Drosophila valois encodes a divergent WD protein that is required for Vasa localization and Oskar protein accumulation

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Summary

***valois* (*vls*) was identified as a posterior group gene in the initial screens for *Drosophila* maternal-effect lethal mutations. Despite its early genetic identification, it has not been characterized at the molecular level until now. We show that *vls* encodes a divergent WD domain protein and that the three available EMS-induced point mutations cause premature stop codons in the *vls* ORF. We have generated a null allele that has a stronger phenotype than the EMS mutants. The vls^{null} mutant shows that vls^{+} is required for high levels of Oskar protein to accumulate during oogenesis, for normal posterior localization of Oskar in later stages of oogenesis and for posterior localization of the Vasa protein during the entire process of pole plasm assembly. There is no evidence for *vls* being dependent on an upstream factor of the posterior pathway, suggesting that Valois protein (Vls) instead acts as a co-factor in the process. Based on the structure of Vls, the function of similar proteins in different systems and our phenotypic analysis, it seems likely that *vls* may promote posterior patterning by facilitating interactions between different molecules.**

Key words: *Drosophila*, Posterior development, WD protein, Valois, Vasa, Oskar

Introduction

The embryonic body axes are specified in *Drosophila melanogaster* during oogenesis, when cytoplasmic determinants localize to different regions of the developing oocyte. This initiates the formation of positional information centers, which define polarity and pattern the body plan along the anteroposterior (AP) and dorsoventral (DV) axes during embryogenesis. This developmental control mechanism is based on mRNA localization and anchoring to specific subcellular compartments. In conjunction with tight translational control of localized mRNAs this is an efficient means with which to generate a local source of polarity determinants, one that is widely used throughout phyla for various purposes (Kloc et al., 2002). In *Drosophila* oocytes, posteriorly localized *oskar* (*osk*) mRNA is locally translated starting in mid-oogenesis (stage 8-9) and nucleates the assembly of the pole plasm (or germ plasm). The pole plasm specifies the germline at the posterior end of the embryo, and it patterns the abdomen along the AP axis (Johnstone and Lasko, 2001; Riechmann and Ephrussi, 2001).

The *osk* ribonucleoprotein (RNP) complex has been characterized, and many conserved factors are known to function in mRNA localization and/or translational control in different systems across phyla (Farina and Singer, 2002; Hachet and Ephrussi, 2004; Roegiers and Jan, 2000; Wilhelm et al., 2000). Because restriction of *osk* activity to the posterior is crucial for normal development (Ephrussi and Lehmann, 1992), both pre- and post-translational control mechanisms regulate Osk protein accumulation. Osk protein is actively degraded by the ubiquitin-proteasome pathway, but protected from it by phosphorylation by Par-1 specifically at the posterior (Riechmann et al., 2002). Translational control of *osk* involves the coordinate action of repressors and derepressors interacting with discrete elements of *osk* transcripts during transport and at the posterior pole (Gunkel et al., 1998; Kim-Ha et al., 1995; Webster et al., 1997; Yano et al., 2004). Additional factors that do not function as derepressors are also required for stimulating *osk* translation (Wilson et al., 1996). In addition, Oo18 RNA-binding protein (Orb) polyadenylates *osk* transcripts at the posterior pole once derepression has been achieved (Castagnetti and Ephrussi, 2003).

Two isoforms of Osk (Long and Short Osk) are produced by initiation at two different in-frame start codons. Short Osk has long been known as the active isoform for pole plasm assembly which recruits downstream components of the pathway such as Vasa (Vas) (Markussen et al., 1995), and recently, Long Osk has been shown to be responsible for anchoring *osk* mRNA and Short Osk at the posterior (Vanzo and Ephrussi, 2002). Short Osk is likely to anchor Vas directly at the posterior (Breitwieser et al., 1996; Vanzo and Ephrussi, 2002). Vas is an ATP-dependent RNA-helicase from the DEAD-box family and has been implicated in translational activation of several maternal transcripts, including *osk* (Styhler et al., 1998; Tinker et al., 1998; Tomancak et al., 1998). *tudor* (*tud*) acts downstream of *vas* and is followed in the cascade by additional genes whose products localize to the pole plasm and mark the separation of

germline establishment and abdominal patterning activities (Golumbeski et al., 1991). Pole cell formation depends on the localization of *germ cell less* (*gcl*) mRNA (Leatherman et al., 2002) and *mitochondrial large ribosomal RNA* (Iida and Kobayashi, 1998). Abdominal patterning relies on the *vas*-dependent translation of *nanos* (*nos*) mRNA at the posterior pole. This results in a concentration gradient of Nos protein along the AP axis, which acts as the primary posterior morphogen (Riechmann and Ephrussi, 2001).

One more posterior group gene, *valois* (*vls*), had been identified in the initial screen for maternal-effect steriles (Schupbach and Wieschaus, 1986), but has neither been cloned nor studied genetically in detail yet. Based on three EMS-induced alleles of *vls*, it was classified as a member of the 'grandchildless-knirps-like' group that also includes *vas, stau* and *tud*. Their phenotype is characterized by a lack of pole cells at the posterior and various degrees of abdominal segment deletions. Pole cell transplantation experiments demonstrated that *vls* functions in the germline (Schupbach and Wieschaus, 1986) and *vls* mutants were shown to have a non-functional pole plasm (Lehmann and Nusslein-Volhard, 1991). Until now, the position of *vls* in the posterior pathway has remained controversial. *vls* was tentatively placed downstream of *osk* and *vas,* but upstream of *tud.* This was based on the observation that *osk* mRNA and Vas protein are initially correctly localized to the posterior of the oocyte in vls^{EMS} mutants. Vas then detaches from the posterior of the embryo soon after fertilization (Ephrussi et al., 1991; Hay et al., 1990; Lasko and Ashburner, 1990) and Tud localization is disrupted in embryos from *vls* mothers (Bardsley et al., 1993). However, conflicting data were reported subsequently. Assembly of an ectopic pole plasm at the anterior of the oocyte, caused by overexpressing *osk* (*6xosk*) (Smith et al., 1992) or by targeting *osk* transcripts specifically to the anterior margin (*osk-bcd3′UTR*) (Ephrussi and Lehmann, 1992), results in progeny embryos with ectopic pole cells and duplication of the abdomen at the anterior. *vls* function was found to be required for the expression of the *6xosk* phenotype, confirming its position downstream of *osk*, but not for the expression of the *osk-bcd3′UTR* phenotype (Ephrussi and Lehmann, 1992; Smith et al., 1992).

Here, we report the cloning and characterization of *vls*. We have created a null mutant for *vls* that shows stronger phenotypes than the presently available vls^{EMS} alleles. In contrast to previous models, this tool allows us to demonstrate that *vls* acts upstream of *vas.* Furthermore, *vls* dramatically affects the levels of Osk protein, even though localization of *osk* mRNA and initial accumulation of Osk do not require *vls* function. *vls* encodes a novel protein with significant similarity to WD domain proteins. The presented data suggest that Vls may act as a co-factor in assembling protein-protein and/or protein-RNA complexes.

Materials and methods

Fly stocks and transgenes

w; *Df(2L)pr2b / Cy Roi*, *l(2)k08103 (barr)*, vls^{PG65}, vls^{RB71} and vls^{HC33} (vls^{EMS}) have been described previously (Bhat et al., 1996; Butler et al., 2001; Schupbach, 1986). vls^{PE36} was not sterile anymore and thus considered lost. The *P[w⁺, barren⁺]* and *P[w⁺, vls⁺/CG10728⁺]* rescue transgenes have been described previously (Masrouha et al., 2003). The *vls-eGFP* transgene was constructed from the $vls^{+}/CG10728^{+}$ genomic rescue construct, using the mutagenic primer: 5′ GTTCGGCAGCAAAATTAAGATCTTTTGATAGCGTG-CGTC 3′ to insert a *Bgl*II site immediately before the *vls* stop codon. The *eGFP* gene with the S65T mutation (Heim et al., 1994) was inserted into this *Bgl*II site using *Bam*HI.

Generation of anti-Vls antibody

A partial *vls* cDNA was recovered by PCR amplification from an ovarian cDNA library (Larochelle and Suter, 1995). Rabbit polyclonal antibodies were raised against an *E. coli* expressed Vls polypeptide (amino acids 166-367; Fig. 1) that contained an N-terminal 6xHis tag. The fusion protein was purified by affinity chromatography and gel electrophoresis prior to injection. Antiserum was affinity purified with a MBP::Vls fusion protein (expressed from the same *vls* cDNA) that was coupled to CnBr-activated sepharose beads (Pharmacia Biotech).

Western blotting

Ovary, embryo, larvae and whole fly extracts were prepared as described previously (Masrouha et al., 2003). Protein samples were resolved by 10% SDS-PAGE electrophoresis, transferred to nitrocellulose membranes and probed with antibodies. Horseradish-peroxidase-conjugated anti-rabbit/mouse IgG were used at 1:2000 (Amersham Pharmacia Biotech).

RNA in situ and immunostaining

In situ hybridizations to *osk* mRNA on ovaries were performed as described previously (Suter and Steward, 1991). The *osk* probe was generated by random priming with the DIG High Prime digoxigenin labeling system (Roche Applied Science). Immunostaining on ovaries were performed as described previously (Findley et al., 2003) with α-Osk at 1:3000 and secondary Alexa Fluor anti-rabbit 488nm.

Vls-eGFP and Vas-eGFP observations

A Zeiss confocal microscope was used for Vls-eGFP and a Leica confocal microscope for Vas-eGFP observations. Two- to three-day-old females were used for ovaries and embryo collections. For live observation of Vls-eGFP, ovaries were dissected in halocarbon oil 27, separated and dragged onto a cover slip. They were then covered with petriPERM 50 hydrophobic membrane dishes (Vivascience). Samples were used for no more than 15 minutes after dissection. *vas-eGFP* ovaries were dissected in Ringer's buffer, fixed for 20 minutes in phosphate-buffered saline (PBS) with 4% paraformaldehyde, rinsed three times, washed twice for 5 minutes in PBST (PBS + 0.2% Tween 20) and mounted in 60% glycerol. Embryos were collected for 1 hour periods, aged accordingly, dechorionated by rolling them over double-sided sticky tape and mounted on slides in halocarbon oil 27. Background signal was evaluated for all types of samples by using yw^{118} ovaries or embryos as a control. The wavelength window of detection was adjusted to reduce background signal produced by auto-fluorescent particles.

Results

Cloning and identification of *vls*

Df(2L)be408 removes part of the *barr* gene, and the entire coding sequences of *chk2* and *CG10728* (Fig. 1) (Masrouha et al., 2003). This deficiency does not complement vls^{EMS} alleles, indicating that one of these three transcription units corresponds to *vls*. A $barr^{+}$ transgene rescues the *barr* but not the *vls* phenotypes. Similarly, a transgene containing *chk2* alone does not rescue the *vls* phenotypes either. By contrast, a transgene containing a wild-type copy of *CG10728* (Fig. 1) was able to rescue the maternal-effect lethal phenotype of $chk2^{null}$ $CG10728^{null}$ double mutants and the grandchildless phenotype associated with $vls^{EMS}/Df(2L)be408$ mutants. These

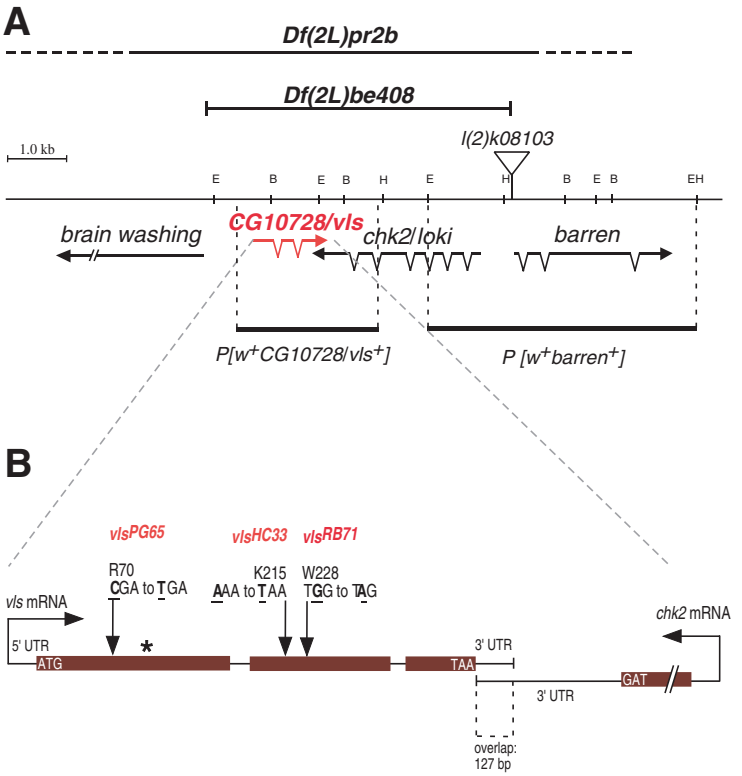

Fig. 1. *vls* genomic region. (A) Four transcription units are found in a 12 kb stretch of DNA in region 38B (Butler et al., 2001). *Df(2L)be408* uncovers *CG10728*, *chk2* and a small region of the *barr* 5′ end. *Df(2L)pr2b* deletes 38B1-2 to 38D2-E1. The fragments indicated as *P[ ]*were reintroduced by P-element-mediated transformation and recombined onto the *Df(2L)pr2b* or *Df(2L)be408* chromosomes. $chk2^{null}$ flies (*Df(2L)be408*, $P[w^+$ *CG10728*$^+/vls^+]$ / *Df(2L)pr2b*, $P[w^+$ $barren^+]$) are viable and fertile, demonstrating that *chk2* does not have any essential function for development. E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII. (B) Magnification of the *vls* gene drawn to scale. Exons are shown as dark boxes, introns and untranslated regions (UTRs) as thin lines. The position of premature stop codons in the three vls^{EMS} alleles is indicated with the corresponding nucleotide substitution and codon change. The predicted 3′ UTRs of *vls* and *chk2* overlap over 127 bp. The asterisk indicates the N-terminal position of the polypeptide used for antibody production.

results strongly suggest that *CG10728* corresponds to *vls*. Indeed, sequencing this genomic region in the three EMS alleles vls^{PG65}, vls^{RB71} and vls^{HC33} finds a single nucleotide substitution in each of them, resulting in premature stop codons in the predicted open reading frame (ORF) of *CG10728* (Fig. 1). This identifies *CG10728* as *vls* and *Df(2L)be408* / *Df(2L)pr2b, P[w+, barr+]* constitutes a true null mutant for *vls* and *chk2*. As the $chk2^+$ construct does not rescue any of the *vls* phenotypes, but the vls^+ transgene rescues all of them, we will refer to this mutant as vls^{null} hereafter.

Overlapping genes and repression of *chk2* by *vls*

Interestingly, *chk2* and *vls* are encoded by opposite strands and cDNA sequence data shows that their 3′UTRs are complementary over 127 nucleotides (Fig. 1). *chk2* is translationally repressed by *orb* during oogenesis (Masrouha et al., 2003), and because translational control often relies on the binding of *trans*-acting factors to sequences in the 3′UTR of mRNAs, we were curious to know whether *vls* could also play a role in *chk2* translational control. Indeed, Chk2 levels increase about 6-fold in $vls^{PG65/HC33}$ and $vls^{PG65/RB71}$ ovaries compared with wild type (Fig. 2) and this is close to the 10-fold upregulation reported for *orb* mutants (Masrouha et al., 2003). This indicates that *vls* is also involved in the regulation of Chk2 levels. However, *orb* does not simply function to control Vls levels because these are normal in *orb* mutants (data not shown).

This peculiar genomic organization of *chk2* and *vls* appears somewhat conserved among *Drosophilidae* because in *D. pseudoobscura*, *vls* and *chk2* are also on opposite strands next to each other. However, based on the gene predictions only (no cDNA sequences are available), there is no evidence that the mRNAs overlap. In the more distant diptera *Anopheles gambiae*, the genes are still on the same chromosome, but much further apart. In the human genome, finally, the predicted homologues of these genes are unlinked (data not shown).

Valois belongs to a family of divergent WD domain proteins

vls encodes a novel protein and PROSITE predicted the existence of two WD domains. Database searches reveal the best sequence similarity with the human methylosome protein 50 (MEP50; 20.4% identity; Fig. 3 and Table 1) and alignment of Vls and MEP50 shows that the two predicted WD domains of Vls correspond closely to the predicted WD domains 2 and 3 of MEP50. With the exception of the WD domain 5, the predicted WD domains of MEP50 show elevated similarity with corresponding Vls regions compared to the alignment of the entire proteins (Table 1). This suggests that Vls may have five to six domains that have a similar structure or function as WD domains, and it may mean that Vls has evolved from a WD domain protein. The six WD domains of MEP50 are thought to fold into a β-propeller structure, which serves as a platform for recruiting the Arg-methyltransferase JBP1/PRMT5 and its substrates, the Sm proteins (Friesen et al., 2002). This event is required for assembling the splicing

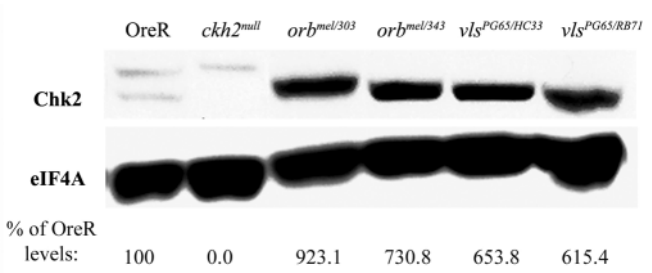

Fig. 2. *chk2* is translationally repressed by *orb* and *vls* during oogenesis. The indicated ovary extracts were probed with α-Chk2 (Masrouha et al., 2003) and α-eIF4A antibodies as a loading control. Protein levels were quantified using a phosphorimager. The upper band in *OreR* and $chk2^{null}$ mutants is crossreacting material of unknown identity. Chk2 levels were normalized to eIF4A levels and expressed as a percentage of normalized Chk2 levels in wild type (OreR), which is arbitrarily set at 100%.

Fig. 3. *Drosophila* Vls resembles *Homo sapiens* methylosome protein 50 (MEP50). The alignment of the two predicted protein sequences was made using ALIGN Query (GENESTREAM SEARCH network server IGH Montpellier, France). Identical residues are highlighted in black and conserved residues in gray. The WD domains of MEP50 (Friesen et al., 2002) are boxed and numbered in blue, the predicted WD domains of Vls (PROSITE) in green.

machinery prior to import into the nucleus (Friesen et al., 2001).

To test whether Vls may be the *Drosophila* ortholog of MEP50, we searched by BLAST the translated *Drosophila* genome for sequences similar to MEP50 and used the CLUSTALW multiple alignments tool to analyze the results. The search revealed an entire family of *Drosophila* WD domain proteins with comparable sequence similarity to MEP50 and clearly a greater level of conservation over the WD domains than in regions outside. Although different algorithms give slightly different alignments, we find that the protein products of *CG6486, Lis-1* and *vls* have the highest levels of similarity over the WD domains of MEP50 (Table 1 includes the six most similar proteins). The observed differences are too small to predict which one of the *Drosophila* proteins is more likely to be the ortholog of MEP50.

Valois is a maternal product

Northern analysis detected a transcript of 1.5 kb for *vls* expressed in ovaries, early embryos and adult females, but absent from pooled larval instars and adult males (Butler et al., 2001). In situ hybridization to OreR ovaries with a *vls* probe detected signal throughout the germ cell cytoplasm from early oogenesis onwards. The signal showed no specific localization pattern. Surprisingly, we detected an equally strong signal in $vls^{PG65/RB71}$ ovaries, and only in the vls^{null} ovaries the signal is at background levels (data not shown).

On Western blots, polyclonal anti-Vls antibodies do not detect any Vls in vls^{null}, vls^{PG65}, vls^{RB71} and vls^{HC33} ovary extracts (Fig. 4A). This shows that the antibody specifically recognizes the Vls protein and that the EMS mutants do not make significant levels of stable full length Vls. However, because we do not know which epitopes are recognized by the polyclonal antibody, it is still possible that the EMS alleles produce truncated forms of Vls. In wild-type flies, Vls is abundant in ovaries, early embryos and adult females, but reduced in adult males (Fig. 4B). The fact that it is present in ovaries and in 0- to 1-hour-old embryos indicates that Vls is a maternally provided protein and this is consistent with the maternal-effect phenotype of *vls* mutants.

Vls as a co-factor of the posterior pathway

Comparing OreR and control vls^{null} ovaries stained with α-Vls antibodies reveals Vls signal at low levels along parts of the oocyte cortex of wild-type stage 10 egg chambers, and also a stronger signal in the nurse cells, where it appears to be concentrated in nuage (data not shown). Because our anti-Vls antibodies do not work well for immunostaining, we also

Table 1. Sequence similarity comparison between MEP50 and *Drosophila* WD domain proteins

Gene/synonym	Predicted or confirmed function	Reference	Percentage identity with the regions of MEP50 indicated							
			Whole alignment	WD domains combined	WD domains separately					
					1	2	3	4	5	6
CG6486	Peroxisome organization and biogenesis	FlyBase	23.2	26.9	15.6	30.3	29	21.9	31.2	33.3
CG8440/Lis-1	Dynein-dependent microtubule transport	Swan et al., 1999	20.2	26.7	27.3	38.7	25	12.5	37.5	19.2
CG10728/vls	Posterior development	This paper	20.4	26.1	22.6	40.6	25.8	32.4	14.3	28
CG4236/chromatin assembly factor 1 subunit (caf1)	Histone lysine N-methylation	Martinez-Balbas et al., 1998	21.8	25.7	18.8	25	29.7	24.2	34.4	22.2
CG3000/retina aberrant in pattern (rap)	Cyclin catabolism, proteolysis and peptidolysis	Karpilow et al., 1989	18.3	24.9	27.3	28.1	34.4	11.4	21.2	26.9
CG3436	Pre-mRNA splicing factor, component of snRNP U5	FlyBase	20.2	24.3	12.5	40.6	32.3	15.2	22.2	23.1
Average			21.1	25.8	19.1	33.9	29.0	18.8	28.1	26.0

generated transgenic flies that express the fusion gene *vls-eGFP*. The *P[w+ vls-eGFP]* transgene rescues the female sterile phenotype of vls^{null} mutants, proving that Vls-eGFP possesses vls^{+} activity (data not shown). Vls-eGFP localization in vls^{null} background is indistinguishable from that in wild-type background (data not shown). Vls-eGFP signal is cytoplasmic and stronger in the germline than in somatic cells, but in contrast to the immunostaining, specific localization patterns of Vls-eGFP were usually not observed (Fig. 5). Only in the germarium did we occasionally observe Vls-eGFP concentrating in perinuclear aggregates that disappear by stage 2 of oogenesis. At later stages, Vls-eGFP signal is uniformly distributed in the nurse cells and oocyte, as well as in young embryos, with no particular enrichment at the posterior or inside the pole cells.

Western blot analysis of Vls as well as localization studies of Vls-eGFP in other posterior group mutants (*osk*, *vas*, *tud*, *gus* and *orb*) and in *grk* failed to identify potential upstream factors of *vls* that could control its expression levels, potential post-translational modifications, or its spatiotemporal distribution patterns (data not shown). Together with the uniform distribution pattern of Vls-eGFP, this argues that Vls may act as a co-factor in the posterior pathway.

Posterior localization of Osk protein in late oogenesis depends on *vls*

To investigate further the position of *vls* in the pathway, we examined the distribution of posterior products in vls^{null} ovaries. *osk* mRNA is efficiently localized at the posterior of vls^{null} mutant oocytes (Fig. 6A), consistent with previous reports for embryos from vls^{PE36} mothers (Ephrussi et al., 1991). Osk protein accumulates at the posterior pole of the oocyte during stages 8-10. During this phase, we observe similar patterns in wild type and *vls* mutants (Fig. 6B-E,B′-E′). However, at later stages (stage 11, Fig. 6B″-E″), Osk levels at the posterior seem somewhat reduced in vls^{null} oocytes compared with OreR and vls^{null} vls^{+}, and we often do not detect any signal for Osk in vls^{null} oocytes. This reduction of Osk levels at the posterior is also observed in hemizygous vls^{PG65}, albeit to a lesser extent (Fig. 6D″).

Confirming these observations, Western blot analyses revealed lower levels of Osk in vls^{null} mutants and vls^{PG65} hemizygotes compared with OreR and vls^{null} vls^{+} ovary extracts, and vls^{null} mutants again show a stronger reduction than vls^{PG65} hemizygotes do (Fig. 7). A similar decrease of Osk levels was reported previously for $vls^{PE36/RB71}$ (Rongo et al., 1995). The Long and Short Osk isoforms are affected differently in *vls* mutants. Whereas Long Osk is only slightly reduced, the Short, indispensable form of Osk, is strongly reduced in vls^{null} and vls^{PG65} hemizygotes. Moreover, we observe an isoform-specific reduction of the hyperphosphorylated (upper) Short Osk compared with the hypophosphorylated (lower) form in *vls* (and *vas*) mutants. This difference is more clearly seen in hemizygous vls^{PG65} than in vls^{null}, as the hypophosphorylated form of Short Osk is also practically undetectable in vls^{null}. This effect had been described previously for *vas* mutants (Markussen et al., 1995). We note that *vls* mutants cause a clear reduction of Long Osk compared with *vas* mutants, which have relatively normal levels. Furthermore, tud^{1} mutant extracts contain lower levels of both isoforms of Short Osk with no isoform-specific

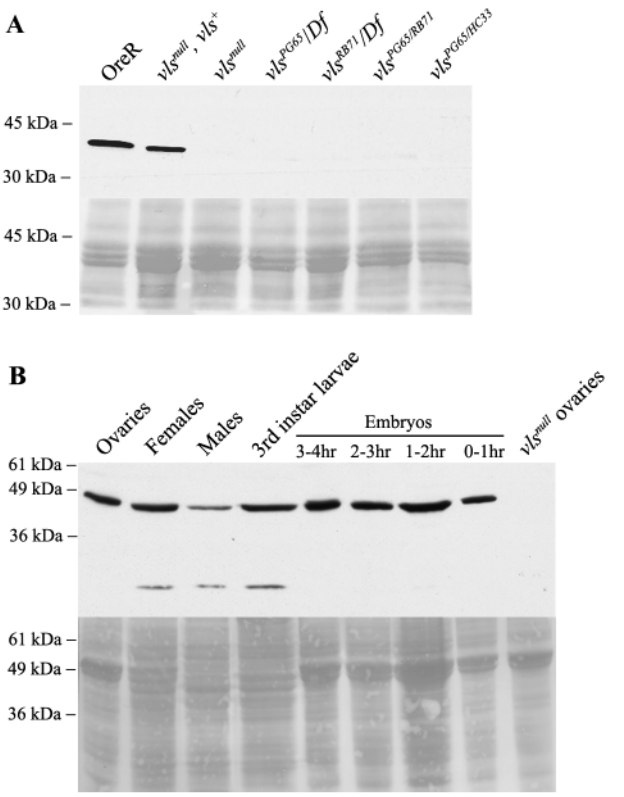

Fig. 4. Western analysis of Vls. Ponceau Red staining serves as a loading control and is shown below the corresponding blot. (A) Anti-Vls antibodies detect a protein of ~42 kDa in wild-type ovaries and in ovaries expressing one copy of the $CG10728^{+}/vls^{+}$ rescue construct. This antigen is not detected in vls^{null} mutants, nor in vls^{EMS} hemizygotes and trans-heterozygotes. Genotypes: vls^{null} – *Df(2L)be408 / Df(2L)pr2b, P[w+ barr+]*; vls^{null} vls^{+} – *Df(2Lbe408, P[w+ CG10728+/vls+] / Df(2L)pr2b, P[w+ barr+]*; and *Df – Df(2L)be408*. (B) Developmental expression profile of Vls. vls^{null} ovary extracts serve as a control for the specificity of the α-Vls antibodies. Other protein extracts were collected from wild-type flies. The faint low molecular weight band does not appear to originate from ovaries or embryos.

reduction, and it seems that the Long isoform might also be slightly reduced although it did not appear to be affected in another study (Markussen et al., 1995).

Taken together, anti-Osk immunostaining and western analyses suggest that *vls* is required for normal levels of Osk to accumulate at the posterior pole while the pole plasm is assembling. Starting around stage 11, Osk signal progressively disappears from the posterior in the absence of *vls* function, and by later stages, Osk accumulation at the posterior is probably greatly reduced, explaining the drastic reduction of overall Osk levels observed on western blots.

vls is essential for posterior localization of Vasa

Vas protein is the next factor in the posterior pathway to localize to the posterior end of the oocyte after *osk* mRNA and protein. This *osk*-dependent Vas localization remains stable at the posterior pole during the early stages of embryogenesis and Vas is later incorporated into pole cells (Lasko and Ashburner, 1990). In vls^{null} ovaries and in embryos from vls^{null} mothers,

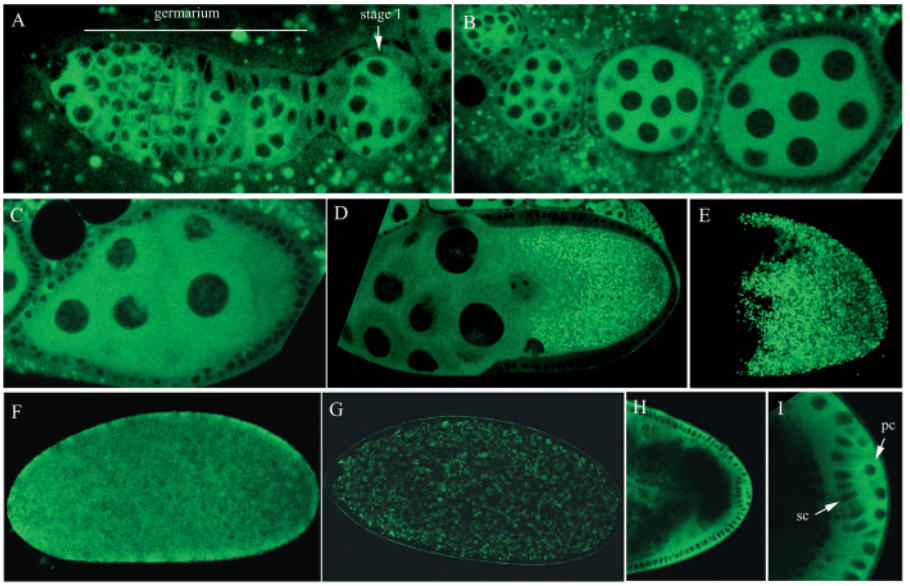

Fig. 5. Distribution of Vls-eGFP in ovaries and embryos. Transgenic flies express two copies of *vls-eGFP*. Anterior is towards the left, posterior towards the right. Vls-eGFP is cytoplasmic. (A) In the germarium, Vls-eGFP signal is found predominantly in the germline and at lower levels also in the somatic follicle cells, and occasionally accumulates in aggregates up to stage 1 egg chambers. Vls-eGFP is uniform in the nurse cells and the developing oocyte throughout the rest of oogenesis. (B) Stage 1-4 egg chambers, (C) stage 8 and (D,E) stage 10. Vls-eGFP signal is generally fairly weak in the oocyte and more difficult to detect against the autofluorescent yolk particles (D). However, the signal is still significantly higher than in control *yw* ovaries without *vls-eGFP* (E). (F,G) 0- to 1-hour-old embryos from *vls-eGFP* (F) and *yw* (G) mothers. (H,I) 1- to 2-hour-old embryos from *vls-eGFP* mothers. Vls-eGFP signal is detected in somatic and germ cell cytoplasm. pc, pole cells; sc, somatic cells.

anti-Vas antibody staining showed very little or no accumulation of Vas at the posterior end (data not shown). This observation was further confirmed by analyzing the distribution of Vas-eGFP (Styhler et al., 1998) in vls^{null} and vls^{PG65} ovaries and embryos. Although the early localization pattern of Vas in nuage of the mutant nurse cells is normal (Fig. 8A-D), the posterior localization in stage 10 oocytes is not observed in the null mutants, and appears very weak in vls^{PG65} hemizygotes (Fig. 8A′-D′).

Later in development, Vas-eGFP signal is detected at the posterior end and then inside the pole cells of embryos from wild-type and vls^{null} vls^{+} mothers, but not from vls^{PG65} hemizygotes and vls^{null} mothers (Fig. 8E-H′). These results contrast with previous reports where Vas localization defects in *vls* mutants (vls^{PE36} and vls^{RB71}) were observed only slightly after fertilization, before pole cell formation (Hay et al., 1990; Lasko and Ashburner, 1990). Our data for the vls^{null} and vls^{PG65} alleles implicate *vls* in the late localization or anchoring of Vas to the posterior cortex during oogenesis.

Discussion

The posterior gene *vls* encodes a maternal protein and is essential for the late localization of Vas to the posterior of the oocyte as well as for the accumulation of Osk, which orchestrates pole plasm assembly. Unlike many other members of the posterior pathway, *vls* transcripts and Vls protein are not localized to the posterior but accumulate uniformly in the nurse cells and oocyte throughout oogenesis. Similar to *vas*, *vls* transcripts and proteins are also detected in adult males even though they have no essential function in males or fly spermatogenesis (Lasko and Ashburner, 1990; Snee and Macdonald, 2004). By contrast, in mice and probably other mammals, *vas* is important for male gametogenesis and has no essential function in female fertility (Raz, 2000). It would thus be interesting to know whether the same is true for vertebrate *vls*.

Nature of *vls* mutants

Specification of the germline in *Drosophila* is more sensitive to pole plasm activity than is abdominal patterning. This is illustrated by the fact that weak alleles of posterior group mutants display a grandchildless phenotype caused by the lack of pole cells, while stronger alleles cause additional abdominal patterning defects that result in embryonic lethality (Lehmann and Nusslein-Volhard, 1991). In our hands, the hemizygous EMS alleles vls^{PG65}, vls^{RB71} and vls^{HC33} are only partially maternal-effect lethal and 100% grandchildless. vls^{null}, however, is 100% maternal-effect lethal. The stronger phenotype of the null mutant suggests that the EMS alleles may be hypomorphs. However, the initial work on *vls* produced strong genetic evidence that the EMS alleles are actually nulls (Schupbach, 1986). It is therefore also possible that the EMS allele stocks accumulated maternal-effect modifiers that allow them to survive to adulthood.

vls mRNA escapes NMD

Although vls^{EMS} alleles contain premature stop codons in the *vls* ORF, the corresponding mutant mRNAs seem to escape nonsense-mediated mRNA decay mechanisms (NMD). Even though premature stop codons are recognized differently in *Drosophila* and vertebrates, the NMD components are conserved (Gatfield et al., 2003). Given that vls^{+} is translated during oogenesis, it seems unlikely that the mutants are protected because of lack of translation (Dreyfuss et al., 2002). It would thus be interesting to find out why vls^{EMS} transcripts accumulate to normal levels.

Collapse of the pole plasm in the absence of *vls* function

Because all aspects of the *vls* mutant phenotype observed in embryos, including abdominal segment deletions, lack of pole cells, gastrulation defects and weak ventralization are rescued completely by a *vls* transgene and not even partially by a *chk2* transgene, we concluded that *vls* alone has a developmental requirement. Furthermore, we have demonstrated elsewhere that *chk2* function is only clearly required upon activation of cell cycle checkpoints (Masrouha et al., 2003). The *vls* phenotypes are reminiscent of a collapse of pole plasm

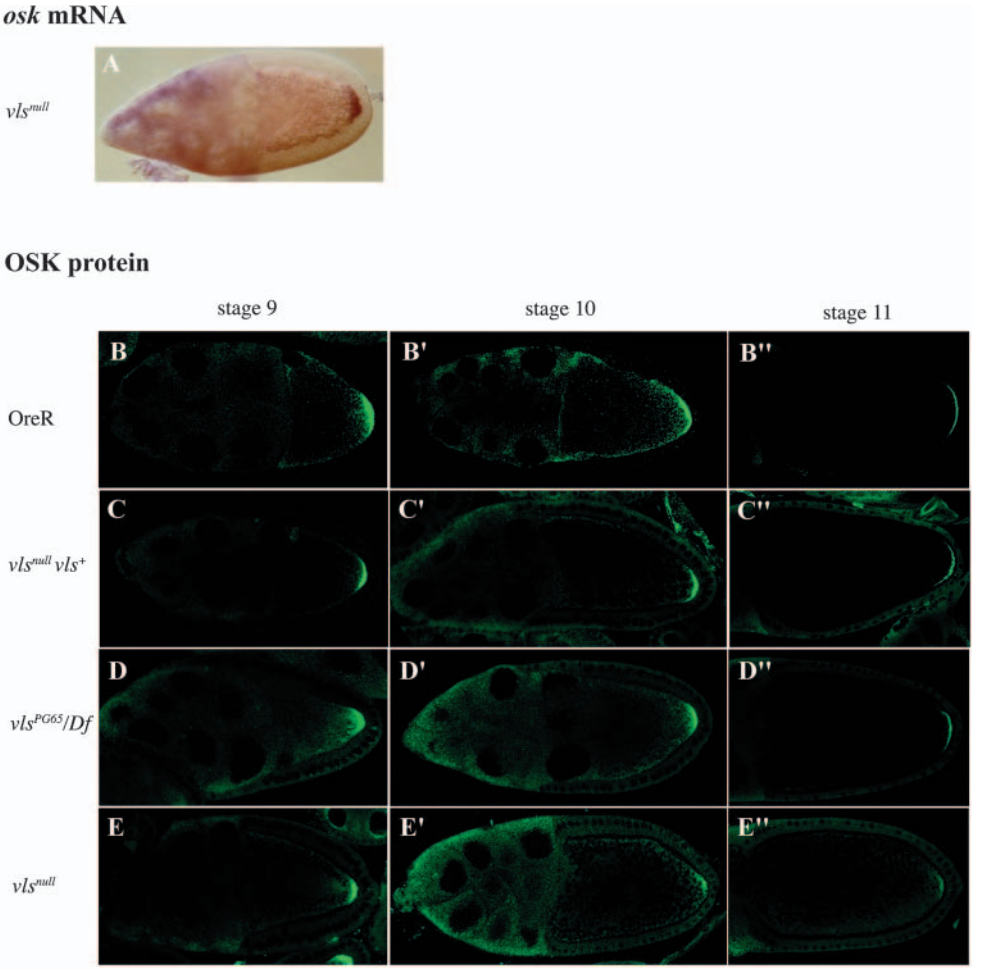

Fig. 6. *osk* mRNA and Osk protein distribution appear normal initially in *vls* mutant ovaries but Osk protein disappears from the posterior pole after stage 11. (A) In situ hybridization with an *osk* probe to a vls^{null} stage 10 egg chamber. *osk* mRNA is correctly concentrated at the posterior of the oocyte in vls^{null} mutants. (B-E″) Immunostaining with α-Osk antibodies on wild-type and *vls* mutant ovaries. Osk signal is tightly concentrated at the posterior of stage 9-10 oocytes of wild type (B-B′) and vls^{null} vls^{+} (C-C′), as well as in vls^{PG65} (D-D′) and vls^{null} (E-E′) females. However, around stage 11, Osk signal appears slightly weaker at the posterior of vls^{PG65} (D″) and significantly weaker in vls^{null} oocytes (E″). This defect is even more pronounced in vls^{null} after stage 11, where usually no posterior Osk signal is detected (not shown).

assembly that seems to occur around stage 10 of oogenesis in our vls^{null} mutants. *vas* is crucial for the pole plasm to assemble properly and recruit the mRNAs and proteins required for pole cell specification and abdominal patterning. Genetic evidence implicates *vas* in the translational activation of several targets during oogenesis, including *osk*, *grk* and, in particular, *nos* at the posterior pole of the embryo (Carrera et al., 2000; Gavis et al., 1996; Johnstone and Lasko, 2004; Markussen et al., 1995; Rongo et al., 1995; Styhler et al., 1998; Tomancak et al., 1998). Vas levels directly correlate with pole plasm activity, pole cell formation being more vulnerable to decreased Vas levels than abdominal patterning is (Ephrussi and Lehmann, 1992). Previous immunostaining for Vas has been reported to show indistinguishable Vas accumulation at the posterior pole of *vls* mutant and wild-type oocytes, and young embryos. These studies, performed with the homo- and hemizygous EMS mutants, showed a loss of posterior localization in the embryos from *vls* mothers sometime between fertilization and pole cell formation (Hay et al., 1990; Lasko and Ashburner, 1990). We used *vas-eGFP* transgenes to assess the posterior localization of Vas in vls^{null} and hemizygous EMS alleles in detail. Maximal localization was still very weak and was found in oocytes and embryos from vls^{EMS} mothers. In vls^{null} mutants we observed a nearly complete failure to localize Vas-eGFP at the posterior pole. This failure coincides with the collapse of the pole plasm and is probably the cause for the various embryonic phenotypes mentioned above. Consistent with this, the observed Vas localization defects parallel the severity of the phenotypes that we report for these *vls* alleles. The weak accumulation of Vas at the posterior of vls^{PG65} hemizygous oocytes gives rise to a grandchildless phenotype, whereas the almost complete absence of Vas from the posterior of vls^{null} oocytes results in a fully penetrant maternal-effect lethal phenotype.

vls is thus required during oogenesis for the localization (transport or anchoring) of Vas to the posterior cortex of the oocyte. The fact that Vls is not specifically enriched at the posterior may suggest that it acts to modify or transport pole plasm components before they reach the posterior pole. Preliminary experiments also failed to produce evidence that Vls and Vas are part of the same protein complex (not shown). This suggests that the mode of action of *vls* on Vas localization is transient or indirect. The fact that *osk* mRNA and protein are initially correctly localized implies that oocyte polarity is normal in *vls* mutants and that *vls* is not required for *osk* mRNA localization. Levels of Osk protein isoforms are then reduced in later stages and western analysis reveals a much more drastic decrease of overall Osk levels than immunostaining does for both types of *vls* alleles. This suggests that most of the drop in Osk levels occurs during the late stages of oogenesis, when the vitelline membrane prevents antibody staining for oocyte Osk. Therefore, it seems that shortly after initiating pole plasm assembly, Osk fails to be maintained at the posterior of *vls* mutants and progressively disappears, concurrent with a complete collapse of the pole plasm.

vls acts upstream of Vas and Osk

Several lines of evidence implicate the Short Osk isoform in directly anchoring Vas. Short Osk interacts strongly with Vas

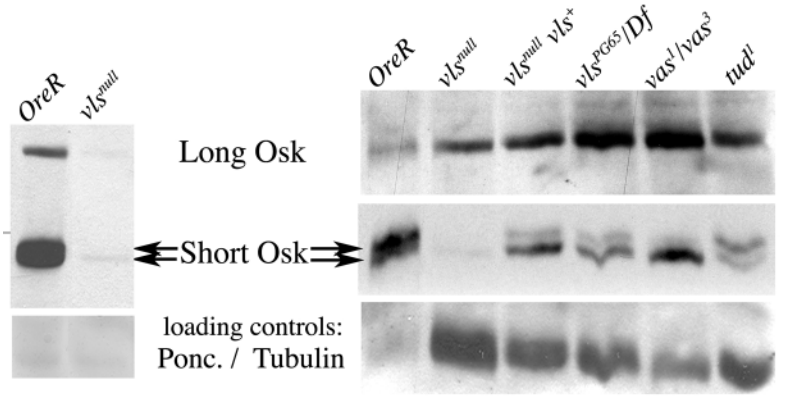

Fig. 7. *vls* is required for normal accumulation of Osk isoforms. Western blots of the indicated ovary extracts probed with α-Osk antibodies. Loading of approximately equal amounts of proteins shows that vls^{null} ovaries contain only very little Osk compared with wild type (*OreR*; left blot). α-Osk antibodies recognize the Long isoform of Osk, as well as the hyperphosphorylated (~57 kDa) and hypophosphorylated (~55 kDa) forms of Short Osk (arrows). For the blot on the right, about ten times as much protein extracts were loaded onto the vls^{null} lane compared with the loading for the *OreR* lane. Ponceau staining of the membrane (Ponc., left blot) and reprobing of the blot with α-Tubulin antibodies (right blot) were used as loading controls.

in the two-hybrid system and recruits Vas when ectopically localized in the oocyte (Breitwieser et al., 1996; Cha et al., 2002; Vanzo and Ephrussi, 2002). Because Vas-eGFP mis-localization patterns in stage 10 oocytes are indistinguishable in *vls* and osk^{54} mutants (not shown), *vls* could act directly at the level of Osk accumulation (e.g. in stimulating translation of *osk*), which is necessary for anchoring Vas at the posterior pole. On the other hand, it is also possible that *vls* acts primarily on Vas protein localization. Because Vas also seems to act in a positive feedback loop back on Osk protein accumulation (Markussen et al., 1995), the lack of Vas localization in *vls* mutants would then also preclude maintenance of posterior accumulation of Osk protein. In *vls* mutants, Osk levels appear to decrease just slightly after Vas should have localized to the posterior pole, thus it appears that the failure to localize Vas could be the cause of the pole plasm collapse in *vls* mutants. To investigate these issues further, we compared Osk levels in *vas* and *tud* mutants with those in *vls* mutants by western analysis where we detect a more significant drop than by immunostaining. This analysis revealed generally stronger phenotypes for *vls* than for *vas* and *tud* mutants. We observed a comparable decrease of Short Osk levels on western blots of *vls*, *vas* and *tud* mutant extracts, but with slight differences in the extent of reduction of the hyper- and hypophosphorylated forms, both of which are more severely affected in *vls* mutants. In addition, we observed a clear reduction of Long Osk levels in *vls*, a minor reduction in *tud,* but none in *vas* mutant extracts (Fig. 7). However, this analysis is complicated by the fact that the *vas* and *tud* alleles that are useful and available, respectively, for these experiments are not nulls (Bardsley et al., 1993; Hay et al., 1988; Lasko and Ashburner, 1990). Their residual activity may therefore maintain Osk at the posterior for a longer period of time. These data are thus consistent with the idea that *vls* acts on either pathway target, Vas or Osk, in a process which could involve additional intermediates that remain to be identified.

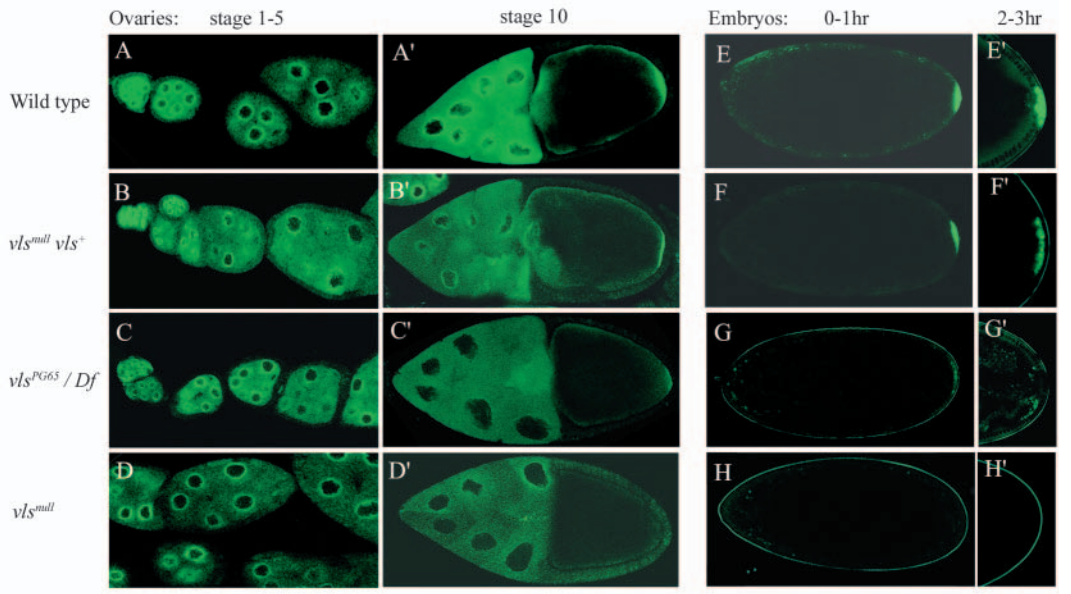

Fig. 8. Posterior Vas localization requires *vls*. Wild type has two copies of *vas-eGFP*. vls^{null} vls^{+}, vls^{PG65} and vls^{null} have one copy of *vas-eGFP*. Fixed ovaries are shown here. Live ovaries show a similar pattern although nuage signal is generally stronger than in fixed ovaries. (A-D) Stage 1-5 egg chambers, (A′-D′) stage 10 egg chambers. (A,A′) Wild-type localization of Vas-eGFP to nuage and to the posterior of the oocyte is observed in Sp/SM1 background. Nuage localization in *vls* mutants appears normal initially (C,D) and slightly reduced in stage 10 egg chambers (C′,D′); however, we did not observe this reduction in live ovaries (data not shown). Posterior localization of Vas-eGFP in the oocyte is undetectable in vls^{null} mutants (D′) and dramatically reduced in vls^{PG65} hemizygotes (C′). This defect is rescued by the introduction of the vls^{+} transgene (B′). The levels of posterior Vas-eGFP appear reduced in vls^{null} vls^{+} oocytes, most probably because of the lower copy number of *vas-eGFP.* (E-H′) Vas-eGFP is not detected at the posterior of young embryos from *vls* mutant mothers. (E-H) 0- to 1-hour-old embryos, (E′-H′) 2- to 3-hour-old embryos. Vas-eGFP accumulates at the posterior of embryos (E,F) and then inside newly formed pole cells (E′,F′) in wild-type and vls^{null} vls^{+} background, but not in embryos from vls^{PG65} hemizygous (G,G′) and vls^{null} mutant (H,H′) mothers.

Why is *vls* not required for expression of the *osk-bcd 3′UTR* phenotype?

vls was tentatively placed downstream of *vas* in the posterior pathway based on studies reporting that Vas localization is correct initially in vls^{EMS} mutants (Hay et al., 1990; Lasko and Ashburner, 1990), and because *vls* was found to be required for the expression of the *6xosk* phenotype (Smith et al., 1992). Surprisingly, however, *vls* is not required for the expression of the *osk-bcd 3′UTR* phenotype (Ephrussi and Lehmann, 1992). As the 3′UTR is present in the *6xosk* transgenes but not in the *osk-bcd 3′UTR* transgene, one explanation for this discrepancy could be that *vls* is required to relieve translational repression mediated by the *osk* 3′UTR.

It is also possible that differences in *osk* mRNA levels and concentration at the anterior between the two systems might explain the discrepancy. In fact, Vas protein accumulation at the posterior pole and the number of pole cells that develop afterwards correlate directly with the *osk* gene copy number (Ephrussi and Lehmann, 1992). Besides, *6xosk* produces lower levels of *osk* mRNA at the anterior than *osk-bcd3′UTR* (Smith et al., 1992). Therefore, the ectopic pole plasm induced by *osk-bcd3′UTR* mRNA is probably more resistant to defects in localization/anchoring of downstream components such as Vas or to defects in the maintenance of Osk itself. By contrast, the *6xosk* system seems to represent a more sensitized background where the collapse of an ectopic pole plasm is more likely to occur in the absence of *vls*. Supporting this idea, the bicaudal phenotype of the progeny from transgenic mothers is 100% penetrant with *osk-bcd 3′UTR* (Ephrussi and Lehmann, 1992), but only 73% penetrant with *6xosk* (Smith et al., 1992). *vls* might thus function as an enhancer of pole plasm assembly, which is dispensable when *osk* pole plasm-inducing activity is already extensively deployed at the anterior. This is consistent with our observation that *vls* dose also correlates with pole plasm activity in the same way that *osk* does. One copy of a wild-type vls^{+} transgene rescues almost completely the phenotypes described for vls^{null}, but we sometimes noted minor defects compared with wild-type flies, and reduced hatching rates of embryos (not shown).

Speculations on the molecular function of Vls

vls differs in many respects from the other long-known members of the posterior pathway and seems to encode a co-factor acting on Osk protein accumulation, Vas localization and possibly on another, yet unknown, component of this pathway. Two lines of evidence suggest that *vls* facilitates the process of pole plasm assembly but is not absolutely essential: some residual Vas localization is possible even in the null mutant; and an ectopic pole plasm can assemble in the absence of *vls* function provided that the system is set up excessively or through different 3′UTR control elements (*osk-bcd3′UTR* vs. *6xosk)*. How could Vls perform this function at the molecular level?

Vls is a divergent WD domain protein. The β-propeller structure of WD proteins is thought to arise from the folding of at least four WD domains and to promote several simultaneous protein-protein interactions (Smith et al., 1999). Because computer predictions only found two or three such domains in Vls, we tested in preliminary experiments whether Vls forms homodimers. However, we did not detect any untagged Vls in immunoprecipitations performed with functional Vls-eGFP and Vls-6xHis fusion proteins (not shown). Whether Vls forms heterodimers with other WD domain-containing proteins remains to be tested. Sequence alignments point to a more likely interpretation. Vls and a whole family of *Drosophila* WD domain proteins show similarities to MEP50, which contains six WD domains and facilitates the interactions between a methyltransferase and its substrates, the Sm proteins (Friesen et al., 2002). Notably, the regions corresponding to the WD domains of MEP50 are better conserved than the others, suggesting that these domains are under greater selection pressure and may therefore fold in similar structures that can fulfill similar functions. This sequence comparison also shows that Vls might not be the ortholog of MEP50 and that different members of this family might fulfill the function of MEP50 in different *Drosophila* tissues.

It is therefore possible that Vls also acts as a mediator of molecular interactions between proteins and possibly also mRNAs. Future experiments will have to focus on identifying the interactors of Vls to determine how precisely *vls* facilitates the pole plasm assembly process. The Vls interactions may turn out to represent an activating step in pole plasm assembly that involves a methyltransferase or another protein modification enzyme and their substrates. With this information it should then also be possible to clarify how directly this mechanism acts on the targets Vas and Osk.

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