The Crystal Structure of the Drosophila Germline Inducer Oskar Identifies Two Domains with Distinct Vasa Helicase- and RNA-Binding Activities

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SUMMARY

In many animals, the germ plasm segregates germline from soma during early development. Oskar protein is known for its ability to induce germ plasm formation and germ cells in Drosophila. However, the molecular basis of germ plasm formation remains unclear. Here, we show that Oskar is an RNA-binding protein in vivo, crosslinking to nanos, polar granule component, and germ cell-less mRNAs, each of which has a role in germline formation. Furthermore, we present high-resolution crystal structures of the two Oskar domains. RNA-binding maps in vitro to the C-terminal domain, which shows structural similarity to SGNH hydrolases. The highly conserved N-terminal LOTUS domain forms dimers and mediates Oskar interaction with the germline-specific RNA helicase Vasa in vitro. Our findings suggest a dual function of Oskar in RNA and Vasa binding, providing molecular clues to its germ plasm function.

INTRODUCTION

Propagation and survival of metazoan species depend on the maintenance of the germline. Germ cell formation is an integral part of sexual reproduction. In many animals, a specialized cytoplasm—the germ plasm—is essential for germ cell formation. The germ plasm of Drosophila (pole plasm) forms during oogenesis at the posterior pole of the oocyte. In this organism, the process of germ cell formation is coupled to posterior patterning of the embryo and involves a series of mRNA and protein localization events. At the top of a genetic hierarchy controlling these processes resides the maternal effect gene oskar (Lehmann and Nüsslein-Volhard, 1986; Ephrussi et al., 1991; Kim-Ha et al., 1991; Ephrussi and Lehmann, 1992). Germ cell formation is achieved by the Oskar-protein-dependent accumulation of pole plasm components such as germ cell-less (gcl), nanos (nos), and polar-granule component (pgc) mRNAs (Mahowald, 2001). Abdominal patterning results from Oskar-dependent localization and translation of nanos mRNA and formation of a gradient of the posterior determinant Nanos (Ephrussi et al., 1991; Barker et al., 1992; Wang and Lehmann, 1991; Wang et al., 1994; Gavis and Lehmann, 1994).

oskar mRNA is produced by the nurse cells and transported to the posterior of the maturing oocyte within the germline syncytium during oogenesis (Ephrussi et al., 1991; Kim-Ha et al., 1991). At the posterior pole, two Oskar protein isoforms (Long and Short Oskar) are synthesized by alternative translation initiation from two in-frame start codons in oskar mRNA (Figure 1A) (Markussen et al., 1995). The more-abundant, Short Oskar isoform is necessary and sufficient to induce pole cell formation and posterior patterning in the embryo (Markussen et al., 1995; Breitwieser et al., 1996; Vanzo and Ephrussi, 2002). In contrast, Long Oskar is dispensable for germ cell and abdomen formation per se but is essential for proper anchoring of both oskar mRNA and Short Oskar to the posterior cortex of the oocyte and thus for effective germ plasm accumulation (Markussen et al., 1995; Vanzo and Ephrussi, 2002).

The fact that Short Oskar is sufficient to induce a functional germ plasm, whereas Long Oskar is not, has been attributed to the Short Oskar isoform-specific recruitment of Vasa protein to the germ plasm (Breitwieser et al., 1996). Vasa is a conserved DEAD-box RNA helicase involved in metazoan germ line development (reviewed in Lasko, 2013). In the Drosophila germline, Vasa is required for formation of the perinuclear nuage in nurse cells, where it constitutes an essential component of the secondary piRNA synthesis pathway (Liang et al., 1994; Malone et al., 2009; Xiol et al., 2014). In the oocyte, Vasa has been implicated in mRNA translation activation via its direct interaction with the initiation factor eIF5B (Carrera et al., 2000; Johnstone and Lasko, 2004).

Oskar is the only protein shown so far to be capable of inducing the formation of a functional germ plasm in an organism. Despite Oskar’s established importance during development, the molecular properties of the protein have remained elusive. Here, we present the crystal structures of the two Oskar domains and address the molecular activities of the germ plasm inducing short isoform in vitro and in vivo. Our analyses reveal that Short Oskar physically interacts with RNA and with the...
Oskar Is an RNA-Binding Protein

(A) Long and Short Oskar domain organization. Residue numbers correspond to the long isoform. The domain boundaries match the crystallization constructs (see also Figure S3).

(B) Embryos were UV irradiated (“+ UV”) and lysed, and mRNA was purified using oligo(dT) beads (“+ oligo(dT)”). Control experiments involved either no UV irradiation of the embryos (“− UV”) or no oligo(dT) on the beads (“− oligo(dT))”. Presence of PABPC, Oskar, and actin in the eluates was tested by western blot using the respective antibodies. (Determination of the total amount of mRNA and protein in the eluates is shown in Figures S1A and S1B.)

(C) Embryos were UV irradiated (“crosslink”), lysed, and subjected to immunoprecipitation with anti-Oskar antibody. After stringent washing, bound RNA was reverse transcribed and the cDNA analyzed by real-time PCR analysis using oligonucleotides specific for the genes indicated on the abscissa. In three biological replicates, the fold enrichment of the mRNAs was determined by the difference of the Ct values resulting from the anti-Oskar immunoprecipitation and those from a control experiment, from which the anti-Oskar antibody was omitted (“control”). A parallel experiment with embryos that did not receive UV irradiation (“no crosslink”) serves as specificity control. The data are represented as mean ± SD, and their p values were calculated with Student’s t test and are as follows: *p = 0.019; **p = 0.0056; ***p = 0.00048. The Oskar-specific enrichment of the mRNAs does not correlate with their relative amounts in the inputs (see Figure S1D).

(D) GST or the indicated GST fusion proteins were incubated with embryo extract and subjected to UV crosslink/mRNA pull-down. Eluates were treated with RNase, and proteins were detected by western blot using anti-GST antibody. The asterisk indicates cross-reaction of the secondary antibody with RNase. Protein markers are indicated on the left (kDa).

(E and F) Nitrocellulose-filter-binding assays using a short ssRNA oligo together with GST and GST fusion of Short Oskar (E) or with untagged LOTUS and Osk domain (F). In (E), the GST tag was necessary to obtain soluble Short Oskar.

See also Figures S1 and S3.

RESULTS

Oskar Is an RNA-Binding Protein

Short Oskar has been shown to localize to polar granules (Breitwieser et al., 1996; Vanzo et al., 2007), electron-dense ribonucleoprotein particles associated with germ plasm in many organisms (Mahowald, 2001; Voronina et al., 2011). Given that several maternal mRNAs require Oskar protein for their localization to the posterior pole of the oocyte (Ephrussi et al., 1991; Jongens et al., 1992; Ding et al., 1993; Nakamura et al., 1996), we tested whether Oskar is able to bind RNA in vivo. To do so, we applied an established UV crosslinking and mRNA pull-down strategy that allows identification of proteins directly bound to mRNAs (Castello et al., 2013). UV crosslinking of RNA to proteins requires a so-called “zero-distance” length between nucleotide bases and protein side chains (Pashev et al., 1991; Zhang et al., 2004), allowing the identification of direct RNA-binding proteins. As Oskar protein is present in maturing oocytes and early embryos, we adapted the protocol for use with Drasophila embryos, which are easy to collect in large quantities. A population of 0- to 2-hr-old embryos was irradiated with 254-nm UV light, resulting in crosslinking of direct RNA-binding proteins to their target RNAs, within their native environment. After lysis of the embryos, mRNAs and crosslinked proteins were purified from the lysate using oligo(dT) beads. The covalent link between mRNA and RNA-binding proteins allows high stringency washing, resulting in removal of proteins not crosslinked to mRNA. Background controls included mRNA pull-downs from lysates of UV-irradiated embryos using beads lacking oligo(dT) and mRNA purification from embryos not treated with UV light. Eluates from experiments performed with oligo(dT) beads contained comparable amounts of mRNA, whereas only background levels of mRNA were eluted from the mock bead control, showing that the mRNA purification was specific for oligo(dT) (Figure S1A). Proteins only co-purified with mRNA under conditions where UV light was applied and oligo(dT) beads were used, demonstrating the specificity of the method (Figure S1B). Western blot analyses of the eluates revealed that Oskar was specifically crosslinked to mRNA, as was the cytoplasmic poly(A)-binding protein (PABPC), which served as a positive control (Figure 1B). Actin, a known non-RNA-binding protein was not crosslinked to mRNA (Figure 1B). This experiment demonstrates that Oskar is a direct RNA-binding protein. Given that Long Oskar was not detectable in the crosslinked eluates.
Oskar Binds to nos, pgc, and gcl mRNAs In Vivo
It has been shown genetically that several mRNAs, such as nos, gcl, pgc, and Hsp83, require Oskar function for their posterior localization during oogenesis (Ephrussi et al., 1991; Jongens et al., 1992; Ding et al., 1993; Nakamura et al., 1996). Oskar protein is also required to maintain localization of its own transcript (Ephrussi et al., 1991; Kim-Ha et al., 1991; Vanzo and Ephrussi, 2002). In early embryos, Oskar plays an additional role in translational de-repression of nos mRNA (Dahanukar et al., 1999; Zaessinger et al., 2006; Jeske et al., 2011).

To test whether Oskar binds directly to one of the aforementioned mRNAs, we immunoprecipitated Oskar protein from lysates prepared from UV-irradiated embryos. After stringent washes, the enrichment of mRNAs crosslinked to Oskar was determined by quantitative real-time PCR analysis. All values were normalized to those derived from parallel control immunoprecipitations from which the anti-Oskar antibody was omitted (Figure 1C). Among the putative mRNA targets tested, nos, pgc, and, to a lesser extent, gcl mRNAs were significantly enriched. In control experiments lacking the crosslinking step, the same mRNAs were either not or only slightly enriched, showing that enrichment of the mRNAs depends on direct contact with Oskar. Oskar does not bind generally to posterior localizing mRNAs, as osk, cyclin B (cycl8), Hsp83, or oo18 RNA-binding protein (orb) mRNAs were not enriched (Figure 1C). We also did not detect significant Oskar binding to the anterior localized bcd mRNA or to the evenly distributed Actn 42A mRNA.

The OSK Domain Binds to RNA
To gain insight into the RNA-binding ability of Oskar, we explored the biochemical properties of the protein. The two Oskar protein isoforms harbor two defined domains of so far unknown function (Figure 1A). The N-terminal region of Oskar contains a LOTUS (Limkain, Oskar, and Tudor containing proteins 5 and 7) or OSTMH (Oskar-TDRD5/TDRD7-helix-turn-helix) domain, which was identified through bioinformatics and suspected to represent an RNA-binding domain (Anantharaman et al., 2010; Callebaut and Moron, 2010). The LOTUS domain is conserved from bacteria to higher eukaryotes. In the animal kingdom, LOTUS domains are found in germline-specific proteins, such as Oskar, the Tudor domain-containing proteins (TDRD) 5 and 7, and the meiosis arrest femal e1 (MARF1) (also known as Limkain-b1). The C-terminal region of Oskar contains a domain related to SGNH hydrolases but also bears features unique to Oskar proteins (see below). We therefore named it the OSK domain.

To assess which of the domains mediates the interaction of Oskar with RNA, we performed a modified UV crosslink/mRNA pull-down experiment. Bacterially expressed Oskar LOTUS (aas 139–240) or OSK domain (aas 401–606) fused to GST was added to extracts of 0- to 2-hr Drosophila embryos and incubated to allow protein-RNA complex formation. Subsequently, the lysates were treated with UV light and subjected to oligo(dT)-mediated mRNA pull-down. The GST tag allowed detection of the different recombinant proteins with equal sensitivity in western blots of the eluates. Whereas the GST-LOTUS domain did not co-purify with the mRNA (Figure 1D), the OSK domain unexpectedly showed RNA-binding properties.

RNA crosslinking to proteins requires the presence of specific amino acid residues (mainly Cys, Lys, or aromatic residues) at zero-distance length to the ribonucleotide bases (Hocksmith et al., 1986; Zhang et al., 2004). Considering that such amino acids might not be present or ideally positioned in the Oskar LOTUS domain, we tested the ability of recombinant Short Oskar or its individual domains to bind to an RNA oligonucleotide in a nitrocellulose filter-binding assay. Not knowing what, if any, specific RNA motif is bound by Oskar, the unspecific RNA oligo we used in the filter-binding assay solely serves to measure the intrinsic affinity of the individual Oskar domains for RNA, independently of a crosslinking reaction.

Consistent with the UV crosslinking results, GST-Short Oskar as well as the untagged OSK domain bound to the RNA oligo, whereas the untagged LOTUS domain did not interact with RNA in this assay (Figures 1E and 1F). The affinity of both GST-Short Oskar and the OSK domain alone for the RNA oligo used is in the low micromolar range but might be significantly higher for one of the genuine substrates.

Taken together, our experiments demonstrate that Oskar is an RNA-binding protein in vivo. Our in vitro experiments indicate that the Oskar domain has the capacity to bind RNA and suggest it is an RNA-binding domain. That said, further work is needed in the future to obtain information on Oskar’s RNA-binding specificity through unbiased identification of its in vivo RNA-binding sites, using methods such as crosslink immunoprecipitation (iCLIP).

Crystal Structure of the OSK Domain
To gain insight into the structure of the OSK domain, we crystallized the carboxy-terminal portion of Oskar (aas 401–606). The structure was solved by single isomorphous replacement with anomalous scattering (see Supplemental Experimental Procedures) and subsequently refined to R/Rees values of 16.6%/19.5% at 1.7 Å resolution (Table S1). The RNA-binding OSK domain folds into an α-β sandwich structure consisting of a central β sheet surrounded by α helices (Figure 2A), resembling SGNH hydrolases. SGNH hydrolases catalyze the hydrolysis of diverse types of ester bonds (Akoh et al., 2004) and are classified by the presence of four invariant catalytic residues Ser, Gly, Asn, and His, each located in one of four conserved sequence blocks (Figure 2B, white letters). Of these, Ser and His, together with another Asp, form a serine triad (Figure 2B, violet highlights; Figure 2C, left, violet residues).

The OSK domain shows highest structural similarity to several SGNH hydrolases (Figure S2A), including the highly substrate-specific mammalian platelet-activating factor acetylhydrolase (PAF-AH) and the more-promiscuous E. coli enzyme.
TAP, which can act as an esterase, thioesterase, arylesterase, lysophospholipase, and even as a protease (reviewed in Akoh et al., 2004). However, the OSK domain lacks three of the four residues of the SGNH motif, as well as the serine triad (Figures 2B and 2C), arguing against an enzymatically active domain. Furthermore, in the OSK domain, the loop—which harbors the catalytic triad residues Asp and His in SGNH enzymes—contains an 11-amino-acid residue insertion that engages in long-range contacts with the OSK domain body (Figures 2B and 2C). The length of the insertion and several of the loop residues are conserved in all Oskar orthologs (Figure S3), suggesting a possible role of the loop in OSK domain function. BLAST search revealed that such an SGNH hydrolase fold lacking the active site residues is unique to Oskar orthologs.

Over the years, numerous oskar alleles have been identified in EMS mutagenesis screens for mutants displaying posterior patterning defects (Lehmann and Nüsslein-Volhard, 1986; Tearle and Nüsslein-Volhard, 1987; Ephrussi et al., 1991; Rongo et al., 1995). These oskar alleles fall into two classes: nonsense alleles that contain premature stop codons and therefore lack the OSK domain (Figure 3A, empty arrowheads) and a dozen missense alleles harboring point mutations, all of which lie within the OSK domain (Figure 3A, filled arrowheads) (Kim-Ha et al., 1991; Rongo, 1996). Together, these mutants point toward a possible essential role for the OSK domain in Oskar protein function. The crystal structure allowed us to map the positions of the different missense mutations in the OSK domain. All 12 missense mutations affect amino acids located within one-half of the OSK domain (Figure 3B). Introduction of any of these mutations dramatically decreased solubility of recombinant GST-OSK (M.J., unpublished data), suggesting that they may interfere with proper protein folding and stability. Consistent with our observation, several of these mutations prevent accumulation of Oskar protein during oogenesis (Markussen et al., 1997).

Finally, we analyzed the crystal structure of the OSK domain with respect to its RNA-binding activity. About two-thirds of the OSK domain surface is positively charged or hydrophobic (Figure S3C) and hence shows chemical properties typical of RNA-binding proteins. Furthermore, the OSK domain crystallized in the presence of high concentrations of ammonium sulfate, and six sulfate ions, tightly coordinated with basic residues, could be inferred from the electron density map (Figures S2B–S2D). Sulfate and phosphate ions are chemically similar and are frequently present in crystal structures of nucleic-acid-binding domains, where they occupy protein areas with a potential to contact the sugar-phosphate backbone of nucleic acids. Thus, residues in these areas might be good candidates for mutational analysis with respect to RNA binding in the future.

Taken together, we conclude that the crystal structure of the RNA-binding OSK domain resembles an inactive SGNH hydrolase domain with surface properties characteristic of RNA-binding domains.
Crystal Structure of the Oskar LOTUS Domain Dimer

In view of the absence of experimental support for an RNA-binding function of the Oskar LOTUS domain, we wished to gain insight into its function. We therefore analyzed the purified LOTUS domain and found that it did not behave as a monomer during size exclusion chromatography. Subsequent static light scattering (SLS) measurement revealed that the LOTUS domain forms dimers (Figure S5A). Due to the high affinity of the monomers, we were not able to determine the dissociation constant of the dimer. However, we estimate its $K_D$ to be in the nM range, as analytical gel filtration analysis of the protein at dilutions close to the detection limit (2 μM) as well as analyses by analytical ultracentrifugation did not result in detectable dimer dissociation.

We crystallized two LOTUS domain constructs, comprising amino acids 139–222 and 139–240 (Figures S3 and S4). Crystals of the first construct diffracted to 2.1 Å and the second to 2.35 Å resolution, and both structures were solved by molecular replacement (see Supplemental Experimental Procedures). The structures were subsequently refined to $R/R_{ref}$ values of 18.6%/23.4% and 19.4%/21.5%, respectively (Table S1). The Oskar LOTUS domain represents a winged helix-turn-helix domain consisting of three $\alpha$ helices and two $\beta$ strands, folded into a three-helix bundle, with a two-stranded antiparallel $\beta$ sheet packed on top (Figure 4A). Our structures of the Drosophila protein revealed an additional N-terminal $\alpha$ helix that is poorly conserved in other insect orthologs ($\alpha$ helix α1; Figures 4A and S3). In contrast, the presumably unstructured C-terminal extension of the LOTUS domain (residues 220–240; Figure S4A) is highly conserved in Oskar (Figure S3). For these reasons, in all our assays addressing Oskar LOTUS domain function, we made use of the longer N-terminal construct of Short Oskar (aas 139–240) spanning the N-terminal $\alpha$ helix, the LOTUS domain core, and the conserved C-terminal amino acids (Figures 1A and S3).

The interface area and the buried surface area of the dimer measure 742 Å² and 1,480 Å², respectively (PISA analysis; Krissinel and Henrick, 2007). The LOTUS domain dimer is mainly stabilized by hydrogen bonds. The $\beta$ strands of each monomer interact via six hydrogen bonds formed between main-chain atoms, resulting in a central extended four-stranded antiparallel $\beta$ sheet (Figure 4A). Beneath the extended $\beta$ sheet, the dimer is further stabilized by two symmetric hydrogen bond networks, each involving Arg215 of the $\beta$1 strand in one monomer and Asp197 of $\alpha$ helix 4 in the other monomer (Figure 4B). In addition to the polar interactions, the dimer interface involves hydrophobic contacts between Leu200 of each monomer. To verify the assigned dimer interface, we created a mutation (R215E) designed to interfere with hydrogen bonding between Arg215 and Asp197. Analytical gel filtration and SLS analysis of the LOTUS domain confirmed that the R215E mutation indeed prevents dimerization (Figure 4C; V.R. and M.J., unpublished data).

The surface involved in LOTUS domain dimerization, including the residues Asp197, Leu200, and Arg215, is highly conserved among Oskar proteins of all drosophilids, but not other insects (Figures 5A and 5B). To assess whether the ability to form dimers is evolutionarily conserved, we attempted to analyze the oligomerization state of the LOTUS domains of Oskar orthologs in ten non-drosophilid insects. Out of the ten identified domains, eight could be expressed in a soluble fashion and were subjected to SLS analysis (Figure S5). As in the case of Drosophila, we observed dimer formation with the Oskar LOTUS domains of the Mediterranean fruit fly Ceratitis capitata and the parasitic wasp Nasonia vitripennis. Given that the Drosophila dimer interface involves several hydrogen bonds between main-chain atoms within the extended $\beta$ sheet, it seems likely that a similar interface also forms in these two insects. In contrast, the LOTUS domains of the six other non-drosophilids are monomers in solution (Figure 5C), showing that dimer formation is not a generally conserved feature of Oskar LOTUS domains. In line with this observation, the N-terminal LOTUS domain of human TDRD5...
The LOTUS Domain Binds the RNA Helicase Vasa

Posterior patterning of the Drosophila embryo requires Oskar, as well as several other proteins, including the RNA helicase Vasa. Vasa is recruited to the posterior pole of the oocyte by Short Oskar protein, and it was inferred from yeast two-hybrid assays and immunoprecipitations from oocyte lysates that the two proteins physically interact (Breitwieser et al., 1996). However, these experiments could not rule out that the observed interaction was mediated by RNA. We therefore re-evaluated the Oskar-Vasa interaction by pull-down experiments in an RNA-free environment, using purified GST-Short Oskar fusion protein in combination with the soluble helicase core of Vasa (aa200–623; Sengoku et al., 2006). This approach confirmed that GST-Short Oskar displays a strong and specific interaction with Vasa (Figure 6A, lanes 1–7). Furthermore, we tested GST fusions of the individual Oskar domains and found that the Vasa helicase core co-purified with the LOTUS, but not with the OSK domain (Figure 6A, lanes 8–13). Additional isothermal titration calorimetry (ITC) experiments revealed a dissociation constant for the LOTUS-Vasa complex in the low μM range (Figure 6B). As the obtained stoichiometry supports a complex composition consisting of one LOTUS dimer and two Vasa molecules, Vasa interaction is unlikely to interfere with dimer formation.

Previous analyses suggested that Vasa binds to the C-terminal half of Oskar, which comprises part of the intrinsically disordered region and the OSK domain but does not include the LOTUS domain (aa290–606; Breitwieser et al., 1996). In contrast, in our GST pull-down assay, we exclusively observed interaction of Vasa with the LOTUS domain. However, our experiment only involved the helicase core of Vasa and would not have revealed interactions of other regions of the protein with Oskar. As full-length Vasa was prone to precipitation in the GST pull-down assay, we assessed its interaction with different portions of Oskar in a split-ubiquitin-based yeast two-hybrid assay (Stagljär et al., 1998; Mockl et al., 2007). As with the helicase core, the full-length Vasa interacted only with Short Oskar and the LOTUS domain. In contrast, neither the region that is predicted to be disordered (aa240–400; Figure 1A), nor the OSK domain, nor the combination of the two showed any interaction with Vasa (Figures 6C and 6D).

Taken together, our findings show that the LOTUS domain mediates the direct, RNA-independent interaction of Oskar with Vasa in vitro and we propose that the Oskar LOTUS domain functions as a key protein-protein interaction module in vivo.

DISCUSSION

Oskar is the only protein known to date to be capable of inducing the formation of functional germ cells in an organism. Genetic epistasis experiments have placed Oskar at the top of a hierarchy in germ plasm formation. As such, and due to the lack of domains with obvious functions, Oskar was viewed as a scaffold...
Figure 5. Dimerization of the Oskar LOTUS Domain Is Conserved in Drosophilids, but Not in Other Insects

(A–D) Surface representation of the Oskar LOTUS domain monomer colored according to residue conservation, considering either aligned sequences from drosophilids (A) or alignments between Drosophila melanogaster and non-drosophilid sequences (B) as shown below the structures (C and D). The cartoon representation shows the orientation of the LOTUS domain. Sequences were aligned using MUSCLE (Edgar, 2004) and visualized with the help of ESPript (Robert and Gouet, 2014); residue numbers refer to D. melanogaster Long Oskar. Residues involved in dimerization (green inverted triangle) of the Drosophila Oskar

(legend continued on next page)
upon which the germ plasm is assembled, by stepwise recruitment of other components. Although the oskar gene is only present in some insects, germ plasm has been described in many species (Juhn and James, 2006; Juhn et al., 2008; Lynch et al., 2011; Ewen-Campen et al., 2012; Voronina et al., 2011; Schisa, 2012). In Danio rerio, Bucky ball, and in C. elegans, PGL-1 and PGL-3 have been shown to have central roles in germ plasm formation (Marlow and Mullins, 2008; Bontems et al., 2009; Updike and Strome, 2010). However, the precise molecular functions of these proteins are not understood.

Our study sheds light on the molecular properties of the Drosophila germ plasm inducer. We show that Oskar is an RNA-binding protein and that it interacts with nos, pgc, and gcl mRNAs in vivo. Furthermore, our structural and functional characterization of the two Oskar domains reveals several unexpected features. First, the C-terminal OSK domain binds RNA in vitro. Second, the LOTUS domain, which was previously predicted to be an RNA-binding domain, does not bind RNA in our assays but constitutes a dimerization domain and physically interacts with the RNA helicase Vasa in vitro.

The protein domains responsible for Vasa and RNA interaction are present in both Oskar isoforms. However, in contrast to Short Oskar, Long Oskar is not able to recruit Vasa (Breitwieser et al., 1996) and our in vivo data suggest that Long Oskar also lacks RNA-binding activity. We suggest that these fundamental differences might be explained by an inhibitory effect of the Long-Oskar-specific N-terminal extension (NTE) on the LOTUS and OSK domain functions (Figure 1A). The effect of the NTE on the domain functions could not be tested in vitro, as the NTE is insoluble (as is Long Oskar) and causes insolubility when fused to the different domains (M.J., unpublished data). Alternatively, the apparent functional difference between Short and Long Oskar may simply reflect their localization in distinct cellular compartments (Vanzo et al., 2007), which might result in different accessibility of the Oskar isoforms to potential interaction partners.

LOTUS-Vasa Interaction in the Germline

Previously, an interaction of Vasa with the C-terminal half of Oskar was reported; however, the LOTUS domain in isolation was not tested and the methods used could not exclude that the interaction was mediated by RNA (Breitwieser et al., 1996). Our finding that the C-terminal OSK domain binds RNA and our ability to produce soluble Oskar domains prompted us to re-evaluate the Oskar-Vasa interaction in an RNA-free environment. Our experiments using purified proteins demonstrate the direct interaction of Oskar with Vasa and define the LOTUS domain as the Vasa-interacting module in vitro.

LOTUS domains are present in important germline-specific proteins, such as Oskar, TDRD5, and TDRD7 (Anantharaman et al., 2010; Callebaut and Mornon, 2010). In different animals, these proteins co-localize in germ granules with Vasa, a highly

**LOTUS domain (in addition to the β sheets) are highlighted above the Drosophila sequence. The secondary structure above the alignment refers to the D. melanogaster protein.**

(E) Phylogenetic tree of 11 insects that contain an oskar gene highlighting the oligomeric status of their individual LOTUS domains, which was determined by static light scattering (see Figure S5). Monomer and dimer are depicted by one and two purple spheres, respectively. See also Figure S5.
conserved RNA helicase required for germline development in metazoans (Yabuta et al., 2011; Patil and Kai, 2010; Hosokawa et al., 2007; Strasser et al., 2008; Zhang et al., 2013; Vanzo et al., 2007, Lasko, 2013). The evolutionarily conserved co-existence of Vasa and LOTUS-domain-containing proteins in germ granules raises the possibility of a general function of LOTUS domains in Vasa interaction.

In the Drosophila oocyte, Oskar recruits Vasa to initiate germ plasm assembly (Ephrussi and Lehmann, 1992). Mouse TDRD7 has been shown to co-precipitate the Vasa homolog (MVH) from adult mouse testis (Hosokawa et al., 2007), and Drosophila TDRD5 (Tejas) and TDRD7 (Tapas) have been shown to co-precipitate Vasa from transfected cell lysates (Patil and Kai, 2010; Patil et al., 2014). Although these experiments do not show that the TDRD-Vasa interactions are direct, one study shows that it is the LOTUS domain that mediates the interaction (Patil and Kai, 2010). Our present work shows that the Oskar LOTUS domain binds Vasa directly in vitro. Vasa has a function in the germline-specific secondary piRNA biogenesis pathway, where it binds directly to transposon RNAs (Malone et al., 2009; Xiol et al., 2014). It is tempting to speculate that LOTUS domain proteins might modulate Vasa activity.

**Oskar Function in RNA Regulation**

Several mRNAs have been shown to require Oskar for their localization to the posterior of oocytes and embryos, which led to speculation that the protein might bind RNA. Our study demonstrates that Oskar is an RNA-binding protein and that a subset of posterior localized RNAs, namely nos, pgc, and gcl mRNAs, associates directly with Oskar in the Drosophila embryo. The precise role of Oskar interaction with these RNAs remains unclear, but it may be involved in RNA localization to the pole and/or RNA regulation. All three target mRNAs are subject to translation control (e.g., Rangan et al., 2009) and, although the underlying mechanisms for pgc and gcl mRNA regulation are poorly understood, indication of an active role of Oskar comes from its known involvement in nos mRNA regulation. Regulated translation of nos mRNA leads to a Nos protein gradient that is essential for posterior patterning of the embryo (Wang and Lehmann, 1991; Gavis and Lehmann, 1994). Whereas the majority of nos mRNA is evenly distributed throughout the embryo and repressed by a Smaug-protein-dependent process (reviewed in Pinder and Smibert, 2013), the portion of nos mRNA localized at the embryo posterior escapes from repression by a mechanism that involves Oskar (Dahanukar et al., 1999; Zaessinger et al., 2006). Further studies indicated that Oskar acts on Smaug, preventing it from binding to nos mRNA (Zaessinger et al., 2006; Jeske et al., 2011). Our finding that Oskar directly binds nos mRNA adds a new component for consideration in future studies on the mechanisms of nos recruitment and translational activation by Oskar. Understanding Oskar’s selective binding to specific RNAs will require further investigation, both with respect to the RNA sequence elements bound and the Oskar amino acid residues involved in the interaction.

Our in vitro analysis shows that the C-terminal OSK domain binds RNA, and the large basic and hydrophobic patches on the surface of the domain support a function in RNA binding. Basic and hydrophobic surface residues of membrane-binding proteins have also been shown to contact lipids (Lemmon, 2008; Stahelin, 2009). However, the OSK domain is unlikely to function in lipid interaction, as it does not localize to membranes in cultured Drosophila cells or in yeast (M.J., unpublished data). Evolutionary analysis revealed that the OSK domain sequence is more similar to bacterial than insect SGNH hydrolases, suggesting that the OSK domain is of bacterial origin and arose by horizontal gene transfer (Lynch et al., 2011). The OSK domain might have evolved from an ancient, intact hydrolase that lost its enzyme activity and acquired a new function in RNA binding. Such a functional transition is conceivable, as several enzymes have been described that are known to possess “moonlighting” proteins.

**Figure 7. Molecular Model of Oskar Function**

Long and Short Oskar are synthesized at the posterior pole of the Drosophila oocyte. There, Short Oskar initiates the formation of germ plasm. Short Oskar interacts with the RNA helicase Vasa via its LOTUS domain (1) and binds to RNA via its OSK domain (2). Oskar’s RNA targets in the embryo include nos, pgc, and gcl mRNAs. Vasa might contribute to Oskar’s function in RNA metabolism (3). In addition, Oskar might regulate Vasa activity. Long Oskar contains an N-terminal extension that confers activities distinct from those of Short Oskar. Long Oskar is essential for proper anchoring of the germ plasm to the posterior cortex of the oocyte. The N-terminal extension inhibits the potential Vasa and RNA-binding activities of Long Oskar by a yet unknown mechanism. The drawing of Vasa was generated with the help of the published structure (PDB: 2DB3; Sen-goku et al., 2006).
RNA interaction activities (Hentze and Preiss, 2010; Castello et al., 2012).

It is possible that other regions of Oskar protein contribute to its RNA-binding function. For example, the region linking the LOTUS and OSK domains, which is predicted to be intrinsically disordered, is enriched in basic residues and might also participate in RNA interaction. Finally, the fact that the LOTUS domain directly associates with Vasa suggests that Oskar and Vasa may function jointly in achieving RNA-binding specificity and possibly regulation. A proposed model of the molecular function of Oskar protein is shown in Figure 7. The model highlights the distinct activities of Short and Long Oskar in germ plasm function.

**EXPERIMENTAL PROCEDURES**

All methods are described in detail in the Supplemental Experimental Procedures.

**RNA Binding Assays**

The UV crosslink-mRNA pull-down experiment was performed as described (Castello et al., 2013) with some modifications for Drosophila embryos.

For the identification of mRNAs that are bound by Oskar, a crosslink/immuno precipitation was performed. Anti-Oskar antibody was coupled to Protein G Sepharose (GE Healthcare), and beads were incubated with extracts prepared from 0- to 2-hr Drosophila embryos that have been subjected to UV irradiation. The control experiment was performed with lysates from embryos that were not UV irradiated. After stringent washing, the protein in the eluate was digested with Proteinase K, the RNA was isolated, and reverse-transcribed using random hexamer primers, and the cDNA was analyzed by real-time PCR using mRNA-specific primers. The results were analyzed using the ΔΔCt method, where the fold enrichment is determined from the values of the condition where the anti-Oskar antibody was used and a control condition from which the anti-Oskar antibody was omitted.

In the second UV crosslink-mRNA pull-down experiment, recombinant GST fusion proteins were incubated with lysates from 0- to 2-hr embryos (prepared as in Jeske et al., 2014) for 30 min at 22°C and the mixture irradiated with 200 mJ/cm² UV and subjected to mRNA pull-down as in Castello et al. (2013).

For the filter-binding assay, a 5-32P-labeled RNA oligonucleotide (5’ AAAAA AAAAA AAGGC AAGGG UUG 3’) was incubated with increasing amounts of recombinant proteins and filtered, and the radioactivity retained on the filter was quantified by Cerenkov counting.

**Crystallization and Structure Determination**

Protein domains used for crystallization were expressed and purified from E. coli. Oskar 139–222 and 139–240 were crystallized using the sitting-drop vapor diffusion method, and diffraction data of the crystals were collected at ESRF beamlines ID23-1 and ID14-4 (Grenoble), respectively. Initial phases were obtained by molecular replacement and subsequently refined using the model. Oskar 401–606 crystals were obtained by the hanging-drop vapor diffusion method and diffraction data collected at the PXIII beamline of the Swiss Light Source. For derivativization, native crystals were soaked with 1 mM p-chloromercuribenzenesulfonic acid and the data set was collected using an in-house Rigaku rotating anode. The structure was solved by single isomorphous replacement with anomalous scattering and refined against the native data set.

**Protein-Protein Interaction Studies**

GST pull-down assays, yeast-two hybrid experiments, and ITC measurements are described in the Supplemental Experimental Procedures.

**ACCESSION NUMBERS**

The structures reported here have been deposited to the Protein Data Bank and are available under accession numbers PDB: 5A48 (LOTUS domain comprising aas 139–240), PDB: 5A49 (LOTUS domain comprising aas 139–222), and PDB: 5A4A (OSK domain).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.06.055.

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**REFERENCES**

Akoh, C.C., Lee, G.-C., Liaw, Y.-C., Huang, T.-H., and Shaw, J.-F. (2004). GDSL family of serine esterases/lipases. Prog. Lipid Res. 43, 534–552.

Anantharaman, V., Zhang, D., and Aravind, L. (2010). OST-HTH: a novel predicted RNA-binding domain. Biol. Direct 5, 13.

Aravind, L., Anantharaman, V., Balaji, S., Babu, M.M., and Iyer, L.M. (2005). The many faces of the helix-turn-helix domain: transcription regulation and beyond. FEMS Microbiol. Rev. 29, 231–262.

Barker, D.D., Wang, C., Moore, J., Dickinson, L.K., and Lehmann, R. (1992). Pumilio is essential for function but not for distribution of the Drosophila abdominal determinant Nanos. Genes Dev. 6 (124), 2312–2323.

Bontems, F., Stein, A., Marlow, F., Lyautey, J., Gupta, T., Mullins, M.C., and Dosch, R. (2009). Bucky ball organizes germ plasm assembly in zebrafish.Curr. Biol. 19, 414–422.

Breitwieser, W., Markussen, F.H., Horstmann, H., and Ehrussi, A. (1996). Os- kar protein interaction with Vasa represents an essential step in polar granule assembly. Genes Dev. 10, 2179–2188.

Callebaut, I., and Monon, J.-P. (2010). LOTUS, a new domain associated with small RNA pathways in the germline. Bioinformatics 26, 1140–1144.

Carrera, P., Johnstone, O., Nakamura, A., Casanova, J., Jäckle, H., and Lasko, P. (2000). VASA mediates translation through interaction with a Drosophila yfl2 homolog. Mol. Cell 5, 181–187.

Castello, A., Fischer, B., Eichelbaum, K., Horos, R., Beckmann, B.M., Strein, C., Davey, N.E., Humphreys, D.T., Press, T., Steinmetz, L.M., et al. (2012). Insights into RNA biology from an atlas of mammalian RNA-binding proteins. Cell 149, 1393–1406.

Castello, A., Horos, R., Strein, C., Fischer, B., Eichelbaum, K., Steinmetz, L.M., Kriegsveld, J., and Hentze, M.W. (2013). System-wide identification of RNA-binding proteins by interactome capture. Nat. Protoc. 8, 491–500.
Dahanukar, A., Walker, J.A., and Wharton, R.P. (1999). Smaug, a novel RNA-binding protein that operates a translational switch in Drosophila. Mol. Cell 4, 209–218.

Ding, D., Parkhurst, S.M., Halsell, S.R., and Lipshitz, H.D. (1993). Dynamic Hsp83 RNA localization during Drosophila oogenesis and embryogenesis. Mol. Cell. Biol. 13, 3773–3781.

Edgar, R.C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792–1797.

Ephrussi, A., and Lehmann, R. (1992). Induction of germ cell formation by oskar. Nature 358, 387–392.

Ephrussi, A., Dickinson, L.K., and Lehmann, R. (1991). Oskar organizes the germ plasm and directs localization of the posterior determinant nanos. Cell 66, 37–50.

Ewen-Campen, B., Srouji, J.R., Schwager, E.E., and Extavour, C.G. (2012). Oskar predates the evolution of germ plasm in insects.Curr. Biol. 22, 2278–2283.

Gavis, E.R., and Lehmann, R. (1994). Translationally regulated nanos by RNA localization. Nature 369, 315–318.

Hentze, M.W., and Preiss, T. (2010). The REM phase of gene regulation. Trends Biochem. Sci. 35, 435–446.

Hockensmith, J.W., Kubasek, W.L., Vorachek, W.R., and von Hippel, P.H. (1986). Laser cross-linking of nucleic acids to proteins. Methodology and first applications to the phage T4 DNA replication system. J. Biol. Chem. 261, 3512–3518.

Hosokawa, M., Shoji, M., Kitamura, K., Tanaka, T., Noce, T., Chuma, S., and Nakatsuji, N. (2007). Tudor-related proteins TDRD1/MTR-1, TDRD6 and TDRD7/TRAP: domain composition, intracellular localization, and function in male germ cells in mice. Dev. Biol. 301, 38–52.

Jongens, T.A., Hay, B., Jan, L.Y., and Jan, Y.N. (1992). The germ cell-less gene product: a posteriorly localized component necessary for germ cell development in Drosophila. Cell 70, 569–584.

Juhn, J., and James, A.A. (2006). oskar gene expression in the vector mosquitoes, Anopheles gambiae and Aedes aegypti. Insect Mol. Biol. 15, 363–372.

Juhn, J., Mariniotti, O., Calvo, E., and James, A.A. (2008). Gene structure and expression of nanos (nos) and oskar (osk) orthologues of the vector mosquito, Culex quinquefasciatus. Insect Mol. Biol. 17, 545–552.

Kim-Ha, J., Smith, J.L., and Macdonald, P.M. (1991). oskar mRNA is localized to the posterior pole of the Drosophila oocyte. Cell 66, 23–35.

Krisstel, E., and Henrick, K. (2007). Inference of macromolecular assemblies from crystalline state. J. Mol. Biol. 372, 774–797.

Lasko, P. (2013). The DEAD-box helicase Vasa: evidence for a multiplicity of functions in RNA processes and developmental biology. Biochim. Biophys. Acta 1829, 810–816.

Lehmann, R., and Nüsslein-Volhard, C. (1986). Abdominal segmentation, pole cell formation, and embryonic polarity require the localized activity of oskar, a maternal gene in Drosophila. Cell 47, 141–152.

Lehmann, R., and Nüsslein-Volhard, C. (1986). Abdominal segmentation, pole cell formation, and embryonic polarity require the localized activity of oskar, a maternal gene in Drosophila. Cell 47, 141–152.

Lemmon, M.A. (2008). Membrane recognition by phospholipid-binding domains. Nat. Rev. Mol. Cell Biol. 9, 99–111.

Liang, L., Diehl-Jones, W., and Lasko, P. (1994). Localization of vasa protein to the Drosophila pole plasm is independent of its RNA-binding and helicase activities. Development 120, 1201–1211.

Lynch, J.A., Oziak, O., Khila, A., Abouheif, E., Desplan, C., and Roth, S. (2011). The phylogenetic origin of oskar coincided with the origin of maternally provisioned germ plasm and pole cells at the base of the Holometabola. PLoS Genet. 7, e1002029.

Mahowald, A.P. (2001). Assembly of the Drosophila germ plasm. Int. Rev. Cytol. 203, 187–213.

Malone, C.D., Brennecke, J., Dus, M., Stark, A., McCombie, W.R., Sachidanandam, R., and Hannon, G.J. (2009). Specialized piRNA pathways act in germline and somatic tissues of the Drosophila ovary. Cell 137, 522–535.

Markussen, F.H., Michon, A.M., Breitwieser, W., and Ephrussi, A. (1995). Translational control of oskar generates short OSK, the isoform that induces pole plasma assembly. Development 121, 3723–3732.

Markussen, F.H., Breitwieser, W., and Ephrussi, A. (1997). Efficient translation and phosphorylation of Oskar require Oskar protein and the RNA helicase Vasa. Cold Spring Harb. Symp. Quant. Biol. 62, 13–17.

Marlow, F.L., and Mullins, M.C. (2008). Bucky ball functions in Balbiani body assembly and animal-vegetal polarity in the oocyte and follicle cell layer in zebrafish. Dev. Biol. 321, 40–50.

Moccì, N., Deplazes, A., Hassa, P.O., Zhang, Z., Peter, M., Hottiger, M.O., Stagljar, I., and Auerbach, D. (2007). Yeast split-ubiquitin-based cytosolic screening system to detect interactions between transcriptionally active proteins. Biotechniques 42, 725–730.

Nakamura, A., Amikura, R., Mukai, M., Kobayashi, S., and Lasko, P.F. (1996). Requirement for a noncoding RNA in Drosophila polar granules for germ cell establishment. Science 274, 2075–2079.

Pashev, I.G., Dimitrov, S.I., and Angelov, D. (1991). Crosslinking proteins to nucleic acids by ultraviolet laser irradiation. Trends Biochem. Sci. 16, 323–326.

Patil, V.S., and Kai, T. (2010). Repression of retroelements in Drosophila germline via piRNA pathway by the Tudor domain protein Tejas. Curr. Biol. 20, 724–730.

Patil, V.S., Anand, A., Chakrabarti, A., and Kai, T. (2014). The Tudor domain protein Tapas, a homolog of the vertebrate Tdrd7, functions in the piRNA pathway to regulate retrotasposons in germline of Drosophila melanogaster. BMC Biol. 12, 61.

Pinder, B.D., and Smibert, C.A. (2013). Smaug: an unexpected journey into the mechanisms of post-transcriptional regulation. Fly (Austin) 7, 142–145.

Rangan, P., DeGennaro, M., Jaime-Bustamante, K., Coux, R.X., Martinho, R.G., and Lehmann, R. (2009). Spatial and temporal control of germ-plasm RNAs. Curr. Biol. 19, 72–77.

Robert, X., and Gouet, P. (2014). Deciphering key features in protein structures with the new ENDscript server. Nucleic Acids Res. 42, W320-4.

Rongo, C.G. (1996). The role of RNA localization and translational regulation in Drosophila germ cell determination. PhD thesis (Cambridge: Massachusetts Institute of Technology).

Rongo, C., Gavis, E.R., and Lehmann, R. (1995). Localization of oskar RNA regulates oskar translation and requires Oskar protein. Development 121, 2737–2746.

Schisa, J.A. (2012). New insights into the regulation of RNP granule assembly in oocytes. Int Rev Cell Mol Biol 295, 233–289.

Sengoku, T., Nureki, O., Nakamura, A., Kobayashi, S., and Yokoyama, S. (2006). Structural basis for RNA unwinding by the DEAD-box protein Drosophila Vasa. Cell 125, 287–300.

Stagljar, I., Korostensky, C., Johnsson, N., and te Heesen, S. (2006). A genetic system based on split-ubiquitin for the analysis of interactions between membrane proteins in vivo. Proc. Natl. Acad. Sci. USA 95, 5187–5192.

Stahlín, R.V. (2009). Lipid binding domains: more than simple lipid effectors. J. Lipid Res. 50, S299–S304.

Strasser, M.J., Mackenzie, N.C., Dumstrei, K., Nakkrasae, L.-I., Stebler, J., and Raz, E. (2008). Specialized piRNA pathways act in germplasm localization and chromatin remodeling in the Drosophila germline. Proc. Nat. Acad. Sci. USA 105, 11083–11088.

Tearle, R.G., and Nusslein-Volhard, C. (1987). Tubingen mutants and stock list. Tubingen Germ Cell Mutants and Stock List. Publications of the Institute of Technology) of the University of Tubingen, 157 pages.

Teichmann, M., Dumay-Odelot, H., and Fribourg, S. (2012). Structural and functional aspects of winged-helix domains at the core of transcription initiation complexes. Transcription 3, 2–7.
Updike, D., and Strome, S. (2010). P granule assembly and function in Caenorhabditis elegans germ cells. J. Androl. 31, 53–60.

Vanzo, N.F., and Ephrussi, A. (2002). Oskar anchoring restricts pole plasm formation to the posterior of the Drosophila oocyte. Development 129, 3705–3714.

Vanzo, N., Oprins, A., Xanthakis, D., Ephrussi, A., and Rabouille, C. (2007). Stimulation of endocytosis and actin dynamics by Oskar polarizes the Drosophila oocyte. Dev. Cell 12, 543–555.

Voronina, E., Seydoux, G., Sassone-Corsi, P., and Nagamori, I. (2011). RNA granules in germ cells. Cold Spring Harb. Perspect. Biol. 3, pii: a002774.

Wang, C., and Lehmann, R. (1991). Nanos is the localized posterior determinant in Drosophila. Cell 66, 637–647.

Wang, C., Dickinson, L.K., and Lehmann, R. (1994). Genetics of nanos localization in Drosophila. Dev. Dyn. 199, 103–115.

Xiol, J., Spinelli, P., Laussmann, M.A., Homolka, D., Yang, Z., Cora, E., Couté, Y., Conn, S., Kadlec, J., Sachidanandam, R., et al. (2014). RNA clamping by Vasa assembles a piRNA amplifier complex on transposon transcripts. Cell 157, 1698–1711.

Yabuta, Y., Ohta, H., Abe, T., Kurimoto, K., Chuma, S., and Saitou, M. (2011). TDRD5 is required for retrotransposon silencing, chromatoid body assembly, and spermiogenesis in mice. J. Cell Biol. 192, 781–795.

Zaessinger, S., Busseau, I., and Simonelig, M. (2006). Oskar allows nanos mRNA translation in Drosophila embryos by preventing its deadenylation by Smaug/CCR4. Development 133, 4573–4583.

Zhang, L., Zhang, K., Prändl, R., and Schöffl, F. (2004). Detecting DNA-binding of proteins in vivo by UV-crosslinking and immunoprecipitation. Biochem. Biophys. Res. Commun. 322, 705–711.

Zhang, Q.-J., Luo, Y.-J., Wu, H.-R., Chen, Y.-T., and Yu, J.-K. (2013). Expression of germline markers in three species of amphioxus supports a preformation mechanism of germ cell development in cephalochordates. Evodevo 4, 17.