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; Gunkei 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 (Erdeyli 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; Erdeyli 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; Oha 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, thereby promoting localization 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...
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 (Filaro and Ephrussi, 2003). Recently, it was shown that Cup protein, which immunoprecipitates with elf4E 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 hnrRNA A/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/translational 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; Siegel et al., 1994).

To confirm that p50 is Hrp48, we immunoprecipitated p50 UV-crosslinked to oskar mRNA probes. Labeled
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
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 nanelectrospray mass spectrometry. The 11 peptides (underlined), some of which overlapped, were all matched to Hrp48.

(B) Identification of p50 as Hrp48. Immuno-precipitation 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 hrp48K16203 germ-line clones, indicating the specificity of the signal. In the follicle cells, Hrp48 is predominantly cytoplasmic (C), and is not detected in hrp48K16203 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 grk2B6/2E12, 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)wedeo5, are fully lethal (see Experimental Procedures). We therefore examined the viability of flies transheterozygous for three different alleles and the hypomorphic allele hrp48K0413 (Hammond et al., 1997). The hrp48K02814/hrp48K10413 combination was fully lethal. In contrast, hrp48K10413/hrp48K02814 and hrp48K02814/hrp48K10413 combinations were semilethal, with a more reduced viability in the case of hrp48K10413/hrp48K02814 (7% male and 36% female progeny) than of hrp48K02814/hrp48K10413 (24% male and 40% female progeny). Thus, with regard to lethality, hrp48K02814 is the strongest, hrp48K10413 is the intermediate, and hrp48K0413 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 hrp48K02814/hrp48K10413, hrp48K02814/hrp48K02647, and hrp48K10413/hrp48K02647, using the ovoD1 dominant female-sterile technique (Chou et al., 1993). The majority of hrp48K02814 germ-line 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, hrp48K10413 and hrp48K16203, developed egg chambers and laid eggs, although in the case of hrp48K16203 many of the eggs had a shape characteristic of dumping defects during the latest stages of oogenesis and were not fertilized. As hrp48K16203 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 fluorescent in situ hybridization in wild-type and hrp48K16203 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 hrp48K16203, 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 hrp48K16203 show normal bicoid (E) and gurken (F) mRNA localization.

(G–I) Staufen protein (green) is mislocalized in hrp48K16203 germline clones at stages 8 (G), 9 (H), and 10 (I). See Table 1 for distribution frequencies.

Hrp48 Regulates oskar mRNA Translation

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

<table>
<thead>
<tr>
<th>Genotype of Females</th>
<th>Posterior Signal (as in wild-type)</th>
<th>Signal Close to the Posterior</th>
<th>Posterior and Central Signal</th>
<th>Central Signal</th>
<th>Diffuse Signal</th>
<th>No Signal</th>
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<tbody>
<tr>
<td>A. Staufen Mislocalization</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Wild-type</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>hrp48&lt;sup&gt;K16203&lt;/sup&gt; glc</td>
<td>8</td>
<td>17</td>
<td>7</td>
<td>62</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>hrp48&lt;sup&gt;K16203&lt;/sup&gt; glc + hs-hrp48</td>
<td>48</td>
<td>4</td>
<td>11</td>
<td>32</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>B. Kin-β-gal Mislocalization (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Wild-type</td>
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<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>hrp48&lt;sup&gt;K16203&lt;/sup&gt; glc</td>
<td>38</td>
<td>7</td>
<td>6</td>
<td>43</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
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<td>75</td>
<td>0</td>
<td>7</td>
<td>11</td>
<td>0</td>
<td>7</td>
</tr>
</tbody>
</table>

Penetration of the Staufen (A) and Kin-β-gal fusion protein (B) mislocalization defects in hrp48<sup>K16203</sup> 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<sup>K16203</sup> 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<sup>K16203</sup> 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<sup>K16203</sup> (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<sup>K16203</sup> oocytes (Figures 5C and 5D). The frequencies of mislocalized Oskar and Staufen are similar and, for both, the penetration of the defect is significantly greater at stage 9 than stage 10 (see Figure 5 legend). Therefore, oskar mRNA is translated ectopically in hrp48<sup>K16203</sup> 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.
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 germline clones in an osk104/osk44 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 germline clones, osk104/osk44 oocytes is indistinguishable from that observed in hrp48 alone (Figures 5E and 5F). However, the concentrated dot of Staufen detected at the center of hrp48 oocytes (Figure 5E) is not detected in hrp48/ osk104/oosk44 oocytes (Figure 5F). Therefore, the formation of Staufen/ oskar mRNA aggregates in hrp48 oocytes is dependent on Oskar, demonstrating that oskar is translated ectopically in hrp48 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 m1414lacZ into hrp48 heterozygous flies and compared its expression in the heterozygous mutant background and in a wild-type background. In wild-type ovaries, Hrp48 and oocyte cytoplasm, as well as at the posterior pole. (I) Egg-chambers of females heterozygous for hrp48, in which a P{lacW} element is inserted in the hrp48 promoter region, express no β-galactosidase.

Figure 5. Ectopic Expression of Oskar Protein in hrp48 Oocytes. (A–D) Antibody detection of Oskar protein in wild-type (A and B) and hrp48 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 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 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 germline clones. After 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 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). (G) In hrp48 germline clones, Staufen protein enrichment in the oocyte is similar to that observed in hrp48 germline clones 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.
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被告

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-osk3 UTR; nosGAL4VP16 females, with hrp48被告

the strongest allele, causing the strongest enhancement (Figure 6). Although the vast majority (88%) of embryos produced by hrp48被告

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 aretIG3/ UASP-osk3 UTR; nosGAL4VP16/+ females. We observed no oskar mRNA localization defects in hrp48/UASP-osk3 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 gerken 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 nucleius 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 gerken 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. Theumping defects of some hrp48 mutant egg chambers suggest that Hrp48 may also regulate the function of the actin cytoskeleton.
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 (χ² 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<sup>1620</sup> germine clones show A/P patterning defects consistent with mis-regulation 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 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 to 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 S′ 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 (5'24/3'25) (Gunkel et al., 1998). An oskar-lacZ reporter transgene bearing these deletions (m11+1 lac5 24/3'25) 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 (m11+1 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 elf4E 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 Kinesis 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 mRNA (Cha et al., 2002; Palacios and St Johnon, 2002). However, it is still unclear how the oskar mRNA associates with the motor, as Kinesin light chain is not required for oskar mRNA localization in the oocyte (Palacios and St Johnon, 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 hpr48(02647), hpr48(02814), and hpr48(16203), respectively. grk mutant oocytes were from grk2129/grk2128 females. The following transgenic stocks were also used in this study: yw; P{In(1)CyO} KS230 (Kim-P; g2-1) (Clark et al., 1994), w; UASp-osk3'UTR/GAL4VP16 (Filardo and Ephrussi, 2003). The osk-lacZ reporter transgenes are from Gunkel et al. (1998).

Complementation of the hpr48 alleles was performed by crossing hpr48(02647)/CyO, hpr48(02814)/CyO, hpr48(16203)/CyO, and Df(2L)wee w2/2 CyO or hpr48(16203)/CyO flies at 25 degrees. The viability of the transheterozygous progeny was compared with the viability of their heterozygous siblings. For clonal analysis, hpr48 mutations were first recombined with P{n[nefT4O]} (Chou and Perrimon, 1996), and germline clones of the hpr48 mutants were then generated and selected using FLP/FRT dominant female sterile (ovoD1) technique (Chou et al., 1993).

For rescue of hpr48(16203) by the hs-hpr48 transgene (P[w+; hsp70- hpr48 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 hpr48 germline clones with or without the hs-hpr48 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 CBnR-activated Sepharose 4B
Hrp48 Regulates oskar mRNA Translation

(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 7.6), 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 9 derepression regions of oskar (+1955–2042 and +34–202 of the cDNA, respectively) were transcribed by T7 polymerase, in the presence of 3.7 MBq [32P]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 anti-serum 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.

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