Oskar controls morphology of polar granules and nuclear bodies in *Drosophila*

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Germ cell formation in *Drosophila* relies on polar granules, which are large ribonucleoprotein complexes found at the posterior end of the embryo. The granules undergo characteristic changes in morphology during development, including the assembly of multiple spherical bodies from smaller precursors. Several polar granule components, both protein and RNA, have been identified. One of these, the protein Oskar, acts to initiate granule formation during oogenesis and to recruit other granule components. To investigate whether Oskar has a continuing role in organization of the granules and control of their morphology, we took advantage of species-specific differences in polar granule structure. The polar granules of *D. immigrans* fuse into a single large oblong aggregate, as opposed to the multiple, distinct, spherical granules of *D. melanogaster* embryos. *D. immigrans oskar* rescues the body patterning and pole cell defects of embryos from *D. melanogaster oskar*⁻ mothers, and converts the morphology of the polar granules to that of *D. immigrans*. The nuclear bodies, which are structures that appear to be closely related to polar granules, are also converted to the *D. immigrans* type morphology. We conclude that *oskar* plays a persistent and central role in the polar granules, not only initiating their formation but also controlling their organization and morphology.

KEY WORDS: *Drosophila*, Oskar, Pole cell, Polar granules, Nuclear bodies

INTRODUCTION

Pole plasm is a specialized cytoplasm found at the posterior pole of *Drosophila* oocytes. Polar granules form within this cytoplasm by stage 10 of oogenesis and are incorporated into the pole cells (germ cells) by stage 3 of embryogenesis. Classical experiments demonstrated the necessity of the pole plasm for forming germ cells (Geigy, 1931), and the ability of transplanted pole plasm to form ectopic pole cells (Illmensee and Mahowald, 1974). Stronger evidence that the pole-cell-forming activity was contained within the polar granules, rather than posterior cytoplasm, was obtained once polar granule components were identified. The isolation of maternal effect mutants defective in embryonic body patterning revealed one group, the grandchildless-knirps genes, that have posterior patterning defects and fail to form pole cells (Boswell and Mahowald, 1985; Lehmann and Nüsslein-Volhard, 1986; Schüpbach and Wieschaus, 1986). At least four members of this group, *aubergine*, *oskar*, *tudor* and *vasa*, encode proteins that are concentrated in polar granules (Bardsley et al., 1993; Hay et al., 1988; Lasko and Ashburner, 1990; Breitwieser et al., 1996; Harris and Macdonald, 2001). Notably, mislocalization of Osk protein to the anterior, using a transgene containing the *osk* coding region and *bicoid* anterior mRNA localization signal, induces the formation of anterior polar granules and pole cells. All of the known polar granule components are recruited to the site of ectopic polar granules, and the pole cells that form are functional germ cells (Ephrussi and Lehmann, 1992; Nakamura et al., 1996; Megosh et al., 2006). The behavior of the mislocalized Osk protein clearly demonstrates that Osk can nucleate polar granule formation. However, it remains uncertain if Osk serves only to recruit other factors, or if it has a more active or persistent role in the structure and organization of polar granules.

Polar granule organization has been revealed by ultrastructural studies (Mahowald, 1962; Mahowald, 1968; Mahowald et al., 1976). The electron-dense granules first appear at the posterior end of *D. melanogaster* oocytes as an abundance of small particles. These particles persist in the embryo, and as the pole cells mature they fuse to form larger, spherical structures with electron-lucid cores. By gastrulation, each pole cell contains a few dispersed polar granules, which remain until the pole cells migrate through the midgut primordium. In parallel, nuclear bodies, with the same spherical structure, form in nuclei (Mahowald et al., 1976; Harris and Macdonald, 2001). During pole cell migration, the polar granules begin to fragment and associate with the outer nuclear envelope, resembling nuage (Mahowald, 1971). The organization of polar granules differs among *Drosophila* species (Counce, 1963; Mahowald, 1968). Most notably, in *D. immigrans*, the polar granules fuse into one large aggregate (Mahowald, 1968; Mahowald et al., 1976), as opposed to the multiple, spherical, individual granules that are characteristic of *D. melanogaster*.

Here we show that Osk is persistently present in both polar granules and nuclear bodies from their formation to their fragmentation. Expression in *D. melanogaster* of the *osk* gene from *D. immigrans* transforms polar granules and nuclear bodies to the *D. immigrans* type. Our results show that Osk plays a continuing role in polar granule organization, and may be the primary determinant of the underlying arrangement of both polar granules and nuclear bodies.

MATERIALS AND METHODS

Fly stocks
D. immigrans flies were obtained from the Tucson *Drosophila* Stock Center. *P[uas-gfp-aub]* and *P[osk⁺]* have been described previously (Harris and Macdonald, 2001; Kim-Ha et al., 1995).

Transgenes
D. immigrans osk (GenBank accession number: DQ823083) was isolated from a genomic library prepared from *D. immigrans* DNA in Lambda Fix II (Stratagene). *P[oskimm]* contains an 8.7 kb *Not*I-*Asp*718 genomic fragment including 5 kb of DNA upstream of the start codon, and 1 kb downstream of

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the polyadenylation site, all in a pCaSpeR vector (Pirotta, 1988) modified by insertion of *Not*I and *Asp*718 linkers at filled-in *Pst*I and *Eco*RI sites, respectively. In *P[osk^{imm3'mel}]* the *osk^{imm}* 3′UTR and 3′ flanking sequences are replaced with the equivalent region (a 1.6 kb *Hin*dIII-*Asp*718 fragment) from *D. melanogaster osk*. The *P[M1L-osk^{imm3'mel}]*, *P[M103L-osk^{imm3'mel}]* and *P[M103,106L-osk^{imm3'mel}]* transgenes were all modified from *P[osk^{imm3'mel}]* using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene).

Sequence analysis

Oskar protein sequences from different *Drosophila* species (http://flybase.net/blast/) were compared using ClustalW (http://www.ebi.ac.uk/clustalw/). To form the graph, each amino acid was given a score from 0-8 depending on how many sequences were identical. The average of every 5 amino acids was plotted in Excel (Microsoft).

Immunostaining and imaging

Embryos were collected and stained as described previously (Macdonald and Struhl, 1986; Macdonald et al., 1991) using secondary antibodies coupled to Cy5 (Jackson Immunoresearch Laboratories). Osk antibodies were used at 1:3000, with incubation at room temperature overnight. Stained embryos were mounted in Vectashield medium (Vector Labs) and imaged using a Leica TCS-SP confocal microscope.

RESULTS AND DISCUSSION

To determine if Osk has an ongoing role in polar granule organization beyond the initial recruitment of other factors, we asked if replacing Osk with *D. immigrans* Osk (Osk^{imm}) would convert the polar granules of *D. melanogaster* to the *D. immigrans* type. The *D. immigrans osk* transgene P[osk^{imm}] was expressed in *D. melanogaster* flies lacking endogenous Osk protein

[*osk^{54}/Df(3R)pXT103*; referred to below as *osk^{54}/Df*]. *osk^{imm}* mRNA was localized to the posterior of the embryo (although not as tightly as *osk* mRNA; see Fig. S1 in the supplementary material), and rescued the body patterning and pole cell formation defects of *osk* mutants; fewer pole cells formed, but they were functional. A related transgene, *P[osk^{imm3'mel}]*, bearing the 3′UTR and associated regulatory sequences of *osk*, behaved almost identically (see Fig. S2 in the supplementary material). These phenotypes, and those described below, are dependent only on the maternal genotype; for simplicity, the embryos are described by their mother's genotype.

In pre-cellular blastoderm *D. melanogaster* embryos, polar granule components, including Osk and the granule marker GFP-Aub, appear at the posterior in a crescent of small sand-like granules, approximately 0.2-0.3 μm in diameter. At cellular blastoderm stage, the small granules have begun to coalesce to form spherical particles (0.7-1.4 μm in diameter). These spheres appear in confocal optical sections as donut shapes (Harris and Macdonald, 2001) (see Fig. S3 in the supplementary material).

In early *P[osk^{imm}]/+; osk^{54}/Df* embryos, the progression of polar granule development was like that of *D. melanogaster* until the cellular blastoderm stage (Fig. 1I), when the smaller granules began to accumulate in one area of the cell, so that by gastrulation each pole cell contained a single large aggregate of granule material (Fig. 1J), as in *D. immigrans* embryos. This aggregate appeared in confocal sections as a series of loosely connected particles (Fig. 1L), whose linkage to one another was clearly revealed in a *z* series of sections (data not shown). This polar granule morphology persisted until the pole cells began to migrate. Expression of *P[osk^{imm3'mel}]* in *osk^{54}/Df* embryos had essentially the same effect (data not shown).
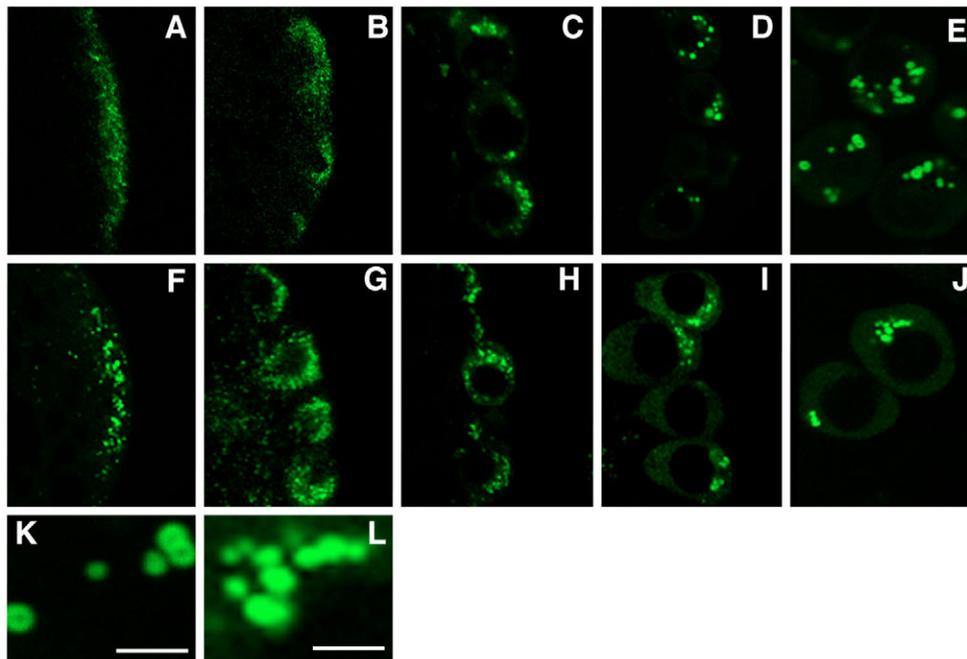

Fig. 1. Osk^{imm} dictates polar granule morphology. Granule morphology revealed by signal from GFP-Aub at (**A**,**F**) egg-lay, (**B**,**G**) pole bud formation, (**C**,**H**) syncytial blastoderm, (**D**,**I**) cellular blastoderm and (**E**,**J**) gastrulation. A and F are at lower magnification to show the majority of the polar plasm. (**K**,**L**) Enlargements of granules shown in E and J, respectively. Maternal genotypes: A-E, K, *osk^{54}/+*; F-J, L, *P[osk^{imm}]/+; osk^{54}/Df*. In early embryos, the granules of all genotypes appear essentially the same: small, sand-like and spread throughout the cytoplasm. At cellular blastoderm and gastrulation, polar granules in control embryos with either a single endogenous copy of *osk^+* (D,E) or no endogenous *osk* and a single copy of the *P[osk^+]* transgene (data not shown) have the characteristic wild-type 'donut' appearance. In *P[osk^{imm}]/+; osk^{54}/Df* (I,J) or *P[osk^{imm3'mel}]/+; osk^{54}/Df* (data not shown) embryos, the granules fail to form 'donuts' and fuse into one area of granule material per cell. When seen as serial projections, these areas of granule material appear as a single continuous aggregate. Scale bar: 2 μm for K,L.

Thus, Osk[imm] confers the *D. immigrans* morphology on polar granules formed in a background where all components but Osk are of the *D. melanogaster* type. The control embryos, in which polar granule formation relies on a single copy of *osk*, had the typical appearance of *D. melanogaster* polar granules at all stages (Fig. 1A-E).

Osk appears not only in polar granules in the pole cell cytoplasm, but also in structures identified as nuclear bodies (on the basis of morphology and the presence of Vas), and persists in the nuclear bodies until their decay (Harris and Macdonald, 2001) (see Fig. S3 in the supplementary material). In *D. melanogaster*, the nuclear bodies first appear at syncitial blastoderm stage. They persist until the pole cells migrate to the gonad when they are no longer detectable. The nuclear bodies of *D. immigrans* are initially similar to those of *D. melanogaster*, displaying the characteristic donut appearance in cross-section. However, during the cellular blastoderm stage they develop discontinuities and distortions. Often, portions of multiple spheres aggregate (Mahowald et al., 1976).

To determine if Osk[imm] would alter *D. melanogaster* nuclear body morphology, the transgenes expressing the protein were tested in a wild-type background (this is necessary because immunodetection of Osk is used to visualize the bodies; GFP-Aub is only cytoplasmic and we have no antibodies to detect Osk[imm]). In the presence of Osk[imm], the nuclear bodies initially appeared with the regular spherical morphology shared by both species up to the cellular blastoderm stage (Fig. 2C). Subsequently, the nuclear bodies developed fissures, as seen in the donut cross-sections, and the

spheres became irregular and flattened (Fig. 2F). Thus, the nuclear bodies had been transformed towards the *D. immigrans* morphology. It is not surprising that the transformation was not complete, as endogenous Osk was also present. We conclude that Osk[imm] is a determinant for the morphology of polar granules and nuclear bodies.

Osk protein is made in two forms, Long and Short, initiated from different methionine codons (Markussen et al., 1995; Vanzo and Ephrussi, 2002). To determine which Osk[imm] isoform is responsible for polar granule morpology, three transgenes based on *P[osk^{imm3'mel}]*, but with mutations to direct synthesis of the individual Osk[imm] isoforms, were tested in wild-type flies.

The *P[M1L-osk^{imm3'mel}]* transgene (with the first methionine codon mutated to leucine, and thus unable to make Long Osk[imm]) transformed granules to the *D. immigrans* type (Fig. 3B). Thus, the Short isoform of Osk[imm] is sufficient to specify polar granule morphology. The *P[M103L-osk^{imm3'mel}]* and *P[M103,106L-osk^{imm3'mel}]* transgenes differed in their activities. When only the first of the two methionines near the predicted start of Short Osk[imm] was mutated (M103L), the transgene could still transform the polar granules to the *D. immigrans* type (Fig. 3C). However, when both methionine codons that could initiate Short Osk[imm] were mutated (M103L and M106L), the ability to transform polar granule morphology was lost (Fig. 3D). These results strongly suggest that either methionine can initiate translation of Short Osk[imm], and that the Long Osk[imm] isoform does not contribute to specification of polar granule morphology.
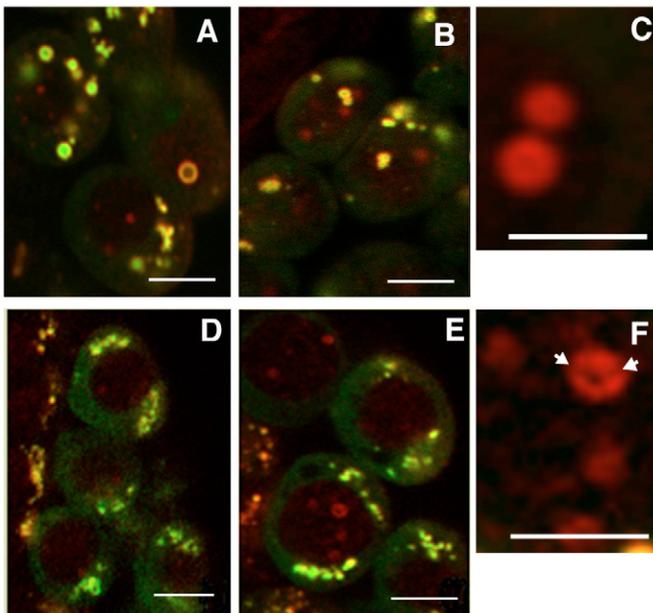

Fig. 2. Osk[imm] dominantly influences polar granule and nuclear body morphology. Pole cells displaying GFP-Aub (green) and immunostained for Osk (red) from cellular blastoderm stage (**A**,**D**) or gastrulation stage (**B**,**C**,**E**,**F**) embryos, with enlarged views of nuclear bodies in C and F. Embryos are from otherwise wild-type mothers bearing a single copy of the following transgenes: A-C, *P[osk⁺]*; D-F, *P[osk^{imm}]*. *P[osk⁺]* polar granules are spherical (A,B). In the presence of both endogenous Osk and Osk[imm] (D,E), the polar granules fuse into one to three large aggregates. Nuclear bodies are normally spherical (C), but in *P[osk^{imm}]* embryos (F) the nuclear bodies show deformations and discontinuities (arrows). Scale bars: A,B,D,E, 5 μm; C,F, 2 μm.
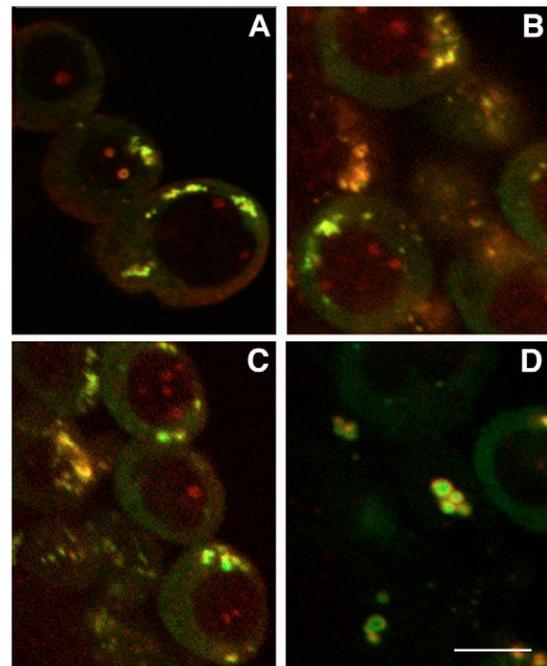

Fig. 3. Short Osk[imm] controls polar granule morphology. Polar granule and nuclear body morphology in gastrulating embryos revealed by GFP-Aub (green) and immunostaining for Osk (red). Embryos are from otherwise wild-type mothers bearing a single copy of the following transgenes: (**A**) *P[osk^{imm3'mel}]*; (**B**) *P[M1L- osk^{imm3'mel}]*, (**C**) *P[M103L- osk^{imm3'mel}]*; (**D**) *P[M103,106L-osk^{imm3'mel}]*. Polar granule morphology is shifted towards the *D. immigrans* type in A-C, but not D. Although there are spherical bodies in A-C, they are all in nuclei (i.e. nuclear bodies), and the cytoplasmic polar granules are not spherical. Scale bar: 5 μm.

DEVELOPMENT

Alignment of Osk and Osk[imm], as well as Osk from other species, revealed regions of higher and lower similarity (see Fig. S4 in the supplementary material). The variation between Osk and the predicted Osk[imm] protein sequences is similar to that seen in comparison of other species of similar evolutionary divergence. Thus, the feature responsible for the striking difference in the polar granules of *D. melanogaster* and *D. immigrans* cannot be readily predicted.

The Osk, Vas, Aub and Tud proteins are present in polar granules and are required for their formation. Only for Tud has a specific function in polar granule assembly been suggested. Several mutants in Tud result in smaller and fewer granules, raising the possibility of a role in the fusion of granule material (Boswell and Mahowald, 1985; Thomson and Lasko, 2004). Recently, a detailed dissection of Tud revealed a crucial role for Tud domain 1 (*tud^{A36}* mutant) in germ cell formation and polar granule formation, and led to the proposal that the Tud domains serve as docking platforms for polar granule assembly. Notably, mutation of a single Tud domain altered polar granule morphology from the small, sand-like spherical granules seen in wild-type embryos at egg-lay, to string-like granules (Arkov et al., 2006). Thus, Osk, with its central role in specifying granule morphology, may perform this role via interactions with Tud. We compared, by yeast two-hybrid assays, the interactions of the short isoforms of Osk and Osk[imm] proteins with Tud and with the other known *D. melanogaster* polar granule components (Breitwieser et al., 1996), but found no substantial differences in the level of interactions displayed by the Osk and Osk[imm] proteins (data not shown).

Evidence of a central role for Osk in organizing polar granules and nuclear bodies focuses attention on the question of how it performs these functions, and on what features of Osk determine which type of granule will form. The answer to the first question must involve the interactions of Osk with other granule components, but how molecular interactions of proteins whose volume is measured in Angstroms can direct formation of seemingly perfect spheres that are hundreds of nanometers in diameter is uncertain. The extreme regularity of the structures invites comparisons to other highly regular spherical structures, such as viral capsids. The principles underlying the assembly of icosahedral virions could also apply to spherical polar granules, with an important difference: virions are assembled from a well-defined number of subunits, usually organized as a series of hexamers and pentamers (Morgan, 2003). A similar assembly on the scale of polar granules would require subunits several orders of magnitude larger than the viral capsid proteins. Thus the subunits would have to consist of a very large number of individual polypeptides; these subunits could possibly correspond to the large number of small, sand-like polar granules seen very early in embryogenesis. Extending the analogy to viral capsid assembly to address the different morphologies of polar granules, the spherical polar granules could be comparable to wild-type viral capsids, with the *D. immigrans*-type granules comparable to polymorphic aggregates of the coat proteins. Whereas only the spherical assemblies could function to encase a viral genome, either type could contain integral germ cell determinants. Our results show that either type of polar granule can mediate germ cell formation in *D. melanogaster*.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/2/233/DC1

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