Developmental Cell, Vol. 6, 637–648, May, 2004, Copyright ©2004 by Cell Press

Hrp48, a *Drosophila* hnRNPA/B Homolog, Binds and Regulates Translation of *oskar* mRNA

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Summary

Establishment of the *Drosophila* embryonic axes provides a striking example of RNA localization as an efficient mechanism for protein targeting within a cell. *oskar* mRNA encodes the posterior determinant and is essential for germline and abdominal development in the embryo. Tight restriction of Oskar activity to the posterior is achieved by mRNA localization-dependent translational control, whereby unlocalized mRNA is translationally repressed and repression is overcome upon mRNA localization. Here we identify the previously reported *oskar* RNA binding protein p50 as Hrp48, an abundant *Drosophila* hnRNP. Analysis of three *hrp48* mutant alleles reveals that Hrp48 levels are crucial for polarization of the oocyte during mid-oogenesis. Our data also show that Hrp48, which binds to the 5′ and 3′ regions of *oskar* mRNA, plays an important role in restricting Oskar activity to the posterior of the oocyte, by repressing *oskar* mRNA translation during transport.

Introduction

Establishment of the antero-posterior (A/P) and dorso-ventral (D/V) axes of the *Drosophila* embryo is controlled by maternal mRNAs localized in the oocyte during oogenesis (Riechmann and Ephrussi, 2001). *oskar* mRNA, encoding the posterior determinant, is localized at the posterior pole of the oocyte, and its localization-dependent translation generates the source of Oskar protein required for posterior patterning of the embryo (Ephrussi et al., 1991; Gunkel et al., 1998; Kim-Ha et al., 1995, 1991; Rongo et al., 1995). Oskar nucleates assembly of the pole plasm, which contains the abdominal and germline determinants. *oskar* is both necessary and sufficient for pole plasm formation (Ephrussi and Lehmann, 1992; Lehmann and Nüsslein-Volhard, 1986). *oskar* mutants and mutants with a defect in *oskar* mRNA localization or translation develop no abdominal structures or pole cells, the primordial germ cells of the fly (Erdélyi et al., 1995; Lehmann and Nüsslein-Volhard, 1986; St Johnston et al., 1991; van Eeden et al., 2001). In contrast, ectopic expression of *oskar*, even at low levels, causes severe patterning defects and, in extreme cases, bicaudal embryos develop, bearing pole cells and an ectopic abdomen of reverse polarity in the place of the head (Ephrussi et al., 1991; Ephrussi and Lehmann, 1992; Smith et al., 1992). Hence, correct embryonic patterning requires tight restriction of *oskar* activity.

During oogenesis, the *Drosophila* oocyte and 15 nurse cells develop as a syncytium surrounded by a follicular epithelium (Spradling, 1993). *oskar* mRNA is synthesized in the nurse cells, and its localization is an elaborate process involving movement of the transcript from the nurse cells into the oocyte, and through the oocyte to the posterior pole (Ephrussi et al., 1991; Kim-Ha et al., 1991). Analysis of genetic mutants, as well as cytoskeletal inhibitor experiments, have shown that both actin and microtubules play an important role in *oskar* mRNA localization (Clark et al., 1994; Erdélyi et al., 1995). Moreover, *oskar* mRNA transport correlates with polarity of the microtubule cytoskeleton (Theurkauf et al., 1992). During early oogenesis, when *oskar* mRNA is enriched in the oocyte (stages 1–7), the single microtubule organizing center (MTOC) of the nurse cell-oocyte syncytium lies at the oocyte posterior pole. During mid-oogenesis (stages 8/9), polarity of the microtubule network is reversed (González-Reyes et al., 1995; Roth et al., 1995), and *oskar* mRNA has been proposed to travel with Kinesin heavy chain (Khc) to the microtubule plus ends, localizing at the posterior pole (Brendza et al., 2000; Cha et al., 2002; Palacios and St Johnston, 2002). In *gurken (grk)*, *protein kinase A (pka)*, and *par-1* mutant oocytes, which fail to correctly repolarize at mid-oogenesis, microtubules nucleate from the entire cortex and focus their plus ends in the center of the oocyte, where *oskar* mRNA localizes ectopically (González-Reyes et al., 1995; Lane and Kalderon, 1994; Roth et al., 1995; Shulman et al., 2000; Tomancak et al., 2000). Although posterior localization of *oskar* mRNA involves microtubules and Khc (Brendza et al., 2000; Clark et al., 1994), Kinesin light chain is not required, and thus the mechanisms underlying the transport of *oskar* mRNA to the posterior pole remain unclear (Palacios and St Johnston, 2002). It is not known whether *oskar* mRNP particles are directly coupled to the Kinesin motor, nor whether *oskar* mRNA transport is more indirect, for instance promoted by motor-dependent cytoplasmic flows and binding of *oskar* mRNA to a posterior localized anchor (Glotzer et al., 1997), or whether both mechanisms are involved.

It is also not known which are the RNA binding proteins specifically linking *oskar* mRNA and the transport machinery. Staufen (Stau) is a double-stranded RNA

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binding protein whose activity is required for *oskar* mRNA localization (Ramos et al., 2000; St Johnston et al., 1992). Staufen and *oskar* mRNA colocalize in both wild-type and mutant oocytes and are mutually interdependent for their localization, suggesting that they interact directly in vivo (Ephrussi et al., 1991; St Johnston et al., 1991). However, a specific affinity of Staufen for *oskar* mRNA has not been shown. *Drosophila* Y14/Tsunagi (Hachet and Ephrussi, 2001; Mohr et al., 2001) and Mago nashi (Micklem et al., 1997; Newmark and Boswell, 1994; Newmark et al., 1997), interacting components of the recently identified Exon Junction Complex (EJC) of proteins (Kataoka et al., 2001, 2000; Le Hir et al., 2001, 2000; Zhao et al., 2000), are enriched in the nurse cell nuclei and cytoplasm and are required for localization of *oskar* mRNA, with which they colocalize at the posterior pole (Hachet and Ephrussi, 2001; Mohr et al., 2001). The involvement of these nuclear shuttling proteins in *oskar* mRNA localization suggests that events in the nucleus play an instructive role in *oskar* mRNA localization at posterior pole cytoplasm.

oskar mRNA is translationally repressed during transport, and its translation is activated upon localization of the mRNA at the posterior pole (Gunkel et al., 1998; Kim-Ha et al., 1995; Markussen et al., 1995; Rongo et al., 1995). Translational repression of *oskar* mRNA is mediated by the product of the *arrest* locus, Bruno (Castagnetti et al., 2000; Lie and Macdonald, 1999; Webster et al., 1997). Bruno is an ovarian RNA binding protein that binds to conserved elements in the *oskar* 3′UTR, the Bruno Response Elements (BRE) (Kim-Ha et al., 1995). Bruno has multiple roles in oogenesis (Webster et al., 1997), including translational regulation of *gurken* mRNA (Filardo and Ephrussi, 2003). Recently, it was shown that Cup protein, which immunoprecipitates with eIF4E and Bruno in ovarian extracts, is required for *oskar* translational repression (Nakamura et al., 2004; Wilhelm et al., 2003). At the posterior pole, translational derepression of *oskar* is mediated by the 5′ region of the mRNA, which also functions to repress translation in the nurse cells (Gunkel et al., 1998) (and unpublished data). Two proteins, p50 and p68, bind specifically to this region. p50 also binds to the *oskar* 3′UTR, within the translational repressor region containing the BREs, and mutations that specifically reduce binding of p50 to the 3′UTR, without affecting the binding of Bruno, cause precocious translation of an *oskar* reporter, suggesting that p50 might be involved in *oskar* translational repression (Gunkel et al., 1998).

We have now purified p50 and show that it is Hrp48, a *Drosophila* member of the hnRNPA/B family of RNA binding proteins (Matunis et al., 1992a, 1992b). Our analysis of three hrp48 alleles shows that Hrp48 acts in the germline in establishment of oocyte polarity and in *oskar* mRNA regulation, repressing translation of *oskar* mRNA during localization. In a complementary, genetic, approach, Huynh et al. have identified alleles of *hrp48* that specifically affect Hrp48 *oskar* mRNA localization (Huynh et al., 2004). Taken together, our data indicate that the *oskar* mRNA binding protein Hrp48 has a central role in *oskar* mRNA localization and translational control, suggesting that these processes might be coupled and mediated by a bifunctional *oskar* mRNA localization/translation complex comprising Hrp48.

Results

Identification of the *oskar* mRNA Binding Protein p50 as Hrp48

We originally identified p50 in ovarian extracts as a protein that displayed specific binding to both the 3′ and 5′ translational control regions of *oskar* mRNA (Gunkel et al., 1998). A protein with similar RNA binding properties was detected in embryo (Castagnetti et al., 2000) and in *Drosophila* Schneider S2 cell extracts (Figure 1A). To understand the role of p50 in *oskar* regulation, we carried out the biochemical purification of p50 using S2 cells as the starting material (Figure 1B). The presence of p50 protein was monitored throughout the purification procedure, making use of its capacity to bind *oskar* mRNA.

We first generated S100 extracts, which we fractionated by ammonium sulfate precipitation. Sixty-two percent of the total binding activity present in the S100 was detected in the 0%–30% ammonium sulfate fraction, allowing an approximately 10-fold enrichment of the protein at this purification step (data not shown). The precipitated proteins were separated according to size by Sephacryl S-300 column chromatography (Figure 1C, upper panel). Alignment of the protein elution and RNA binding profiles revealed that, while the vast majority of the proteins eluted from the column in fractions 33 to 51, p50 RNA binding activity was enriched in fractions 50 to 76 (Figure 1C, lower panel). As another closely migrating RNA binding activity was detected in fractions 50 to 64, we discarded these and pooled fractions 65 to 76, for further purification by RNA-affinity column chromatography. To eliminate proteins that bound to the column nonspecifically, we first passed the pooled proteins over the resin alone. The eluted proteins (Figure 1D, total) were then loaded on a column of the same resin coupled to RNA derived from the *oskar* 3′ translational repressor region, which binds p50 efficiently (Gunkel et al., 1998). After extensive washing, retained proteins were eluted and p50 RNA binding activity was evaluated by UV crosslinking of the protein fractions to *oskar* mRNA (Figure 1D, elution). Purified fractions displaying significant, reduced, or no p50 binding activity were pooled in subsets and their protein composition examined by SDS-PAGE and staining with Coomassie Brilliant Blue (Figure 1E). This revealed that the purification procedure yielded a unique band migrating as a 50 kDa protein exclusively in those fractions containing p50 binding activity after RNA purification (Figure 1E, compare lane 1 with 2–4). The 50 kDa protein bands were excised, in-gel digested with trypsin, and analyzed by MALDI peptide mapping (Shevchenko et al., 1996a, 1996b). Eleven peptides were identified. All mapped to, and together covered, approximately 40% of P48809 protein (Figure 2A), a *Drosophila* A/B-type hnRNP, previously referred to as Hrb27C or Hrp48 (Matunis et al., 1992a, 1992b; Siebel et al., 1994).

To confirm that p50 is Hrp48, we immunoprecipitated p50 UV-crosslinked to *oskar* mRNA probes. Labeled

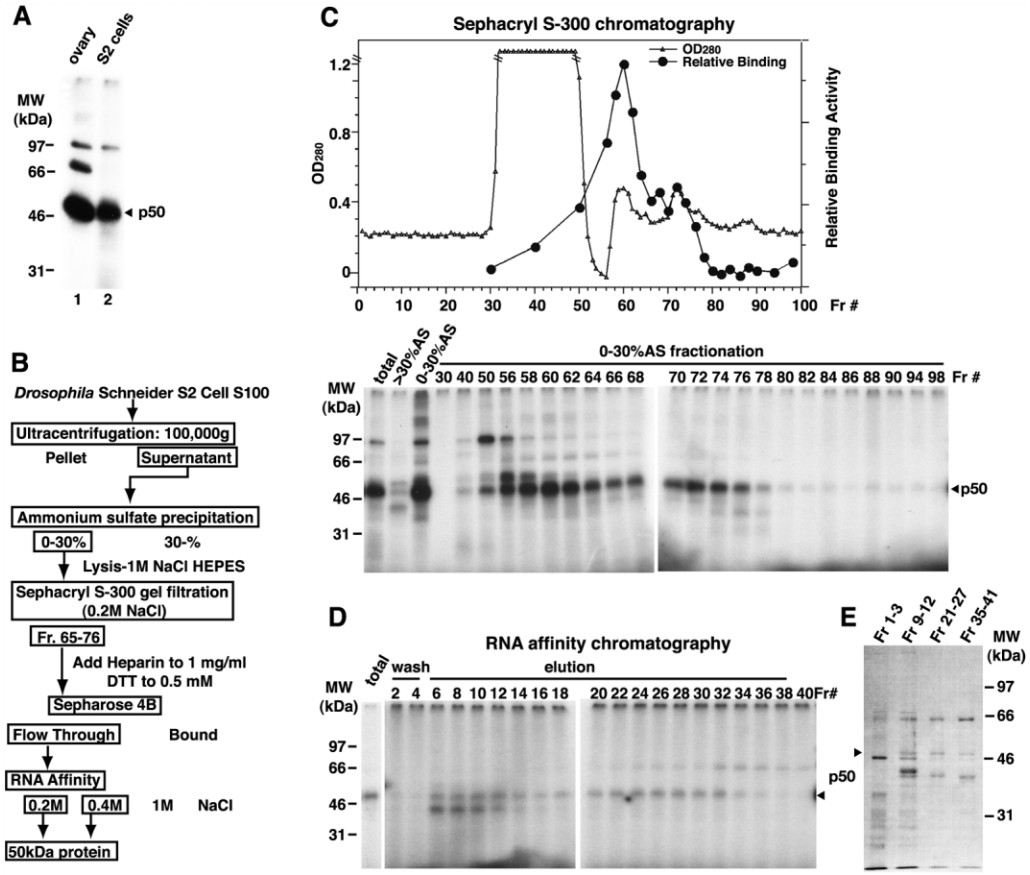

Figure 1. Purification of p50

(A) UV crosslinking analysis of ovary and Schneider cell extracts. An [α-^{32}P]-labeled RNA corresponding to BRE region A (Kim-Ha et al., 1995) was incubated with and UV-crosslinked to proteins in extracts from ovaries (lane 1) or Schneider cells (lane 2). Both extracts contain a protein of approximately 50 kDa (arrowhead) that binds to the BRE region A probe.

(B) Purification flow chart. See Supplemental Data (http://www.developmentalcell.com/cgi/content/full/6/5/637/DC1) for details.

(C) Total protein and p50 binding activity elution profile of ammonium sulfate precipitated proteins subjected to Sephacryl S-300 chromatography (upper panel). UV-crosslinking of proteins in the fractions to *oskar* BRE region A probe (lower panel). Protein elution was monitored by measuring absorbance of the fractions at 280 nm (upper panel, triangles). Relative p50 RNA binding activity (upper panel, circles) was determined by estimating the intensity of the 50 kDa band detected by the UV-crosslinking assay in each fraction (lower panel) and normalization to the 50 kDa band detected in fraction 50. p50 RNA binding activity detected in Schneider cell extract (total) was reduced in the >30% ammonium sulfate fraction, but enriched in the 0%–30% fraction, which was fractionated by Sephacryl S-300 chromatography (lower panel). p50 RNA binding activity (arrowhead) was enriched in fractions 50–76. To avoid contamination by a closely migrating protein in fractions 50–64, fractions 65 to 76 were pooled for further purification.

(D) UV-crosslinking assay of proteins in fractions after Sepharose 4B and RNA affinity chromatography. Pooled flowthough fractions from the Sepharose 4B column (total) were applied to the RNA-affinity column; the column was washed (fractions 1–4) and bound proteins eluted using buffer containing 400 mM NaCl (fractions 6 to 34), and 1 M NaCl (fractions 35 to 40). p50 RNA binding activity (arrowhead) measured by UV-crosslinking of proteins to *oskar* BRE region A probe was not detected in wash fractions (fractions 2 and 4), and was enriched in fractions 6 to 32. Less p50 RNA binding activity was detected in fractions 34 to 40. Molecular weights are indicated on the left.

(E) SDS-PAGE analysis of proteins in pooled fractions of the RNA-affinity chromatography. Fractions 1–3, 9–12, and 21, 23, 25, 27 (21–27), and 35, 37, 39, 41 (35–41) from the RNA-affinity column were pooled and precipitated with Trichloro-acetic acid (final 5%) and analyzed on a 10% SDS-polyacrylamide gel, after staining with Coomassie Brilliant Blue. Bands migrating at 50 kDa in fractions 9–12 and 21–27 were excised for analysis by nanoelectrospray mass spectrometry.

RNAs corresponding to the 5′ or the 3′ translation regulatory regions of *oskar* mRNA were incubated with ovarian extract, UV crosslinked to bound proteins, digested with RNase A, and immunoprecipitated using antibodies specific for either Hrp48 or Bruno or a control preimmune serum (Figure 2B). Whereas Bruno antiserum precipitated the 70 kDa protein specifically (Figure 2B, left panel), the Hrp48 antiserum precipitated the 50 kDa protein specifically, when crosslinked to either the 3′ or the 5′ *oskar* RNA probes (Figure 2B, left and right panels, respectively). This confirmed that the *oskar* RNA binding protein p50 is Hrp48.

Hrp48 Distribution and Role during Oogenesis

To study the relevance of Hrp48 during *Drosophila* oogenesis, we analyzed the phenotype of different alleles of the *Hrb27C* locus, which encodes Hrp48 and to which we will refer as the *hrp48* gene. Several *hrp48* mutant

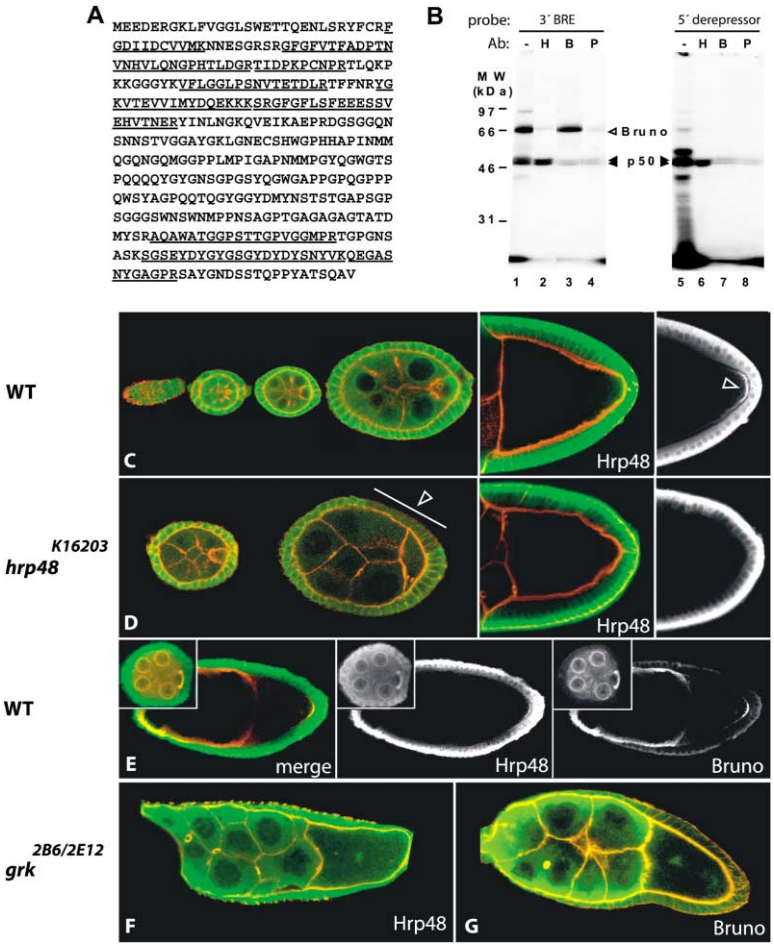

Figure 2. Identification of p50 as Hrp48 and Analysis of Its Distribution during Oogenesis

(A) Amino acid sequence of Hrp48 and of the peptides determined by MALDI peptide mapping from purified p50 (underlined). p50 bands present in Figure 1E, lanes 2 and 3, were excised, in-gel digested with trypsin, and analyzed by nanoelectrospray mass spectrometry. The 11 peptides (underlined), some of which overlapped, were all matched to Hrp48.

(B) Identification of p50 as Hrp48. Immunoprecipitation of UV-crosslinked ovarian proteins to the BRE A region probe (EcoRI-ApaLI) or to the 5′ derepressor region probe (EcoRI-BglII) (Gunkel et al., 1998) of *oskar* mRNA. Three proteins (left panel), p90, Bruno, and p50, were crosslinked to the BRE A RNA probe (lane 1), and anti-Hrp48 antiserum precipitated only p50 specifically (lane 2), while anti-Bruno antiserum precipitated only Bruno (lane 3); preimmune serum (lane 4) is a specificity control. p50 was crosslinked to the 5′ derepressor region RNA probe (right panel, lane 5) and was precipitated by anti-Hrp48 antiserum specifically (lane 6). Anti-Bruno antiserum and preimmune serum (lanes 7 and lane 8, respectively) failed to immunoprecipitate any proteins crosslinked to the 5′ region probe.

(C) Hrp48 protein (green) immunostaining of egg chambers of wild-type egg chambers. Early and until mid-oogenesis, Hrp48 accumulates in the posterior half of the oocyte, between the oocyte nucleus and the plasma membrane. In the nurse cells, Hrp48 is predominantly cytoplasmic and perinuclear. At stage 9, Hrp48 is enriched at the posterior pole of the oocyte (2C, right panel).

(D) These signals are lost in *hrp48*^{K16203} germline clones, indicating the specificity of the signal. In the follicle cells, Hrp48 is predominantly cytoplasmic (C), and is not detected in *hrp48*^{K16203} follicle cell clones (D, left panel, arrow).

(E) Double-staining for Hrp48 and Bruno, showing their colocalization in the nurse cells and oocyte during early oogenesis (insert) and at stage 9, when they are detected at the posterior pole.

(F and G) In egg chambers of the strong *gurken* allelic combination *grk*$^{2B6/2E12}$, both Hrp48 and Bruno accumulate in the center of the oocyte. Egg-chambers are oriented anterior to the left, dorsal to the top.

alleles were previously isolated and characterized, revealing that in these mutants Hrp48 levels are reduced (Hammond et al., 1997). *hrp48* mutations cause lethality at the larval stage (Hammond et al., 1997) and, when placed in *trans* to the deficiency *Df(2L)wee*^{wo5}, are fully lethal (see Experimental Procedures). We therefore examined the viability of flies transheterozygous for three different alleles and the hypomorphic allele *hrp48*^{K10413} (Hammond et al., 1997). The *hrp48*^{K02814}/*hrp48*^{K10413} combination was fully lethal. In contrast, *hrp48*^{K16203}/*hrp48*^{K10413} and *hrp48*^{02647}/*hrp48*^{K10413} combinations were semilethal, with a more reduced viability in the case of *hrp48*^{K16203}/*hrp48*^{K10413} (7% male and 36% female progeny) than of *hrp48*^{02647}/*hrp48*^{K10413} (24% male and 40% female progeny). Thus, with regard to lethality, *hrp48*^{K02814} is the strongest, *hrp48*^{K16203} is the intermediate, and *hrp48*^{02647} is the weakest allele.

We then analyzed the role of *hrp48* specifically in the germline, generating homozygous mutant clones by mitotic recombination in the germline of females heterozygous for *hrp48*^{K16203}, *hrp48*^{02647}, and *hrp48*^{K02814}, using the *ovoD1* dominant female-sterile technique (Chou et al., 1993). The majority of *hrp48*^{K02814} germline clones failed to develop beyond the early stages of oogenesis, suggesting that *hrp48* has an early function in the germline. Germline clones of the two other *hrp48* alleles, *hrp48*^{K16203} and *hrp48*^{02647}, developed egg chambers and laid eggs, although in the case of *hrp48*^{K16203} many of the eggs had a shape characteristic of dumping defects during the latest stages of oogenesis and were not fertilized. As *hrp48*^{K16203} is the stronger of the two alleles, we concentrated most of our analysis on germline clones of this mutant.

Hrp48 is expressed in the germline and follicle cells of developing cysts, beginning during the earliest stages of oogenesis (Figure 2C). In the follicle cells and nurse cells, Hrp48 shows a uniform cytoplasmic distribution, with a distinct perinuclear enrichment (Figure 2C, left panel). In the oocyte during stages 1–6, Hrp48 accumulates and is concentrated at the posterior, between the oocyte nucleus and the follicle cells (Figure 2C, left panel). No perinuclear signal is detected around the oocyte nucleus. During stages 7/8, Hrp48 appears uniformly distributed throughout the oocyte cytoplasm

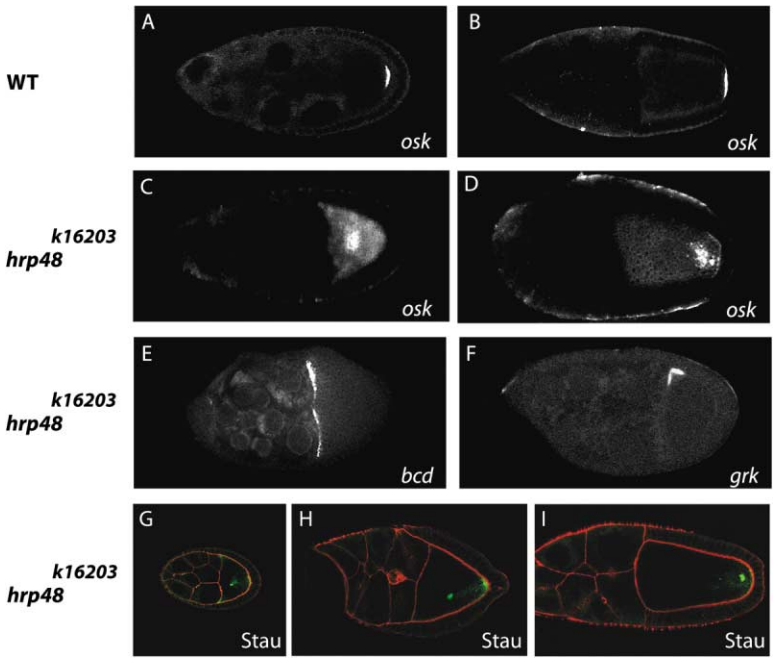

Figure 3. Hrp48 Is Required for *oskar* mRNA Localization at the Posterior Pole of the Oocyte

(A–F) Detection of *oskar, bicoid*, and *gurken* mRNAs by fluoresecent in situ hybridization in wild-type and *hrp48*^{K16203} mutant egg chambers. (A and B) In the wild-type, *oskar* mRNA is tightly localized at the posterior cortex of the oocyte at stage 9 (A) and stage 10 (B). (C and D) In germline clones of *hrp48*^{K16203}, 75% of stage 9 oocytes (C) show ectopic localization of *oskar* mRNA in the center of oocyte; 19% show a diffuse signal, and 6% show posterior localization, as in wild-type. At stage 10 (D), 32% of oocytes show *oskar* mRNA in aggregates, and 52% show a faint or diffuse signal. The remaining oocytes show *oskar* mRNA localized at the posterior as in the wild-type.

(E and F) Germline clones of *hrp48*^{K16203} show normal *bicoid* (E) and *gurken* (F) mRNA localization.

(G–I) Staufen protein (green) is mislocalized in *hrp48*^{K16203} germline clones at stages 8 (G), 9 (H), and 10 (I). See Table 1 for distribution frequencies.

(data not shown) and stage 9 Hrp48 is detected at the posterior pole (Figure 2C, right panel). To assess the specificity of the Hrp48 signal, we performed immunostaining of *hrp48*^{K16203} germline clones (Figure 2D). The perinuclear, cytoplasmic, and posterior signals detected in wild-type cysts were strongly reduced in *hrp48*^{K16203} germline clones and in follicle cell clones (Figure 2D). This confirmed that the protein detected in wild-type ovaries is indeed Hrp48.

The distribution of Hrp48 in the oocyte parallels that of *oskar* mRNA. This, together with the fact that Hrp48 and Bruno bound to *oskar* mRNA can be coimmunoprepitated in an RNA-dependent manner (Gunkel et al., 1998), suggested that Hrp48 might be part of the *oskar* mRNA complex in vivo. Therefore we analyzed the relative distributions of Hrp48 and Bruno in the germline, where Bruno is exclusively expressed (Figure 2E). Hrp48 and Bruno show similar perinuclear and cytoplasmic distributions in the nurse cells, and at the oocyte posterior during early and mid-oogenesis. In *gurken (grk)* mutants, in which *oskar* mRNA is mislocalized to the center of the oocyte (González-Reyes et al., 1995; Roth et al., 1995), both Hrp48 (Figure 2F) and Bruno (Figure 2G) are detected in the center of the oocyte, suggesting that Hrp48 is part of the *oskar* mRNA complex in vivo.

Hrp48 Is Required for Oocyte Polarization and *oskar* mRNA Localization in the Oocyte

Several hnRNPs have been shown to play a role in nucleocytoplasmic or intracytoplasmic transport of mRNAs (Cote et al., 1999; Hoek et al., 1998; Norvell et al., 1999). To test if Hrp48 is required for posterior localization of *oskar* mRNA, we examined the distribution of *oskar* mRNA in *hrp48*^{K16203} mutant oocytes. In situ hybridization revealed that *oskar* mRNA accumulation and distribution are normal until stage 8 of oogenesis in the mutant (data not shown). At stage 9, however, *oskar* mRNA is either diffuse or in aggregates in the oocyte cytoplasm (Figure 3C), rather than at the posterior pole (Figures 3A and 3B; see Figure 3 legend for phenotype frequencies). An intermediate phenotype is also observed, whereby a portion of the mRNA is mislocalized as a dot (or dots) at the center of the oocyte, while the rest of the RNA is localized at the posterior pole. At stages 10a–b, aggregates of *oskar* mRNA are frequently observed near, but not at, the posterior pole (Figure 3D). Localization of *bcd* and *grk* mRNAs does not appear to be affected in *hrp48*^{K16203} oocytes at stages 9 and 10, and the oocyte nucleus is located in its normal position, in the dorsal-anterior corner of the oocyte (Figures 3E and 3F). Staufen protein is required for *oskar* mRNA localization and translation and colocalizes tightly with *oskar* mRNA in wild-type and most mutant situations, suggesting that Staufen is a component of the *oskar* mRNA localization complex. In *hrp48*^{K16203} oocytes, Staufen is mislocalized in one or several dots near the center of the oocyte (Figures 3G–3I; see Table 1A for frequencies of the phenotypes), mimicking the ectopic localization of *oskar* mRNA. Thus, *hrp48* is involved either directly or indirectly in posterior localization of the *oskar* mRNA/Staufen complex.

To see if the defects in *oskar* mRNA/Staufen localization in *hrp48*^{K16203} oocytes might be caused by an improperly polarized microtubule cytoskeleton, we examined the overall polarity of the oocyte microtubules. We made use of a Kinesin-β-galactosidase (Kin-β-gal) fusion protein, whose localization at the posterior pole the oocyte at stage 9 requires its own motor activity and is independent of *oskar* (Clark et al., 1994). Thus, Kin-β-gal can serve as a reporter of oocyte polarity. We examined simultaneously the distribution of Staufen, a reporter of *oskar* mRNA, and of Kin-β-gal in the different *hrp48* mutant alleles. In *hrp48*^{K16203} germline clones, Kin-β-gal was ectopically localized in 62% of the oocytes (Figure 4B and Table 1B), revealing an aberrant organization of the oocyte microtubule cytoskeleton. This phenotype

Table 1. Staufen and Kin-β-gal Fusion Protein Mislocalization Defects in *hrp48* Germline Clones

Genotype of Females	Posterior Signal (as in wild-type)	Signal Close to the Posterior	Posterior and Central Signal	Central Signal	Diffuse Signal	No Signal
A. Staufen Mislocalization						
Wild-type	100	0	0	0	0	0
$hrp48^{K16203}$ glc	8	17	7	62	6	0
$hrp48^{K16203}$ glc + hs-hrp48	48	4	11	32	0	7
B. Kin-β-gal Mislocalization (%)						
Wild-type	100	0	0	0	0	0
$hrp48^{K16203}$ glc	38	7	6	43	6	0
$hrp48^{K16203}$ glc + hs-hrp48	75	0	7	11	0	7

Penetrance of the Staufen (A) and Kin-β-gal fusion protein (B) mislocalization defects in $hrp48^{K16203}$ germline clones. These phenotypes are rescued by expression of a wild-type copy of *hrp48*.

was observed for all three alleles of *hrp48* examined. Importantly, both *oskar* mRNA/Staufen localization and polarity defects of $hrp48^{K16203}$ germline clones were significantly suppressed by expression of *hrp48* from a transgene under control of the heat-shock promoter (Hammond et al., 1997) (Tables 1A and 1B), demonstrating that both phenotypes are caused by the reduction of *hrp48* levels. Hence *hrp48* is required for the establishment or maintenance of oocyte polarity.

The double-detection of Staufen and Kin-β-gal also revealed that, in contrast to wild-type oocytes, in which the two proteins colocalize at the posterior pole (Figure 4A), in *hrp48* oocytes, Staufen and Kin-β-gal do not colocalize (Figures 4B–4D). In some instances, although Kin-β-gal was observed at or near the posterior pole, Staufen protein was dispersed in aggregates throughout the oocyte cytoplasm (Figures 4C and 4D). Simultaneous detection of *oskar* mRNA and Staufen protein revealed that they colocalize in these aggregates, in all three alleles (data not shown). The observed failure of Staufen and Kin-β-gal to colocalize ectopically in *hrp48* oocytes suggests that Hrp48 may play a role in *oskar* mRNA localization. The identification and characterization by Huynh et al. of new alleles of *hrp48* that specifically affect *oskar* mRNA localization to the posterior pole of the oocyte demonstrates that Hrp48 plays an essential role in this process (Huynh et al., 2004).

Hrp48 Represses Translation of *oskar* mRNA

To investigate the role of *hrp48* in *oskar* translation, we examined Oskar protein accumulation in $hrp48^{K16203}$ germline clones. As in the case of the hypomorphic *arrest/Bruno* alleles that allow egg chamber development past the early stages of oogenesis, no Oskar protein is detected during the early stages of oogenesis in $hrp48^{K16203}$ (Webster et al., 1997, and data not shown). At stages 9 through 10b, however, an ectopic dot of Oskar is detected in the center of $hrp48^{K16203}$ oocytes (Figures 5C and 5D). The frequencies of mislocalized Oskar and Staufen are similar and, for both, the penetrance of the defect is significantly greater at stage 9 than stage 10 (see Figure 5 legend). Therefore, *oskar* mRNA is translated ectopically in $hrp48^{K16203}$ oocytes, suggesting a defect in translational repression.

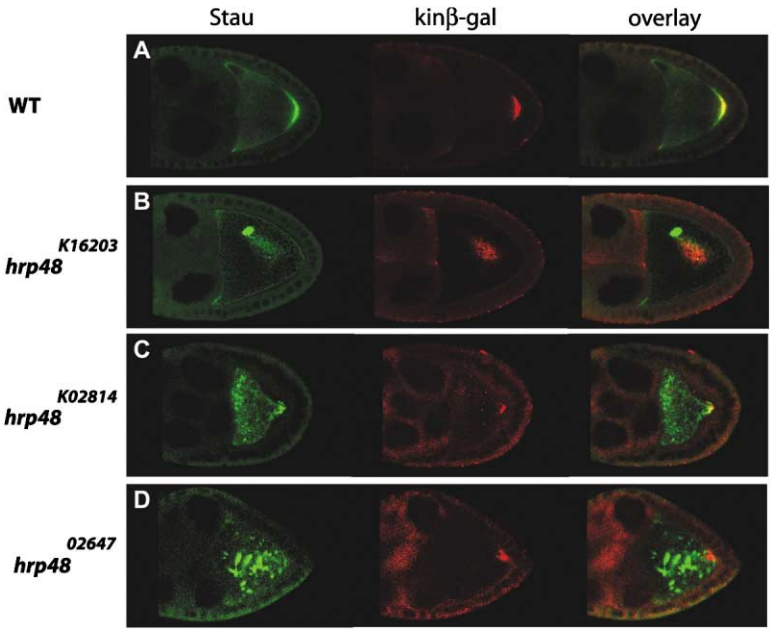

Figure 4. Oocyte Polarity and Staufen Localization Are Abnormal in *hrp48* Mutant Oocytes

(A) Staufen protein (green) and Kin-β-gal (red) colocalize at the posterior pole of wild-type oocytes.

(B–D) Germline clones of *hrp48* alleles show variable polarity defects, as revealed by the Kin-β-gal reporter. In all three alleles, Staufen is mislocalized in the oocyte; however, its distribution does not overlap with that of the polarity reporter Kin-β-gal. Egg-chambers are oriented anterior to the left.

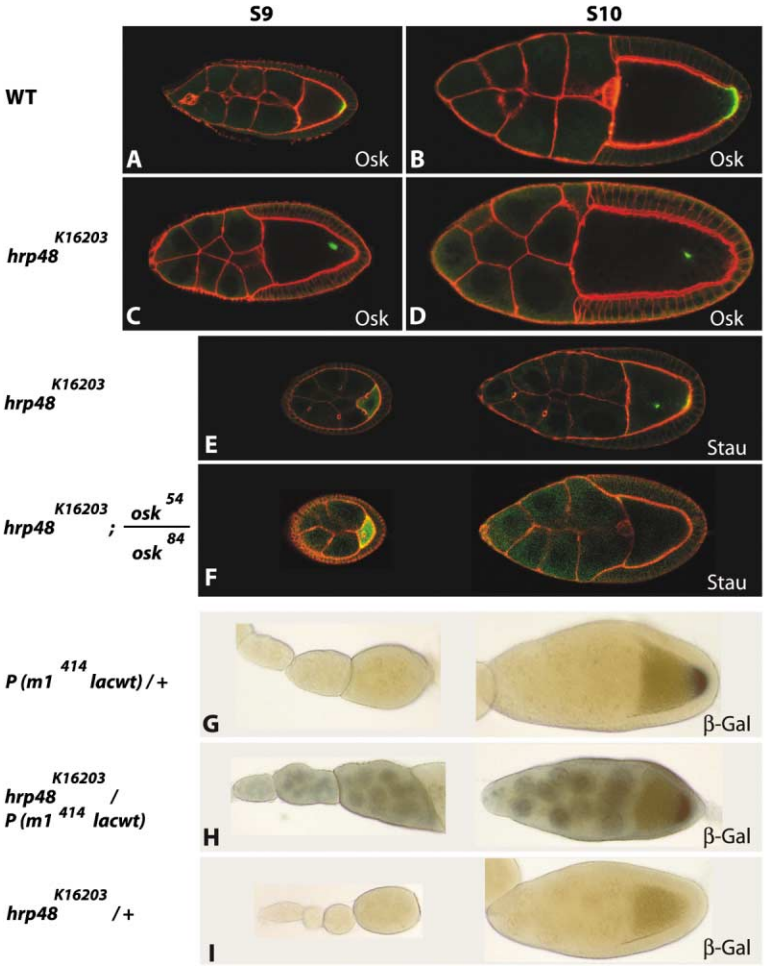

Figure 5. Ectopic Expression of Oskar Protein in *hrp48*^{K16203} Oocytes

(A–D) Antibody detection of Oskar protein in wild-type (A and B) and *hrp48*^{K16203} germline clone (C and D) egg-chambers. In wild-type stage 9 (A) and 10a (B) oocytes, Oskar protein (green) is detected only at the posterior pole. In *hrp48*^{K16203} germline clones at stage 9, 83% of stage 9 oocytes show ectopic localization of Oskar in a dot at the center of the oocyte or away from the posterior pole (C), 7% show a diffuse signal, 4% show a normal posterior signal, and in 6% no signal was detected (data not shown). In *hrp48*^{K16203} germline clones, 63% of stage 10b oocytes show a dot of ectopic Oskar protein in the center or away from the posterior pole (D), and 26% show a faint or diffuse signal, and the rest show essentially normal Oskar localization (data not shown).

(E and F) Formation of Staufen aggregates in the center of the *hrp48*^{K16203} oocytes requires Oskar protein. (E) In *hrp48*^{K16203} germline clones, Staufen protein is transported into and uniformly distributed in the oocyte until stage 7/8 (E, left); at stage 9, Staufen is detected in one or several large aggregates in the center of the oocyte (E, right). (F) In *hrp48*^{K16203} germline clones generated in the Oskar protein null background, *osk*^{54}/*osk*^{84}, Staufen protein enrichment in the oocyte is similar to that observed in *hrp48*^{K16203} germline clone until stage 7/8 (F, left); however, aggregates of Staufen protein are not detected in these oocytes at stage 9, indicating that Oskar protein is required for aggregate formation (F, right).

(G–I) Mutation of *hrp48* interferes with translational repression of an *oskar*-lacZ reporter gene. Ovaries of females the genotype indicated on left were fixed and stained with X-gal to reveal β-galactosidase distribution. (G) Egg-chambers of wild-type females bearing one copy of the *m1*^{414}*lacwt oskar* translation reporter (Gunkel et al., 1998) express β-galactosidase exclusively at the posterior pole of the oocyte, and show no expression in the nurse cells. (H) Egg-chambers of females heterozygous for *hrp48*^{K16203} and bearing the *m1*^{414}*lacwt* reporter express β-galactosidase throughout the nurse cells and oocyte cytoplasm, as well as at the posterior pole. (I) Egg-chambers of females heterozygous for *hrp48*^{K16203}, in which a P{lacW} element is inserted in the *hrp48* promoter region, express no β-galactosidase.

oskar mRNA anchoring to the posterior cortex of the oocyte is mediated by Oskar protein (Ephrussi et al., 1991; Kim-Ha et al., 1991; Markussen et al., 1995; Rongo et al., 1995; Vanzo and Ephrussi, 2002). To determine if the presence of *oskar* mRNA and Staufen in dots in *hrp48* oocytes might be a consequence of ectopic translation and aggregation of the mRNA, we generated *hrp48*^{k16203} germline clones in an *osk*^{54}/*osk*^{84} protein null mutant background (Kim-Ha et al., 1991; Markussen et al., 1995; Rongo et al., 1995) and examined the distribution of Staufen. Until stage 7/8, the distribution of Staufen in *hrp48*^{k16203}*; osk*^{54}/*osk*^{84} oocytes is indistinguishable from that observed in *hrp48*^{K16203} alone (Figures 5E and 5F). However, the concentrated dot of Staufen detected at the center of *hrp48*^{K16203} oocytes (Figure 5E) is not detected in *hrp48*^{K16203}*;osk*^{54}/*osk*^{84} oocytes (Figure 5F). Therefore, the formation of Staufen/*oskar* mRNA aggregates in *hrp48*^{K16203} oocytes is dependent on Oskar, demonstrating that *oskar* is translated ectopically in *hrp48*^{K16203} oocytes.

Hrp48 (p50) binds to the translation regulatory regions of *oskar* mRNA (Gunkel et al., 1998). We previously showed that mutations in the *oskar* 3′UTR that selectively reduce p50/Hrp48 binding cause leaky translational repression of an *oskar* reporter mRNA in the oocyte during stages 6–7, before the mRNA is localized. This indicated an involvement of p50/Hrp48 in translational repression of *oskar* mRNA via the 3′UTR (Gunkel et al., 1998). To test the effect of reducing Hrp48 levels on *oskar* expression, we crossed the *oskar* translation reporter *m1*^{414}*lacwt* into *hrp48*^{K16203} heterozygous flies and compared its expression in the heterozygous mutant background and in a wild-type background. In wild-type ovaries, β-galactosidase is detected exclusively at the posterior pole of the oocyte (Figure 5G). In contrast, in the *hrp48*^{K16203} heterozygous background, from early oogenesis onward, high levels of β-galactosidase activity are detected in the nurse cells and throughout the oocyte, in addition to the posterior pole where the RNA is localized (Figure 5H and data not shown). No β-galactosidase staining is detected in the ovaries of *hrp48*^{K16203} heterozygous control females lacking the *m1*^{414}*lacwt* transgene (Figure 5I). Thus, halving the amount of Hrp48 causes precocious and ectopic translation of

the *oskar* reporter in the nurse cells and oocyte, consistent with a role of Hrp48 in translational repression of unlocalized *oskar* mRNA.

Ectopic expression of Oskar causes anterior patterning defects. We therefore examined the cuticle patterns of the embryos that developed from $hrp48^{K16203}$ germline clones. Although only 3% developed into larvae, about half (47%) of the unhatched eggs (97%) developed into embryos that displayed a wild-type cuticle pattern and 20% failed to develop. Another 18% bore fewer than six segments, revealing reduced levels of posterior patterning activity. The remaining 15% of unhatched embryos showed head defects consistent with posterior patterning activity at the anterior. The phenotype of these embryos indicated a role of *hrp48* in regulation of A/P patterning in the embryo.

To gain further evidence that *hrp48* regulates A/P patterning by controlling *oskar* translation, we made use of females of a genetic background sensitive to Oskar expression levels, due to the overexpression of the *oskar* 3′UTR from a transgene (Filardo and Ephrussi, 2003). A small proportion of embryos produced by these females exhibit anterior defects resulting from titration of translational repressors from endogenous *oskar* mRNA, causing its ectopic translation (Filardo and Ephrussi, 2003). We therefore hypothesized that reduction of Hrp48 levels by removal of one wild-type copy of *hrp48* would increase the severity of the A/P patterning defects. Indeed, each of the *hrp48* alleles enhanced the anterior patterning defects of embryos produced by UASp-*osk*3′UTR; nosGAL4VP16 females, with $hrp48^{K02814}$, the strongest allele, causing the strongest enhancement (Figure 6). Although the vast majority (88%) of embryos produced by $hrp48^{K02814}$/UASp-*osk*3′UTR; nosGAL4VP16/+ females developed, most unhatched embryos showed severe A/P patterning defects, ranging from head defects to a full bicaudal phenotype. As a control, we tested the effect of reducing levels of the *oskar* translational repressor Bruno *(aret)*. A similar enhancement of anterior patterning defects was observed in the progeny of $aret^{QB72}$/UASp-*osk*3′UTR; nosGAL4VP16/+ females. We observed no *oskar* mRNA localization defects in *hrp48*/UASp-*osk*3′UTR; nosGAL4VP16/+ oocytes and, consistent with this, no embryos displayed a posterior group phenotype. Although we cannot rule out that Hrp48 regulation of *oskar* may be indirect, the observed patterning defects, together with our demonstration that Hrp48 binds to *oskar* mRNA and that the *osk-lacZ* transgene is precociously and ectopically expressed when Hrp48 levels are reduced, strongly suggest that Hrp48 represses *oskar* translation via direct binding to the mRNA.

Discussion

Genetic screens have led to the identification of several proteins involved in localization and/or in translational control of *oskar* mRNA. However, none of these proteins has been shown to bind *oskar* mRNA directly, and it has therefore been unclear to what extent the two processes are mechanistically linked. In this study, we have identified the *oskar* mRNA binding protein p50 as Hrp48. We show that Hrp48/p50 binds to several regions in *oskar* mRNA (Gunkel et al., 1998), colocalizes with *oskar* mRNA and its translational repressor Bruno, and is essential for translational repression of *oskar* mRNA during transport. Taken together with the results of Huynh et al. showing that Hrp48 is essential for *oskar* mRNA localization (Huynh et al., 2004), our results strongly suggest that the action of *hrp48* on *oskar* mRNA is direct and that Hrp48 is an essential component of the *oskar* mRNP localization/translation complex. The involvement of this *oskar* mRNA binding protein both in localization and translation of the mRNA suggests the functional coupling of the two processes.

Hrp48 Is Essential for RNA Regulation in the *Drosophila* Germline

HnRNPs are abundant RNA binding proteins involved in many aspects of mRNA regulation. HnRNPs associate with transcripts at their site of synthesis in the nucleus, and can remain associated with the RNA during processing and export, as well as during cytoplasmic processes such as translation and localization (Dreyfuss et al., 2002). Mammalian hnRNP A2 binds to specific sequences in the 3′UTR of myelin basic protein mRNA and mediates its localization and translational control in rat oligodendrocytes (Hoek et al., 1998). In *Xenopus* oocytes, VgRBP60, an hnRNP I-related protein, colocalizes with and binds sequence elements in Vg1 mRNA that are critical for its localization at the vegetal pole (Cote et al., 1999). In the *Drosophila* oocyte, two isoforms of Squid/hrp40 have essential roles in localization and translational control of *gurken* mRNA, the dorsoventral polarity determinant (Norvell et al., 1999).

Drosophila Hrp48, a member of the hnRNPA/B family of proteins, is an abundant hnRNP, bearing two N-terminal RRM-type RNA binding domains and a C-terminal Glycine-rich domain (Matunis et al., 1992a, 1992b). Hrp48 is expressed in somatic and germline cells of the ovary, where it is detected at low levels in the nucleus and predominantly in the cytoplasm (Siebel et al., 1995). Mammalian hnRNP A1, a putative homolog of hrp48, has been shown to function in splice-site selection (Mayeda and Krainer, 1992) and to shuttle between the nucleus and the cytoplasm (Pinol-Roma and Dreyfuss, 1992). Hrp48 is a cofactor in regulation of alternative splicing (Hammond et al., 1997; Siebel et al., 1995, 1994). Mutations reducing *hrp48* expression cause developmental defects and lethality, indicating that *hrp48* is essential in the fly (Hammond et al., 1997). A recent study of strong *hrp48* alleles has identified a role for Hrp48 in *gurken* mRNA localization (Goodrich et al., 2004).

Although *hrp48* affects *oskar* mRNA regulation, it does not appear to regulate *oskar* mRNA processing, as the mRNA is accurately spliced in the mutants (T.Y., data not shown). The *hrp48* mutants we analyzed also show defects in the organization of the oocyte microtubules. These defects are due to low levels of Hrp48 in the mutants, as they are suppressed by expression of *hrp48* from a transgene. Given the demonstrated involvement of Hrp48 in RNA splicing, localization, and translational control, it is likely that polarity defects in *hrp48* mutants are caused by deregulation of RNAs whose products are required for oocyte polarization. The dumping defects of some *hrp48* mutant egg chambers suggest that Hrp48 may also regulate the function of the actin cytoskeleton.

A

genotype	unhatched embryos: wild type % (n=)	unhatched embryos: head defects % (n=)	unhatched embryos: bicaudal % (n=)	hatched % (n=)	total n=
+/+	0.4 (31)	1.0 (74)	0.0 (0)	97 (7475)	7706
hrp48^{02647}/+	0.4 (21)	5.7 (329)*	1.1 (61)*	91 (5248)*	5767
hrp48^{k16203}/+	0.2 (4)	4.1 (90) *	0.4 (8) *	93 (2024)*	2176
hrp48^{02814}/+	0.0 (0)	7.6 (101)*	2.6 (35)*	88 (1175)*	1334
aret ^{QB72}/+	0.1 (3)	3.6 (83) *	0.8 (18)*	95 (2159)*	2273

B

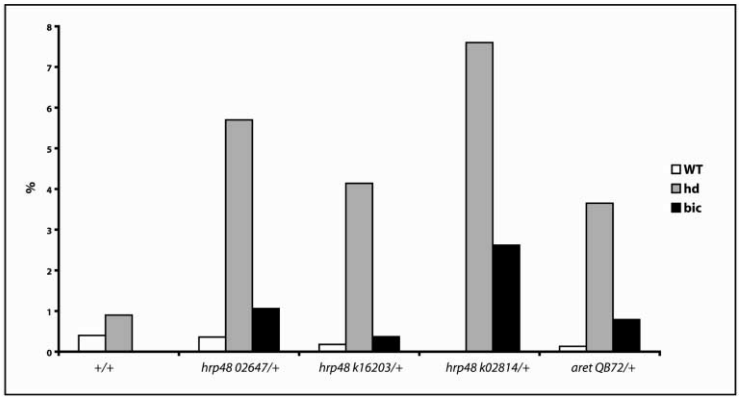

Figure 6. Reduction of *hrp48* Gene Dosage Causes Anterior Patterning Defects in the Sensitized Background

The proportion of embryos showing anterior patterning defects and duplication of posterior structures increases when *hrp48* gene dosage is reduced in females overexpressing the *oskar* 3′UTR. (A) The total number of laid eggs was scored (right column) and their phenotypes quantified as a percentage of the total; the absolute number of embryos of each class is indicated (n=). For each genotype, a small proportion of embryos did not develop a clear cuticular phenotype and could not be scored. The star (*) indicates $p < 0.001$ (χ^2 test). (B) Graphic representation of the distribution of cuticle phenotypes of the unhatched embryos, as quantified and described in (A).

However, the *oskar*-related defects we observe in the mutants do not resemble anchoring defects: their onset occurs before stage 10, when cytoplasmic streaming commences, and they decrease in severity as streaming proceeds. The difference in phenotype of the mutants we analyzed, in which the level of expression of wild-type Hrp48 protein is reduced, causing polarity defects, and those identified by Huynh et al., which express mutant Hrp48 proteins at wild-type levels but show normal oocyte polarity (Hammond et al., 1997), highlights the importance of the threshold of expression of Hrp48 and the involvement of the protein in multiple RNA regulatory events.

Hrp48 as a Repressor of *oskar* mRNA Translation

The only protein shown previously to bind to *oskar* mRNA directly and to repress its translation during transport is Bruno. Null alleles of *arrest* fail to develop beyond the early stages of oogenesis, and hypomorphic alleles that allow development until stage 9 show no precocious translation of *oskar* mRNA. The evidence that Bruno is an *oskar* translational repressor in vivo is extensive and comes from analysis of transgenes in which Bruno binding sites were mutated (Kim-Ha et al., 1995), from the cuticle phenotype of embryos in which *arrest* gene dosage was varied in a sensitized genetic background (Webster et al., 1997), and from overexpression of Bruno in the germline, which causes the development of embryos with a posterior group phenotype (Filardo and Ephrussi, 2003).

Several lines of evidence support a role of Hrp48 in *oskar* translational repression. First, a substantial portion of embryos developing from *hrp48*^{K16203} germline clones show A/P patterning defects consistent with misregulation of posterior patterning activity: reduced abdominal segmentation, presumably due to defects in *oskar* mRNA localization, and head defects. Second, an enhancement of the head defects caused by overexpression of the *oskar* 3′UTR is observed when *hrp48* levels are reduced, and in extreme cases embryos with a bicaudal phenotype develop. Third, we observe translational derepression of the *lacZ-oskar* translational reporter in *hrp48* heterozygous females. The absence of detectable Oskar protein in the cytoplasm of heterozygous or homozygous *hrp48* mutant oocytes during the early stages of oogenesis suggests that the *lacZ* reporter is more sensitive than the antibody, and thus the extent to which *oskar* is derepressed in the mutants is unclear.

Bruno binds to the A, B, and C regions of the *oskar* 3′UTR, each of which contains a pair of U(G/A)U(A/G)U(G/A)U sequence elements defining the BRE consensus (Kim-Ha et al., 1995). Mutations reducing binding of Bruno to BRE region A in vitro cause precocious reporter translation in the nurse cells and in the oocyte, indicating a role of Bruno in *oskar* repression in both cell types (Gunkel et al., 1998). We have found that Hrp48 binds the A, B, and C regions in vitro (S. Castagnetti and A.E., unpublished data) and represses *oskar* translation in vivo. Sequences with homology to the characterized Hrp48 binding site in P element transposase mRNA, the F2 site (UAGGUUAAG), are located in the *oskar* 5′ translation regulatory region, and within 10 nucleotides

of the BREs in regions A and B, and overlapping with the BRE in region C in the 3′UTR. Deletion of these F2-like sequences from an AB region probe selectively disrupts Hrp48 binding in vitro, without affecting Bruno binding (5′Δ24/3′Δ25) (Gunkel et al., 1998). An *oskar-lacZ* reporter transgene bearing these deletions *(m1^{414}lac5′Δ24/3′Δ25)* shows precocious translation in the oocyte, but not in the nurse cells (Gunkel et al., 1998). Hence, the F2-like elements in regions A and B of the 3′UTR may mediate the repressive effect of Hrp48 on translation of unlocalized *oskar* mRNA in the oocyte. Proof that the repressive effect of Hrp48 on *oskar* translation is mediated by binding of the protein to these elements will require a complete mutational analysis, as was performed for Bruno, resulting in the definition of the BREs (Kim-Ha et al., 1995; Webster et al., 1997).

Interestingly, when the region to which Hrp48 binds at the 5′ end of *oskar* mRNA is deleted in the context of the BRE A LS5 mutation *(m1^{117}lacLS5)*, an increase in precocious translation both in the nurse cells and in the oocyte is observed (Gunkel et al., 1998), indicating a function of the 5′ region in translational repression. The recent demonstration that mutations in Cup, an eIF4E binding protein that coprecipitates with Bruno in ovarian extracts, cause precocious *oskar* translation suggests that translational repression may occur at the initiation step (Nakamura et al., 2004). The fact that sequences at the 5′ and 3′ ends of *oskar* mRNA regulate localization-dependent translation, together with our observation that Hrp48 can homodimerize in a yeast two-hybrid assay (O. Hachet and A.E., unpublished data) raises the possibility that Hrp48 may promote circularization of *oskar* mRNA, and thus facilitate Cup-mediated repression.

Assembly of the *oskar* mRNA Localization Complex

Hrp48 binds *oskar* mRNA directly and regulates both its localization and translation, suggesting that these processes might be coupled and mediated by a bifunctional mRNA localization/repression complex comprising Hrp48. We do not know at what stage Hrp48 first associates with *oskar* mRNA. However, the similar distributions of Hrp48 and *oskar* mRNA in the oocyte and the abundance of Hrp48 in the cytoplasm suggest their association in the cytoplasm.

How mRNA localization/translation complexes are assembled and associate with the transport machinery are central questions. The involvement in *oskar* mRNA localization of the EJC components Mago-Nashi and Y14 (Hachet and Ephrussi, 2001; Micklem et al., 1997; Mohr et al., 2001; Newmark and Boswell, 1994; Newmark et al., 1997), which associate with RNAs at exon-exon junctions upon splicing (Kataoka et al., 2001, 2000; Le Hir et al., 2001, 2000; Zhao et al., 2000), indicates that assembly of the *oskar* mRNA localization complex begins in the nucleus. The fact that all the exon-exon junctions in *oskar* mRNA are located in the coding region (Ephrussi et al., 1991; Kim-Ha et al., 1991) and that distinct regions of the *oskar* 3′UTR are required for mRNA localization (Kim-Ha et al., 1993) indicates the involvement of a higher order complex in *oskar* mRNA localization. In this context, it is interesting to speculate that Hrp48 molecules bound to distinct regions of *oskar* mRNA may promote the association of several mRNAs and associated proteins into large transport-competent particles. The perinuclear localization of Hrp48, Barentsz, Bruno, and Cup may indicate that they associate with the mRNA at the time of its nuclear-cytoplasmic transport.

During mid-oogenesis, *oskar* mRNA and associated proteins are localized in a microtubule-dependent manner to the posterior pole of the oocyte, where the plus ends of the oocyte microtubule are focused (Brendza et al., 2000; Clark et al., 1994). In *Kinesin heavy chain* mutants, *oskar* mRNA localization fails and the RNA remains all along the cell cortex, indicating a role of Kinesin heavy chain in polarized transport of the *oskar* mRNP (Cha et al., 2002; Palacios and St Johnston, 2002). However, it is still unclear how the *oskar* mRNP associates with the motor, as Kinesin light chain is not required for *oskar* mRNA localization in the oocyte (Palacios and St Johnston, 2002). Thus, future work will be required to determine the molecules and cellular mechanisms whereby nuclear-associated proteins, such as the EJC components, Barentsz, and Hrp48, assemble an *oskar* mRNA complex competent for transport to the posterior pole of the *Drosophila* oocyte.

Experimental Procedures

Fly Strains and Genetic Analysis

The P element insertion lines in the Hrb27C locus, l(2)02647, and l(2)K02814, were obtained from the Bloomington Stock Center, and l(2)K16203 was a gift from Don Rio. In this paper, we refer to l(2)02647, l(2)K02814, and l(2)K16203 as *hrp48^{02647}*, *hrp48^{K02814}*, and *hrp48^{K16203}*, respectively. *grk* mutant oocytes were from *grk^{2B6}/grk^{2E12}* females. The following transgenic stocks were also used in this study: *yw; Pin/Cyo; KZ503* (Kin-β-gal) (Clark et al., 1994), *w; UASp-osk3′UTR/nanos GAL4VP16* (Filardo and Ephrussi, 2003). The *osk-lacZ* reporter transgenes are from Gunkel et al. (1998).

Complementation of the *hrp48* alleles was performed by crossing *hrp48^{K02814}/CyO*, *hrp48^{K16203}/CyO*, *hrp48^{02647}/CyO*, and Df(2L)wee^{wo5}/CyO or *hrp48^{K10413}/CyO* flies at 25 degrees. The viability of the transheterozygous progeny was compared with the viability of their heterozygous siblings. For clonal analysis, *hrp48* mutations were first recombined with P{neoFRT40A} (Chou and Perrimon, 1996), and germline clones of the *hrp48* mutants were then generated and selected using FLP/FRT dominant female sterile (*ovoD1*) technique (Chou et al., 1993).

For rescue of *hrp48^{K16203}* by the hs-hrp48 transgene (P[w+; *hsp70-hrp48* cDNA]; Hammond et al., 1997), crosses were maintained at 25°C and heat-shocked at 37°C every 12 hr for 30 min, from the 3rd larval instar until the pupal stage. Adult females of *hrp48* germline clones with or without the hs-hrp48 transgene were dissected and their ovaries analyzed.

Purification and Identification of p50

See Supplemental Data (http://www.developmentalcell.com/cgi/content/full/6/5/637/DC1) for details of the purification procedure and peptide identification.

Synthesis of the RNA Affinity Column

An 88 base pair EcoRI-BglII fragment containing *oskar* cDNA bp +1955–2042 (Ephrussi et al., 1991) and containing the 5′-most BRE (A) of the *oskar* 3′UTR was inserted into a modified Bluescript plasmid, bearing an EcoRI site at the transcription start site. Plasmid DNA was linearized using BglII and used as template for transcription by T7 RNA polymerase (Novagen). A standard 150 μl reaction yielded ~800 μg of RNA after digestion of the template DNA with DNase (Promega). The RNA was incubated overnight at 4°C, in 0.4 M MES-KOH (pH 6.0) buffer with the CNBr-activated Sepharose 4B

(Amersham-Pharmacia), which had been washed with 1 M HCl and water. The RNA-coupled resin was then washed three times with DEPC-treated water and 0.1 M Tris-HCl (pH 7.8), and equilibrated with Buffer B.

UV Crosslinking and Immunoprecipitation

UV crosslinking followed by immunoprecipitation was performed as previously described (Gunkel et al., 1998). The RNA probes used and referred to as 3′ repressor and 5′ derepressor regions of *oskar* (+1955–2042 and +34–202 of the cDNA, respectively) were transcribed by T7 polymerase, in the presence of 3.7 MBq [α^{32}P] UTP, and purified on a denaturing polyacrylamide gel. Immunoprecipitations were performed after digestion with 10 μg of RNase A for 20 min at 37°C of the UV-crosslinked reaction mixtures. 2 μl of antiserum raised against Hrp48 or Bruno or the preimmune serum was added to the crosslinked samples and incubated for 1 hr at 4°C. The resulting immuno-complexes were recovered with protein A-Sepharose resin (Amersham-Pharmacia) and analyzed by 10% SDS-PAGE electrophoresis followed by autoradiography.

Immunohistochemistry and In Situ Hybridization

Ovaries were dissected in Grace's medium (Sigma), fixed for 20 min in 4% paraformaldehyde/PBT (PBS with 0.1% Triton X-100), and washed twice in PBT for 5 min. Samples were then incubated for 1 hr in PBS with 1% Triton X-100 and 0.5% BSA. Incubation with primary antibody was carried out overnight (or 36 hr for Hrp48 staining) at room temperature in PBS containing 0.3% Triton X-100 and 0.5% BSA. Primary antibodies were diluted as follows: rabbit anti-Hrp48 (gift of Don Rio) 1/1000 (1/5000 for the double-staining with anti-Bruno); rat anti-Bruno (1:5000 dilution; Filardo and Ephrussi, 2003); rabbit anti-Oskar (1/4000; Vanzo and Ephrussi, 2002); rabbit anti-Staufen (gift of D. St Johnston) 1/500; β-gal, 1/5000 (Promega). After washing twice in PBT for 5 min, and blocking with PBS containing 0.3% Triton X-100 and 0.5% BSA for 10 min, ovaries were incubated with fluorescein- or rhodamine-conjugated Goat anti-rabbit or Goat anti-rat secondary antibodies (1:500; Amersham), and optionally with rhodamine-conjugated phalloidin (1:250, Molecular Probes) for 2 hr. They were then washed three or four times with PBT for 10 min. Samples were mounted in 80% Glycerol containing 2% N-propylgallate and analyzed by confocal microscopy (Leica).

In situ hybridization and simultaneous detection of RNA and protein were performed as previously described (Vanzo and Ephrussi, 2002).

Acknowledgments

We thank Don Rio for his generous gifts of antibodies and *hrp48* transgenic and mutant stocks, and Daniel St Johnston for Staufen antibody. We especially thank Paolo Filardo for fly stocks and suggesting the *oskar* 3′UTR overexpression experiment, Mikita Suyama for help with statistical analysis, and Shoko Yoshida, Anna Cyrklaff, and Olivier Hachet for help with in situ hybridizations and with construction of stocks. We also thank Shoko Yoshida, Stefania Castagnetti, and the other members of the Ephrussi lab for helpful discussions during the course of this work. T.Y. was supported by fellowships from the TOYOBO Biotechnology Foundation and Cell Science Research Foundation and S.L.d.Q. by an EMBO long-term fellowship and a Marie Curie fellowship from the European Union.

Received: August 18, 2003
Revised: March 23, 2004
Accepted: March 30, 2004
Published: May 10, 2004

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