Interaction with eIF5B is essential for Vasa function during development

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Summary

The DEAD-box RNA helicase Vasa (Vas) is required for germ cell development and function, as well as for embryonic somatic posterior patterning. Vas interacts with the general translation initiation factor eIF5B (cIF2, also known as dIF2), and thus may regulate translation of specific mRNAs. In order to investigate which functions of Vas are related to translational control, we have analyzed the effects of site-directed *vas* mutations that reduce or eliminate interaction with eIF5B. Reduction in Vas-eIF5B interaction during oogenesis leads to female sterility, with phenotypes similar to a *vas* null mutation. Accumulation of Gurken (Grk) protein is greatly reduced when Vas-eIF5B interaction is reduced, suggesting that this interaction is crucial for translational regulation of *grk*. In addition, we show that reduction in Vas-eIF5B interaction virtually abolishes germ cell formation in embryos, while producing a less severe effect on somatic posterior patterning. We conclude that interaction with the general translation factor eIF5B is essential for Vas function during development.

Key words: Translation, Germ cells, DEAD-box, Axis-patterning, Gurken, *Drosophila*, cIF2, Vasa

Introduction

Early *Drosophila* development is orchestrated by maternal RNAs and proteins stored within the oocyte and embryo. Coordinated translational regulation and cytoplasmic localization of specific maternal transcripts determine developmental decisions such as axis patterning and germ cell specification (reviewed by Johnstone and Lasko, 2001). During oogenesis, the TGFα signaling molecule Gurken (Grk) establishes polarity within the oocyte along both the anteroposterior and dorsoventral axes (Neuman-Silberberg and Schüpbach, 1993; González-Reyes et al., 1995; Roth et al., 1995) (reviewed by Nilson and Schüpbach, 1999). Subsequently, at the posterior end of the oocyte and early embryo, a specialized region of cytoplasm, called pole plasm, accumulates RNAs and proteins required for germline development (reviewed by Mahowald, 2001). Pole plasm assembly is initiated through the localized translation of *oskar* (*osk*) mRNA (Ephrussi et al., 1991; Kim-Ha et al., 1991). Vasa (Vas) protein and *nanos* (*nos*) mRNA are among several molecules that accumulate in the pole plasm downstream of Osk. Localized translation of *nos* mRNA in the pole plasm produces a Nos protein gradient that is essential to determine abdominal fate, thus directly linking germ cell development to posterior somatic patterning in the embryo (Lehmann and Nüsslein-Volhard, 1991; Wang and Lehmann, 1991).

osk, *grk* and *nos* RNAs are all under complex translational regulation in the developing oocyte, mediated through cis-acting elements in their untranslated regions (UTRs). Genetic and biochemical analyses in *Drosophila* ovaries and embryos have identified several translational regulatory proteins. Bruno (Bru) is involved in repressing translation of *osk* and *grk* (Kim-Ha et al., 1995; Webster et al., 1997; Filardo and Ephrussi, 2003; Nakamura et al., 2004), and Smaug (Smg) is involved in repressing translation of *nos* (Smibert et al., 1996; Dahanukar et al., 1999; Nelson et al., 2004). Although recent work has linked both Bru and Smg to the cap-binding step of translation initiation (Wilhelm et al., 2003; Nakamura et al., 2004; Nelson et al., 2004), translational repression of *osk*, *grk* and *nos* probably targets multiple steps of translation (Lie and Macdonald, 1999; Clark et al., 2000; Nakamura et al., 2004). In general, details of the mechanisms of translational derepression and activation for specific transcripts remain obscure. In several organisms, translational activation of maternal mRNAs involves cytoplasmic polyadenylation. The activity of the *Drosophila* cytoplasmic polyadenylation element-binding protein, called oo18 RNA-binding protein or Orb (Lantz et al., 1992) is implicated in activating translation of *osk* and possibly of other mRNAs (Chang et al., 1999; Castagnetti and Ephrussi, 2003).

Our work addresses the function of the highly conserved DEAD-box RNA helicase Vas, which is required for the progression of oogenesis and for pole plasm assembly (Schüpbach and Wieschaus, 1986; Hay et al., 1988; Lasko and Ashburner, 1988; Liang et al., 1994). Based on sequence similarity with yeast Ded1p (reviewed by Linder, 2003), and the finding that expression of several proteins is reduced in *vas* mutants, Vas has been suggested to function in translational regulation (reviewed by Johnstone and Lasko, 2001). Females bearing hypomorphic *vas* mutations complete oogenesis but produce embryos lacking germ cells and lacking posterior

segments, indicating an essential role for *vas* in both processes (Schüpbach and Wieschaus, 1986). An earlier function for Vas, during oogenesis, was also demonstrated through the study of null mutations that are viable but produce no embryos (Styhler et al., 1998; Tomancak et al., 1998). Although *vas*-null oocytes display minimal disruption of *grk* RNA accumulation, Grk protein levels are severely reduced, leading to the hypothesis that Vas could play a role in *grk* translational control (Styhler et al., 1998; Tomancak et al., 1998). Vas also appears to represent an important link between meiotic cell cycle progression and developmental events such as establishment of polarity. In response to a meiotic checkpoint, activated by a delay in DNA double-strand break (DSB) repair during oogenesis, Vas is post-translationally modified, and this corresponds to a downregulation in Grk protein accumulation (Ghabrial and Schüpbach, 1999).

In previous work, we identified a translation factor dIF2, now called eIF5B, as a Vas-binding protein (Carrera et al., 2000). A genetic interaction between null alleles of *dIF2* and *vas* suggested a functional link between these two proteins. eIF5B/dIF2 has since been demonstrated in mammalian systems to be required for all cellular translation and to act at the 60S ribosomal subunit joining step of translation initiation (Pestova et al., 2000b). Subsequent work has indicated that translation can be regulated at the stage of subunit joining (Ostareck et al., 2001; Searfoss et al., 2001). These results suggest that Vas could function as a translational regulator of specific mRNAs through interaction with eIF5B.

To test this hypothesis, we created specific *vas* mutations that severely reduce its interaction with eIF5B. These mutant forms of Vas still localize correctly, allowing us to investigate which developmental functions of Vas require an interaction with eIF5B, and are therefore likely to involve a translational regulatory role. We found that the Vas-eIF5B interaction was essential for the progression of oogenesis, and for normal expression of Grk. In addition, we found that the interaction between Vas and eIF5B was crucial for germ cell specification, but we observed a much less stringent requirement for this interaction in posterior somatic segmentation. We conclude that interaction with eIF5B is essential for Vas function, and propose that Vas achieves translational regulation in the germline through eIF5B binding. The Vas-eIF5B interaction represents a significant opportunity to investigate how a tissue-specific regulator may control the ribosomal subunit joining step of translation initiation to activate the translation of specific transcripts.

Materials and methods

Plasmid construction and mutagenesis

Amino acid reference numbers used are based on the Vas protein predicted by the genomic sequence. The *vas*-coding region was PCR amplified from the original *vas* cDNA that lacks one copy of a 39 nucleotide tandem repeat, encoding amino acids 141-153 (Lasko and Ashburner, 1988), and inserted into *Xho*I/*Not*I digested pBluescript to use as a template for mutagenesis. Specific deletions in *vas* were generated using PCR-mediated mutagenesis, using the following primers: Δ616-618, 5′ TTTCTACGCACCTGTGGTGCC and 3′ AGTCTGGCCAGATCCCTCCAAG; Δ616, 5′ CCGGACTTTCTACGCACCTGTG and 3′ (same as for Δ616-618); Δ617, 5′ GACTTTCTACGCACCTGTGGTG and 3′ AACAGTCTGGCCAGATCCCTC; Δ618, 5′ (same as for Δ616-618) and 3′ CGGAACAGTCTGGCCAGATCC. Mutagenesis was followed by blunt-end ligation, and was verified by sequencing. Each *vas*-coding region was then digested out of pBluescript using *Xho*I and *Not*I, and subcloned into a *Xho*I/*Not*I digested plasmid *P[w*$^{+}$ *Pvas-gfp]* (Nakamura et al., 2001), derived from pCaSpeR2. In addition, each *vas*-coding region was PCR amplified out of pBluescript and subcloned into a *Nco*I/*Xho*I digested pEG202 vector, used for expression in yeast.

Yeast interaction trap assays

The yeast strain EGY48 was co-transformed with 'bait' constructs cloned in pEG202, 'prey' constructs cloned in pJG4-5, and a *lacZ* reporter plasmid pSH18-34. β-Galactosidase activity was monitored using a plate-based assay as described previously (Golemis et al., 1997) and in liquid culture (Reynolds et al., 1997).

Protein expression and western blotting

Preparation of yeast protein extracts was performed according to the Yeast Protocols Handbook (Clontech). For every transformed strain, a 5 ml overnight culture in selective media was used to inoculate a 50 ml culture in YPD media, incubated at 30°C with shaking (220-250 rpm) until OD_{600}=0.4-0.6. Cells were centrifuged at 1000 ***g*** for 5 minutes at 4°C, resuspended in 50 ml cold H_2O, centrifuged at 1000 ***g*** for 5 minutes at 4°C and frozen in liquid nitrogen. Pellets were thawed in pre-warmed Cracking Buffer (8 M Urea, 5% SDS, 40 mM Tris-HCl [pH6.8], 0.1 mM EDTA, 0.4 mg/ml Bromophenol Blue), supplemented with 1 mM PMSF, 1 × protease inhibitor cocktail (Roche Diagnostics) and 10 μl β-mercaptoethanol/ml buffer. Samples were transferred into microcentrifuge tubes containing glass beads, and heated at 70°C for 10 minutes. They were then vortexed for 1 minute, and centrifuged at 20,000 ***g*** for 5 minutes at 4°C. Supernatants were kept on ice while pellets were boiled at 100°C for 3-5 minutes, vortexed for 1 minute and centrifuged at 20,000 ***g*** for 5 minutes at 4°C, and then combined with the first supernatants. *Drosophila* ovarian proteins were extracted by homogenization in phosphate-buffered saline (PBS)/1 mM PMSF/1 × protease inhibitor cocktail (Roche Diagnostics). Samples were centrifuged at 18,000 ***g*** for 15 minutes at 4°C, and supernatants were combined with SDS loading buffer. For western blotting, proteins were resolved on SDS-PAGE gels and transferred onto nitrocellulose membranes, blocked overnight at 4°C in PBS/2% skim milk/0.05% Tween-20 (PBSTM). Membranes were incubated for 1 hour at room temperature with primary antibodies diluted in PBSTM, washed with PBSTM, then incubated for 1 hour at room temperature with HRP-conjugated secondary antibodies (Amersham Pharmacia) diluted 1:5000 in PBSTM. Membranes were washed with PBSTM and proteins were detected by chemiluminescence (NEN). Rabbit anti-Vas was used at 1:5000. Mouse anti-actin (ICN Biomedicals) was used at 1:5000. Mouse anti-α-Tubulin (Sigma) was used at 1:5000. Rabbit anti-4E-BP was used at 1:2000.

Immunohistochemistry and in situ hybridization

Immunostaining of ovaries and embryos with rabbit anti-Nos (1:1000), rabbit anti-Osk (1:500), rabbit anti-Tud (1:250) and rat anti-Vas (1:2000) was performed as described previously (Kobayashi et al., 1999). Fluorescent antibody staining was detected using goat anti-rabbit Alexa^{546nm}, anti-rat Alexa^{633nm} and anti-mouse Alexa^{568nm} secondary antibodies (Molecular Probes). Immunostaining with mouse anti-Grk (1:10) in ovaries was performed as follows: ovaries were dissected in PBST (PBS/0.3% Triton) and fixed for 20 minutes in 200 μl of 4% formaldehyde/PBS + 600 μl heptane. Samples were rinsed with PBST, and blocked for 1 hour in PBS/1.0% Triton/3% BSA. Samples were incubated with primary antibodies diluted in PBST for 1 hour at room temperature, rinsed and then washed overnight in PBST. Samples were then incubated with secondary antibodies for 1 hour at room temperature, washed in PBST, incubated

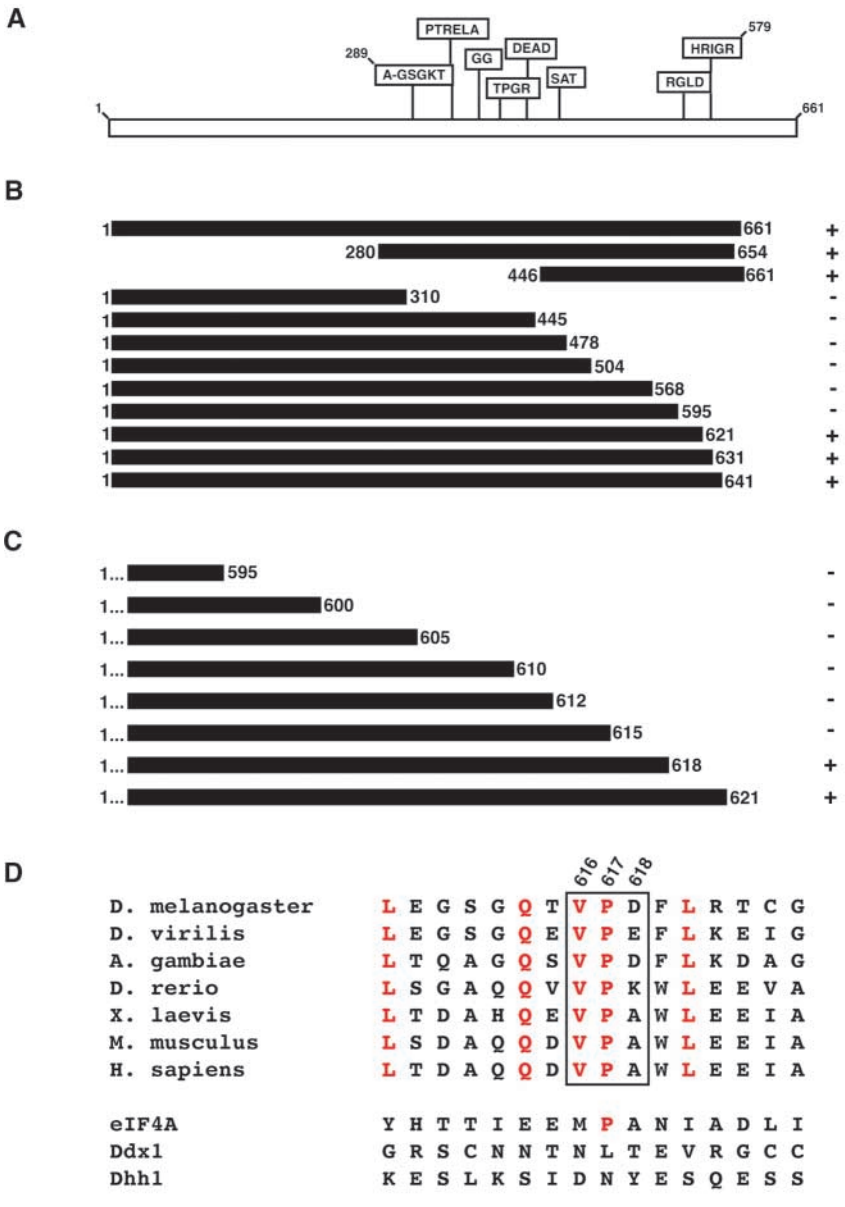

Fig. 1. The Vas C terminus is required for eIF5B interaction. (A) Schematic of Vas, showing motifs characteristic of DEAD-box proteins. (B) Deletions in Vas were tested against eIF5B for direct interaction in the yeast two-hybrid system, using a plate-based assay for β-galactosidase activity. Four replicates were tested for each sample, and were compared to a positive control (full-length Vas, 1-661) and a negative control (Vas 1-310). Interaction with eIF5B is indicated by +, and absence of interaction by –. (C) Further deletions in Vas, ending between residues 595 and 621, were tested against eIF5B in the same system. (D) The region surrounding residues V616, P617 and D618 of Vas (indicated by a box), was compared with homologous proteins in other species, and with more distantly related *Drosophila* DEAD-box proteins (eIF4A, Ddx1 and Dhh1). Residues that are identical between homologous proteins are shown in red.

for 20 minutes in 0.5 μg/ml DAPI, washed in PBST and then mounted in 70% glycerol/PBS. In situ hybridization was performed as described previously (Kobayashi et al., 1999), except that DMSO was omitted during fixation, and PBS/0.1% Tween-20 was used instead of MAB throughout the protocol.

Fly strains and techniques

yw flies were used for P element-mediated germline transformation. *vas* alleles used for subsequent analyses were vas^{PD} (Schüpbach and Wieschaus, 1986) and vas^{PH165} (Styhler et al., 1998). To visualize GFP in ovaries, they were dissected in PBS and fixed in 4% formaldehyde/PBS/0.2% Tween-20. Samples were washed in PBS and mounted in 70% glycerol/PBS. For live GFP visualization in embryos, they were collected on a sieve and washed with H_2O, then dechorionated with bleach and washed again with H_2O. Embryos were then mounted in Halocarbon oil (series 400) and examined immediately. For visualization of dorsal appendages, eggs were collected on a sieve and washed with H_2O, then mounted in Hoyer's medium and incubated overnight at 60°C.

Results

The C-terminal region of Vas is required for interaction with eIF5B

Vas contains many well-defined sequence motifs that are conserved among DEAD-box helicases (Fig. 1A) (Linder et al., 1989). Previous work has shown that interaction with eIF5B involved residues C-terminal to amino acid 310 of Vas (Carrera et al., 2000). In order to map more precisely the region of Vas required for interaction with eIF5B, we created a series of large deletions within Vas and tested these in the yeast two-hybrid system against the clone of eIF5B that we previously isolated in a yeast two-hybrid screen (Fig. 1B). We confirmed that the C-terminal half of Vas was required for interaction with eIF5B, and specifically implicated the region from residue 595 to 621 of Vas in eIF5B-interaction. We then tested a series of Vas constructs that began at the N terminus and ended between residues 595 and 621 (Fig. 1C). This analysis implicated residues 616-618 of Vas as being crucial for eIF5B interaction. We examined whether this region of Vas was conserved in homologous proteins in different species (Fig. 1D). Both V616 and P617 are identical among Vas homologs, while D618 does not appear to be conserved. Some neighboring residues are also invariant among Vas homologs. Although a proline corresponding to P617 is found in eIF4A, in general, more distant *Drosophila* DEAD-box proteins such as eIF4A, Ddx1 and Dhh1 are not highly conserved within this area (Fig. 1D). These results suggest that this region of Vas has been conserved within its homologous proteins for a specific function, and is not a general feature of DEAD-box proteins.

Creation of specific *vas* mutations that reduce or abolish eIF5B interaction

We targeted residues 616-618 of Vas to create specific mutations in an attempt to disrupt eIF5B-interaction. We deleted all three residues (Δ616-618), as well as each residue

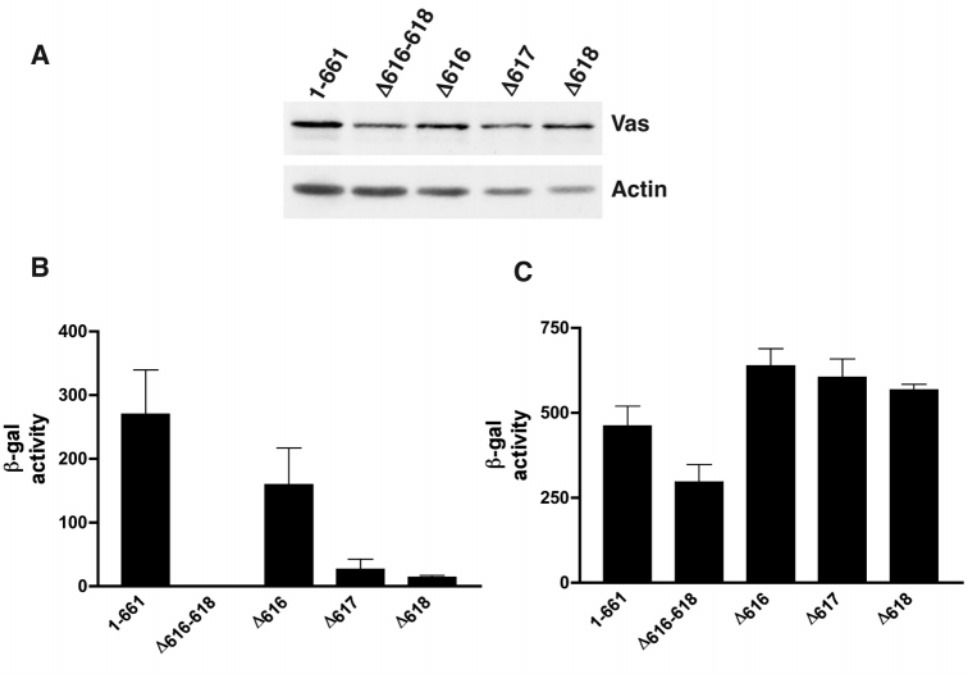

Fig. 2. Deletions within residues 616-618 of Vas impair eIF5B-interaction. (A) Specific Vas deletions, which were transformed into yeast strain EGY48, were tested for their level of protein expression in yeast on a western blot probed with anti-Vas. As a loading control, the blot was also probed with anti-Actin (ICN Biomedicals). (B) Vas deletions were tested for interaction with eIF5B in the yeast two-hybrid system using a liquid assay for β-galactosidase activity. The positive control for interaction is the full-length Vas protein (1-661). The value shown for each sample represents the average of three replicates, and the error bars represent s.d. (C) Vas deletions were also tested for interaction with Osk, using the same system.

individually (Δ616, Δ617 and Δ618), and tested these in the yeast two-hybrid system against eIF5B, using a liquid assay for β-galactosidase activity to quantify the level of interaction. All of these mutant forms of Vas expressed protein efficiently and stably in yeast (Fig. 2A). We found that the Δ616-618 deletion completely eliminated detectable interaction with eIF5B (Fig. 2B). The single amino acid deletions also all showed a reduction in eIF5B-interaction relative to the full-length Vas protein, ~1.7-fold for Δ616, tenfold for Δ617 and 20-fold for Δ618 (Fig. 2B). In order to verify that these mutations in Vas affected eIF5B-interaction specifically and not interaction between Vas and Osk, which is crucial for pole plasm assembly (Breitwieser et al., 1996), we also tested the Vas deletions against Osk in the same assay (Fig. 2C). We found that relative to the full-length Vas protein, Δ616-618 showed a modest (less than twofold) reduction in Osk interaction. The single amino acid deletions, however, showed no reduction of this interaction (Fig. 2C). We conclude that these individual Vas deletions specifically disrupt interaction with eIF5B without affecting the interaction between Vas and Osk. Furthermore, the interaction site of a third known Vas-binding protein, Gustavus (Gus) maps to a region near the N terminus of Vas (Styhler et al., 2002), very distant from the residues we found were crucial for eIF5B interaction. Thus, the *vas* mutations we have generated that affect eIF5B interaction do not affect its interaction with any other known binding protein, and do not affect any conserved motif that is implicated in any function common to DEAD-box proteins, such as ATP binding, ATP hydrolysis, RNA binding and RNA unwinding (Pause and Sonenberg, 1992; Tanner et al., 2003) (reviewed by Tanner and Linder, 2001).

eIF5B interaction is not required for localization of Vas to the pole plasm

During oogenesis, Vas protein is present throughout the cytoplasm of nurse cells and is enriched in nuage particles that are concentrated around the nurse cell nuclei (Lasko and Ashburner, 1990). Within the oocyte, Vas is also distributed throughout the cytoplasm, and in later stages accumulates in the developing posterior pole plasm, where it remains concentrated in the early embryo. The subsequent functions of Vas all require this posterior localization, thus we wanted to establish whether specific mutations that reduce Vas-eIF5B interaction also affected Vas localization. To do this, we expressed GFP-fusions of various forms of Vas in transgenic flies under the control of the endogenous *vas* promoter. We tested the levels of expression of the transgenic fusion proteins on western blots, using endogenous Vas protein as an internal control (Fig. 3). Expression of the wild-type transgenic protein was comparable with that of the endogenous protein (Fig. 3C). Despite generating multiple transgenic lines for each, the Δ616-618, Δ616 and Δ618 proteins were always expressed at lower levels than the wild-type transgenic protein, when each was compared with levels of endogenous Vas (Fig. 3F,I,O). However, the Δ617 transgenic protein was expressed at levels comparable with the wild-type transgenic protein (Fig. 3L).

Using GFP fluorescence, we monitored the localization of the transgenic fusion proteins in ovaries from flies bearing two copies of each transgene in a wild-type background (Fig. 3). We found that in all of the deletion mutants, GFP-Vas protein distribution mimicked that of the endogenous protein, accumulating in the perinuclear region of nurse cells in earlier stages (Fig. 3D,G,J,M), and concentrating in the pole plasm in later stages (Fig. 3E,H,K,N). In flies expressing the Δ616-618 transgenic protein (Fig. 3D,E), the GFP signal was weak because of low protein expression, but even in this case, correct localization could be detected. From these data, we conclude that eIF5B interaction is not required to achieve Vas localization.

To avoid complications resulting from dose effects, we chose to focus the remainder of our analysis on the $vas^{\Delta 617}$ mutation because of its high level of protein expression and the strong

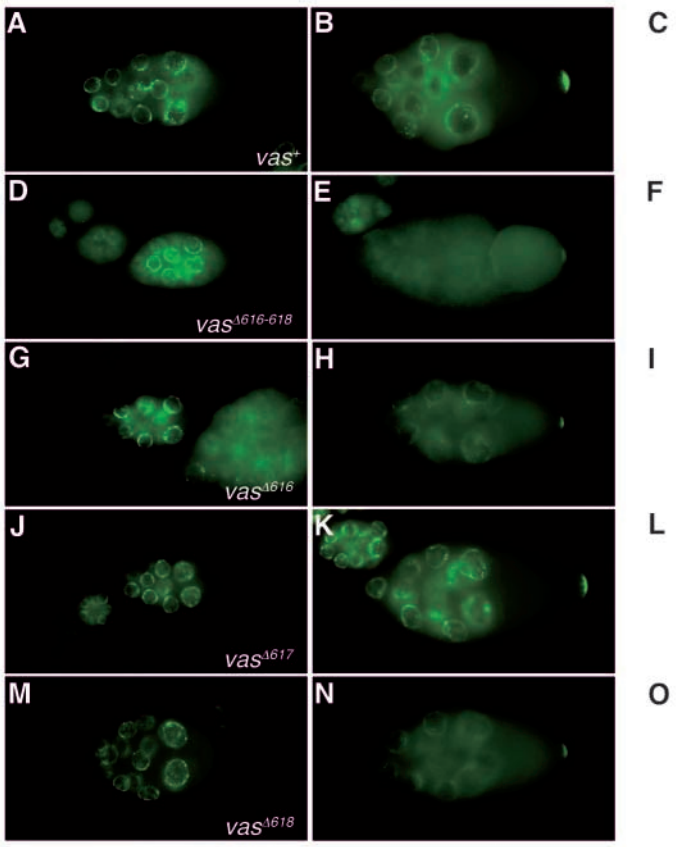

Fig. 3. Localization and expression of transgenic GFP-fusion proteins. For each transgenic genotype, localization of GFP is demonstrated in early and late stages of oogenesis: (A,B) *vas*$^{+}$; (D,E) *vas*$^{\Delta 616\text{-}618}$; (G,H) *vas*$^{\Delta 616}$; (J,K) *vas*$^{\Delta 617}$; (M,N) *vas*$^{\Delta 618}$. Protein expression in ovaries of transgenic flies is demonstrated on western blots probed with anti-Vas. (C) Vas+; (F) VasΔ616-618; (I) VasΔ616; (L) VasΔ617; (O) VasΔ618. Flies contained two copies of each transgene in the wild-type background. For each sample, the faster migrating band represents endogenous Vas and the slower migrating band represents the transgenic GFP-Vas.

effect of this mutation on eIF5B interaction. Comparing the wild-type *vas*$^{+}$ and the *vas*$^{\Delta 617}$ transgenes allowed us to determine the phenotypic consequences of specifically reducing eIF5B interaction, and to separate the functions of Vas that require that interaction from those that depend solely on its localization and expression.

Vas-eIF5B interaction is required for female fertility and for *grk* regulation

In order to investigate the requirement of the Vas-eIF5B interaction during oogenesis, we examined the *vas*$^{\Delta 617}$ transgene in the background of a *vas* null allele, *vas*^{PH165} (Styhler et al., 1998). The wild-type *vas*$^{+}$ transgene, and the *vas*$^{\Delta 617}$ transgene express comparable levels of protein in the *vas* null background (Fig. 4A). Most *vas*^{PH165} egg chambers arrest early in oogenesis, producing few mature eggs, none of which hatches into embryos (Styhler et al., 1998) (Fig. 4B). Strikingly, *vas*^{PH165};P{*vas*$^{\Delta 617}$} females exhibit a very similar arrest in oogenesis to that of *vas*^{PH165} itself, producing few mature eggs that do not hatch (Fig. 4D). In both *vas*^{PH165} and *vas*^{PH165};P{*vas*$^{\Delta 617}$} females of the same age (3-4 days), the number of stage 14 eggs per ovary ranges widely from 0-32 with an average of nine, roughly a third the average of wild-type. Examples of severely atrophied *vas*^{PH165} and *vas*^{PH165};P{*vas*$^{\Delta 617}$} ovaries are shown in Fig. 4B,D. Similar to *vas*^{PH165} (Styhler et al., 1998), eggs produced by *vas*^{PH165};P{*vas*$^{\Delta 617}$} females sometimes exhibit a duplicated micropyle at both the anterior and posterior ends (data not shown). By contrast, ovaries from *vas*^{PH165};P{*vas*$^{+}$} females produce abundant mature eggs that can hatch into viable embryos (Fig. 4C). Thus, the *vas*$^{\Delta 617}$ transgene does not rescue the oogenesis arrest caused by the *vas*^{PH165} mutation, indicating that interaction between Vas and eIF5B is crucial for the progression of oogenesis.

Dorsal appendages provide a sensitive assay for dorsoventral patterning, and in *grk* mutants, ventralization of the eggshell can be observed as a fusion or absence of dorsal appendages. In several *vas* alleles, including *vas*^{PH165}, dorsal appendage defects, indicative of a ventralization phenotype, are observed (Styhler et al., 1998; Tinker et al., 1998; Tomancak et al., 1998). In agreement with previous reports (Styhler et al., 1998), we observed that 63% of eggs produced by *vas*^{PH165} females exhibited one fused or semi-fused dorsal appendage, 12% had no dorsal appendages and 25% had two dorsal appendages. We examined dorsal appendages in our transgenic lines and found that in *vas*^{PH165};P{*vas*$^{\Delta 617}$} females, 67% of eggs exhibited one semi-fused or fused dorsal appendage (Fig. 5C-E), 11% formed no dorsal appendages (Fig. 5F) and 22% had two dorsal appendages (Fig. 5B). Conversely, 82% of control *vas*^{PH165};P{*vas*$^{+}$} eggs had two dorsal appendages (Fig. 5A). Thus, the *vas*$^{\Delta 617}$ transgene does not rescue the ventralization phenotype of the *vas*^{PH165} mutation.

We then assessed *grk* RNA and protein expression in *vas*^{PH165};P{*vas*$^{\Delta 617}$} ovaries, and found that it also was indistinguishable in early stages of development from that observed for *vas*^{PH165} (Fig. 5H,I,K,L) (Styhler et al., 1998). *grk* RNA is strongly enriched in the oocyte (Fig. 5H,I); however, the protein level is severely reduced (Fig. 5K,L). In control *vas*^{PH165};P{*vas*$^{+}$} ovaries, concentration of both *grk* RNA and protein in the oocyte resembles wild type (Fig. 5G,J). Thus, the *vas*$^{\Delta 617}$ transgene does not support efficient expression of Grk in a *vas*-null background. We conclude that the requirements for Vas for the progression of oogenesis, for dorsoventral patterning of the egg chamber, and for *grk* regulation, all rely on its interaction with eIF5B.

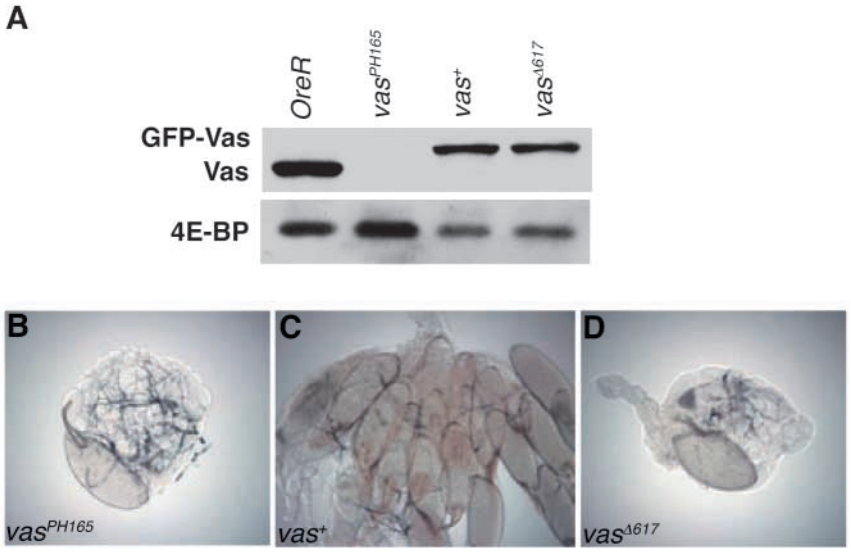

Fig. 4. Vas-eIF5B interaction is required for the progression of oogenesis. (A) Western blot of ovarian extracts from wild-type (OreR), vas^{PH165}, vas^{PH165};P{vas^{+}} and vas^{PH165};P{$vas^{\Delta 617}$} females was probed with anti-Vas. The same blot was probed with anti-4E-BP as a loading control. (B) Ovaries from vas^{PH165} females produce few mature stage 14 eggs owing to a developmental arrest during oogenesis. (C) Expression of a wild-type vas^{+} transgene rescues this defect of the vas^{PH165} mutation, allowing the production of abundant stage14 eggs. (D) Ovaries from vas^{PH165};P{$vas^{\Delta 617}$} females resemble those of vas^{PH165}, exhibiting a similar developmental arrest during oogenesis and producing few mature eggs.

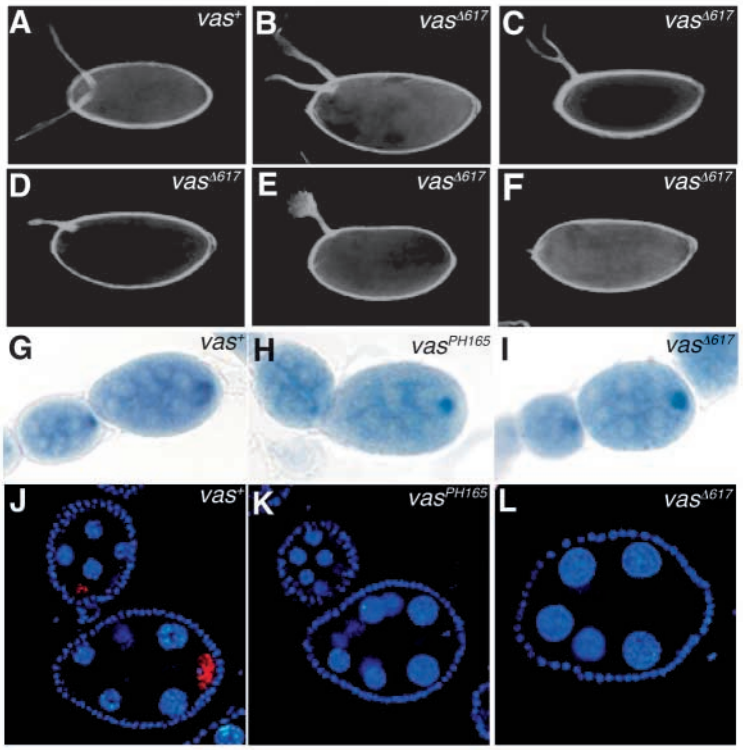

Fig. 5. Vas-eIF5B interaction is important for the establishment of dorsoventral polarity in the egg, and for *grk* regulation. Dark-field photographs of dorsal appendage phenotypes (A-F). Eighty-two percent of vas^{PH165};P{vas^{+}} eggs have two dorsal appendages (A). Dorsal appendages in vas^{PH165};P{$vas^{\Delta 617}$} eggs reveal a range of phenotypes: 22% have two dorsal appendages (B), 67% exhibit one semi-fused or fully fused dorsal appendage (C-E) and 11% have no dorsal appendages (F). (G-L) *grk* RNA and Grk protein were visualized in the early stages of oogenesis, through in situ hybridization and immunostaining. (J-L) Grk protein is shown in red; DAPI staining of DNA is shown in blue. (G,J) In vas^{PH165};P{vas^{+}} ovaries, both RNA and protein are enriched in the developing oocyte. (H-L) In both vas^{PH165} and vas^{PH165};P{$vas^{\Delta 617}$} ovaries, *grk* RNA is enriched in the oocyte (H,I); however, Grk protein is barely detectable (K,L). Grk protein was examined in single confocal sections using the same laser settings for each genotype.

Pole plasm components assemble in vas^{PD};P{$vas^{\Delta 617}$} ovaries

Pole plasm assembly requires the sequential posterior localization of multiple proteins and RNAs (reviewed by Mahowald, 2001). Osk, which is at the top of a complex hierarchy of factors involved in pole plasm assembly, is required for recruitment of Vas to the posterior (Lasko and Ashburner, 1990). Downstream of Vas localization, Tud protein is recruited (Bardsley et al., 1993), and Osk, Vas and Tud are required for pole cell formation and posterior segmentation. In order to investigate the requirement for the Vas-eIF5B interaction in pole plasm assembly, we examined the $vas^{\Delta 617}$ transgene in the background of a hypomorphic *vas* allele, vas^{PD}, as vas^{PH165}; P{$vas^{\Delta 617}$} ovaries produce few late-stage egg chambers. The vas^{PD} allele, in which Vas is detectable only in the germarial stages of oogenesis (Lasko and Ashburner, 1990), completes oogenesis normally, but the embryos produced lack pole cells and posterior segmentation. Sequencing of the vas^{PD} allele did not reveal any alteration in the coding sequence (Liang et al., 1994), thus this mutation is believed to affect only the level of *vas* expression and not the nature of Vas protein.

In wild-type stage 10 egg chambers, strong posterior accumulation of Osk, Vas and Tud protein is evident (Fig. 6B,F,J), while in vas^{PD} ovaries, which have severely reduced levels of Vas protein (Fig. 6A), posterior Osk is abundant, but neither Vas nor Tud is detected at the posterior (Fig. 6C,G,K). We next compared the accumulation of these three proteins in vas^{PD}; P{vas^{+}} and vas^{PD}; P{$vas^{\Delta 617}$} ovaries, which contain comparable levels of Vas protein as assayed by western blotting (Fig. 6A). We found that Osk, Vas and Tud protein can all be readily detected at the posterior of stage 10 oocytes (Fig. 6D-E,H-I,L-M), at apparently equivalent levels for both transgenic

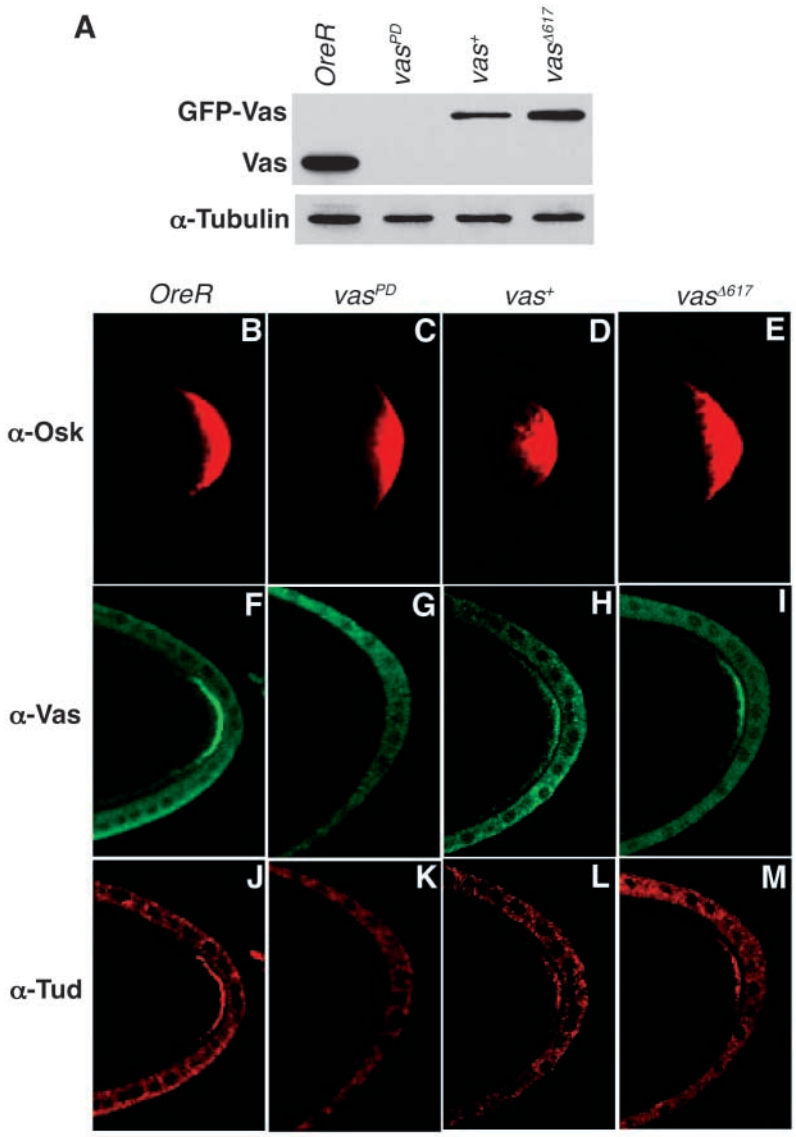

Fig. 6. Pole plasm assembly in the presence of reduced Vas-eIF5B interaction. (A) Western blot of ovarian extracts from wild-type (OreR), vas^{PD}, vas^{PD};P{vas^{+}} and vas^{PD};P{$vas^{\Delta 617}$} females was probed with anti-Vas. The same blot was probed with anti-α-Tubulin as a loading control. (B-M) Stage 10 ovaries from OreR, vas^{PD}, vas^{PD};P{vas^{+}} and vas^{PD};P{$vas^{\Delta 617}$} females were labeled for Osk (B-E), Vas (F-I) and Tud (J-M) by immunostaining.

genotypes. Thus, GFP-VasΔ617 not only localizes correctly to the posterior, but it is also able to recruit the downstream pole plasm component Tud. We conclude that interaction with eIF5B is not required for the role of Vas in the initial assembly of the pole plasm.

Vas-eIF5B interaction is critical for germ cell formation

It has previously been impossible to differentiate whether the embryonic phenotypes produced by hypomorphic *vas* alleles such as vas^{PD} were simply due to a lack of posterior Vas protein in the oocyte, resulting in a failure of recruitment of pole plasm components, or whether Vas has an additional regulatory function within the pole plasm. Analysis of the $vas^{\Delta 617}$ transgene in the vas^{PD} background allowed us to address this question, as Vas protein is expressed abundantly from this transgene, localizes correctly and is able to recruit Tud, an essential factor for germ cell specification (Ephrussi and Lehmann, 1992). We examined pole cell formation in the progeny of flies bearing either the vas^{+} transgene or the $vas^{\Delta 617}$ transgene in the vas^{PD} background. For simplicity, transgenic embryos will be referred to by the genotype of the mother. Using detection of GFP-Vas as a marker in live embryos at the cellular blastoderm stage, we found that the majority of vas^{PD};P{vas^{+} } embryos had formed pole cells at this stage, indicating that the wild-type transgene rescues this *vas* phenotype (Fig. 7A). By contrast, only one vas^{PD};P{$vas^{\Delta 617}$} embryo out of 216 examined exhibited pole cells at the same stage, indicating that expression of the $vas^{\Delta 617}$ transgene could not rescue this *vas* phenotype (Fig. 7B). We verified this result using Nos as an independent marker for pole cells. Although 58% of vas^{PD};P{vas^{+} }embryos exhibited Nos-positive cells at the posterior of the embryo at the cellular blastoderm stage (Fig. 7C), 81% of vas^{PD};P{$vas^{\Delta 617}$} embryos examined did not have any Nos-positive cells (Fig. 7D). Five percent of these embryos formed one to three Nos-positive cells at the posterior (Fig. 7E), while in 14% of embryos, Nos-positive cells were visible, but at inappropriate positions (Fig. 7F). These results demonstrate that, downstream of initial pole plasm assembly, the Vas-eIF5B interaction is vital for embryonic germ cell formation.

Reduction in Vas-eIF5B interaction does not abolish somatic posterior segmentation or Nos deployment

Expression of *fushi tarazu* (*ftz*) RNA serves as a molecular marker for the incipient segmentation pattern of the embryo. The wild-type pattern of *ftz* expression is in seven transverse stripes (Hafen et al., 1984), and mutations in posterior group genes such as *vas* inhibit the expression of stripes 4-6. In contrast to vas^{PD}, in which all embryos exhibit severe posterior segmentation defects (Schüpbach and Wieschaus, 1986), we observed that half of vas^{PD};P{$vas^{\Delta 617}$} embryos have a wild-type segmentation pattern, as inferred from a wild-type *ftz* distribution (Fig. 8A). In fact, when allowed to complete development, many vas^{PD};P{$vas^{\Delta 617}$} embryos hatched into viable larvae. A further 20% of the vas^{PD};P{$vas^{\Delta 617}$} embryos exhibited a weak posterior group phenotype in which stripes 4-6 of *ftz* were present but were reduced in width and intensity relative to the other stripes (Fig. 8B), although the remaining 30% exhibited a stronger phenotype (Fig. 8C), more closely resembling that of vas^{PD} (Schüpbach and Wieschaus, 1986). Over 90% of vas^{PD};P{vas^{+}} embryos expressed *ftz* in the normal pattern of seven stripes (data not shown). From this analysis, we conclude that the $vas^{\Delta 617}$ transgene can partially rescue the abdominal segmentation defect of the vas^{PD} allele, suggesting that the

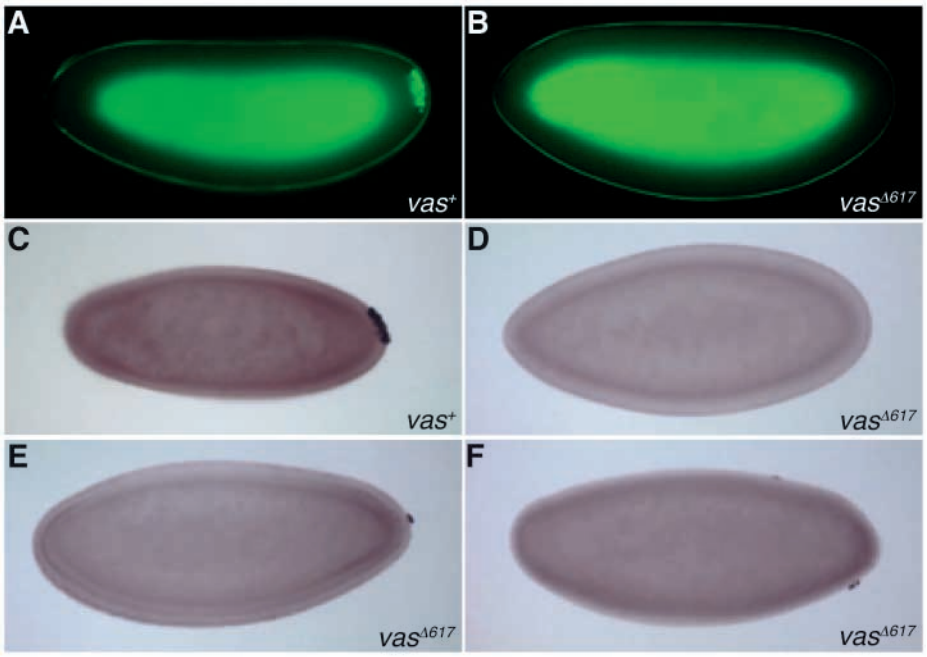

Fig. 7. Vas-eIF5B interaction is vital for pole cell formation. Pole cells were visualized at the cellular blastoderm stage using either GFP-Vas or immunostaining for Nos protein. The pictures shown are representative for each genotype. (A,B) Using GFP-Vas as a marker in live embryos, pole cells could be detected at the posterior of the majority of vas^{PD};P{vas^{+}} embryos (A), but in fewer than 1% of vas^{PD};P{$vas^{\Delta 617}$} embryos (B). (C) Using immunostaining for Nos as a marker for pole cells, Nos-positive cells could be detected at the posterior in 58% of vas^{PD};P{vas^{+}} embryos. (D,E) In 81% of vas^{PD};P{$vas^{\Delta 617}$} embryos, no Nos-positive cells were apparent at the same stage (D), whereas in 5%, one to three Nos-positive cells could be detected at the posterior (E). (F) In 14% of vas^{PD};P{$vas^{\Delta 617}$} embryos, several Nos-positive cells were detected but were positioned elsewhere within the embryo.

Vas-eIF5B interaction is less crucial for posterior patterning than it is for pole cell specification.

nos translation is tightly regulated such that it is repressed outside of the pole plasm and active in the pole plasm (Gavis and Lehmann, 1994). In existing *vas* mutants, *nos* is unlocalized and untranslated, and the absence of a Nos protein gradient prevents abdominal formation (Gavis and Lehmann, 1994; Wang et al., 1994). We examined Nos protein distribution in vas^{PD};P{$vas^{\Delta 617}$} embryos directly. Consistent with the *ftz* expression data, 55% of vas^{PD};P{$vas^{\Delta 617}$} embryos exhibited a detectable Nos gradient (Fig. 8E,F). In control vas^{PD};P{vas^{+}} embryos, a Nos protein gradient could be observed in 95% of the embryos (Fig. 8D). Thus, the $vas^{\Delta 617}$ transgene partially rescues the Nos accumulation defect of the vas^{PD} allele, suggesting that either the Vas-eIF5B interaction is dispensable for Nos translation, or that the low level of interaction between VasΔ617 and eIF5B is sufficient to activate Nos.

Discussion

The Vas-eIF5B interaction is essential for oogenesis, dorsoventral patterning, and Grk expression

We have analyzed a mutant form of Vas, VasΔ617, which has greatly reduced ability to interact with eIF5B. As residue 617 is not involved in binding to any known Vas-interacting protein other than eIF5B, and it is outside the region of Vas that contains the well-characterized catalytic domains that are present in all DEAD-box proteins, we are confident that VasΔ617 specifically disrupts the Vas-eIF5B interaction, and that this mutation can be used to identify developmental processes that are sensitive to an association between Vas and the general translational machinery. We found that the Vas-eIF5B interaction is crucial for the progression of oogenesis, for correct dorsoventral patterning of the egg, and for expression of high levels of Grk in the developing oocyte. These results are most easily explained if *grk* is a target for Vas-mediated translational activation acting through its association with eIF5B.

A role for Vas in positively regulating *grk* translation is consistent with previous work (Styhler et al., 1998; Tomancak et al., 1998; Ghabrial and Schüpbach, 1999). Vas-mediated regulation of *grk* is in turn regulated in response to a meiotic checkpoint, activated when DNA double-strand break (DSB) repair is prevented during meiotic recombination (Ghabrial et al., 1998; Ghabrial and Schüpbach, 1999). In response to this checkpoint, Vas is posttranslationally modified, and Grk accumulation is reduced. It will be important to understand the nature of the DSB-dependent modification of Vas, and to determine whether it affects the Vas-eIF5B interaction, in order to gain insight into the mechanism connecting cell cycle regulation with oocyte patterning.

The RNA-binding and unwinding activities of wild-type Vas and several mutant forms of Vas have previously been assessed through in vitro assays (Liang et al., 1994). Two mutant forms of Vas, encoded by the vas^{O14} and vas^{O11} alleles, were found to be severely reduced for binding to an artificial RNA substrate, and a third form, encoded by vas^{D5}, was defective for RNA unwinding but not for binding. Although vas^{D5} leads to defects in oogenesis, vas^{O11} phenotypically resembles vas^{PD}, and vas^{O14} is a weak temperature-sensitive allele (Lasko and Ashburner, 1990). In the light of our present results, it is surprising that a mutant form of Vas that cannot interact with RNA would nevertheless support oogenesis. Perhaps in vivo, the RNA-binding and helicase activities of Vas are stimulated or enhanced through a co-factor or through posttranslational modifications, and the in vitro assay used in our earlier study may not accurately reflect Vas activity in vivo.

Are there target RNAs for Vas-eIF5B regulation in the pole plasm?

Reduction of the Vas-eIF5B interaction by expressing VasΔ617 severely reduces pole cell formation. This happens despite the

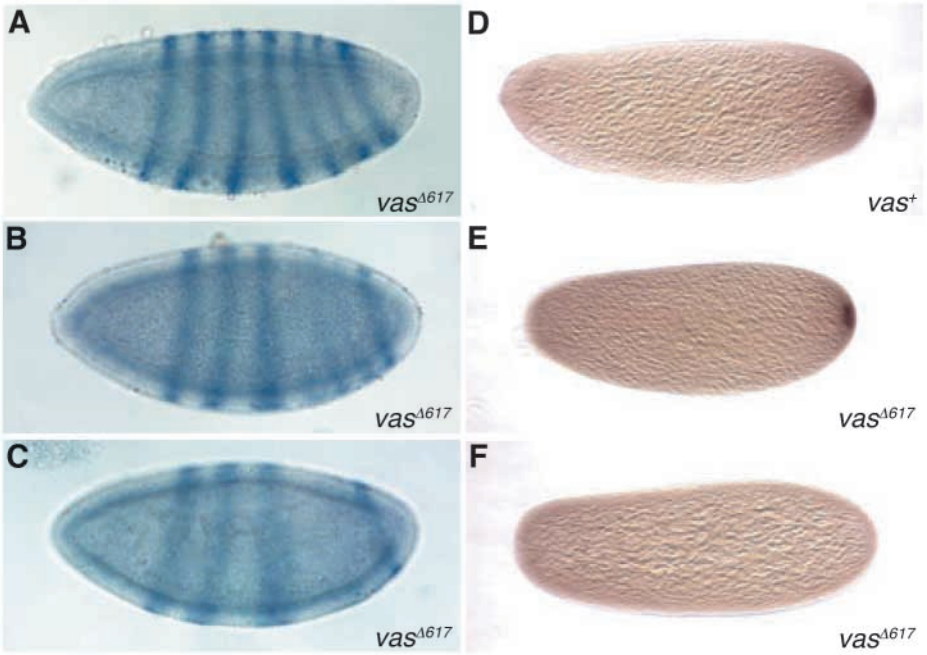

Fig. 8. Reduced Vas-eIF5B binding does not abrogate somatic patterning or Nos deployment. RNA in situ hybridization for *ftz* was used as an indicator of somatic segmentation at the cellular blastoderm stage. (A) Fifty percent of vas^{PD};P{$vas^{\Delta 617}$} embryos revealed a normal *ftz* distribution of seven transverse stripes. (B,C) In 20% of vas^{PD};P{$vas^{\Delta 617}$} embryos, stripes 4-6 were weaker and less defined than the others (B), and in 30%, more severe defects such as deletions and fusions of these segments were apparent (C). (D) Nos protein, visualized through immunostaining in 0- to 2-hour-old embryos, was present at the posterior of 95% of vas^{PD};P{vas^{+}} embryos. (E,F) Posterior Nos was detectable in 55% of the vas^{PD};P{$vas^{\Delta 617}$} embryos (E), and undetectable in the remaining 45% (F).

ability of vas^{PD};P{$vas^{\Delta 617}$} oocytes to accumulate Osk, Vas and Tud at the posterior pole, demonstrating an essential role for Vas in pole cell specification that is dependent upon its association with eIF5B, and that cannot be substituted by Osk and Tud. The simplest interpretation of these results is that Vas derepresses translation of a localized RNA required for pole cell specification, in a manner analogous to what appears to be the case for *grk*.

We considered the possibility that the Vas-eIF5B interaction could target *osk* mRNA. It has previously been shown that whereas Osk protein accumulates normally in *vas* mutant ovaries, Osk levels are severely reduced at the posterior of *vas* mutant embryos (Harris and Macdonald, 2001), suggesting a role for Vas in posterior accumulation of Osk after its initial recruitment, and/or in stabilizing Osk at the posterior. We observed comparable and substantial levels of Osk at the posterior in vas^{PD};P{$vas^{\Delta 617}$} and vas^{PD};P{vas^{+}} embryos (data not shown), arguing against a direct role for the Vas-eIF5B interaction in activating translation of *osk* mRNA. A requirement for Vas in Par1-mediated phosphorylation and stabilization of Osk has been suggested (Breitwieser et al., 1996; Markussen et al., 1997; Riechmann et al., 2002). As VasΔ617 localizes normally and is able to interact with Osk, we would not expect this mutation to have any effect on this Osk modification pathway. Thus, our findings are consistent with a model whereby Vas influences Osk activity through effects on phosphorylation, anchoring and/or stability, perhaps through Par1, rather than directly regulating *osk* translation.

Another candidate target for the Vas-eIF5B interaction is *germ cell-less* (*gcl*), the activity of which is important for pole cell specification but not for posterior patterning (Jongens et al., 1992). Unfortunately, with current reagents, and with new antisera we have generated, we cannot reliably detect Gcl protein even in wild-type embryos prior to pole bud formation, thus we cannot presently address the effects on *gcl* translation of any mutation that abrogates pole cell formation. In addition, effects on *gcl* cannot fully explain the severe consequences of the VasΔ617 mutation on pole cell formation, because the number of pole cells formed in maternal *gcl*-null embryos is somewhat higher than in vas^{PD};P{$vas^{\Delta 617}$} embryos (Robertson et al., 1999). This suggests that even if *gcl* is a target, the Vas-eIF5B interaction may regulate translation of more than one target RNA involved in pole cell formation.

Is the Vas-eIF5B interaction required for posterior patterning?

Although the Vas-eIF5B interaction is vital for pole cell specification, it is perhaps less so for posterior patterning and establishment of the Nos gradient. Previous analysis of hypomorphic mutations in posterior-group genes, including *vas* has indicated that a higher level of activity is required for pole cell specification than for posterior patterning. For example, all embryos produced by females homozygous for vas^{O14} (Lasko and Ashburner, 1990), osk^{301} (Lehmann and Nüsslein-Volhard, 1986) and tud^{WC} (Schüpbach and Wieschaus, 1986), lack pole cells, but some have normal posterior patterning and are able to hatch. Our present results suggest two alternative explanations for these observations. One possibility is that the Vas-eIF5B interaction is required for posterior patterning, but that the residual activity present in VasΔ617 is sufficient to achieve the low activity level that is necessary. Alternatively, the Vas-eIF5B interaction may be dispensable for posterior patterning, and the fact that we do not observe complete rescue of this phenotype with the $vas^{\Delta 617}$ transgene may be due to an indirect effect of this mutation, resulting from a general destabilization of the pole plasm that occurs in embryos that do not form pole cells (Iida and Kobayashi, 2000). In such embryos, pole plasm components localize initially but become fully delocalized by the blastoderm stage (Lasko and Ashburner, 1990). Consistent with this idea, all of the pole plasm components examined that are downstream of Vas, including *nos* RNA, could be detected

at the posterior of *vas*^{PD};P{*vas*$^{\Delta 617}$} embryos, although to variable degrees (data not shown).

Previous work has suggested that *nos* may be a target for Vas-mediated translational regulation (Gavis et al., 1996). Outside of the pole plasm, *nos* translation is repressed through the binding of Smg, and possibly other repressors, to its 3′ UTR (Smibert et al., 1996; Dahanukar et al., 1999; Crucs et al., 2000; Nelson et al., 2004). Smg achieves this regulation at least in part through interaction with the eIF4E-binding protein Cup, thus influencing the cap-binding stage of translation (Nelson et al., 2004). Within the pole plasm, in complexes with Osk and Vas, *nos* translational repression is overcome, potentially through a direct interaction between Osk and Smg (Dahanukar et al., 1999), which may displace Smg-Cup interaction (Nelson et al., 2004). Our analysis of VasΔ617 does not support an important role for the Vas-eIF5B interaction in activating *nos* translation in the pole plasm, as clearly translation of *nos* is far less sensitive to the level of this interaction than is translation of *grk* in early oocytes. The primary function of Vas in *nos* accumulation may therefore be in anchoring *nos* mRNA in complexes within the pole plasm, consistent with recent observations that *nos* mRNA is trapped at the posterior by complexes containing Vas (Forrest and Gavis, 2003). It of course remains possible that the low level of residual eIF5B binding provided by VasΔ617 is sufficient to fulfill a role of Vas in activating translation of this transcript.

How might Vas-eIF5B interaction regulate translation of *grk* and potentially other target mRNAs?

Cap-dependent translation initiation in eukaryotes requires many translation initiation factors, and involves several main steps (reviewed by Pestova et al., 2001). Most known mechanisms of translational regulation impinge on the recruitment of the cap-binding complex eIF4F to the mRNA, which represents the rate-limiting first step of initiation. mRNA circularization through proteins such as Cup serves an important role in translational control by allowing 3′ UTR-bound regulatory factors to influence translation initiation at the 5′ end of the transcript. (Nakamura et al., 2004; Nelson et al., 2004; Wilhelm et al., 2003).

60S ribosomal subunit joining represents the interface between translation initiation and elongation, and the Vas-eIF5B interaction suggests a distinct mechanism of translational control occurring at this last stage of initiation. Although this step has not historically been considered a target for regulation, several examples have emerged to suggest that subunit joining may in fact be subject to regulation. Translational repression of mammalian 15-lipoxygenase (LOX) mRNA is mediated by hnRNP proteins that bind to a specific 3′ UTR regulatory element, and which are thought to act by blocking the activity of either eIF5 or eIF5B (Ostareck et al., 2001). An additional link between mRNA 3′ regulatory regions, and eIF5B activity, comes from analysis of two DEAD-box proteins in yeast, Ski2p and Slh1p (Searfoss et al., 2001). These proteins are required to achieve the selective translation of poly(A)$^+$ mRNAs, relative to poly(A)$^-$ mRNAs, and genetic experiments suggest that they specifically repress the translation of poly(A)$^-$ mRNAs by acting through eIF5 and eIF5B.

Together with these studies, our work suggests that in the *Drosophila* germline, specific translational repression events may target eIF5B and the ribosomal subunit joining step of initiation. Vas, which potentially functions at the 3′ UTR through interaction with specific repressor proteins, may act to alleviate a translation block occurring at this step. Such a model is consistent with what is known about translational regulation of *grk*. For example, *grk* translation is repressed by Bru, which binds to a Bruno-response element within its 3′ UTR (Filardo and Ephrussi, 2003). Vas interacts with Bru (Webster et al., 1997), suggesting that Vas could function as a derepressor by overcoming Bru-mediated repression of *grk* translation. However, the inability of a *vas* transgene to ameliorate the phenotype of *nos*GAL4VP16-driven overexpression of Bru, might argue against this model (Filardo and Ephrussi, 2003). The mechanism by which Bru regulates *grk* remains unclear. Translational repression of *osk* by Bru relies on direct interaction with Cup, linking Bru with eIF4E (Nakamura et al., 2004). However, mutations in *cup* that prevent interaction with Bru do not appear to affect Grk expression, suggesting that Bru may operate through a distinct mechanism to regulate *grk* translation (Nakamura et al., 2004). In addition, in vitro translation assays have suggested that Bru can mediate translational repression through a cap-independent mechanism (Lie and Macdonald, 1999). Thus, Bru may be capable of regulating translation at more than one stage. Based on the observations for the mammalian hnRNP proteins on the LOX mRNA, and the Ski2p and Slh1p helicases in yeast, specific translational repressors such as Bru could target the subunit joining step of initiation.

eIF5B is thought to form a molecular bridge between the two ribosomal subunits, and to play a fundamental role in stabilizing the initiator Met-tRNA$_i^{Met}$ in the ribosomal P site (reviewed by Pestova et al., 2000a). Inhibition of eIF5B activity could occur while the factor is bound to the initiation complex, at the start codon, and block its ability to link or stabilize the ribosomal subunits. Through circularization of the mRNA, this block could be achieved by trans-acting factors at the 3′ UTR, and the Vas-eIF5B interaction may be involved in alleviating these specific repression events, potentially through displacement of a repressor protein. Alternatively, Vas could play a role in recruitment of eIF5B to specific transcripts. As eIF5B is required for all cellular translation, a general mechanism must exist to recruit this factor to all transcripts. However, in a scenario where repressor proteins may be blocking the subunit joining step, either through a direct effect on eIF5B, or another mechanism, it is conceivable that eIF5B could become limiting for translation. In this situation, Vas could play a role in recruiting this factor to specific transcripts.

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