski et al., 2005). 50 µl of a 50% slurry of Glutathione Sepharose (GE Healthcare Life Sciences) beads in GRNA buffer (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 1.5 mM MgSO4, 2 mM DTT, 0.05% NP-40, 10% glycerol and Complete EDTA-free protease inhibitor (Roche)), 30 µg of GST-λN fusion protein (Czaplinski et al., 2005) and 200 pmol of the indicated RNA in a total volume of 400 µl were incubated at 4℃ with shaking for 1 h. The beads were collected by centrifugation (always for 1 min at 3000 rpm) and the supernatant removed. 200 µl 10 mg/ml precleared ovary extract and 300 µl buffer, plus 0.1 mg/ml *E. coli* tRNA and 1µg/ml heparin, were added to the RNA matrix and the mixture incubated at 4℃ with shaking for 1 h. Beads were collected and washed end over end 3 times for 5 min with 1 ml GRNA buffer. After washing, the supernatant was removed and total material was eluted with 100 µl elution

buffer (0.1% SDS, 2.5 mM Tris pH 6.8 and 5 mM DTT) at room temperature. Eluates were concentrated by TCA precipitation and analyzed by SDS-PAGE and silver staining.Gels were ran a short distance to allow the resolution of GST-ëN so that all proteins heavier than GST-ëN could be excised in a minimum number of fractions. Processing of the gel, trypsin digestion and mass spectrometry was carried out by J. R.

Immunoprecipitation

Guinea-pig anti-Syp, rabbit anti-GFP (Invitrogen) and control pre-immune sera were cross-linked to Protein A Sepharose using standard protocols, and the beads equilibrated in IP buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% NP-40, 10% glycerol and Complete EDTA-free protease inhibitor). 20 µl of a 50% slurry was added to 500 µg precleared lysate, and the mixture incubated overnight at 4℃ with mixing. The beads were then rinsed once with cold IP buffer and washed four times for 5 min each at 4℃. After the final wash, the beads were resuspended in 25µl protein sample buffer (Invitrogen), boiled for 5 min, and the supernatant loaded onto a gel for SDS-PAGE and Western blotting. For RNase treatment, the incubation and wash steps were carried out in the presence of 200 µg/ml RNase A or 0.12 U/µl RNasin Ribonuclease Inhibitor (Promega).

For RNA immunoprecipitation all steps were carried out in the presence of RNasin and immunocomplexes were eluted from the beads by the addition of 100 µl extraction buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA and 1.3% SDS) plus 100 U RNasin and incubation for 30 min at 65℃. Following centrifugation, the elution step was repeated and the two supernatants combined. RNA was extracted from input samples and immunoprecipitates using Trizol LS reagent (Invitrogen). Following DNase (Ambion) treatment, immunoprecipitated RNA and 10% total RNA were then used as a template for cDNA synthesis in combination with SuperScript III (Invitrogen) RT and random hexamer primers (Invitrogen). cDNA was then used directly as a template for real time PCR.

Real time quantitative PCR

Real time PCR was carried out using iQ SYBR Green Supermix (Bio-Rad Laboratories) with a Qiagen Rotor-Gene Q according to manufacturer's instructions. 10% of the RT product was used as a template for real time PCR analysis. Cycle threshold ($C_{(T)}$) values were determined by the second differential maximum method as calculated by the Rotor-Gene software. Calculation of relative mRNA levels was done by using the 2 [$-\Delta\Delta C_{(T)}$] method (Livak and Schmittgen, 2001), where the $C_{(T)}$ values of the mRNA level were normalized to the $C_{(T)}$ values of *rp49* mRNA in the same sample and mRNA levels represented as relative fold change over control. $C_{(T)}$ values used were the means of triplicate repeats. Experiments were repeated three times. RNA immunoprecipitation data was also analyzed using the comparative $C_{(T)}$ method, factoring in the 10-fold difference in RNA used for input and immunoprecipitation. For each mRNA and each experimental condition, results are expressed as a percentage of the total input RNA. All primers used in this study are listed in supplementary material Table S4.

Western blotting

Following electrophoresis, proteins were transferred to nitrocellulose (Schleicher and Schuell), or to Polyvinylidene Fluoride (PVDF) (Bio-Rad) membranes using the XCell II Blot Module (Invitrogen) according to the manufacturer's instructions. Western blotting was performed using standard protocols. Visualization of reactive proteins was performed by enhanced chemiluminescence and quantitative infrared imaging (LI-COR Odyssey, LI-COR Biosciences; Lincoln, NE). Intensity of protein bands was quantified using NIH ImageJ and LI-COR software.

Immunohistochemistry and RNA *in situ* hybridization

Ovaries were prepared for immunostaining and *in situ* hybridization as previously described (Wilkie et al., 1999; MacDougall et al., 2003).

Imaging and deconvolution

A Leica SP5 confocal microscope and a widefield DeltaVision microscope (Applied Precision, Olympus IX70, and Roper Coolsnap HQ) were used to image fixed material. DeltaVision images were acquired with Olympus 20 × /0.75, 40 × / 0.95, 60 × /0.9 or 100 × /1.4 objective lenses and then deconvolved (Davis, 2000).

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Competing Interests

The authors declare that there are no competing interests.

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