A late phase of germ plasm accumulation during Drosophila oogenesis requires Lost and Rumpelstiltskin

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SUMMARY
Asymmetric mRNA localization is an effective mechanism for establishing cellular and developmental polarity. Posterior localization of oskar in the Drosophila oocyte targets the synthesis of Oskar to the posterior, where Oskar initiates the assembly of the germ plasm. In addition to harboring germline determinants, the germ plasm is required for localization and translation of the abdominal determinant nanos. Consequently, failure of oskar localization during oogenesis results in embryos lacking germ cells and abdominal segments. oskar accumulates at the oocyte posterior during mid-oogenesis through a well-studied process involving kinesin-mediated transport. Through live imaging of oskar mRNA, we have uncovered a second, mechanistically distinct phase of oskar localization that occurs during late oogenesis and results in amplification of the germ plasm. Analysis of two newly identified oskar localization factors, Rumpelstiltskin and Lost, that are required specifically for this late phase of oskar localization shows that germ plasm amplification ensures robust abdomen and germ cell formation during embryogenesis. In addition, our results indicate the importance of mechanisms for adapting mRNAs to utilize multiple localization pathways as necessitated by the dramatic changes in ovarian physiology that occur during oogenesis.

KEY WORDS: Nanos, Oskar, Germ plasm, mRNA localization, Rumpelstiltskin, Lost, Drosophila

INTRODUCTION
Intracellular mRNA localization is a conserved mechanism for generating protein asymmetries that are necessary for developmental polarity and polarized cell functions. In a variety of organisms, maternally synthesized mRNAs that are localized within the oocyte and embryo produce regionalized distributions of proteins required for patterning of the embryonic axes, specification of germ layers and formation of the germ line (Gavis et al., 2007). Localization of maternal oskar (osk) mRNA to the posterior of the Drosophila oocyte restricts the synthesis of Osk protein to the posterior, where Osk initiates the assembly of the germ plasm (Ephrussi et al., 1991; Markussen et al., 1995; Rongo et al., 1995). This specialized cytoplasm, which contains germ cell fate determinants, persists at the posterior into early embryogenesis, where it induces formation of the pole cells, the germ cell progenitors. The germ plasm is also essential for development of the anterior-posterior body axis, through its role in posterior localization and translational activation of the abdominal determinant nanos (nos) (Ephrussi et al., 1991; Gavis and Lehmann, 1992; Gavis and Lehmann, 1994).

As a maternal mRNA, osk is transcribed in the ovarian nurse cells and is transported from the nurse cells into the oocyte early in oogenesis (stages 1–7 of 14 morphologically defined stages) (Ephrussi et al., 1991; Kim-Ha et al., 1991). During mid-oogenesis (stages 8–10), reorganization of the oocyte microtubule cytoskeleton creates a posterior bias of microtubule plus-ends that allows net posteriorly directed transport of osk by kinesin motors (Theurkauf et al., 1992; Brendza et al., 2000; Zimyanin et al., 2008). After reaching the posterior pole, osk is translated into two functionally distinct Osk isoforms: one recruits additional germ plasm proteins, including the highly conserved RNA helicase Vasa (Vas), whereas the other maintains the localization of osk mRNA and Osk protein through an actin-dependent mechanism (Markussen et al., 1995; Rongo et al., 1995; Breitwieser et al., 1996; Vanzo and Ephrussi, 2002; Vanzo et al., 2007).

A second posterior localization pathway, acting later in oogenesis when the nurse cells initiate apoptosis and extrude or ‘dump’ their contents into the oocyte (stages 11 and 12), mediates localization of nos (Forrest and Gavis, 2003). Microtubule-based transport to the posterior is preemted by the reorganization of microtubules into cortical bundles that mediate the concerted streaming of the oocyte cytoplasm to mix nurse cell and oocyte contents (Theurkauf et al., 1992). Instead, nos moves with the bulk cytoplasm during ooplasmic streaming and becomes trapped by association with germ plasm components at the posterior (Forrest and Gavis, 2003). The integration of nos into the germ plasm activates nos translation and creates a protein gradient that directs abdominal development during embryogenesis (Gavis and Lehmann, 1992). In mutants for germ plasm components such as osk or vas, nos mRNA fails to localize to the posterior, Nos protein is not produced and, consequently, embryos lack abdominal segments (Gavis and Lehmann, 1994; Wang et al., 1994).

The ability of an mRNA to utilize a particular localization pathway is thought to depend on its cadre of associated localization factors. These include proteins that recognize cis-acting localization signals usually found within 3’ untranslated regions (3’UTRs), accessory proteins that package these RNA-protein (RNP) complexes into higher order particles, and adaptors that link the RNP particles to the cytoskeleton for transport and/or anchoring (Gavis et al., 2007; Lewis and Mowry, 2007; Kugler and Lasko, 2009). Genetic and biochemical approaches have identified numerous proteins that interact directly or indirectly with osk mRNA and are required for assembly, transport, and/or anchoring of osk RNP particles. Several of these factors are also involved in the localization of two other mRNAs, bicoid (bcd) and gurken...
(grk), whose respective dynein-dependent transport to the anterior and dorso-anterior regions of the oocyte occurs concomitantly with osk transport (Kugler and Lasko, 2009). These studies and studies of localized mRNAs in other cell types support a model in which localized RNAs are recognized and packaged by a combination of general and RNA-specific factors, with the particular combination of factors dictating how and where the RNP particles are transported and anchored.

We previously identified the Drosophila heterogeneous ribonucleoprotein M (hnRNPM) homolog Rumpelstiltskin (Rump) as a direct-acting nos localization factor (Jain and Gavis, 2008). Here, we report the characterization of a second protein, Lost, which co-purifies with nos and Rump and contributes to nos localization. Surprisingly, our analysis of dump and lost double mutants has uncovered a second phase of osk localization that occurs concomitantly with nos localization during late oogenesis and has not been previously described. This late-acting pathway, but not osk localization during mid-oogenesis, requires the combined functions of Rump and Lost. Moreover, our results reveal that localization of osk during mid-oogenesis is not sufficient for osk-dependent germ plasm function in either anterior-posterior body axis or pole cell formation. Rather, the Rump- and Lost-dependent posterior accumulation of osk during late oogenesis results in an amplification of the germ plasm that ensures wild-type abdominal and germline development. We show that Rump binds directly to osk as well as to nos, suggesting that Rump and Lost are part of a core localization complex that promotes utilization of the late localization pathway by multiple mRNAs in parallel.

**MATERIALS AND METHODS**

**Fly stocks**
The following mutants and transgenic lines were used: y w67C2 (Lindsley and Zimm, 1992), osk497 (Vanzo and Ephrussi, 2002), CG14648ZCL169 (Morin et al., 2001), gfp-vas (Johnstone and Lasko, 2004), osk-mos2/s (Lin et al., 2008), hsp83-MCP-GFP (Forrest and Gavis, 2003), hsp83-MCP-RFP (Weil et al., 2006) and nos-(mos2) (Brecchiel and Gavis, 2008).

**Genetic analysis of lost**
CG14648 is referred to as growl in FlyBase. The lost1 allele was generated by imprecise P element excision of the GFP protein-trap insertion CG14648ZCL169. Individual males, each bearing an excision in trans to Df(3R)ME15, which deletes the lost locus, were screened by PCR using primer pairs to amplify regions immediately upstream or downstream of the P element. Deletion endpoints were then mapped by PCR amplification and sequencing. The lost1 rump1 double mutant was generated by meiotic recombination. The lost rescue transgene consists of a 5.7 kb Xbal-Xhol genomic fragment isolated from BAC clone BACR09G03 (Berkeley Drosophila Genome Project) and inserted into pCaSpeR4 (Pirota, 1988).

**Production of MBP-Lost and anti-Lost monoclonal antibodies**
The lost coding region (minus the initiation codon) was PCR amplified by imprecise P element excision of the GFP protein-trap insertion CG14648ZCL169. Individual males, each bearing an excision in trans to Df(3R)ME15, which deletes the lost locus, were screened by PCR using primer pairs to amplify regions immediately upstream or downstream of the P element. Deletion endpoints were then mapped by PCR amplification and sequencing. The lost1 rump1 double mutant was generated by meiotic recombination. The lost rescue transgene consists of a 5.7 kb Xbal-Xhol genomic fragment isolated from BAC clone BACR09G03 (Berkeley Drosophila Genome Project) and inserted into pCaSpeR4 (Pirota, 1988).

**Immunoprecipitations and immunoblot analysis**
Ovary extract was prepared as described previously (Kalifa et al., 2006), using IP buffer [25 mM HEPEPS (pH 7.4), 150 mM NaCl, 2.5 mM MgCl2, 0.5 mM EDTA, 0.01% Triton X-100, 1× complex protease inhibitor cocktail (Roche) and 10 μg/ml pepstatin] supplemented with phosphatase inhibitors (1 mg/ml sodium vanadate, 20 mM sodium fluoride). Extracts were pre-cleared using Dynabeads Protein G (Invitrogen) for 1 hour at 4°C and then incubated for 2 hours at room temperature with Dynabeads Protein G-bound with Rump 5G4 monoclonal antibody (Jain and Gavis, 2008) or anti-Lost 1D11 monoclonal antibody. Beads were washed six times with IP buffer. Purified protein complexes were fractionated by SDS-PAGE, transferred to nitrocellulose membrane, and detected by immunoblotting and chemiluminescence. Final primary antibody concentrations were: 1:3,000 for rabbit anti-Khc (Cytoskeleton); 1:10,000 for mouse anti-Snf (gift of P. Scheidl, Princeton University, NJ, USA); 1:3000 for rabbit anti-Osk (Vanzo and Ephrussi, 2002); 1:4000 for mouse anti-Rump and 1:1000 for mouse anti-Lost. Mouse TrueBlot Ultra (eBiosciences) secondary antibody was used at 1:1000 to limit detection of heavy chain. Quantification was performed using ImageJ (NIH).

**UV-crosslinking assays**
The osk 5′UTR, 3′UTR and subdivisions of the 3′UTR were amplified from osk cDNA with the addition of a 5′ T7 primer sequence using primers shown in Table S1 in the supplementary material. The nos +2′ element probe has been previously described (Jain and Gavis, 2008). Synthesis of radioabeled RNA probes and UV-crosslinking was performed as described previously (Bergsten et al., 2001).

**In situ hybridization and immunofluorescence**
In situ hybridization for nos, osk and bcd in 0- to 2-hour-old embryos was performed as described previously (Gavis and Lehmann, 1992). Fluorescence in situ hybridization (FISH) for osk in oocytes was performed as described in Vanzo and Ephrussi (Vanzo and Ephrussi, 2002) except that ovaries were fixed in 4% electron microscopy (EM) grade formaldehyde in PBS, subsequent washes and antibody incubations were performed in PBST (PBS + 0.1% Tween-20) and hybridization steps were performed at 55°C. Anti-digoxigenin-rhodamine (1:200, Roche) and DAPI (1:1000, Molecular Probes) were used to visualize the osk probe and nuclei, respectively.

**Anti-Vas immunofluorescence**
was performed on 3- to 5-hour-old embryos as described (Becalska et al., 2011) using rabbit anti-Vasa (1:10,000; gift from P. Lasko, McGill University, Montreal, Canada), goat anti-anti-Rosa 488 (1:1000) and DAPI (1:1000, Molecular Probes). Polyclonal antibodies were screened from an antiserum taken at 1.5 μm intervals throughout the embryo posterior with a Zeiss LSM 510 confocal microscope, 40×/1.3 NA objective. Ooocytoblasts were detected as described previously (Becalska and Gavis, 2010). For visualization of F-actin, ovaries were fixed in 4% EM grade formaldehyde in PBS, washed 3×5 minutes in PBS, blocked in PBST (PBS/1% Tween-20/2% BSA) for 30 minutes, stained with Alexa Fluor 488 phallodin (1:500 in PBT) for 30 minutes and washed 3×10 minutes in PBS. Microtubules and actin were imaged with a Leica SP5 confocal microscope, using a 63×/1.4 NA oil immersion objective.

**Live imaging**
Live imaging of germ plasm components was performed as described in Weil et al. (Weil et al., 2006). Images were collected with a Leica SP5 confocal microscope using a 63×/1.4 NA oil immersion objective. Ooocytic streaming was visualized as described in Becalska et al. (Becalska and Gavis, 2010).

**Fluorescence quantification**
All fluorescence intensity measurements in live oocytes were made using Velocity 5.3 (Improvision). For quantification of osk*GFP and GFP-Vas accumulation at the posterior in live oocytes, confocal z-stacks of 1.5 μm optical sections spanning 25-30 μm at the posterior cortex were taken every 15-20 minutes starting prior to nurse cell dumping (stage 10B) and continuing for 3 hours, after the cessation of nurse cell dumping (late stage 12/early stage 13). For each oocyte, a threshold based on the auto-
fluorescence detected in the oocyte cytoplasm was set and only fluorescence above this threshold was quantified. The total fluorescence at the oocyte posterior at each time point was then normalized to time zero and the net change in fluorescence accumulation was calculated (t3–t0)/t0.

The total volume occupied by oostGFP at the posterior was quantified from z-sections of 1.5 μm thickness taken through the entire posterior of the oocyte at stage 10A or stage late12/early 13. All wild-type and lost rump oocytes of the same stage were imaged in the same imaging session under identical conditions. For each stage, a single fluorescence intensity threshold was then determined for each oocyte.

Fluorescence recovery after photobleaching (FRAP) analysis

Individual oocytes were mounted in glass-bottom dishes as described above. A ‘prebleach’ z-series centered on the oocyte posterior cortex was taken with a 63×/1.4 NA oil immersion objective and a 4X optical zoom. A small 15-25 μm thick region of interest (ROI) at the posterior cortex was bleached to 40-60% of the initial fluorescence by bleaching successive 2 μm sections at maximum 488, 458 and 476 laser intensity for a total of 20 iterations. Recovery was monitored by collecting a z-series every two minutes after bleaching. After background correction, the mean intensity of the ROI for each time point was normalized to the initial fluorescence (100%).

Statistical analysis

All data were analyzed using PRISM 5.0 (Graphpad). For quantification of pole cell and live-imaging data, normal distributions were confirmed using the D’Agostino-Pearson normality test and Student’s t-test was used to determine significance (Zimyani et al., 2008).

RESULTS

Purification and identification of Lost

The nos localization signal, located within the nos 3’UTR, comprises multiple localization elements with partially redundant functions (Gavis et al., 1996; Bergsten and Gavis, 1999). Previously, we performed tandem RNA affinity purification using one of these elements, the 88 nucleotide +2’ localization element, to isolate nos localization factors. Mass spectrometry analysis identified Rump, the Drosophila hnRNP M homolog, which we validated as a direct-acting nos localization factor (Jain and Gavis, 2008). In addition to the peptides corresponding to Rump, 50 peptides were recovered that represent much of the 545 amino acids of the predicted protein encoded by the gene CG14648 (Flybase). CG14648, which we have named lost, was previously identified in a screen of GFP protein-trap insertions for proteins localized to ovarian RNP-containing structures called sponge bodies and has been listed as component of several RNP complexes isolated from cultured cells, but has never been characterized (Blagden et al., 2009; Herold et al., 2009; Snee and Macdonald, 2009; Worringen et al., 2009). The predicted Lost polypeptide contains a putative 5-formyltetrahydrofolate cyclo-ligase domain that is 35% identical to human 5-FTHF cyclo-ligase domains. FTHF cyclo-ligases are essential components of folate metabolism in humans (Dayan et al., 1995; Anguera et al., 2003), but it is currently unknown if and how this domain functions in Drosophila. Notably, there are no conserved protein-protein or RNA-binding domains that would shed light on a role for Lost in mRNA localization.

Lost and Rump interact biochemically

Whereas Rump binds directly to the nos +2’ element used for purification (Jain and Gavis, 2008), we were unable to detect direct binding by recombinant Lost protein to this element or any other region of the nos 3’UTR (see below and data not shown). Inclusion of recombinant Rump in the binding reactions also had no effect. These results suggest that Lost associates indirectly with the nos localization signal, via its interaction with one or more components of the nos RNP.

Because Rump was isolated in the same purification as Lost and binds directly to the nos 3’UTR, we tested whether Lost interacts with Rump. Rump-containing complexes were immunopuriﬁed from oocyte extract using an anti-Rump antibody and analyzed by immunoblotting with the respective antibodies as indicated. The input samples contain 1-2% of the amount of extract used for immunoprecipitation (IP). The enhancement of co-immunoprecipitation in the presence of RNase could be due to protein conformational changes that result in enhanced antibody or increased protein accessibility.

Lost is a cytoplasmic protein that is posteriorly enriched in the oocyte and early embryo

We took advantage of a protein-trap P element inserted in the lost gene (CG14648ZCL3169; Fig. 2A) (Morin et al., 2001) to monitor the distribution of Lost. GFP-Lost flies express lost at near wild-type levels (Fig. 2B), produce protein recognized by an anti-Lost antibody and have no overt phenotype (data not shown), suggesting that the GFP insertion does not disrupt lost function. GFP-Lost is cytoplasmic in the nurse cells and in the oocyte, where it becomes enriched early in oogenesis (Fig. 2C). During mid-oogenesis, GFP-Lost is cortical in late stage oocytes, enriched both anteriorly and posteriorly (Fig. 2E). This cortical distribution...
lost1 blastoderm (F-H) and blastoderm stage (I-K) embryos expressing GFP-Lost (green) and material). Females homozygous for kb deletion that includes the predicted transcriptional start site (Fig. 2). Imprecise excision of this P element resulted in an ~4 kb deletion that includes the predicted CG14648 transcriptional start sites. The region contained in the genomic deletion removes both of the predicted CG14648 transcriptional start sites. The region contained in the genomic lost rescue transgene (glost) is indicated. (B) Northern blot analysis of lost mRNA in wild-type (WT), CG14648ZCL3169 (GFP-lost) and lost1 ovaries. rp49 serves as a loading control. (C-E) Confocal images of GFP-Lost (green) in fixed stage 7-8 (C), stage 10 (D) and stage 13 (E) egg chambers. Arrowheads indicate anterior enrichment, arrows indicate posterior enrichment. nc, nurse cells; oo, oocyte. (F-K) Confocal images showing the posterior poles of live pre-embryos (F-H) and blastoderm stage (I-K) embryos expressing GFP-Lost (green) and nos*RFP (red). pc, pole cells. (L) In situ hybridization to nos mRNA in early wild-type embryos (WT), lost1 embryos or lost1 embryos with the genomic lost rescue transgene (glost, lost1). Anterior is to the left. Right-hand panels show enlargements of the posterior for each genotype. Data from in situ hybridization were used to classify posterior localization as wild type, diffuse or reduced; quantification of these patterns is shown on the right (WT, n=116; lost1, n=292; glost; lost1, n=103).

Isolation of a lost null allele
We generated a lost mutation, lost1, by excision of the protein-trap P element. Imprecise excision of this P element resulted in an ~4 kb deletion that includes the predicted transcriptional start site (Fig. 2A). Northern blot analysis and immunoblotting with anti-Lost antibody show that both lost mRNA and Lost protein are missing from lost1 ovaries (Fig. 2B; see Fig. S1 in the supplementary material). Females homozygous for lost1 are fertile but, similar to the defect seen in rump mutants (Jain and Gavis, 2008), homozygous lost1 males are sterile. Also similarly to rump mutants, lost1 females exhibit a maternal-effect defect, with 59% of embryos produced (n=954) failing to develop. Of those that complete embryogenesis, 7% show mild abdominal segmentation defects (partial deletions or fusions of segments; data not shown).

Although the lost deletion disrupts two additional genes of unknown function, CG14647 and CG9853 (Fig. 2A), all of the lost1 phenotypes described here and below can be rescued by a single copy of a genomic lost transgene (glost, Fig. 2A), indicating that these phenotypes are due to elimination of lost function specifically and not due to disruption of the neighboring genes.

lost mutants exhibit a mild nos localization defect
A role for lost in nos localization was first investigated by performing in situ hybridization to nos in embryos from lost1 mutant females (referred to hereafter as lost1 embryos). In 50% of lost1 embryos, localization of nos appears more diffuse than in wild-type embryos and the wild-type distribution is restored by expression of the lost transgene (Fig. 2L). Like the mild phenotype of rump mutants, the modest but resuable nos localization and abdominal segmentation defects observed in lost1 mutants suggest that Lost acts redundantly with or in parallel to other nos localization factors. Such redundancy among nos localization factors is predicted by the partial functional redundancy exhibited by nos localization signal elements (Gavis et al., 1996; Bergsten and Gavis, 1999).

An unanticipated requirement for Lost and Rump in osk localization
To determine whether the biochemical interaction between Lost and Rump reflects a collaborative role in nos localization, we generated flies mutant for both lost and rump (lost1 rump1).
Similarly to the individual mutants, lost–rump– females exhibit a maternal-effect defect, with 54% of their embryos (n=1905) failing to develop. However, abdominal segmentation defects (primarily loss of one segment or partial deletions and fusions of segments) are more prevalent among the surviving progeny (18%) than for either lost– (7%) or rump– (11%) embryos. Moreover, in contrast to lost or rump mutant embryos, the double mutant embryos show a dramatic decrease in posterior accumulation of nos without affecting overall nos levels (Fig. 3A, data not shown). In 64% of lost–rump– embryos, nos localization is eliminated or reduced to a small posterior disc (Fig. 3A). In addition, Nos protein levels are dramatically reduced compared with wild type (data not shown), consistent with reduced abdominal segmentation. Presumably, many of the embryos with reduced nos localization are among the population that fails to develop, leading to the difference in the frequencies of localization and segmentation defects.

To our surprise, we found that localization of osk is similarly affected in lost–rump– embryos. Despite wild-type osk levels (data not shown), osk localization is reduced to a small disc in 30% of lost–rump– embryos (Fig. 3B). Consequently, in lost–rump– embryos, Osk is reduced to less than 25% of the wild-type level (Fig. 3C). Consistent with previous observations that pole cell formation is highly sensitive to Osk levels, we find that lost–rump– embryos have significantly fewer pole cells than wild-type embryos (27%; wild-type average=22.5, n=26; Fig. 3D). We have previously shown that osk localization is not affected in rump mutant embryos (Jain and Gavis, 2008). Elimination of lost alone has only a minor effect, with osk appearing less tightly localized in 13% of lost– embryos (see Fig. S2 in the supplementary material). Like the more prevalent nos localization defect, this phenotype is rescued by the genomic lost transgene (see Fig. S2 in the supplementary material). Eliminating both lost and rump function thus has a far more dramatic mRNA...
localization phenotype than mutations in either gene alone. These results reveal redundant or overlapping contributions by lost and rump in mediating osk as well as nos localization. The anteriorly localized bcd is unaffected in lost rump embryos (see Fig. S3A in the supplementary material), indicating specificity of lost and rump for particular localization pathways.

Lost and rump are not required for osk mRNA localization during mid-oogenesis

The localized distribution of nos and osk in the embryo is the end result of transport and anchoring events occurring earlier during oogenesis. Thus, we analyzed the distribution of osk mRNA and Osk protein accumulation during oogenesis. Drosophila oogenesis can be divided into 14 morphologically defined stages, with nurse cell dumping occurring at the end of stage 10 (King, 1970). Microtubule-dependent transport of osk to the posterior of the oocyte occurs primarily during stage 9 and the mRNA is subsequently maintained at the posterior by a mechanism involving Osk protein and the actin cytoskeleton (Rongo et al., 1995; Babu et al., 2004; Vanzo et al., 2007; Suyama et al., 2009). In contrast to previously characterized mutants with osk localization defects, posterior localization of osk appears wild type in 100% of stage 9 egg chambers (n=33) and 98% of stage 10 egg chambers (n=52) (Fig. 3E). Consistent with this result, Osk levels are comparable between lost rump and wild-type stage 9/10 egg chambers (Fig. 3F). Neither microtubule organization nor the anterodorsal localization of gsk is obviously affected in lost rump mutant egg chambers (see Fig. S3B-E in the supplementary material).

Furthermore, the cortical actin cytoskeleton, including the F-actin projections induced by Osk at the oocyte posterior, appears to be unaltered in stage 9-10 lost rump mutant oocytes (see Fig. S3G,H in the supplementary material). Taken together, these data indicate that a posterior localization pathway is specifically disrupted in lost rump mutants.

A second phase of osk localization occurs coincident with nurse cell dumping

The decrease in the amount of osk at the posterior of lost rump embryos could reflect a role for Lost and Rump in maintaining osk at the posterior cortex during the later period of oogenesis and/or fertilization. Alternatively, accumulation of osk in the nurse cells through stage 10 (Kim-Ha et al., 1991) suggests that additional osk enters the oocyte during nurse cell dumping. lost and rump might, therefore, function to promote the posterior localization of this population of osk during late oogenesis. Consistent with this idea, a previous study showed that synthetic osk RNA injected into stage 11 oocytes is capable of localizing to the posterior (Glotzer et al., 1997), although the relevance to endogenous osk has never been tested. To distinguish between these possibilities, we first investigated the dynamics of osk mRNA during late oogenesis using the transgenic MS2 tagging system to label osk mRNA with GFP (designated as osk*GFP) in vivo. Expression of ms2-tagged osk rescues the osk mutant oogenesis defect (Lin et al., 2008).

Time-lapse imaging of osk*GFP in cultured wild-type egg chambers reveals a second phase of osk localization during and after nurse cell dumping (Fig. 4A-D). Fluorescence intensity measurements show that, on average, there is a 45% increase in osk*GFP at the posterior as oogenesis progresses from late stage 10 (just prior to nurse cell dumping) to late stage 12 (after nurse cell dumping) (n=6; Fig. 4Q,Q'). Concomitantly, Osk protein levels increase dramatically post-dumping (Fig. 3F). Similarly, a previous study using an Osk-GFP reporter protein showed that Osk levels at the posterior increase during late stages of oogenesis (Snee et al., 2007). Our results suggest that a late phase of osk localization is most likely responsible for the observed accumulation of Osk-GFP in late oocytes. As the osk-(ms2)6 transgene introduces an extra copy of osk, it is possible that the second phase of osk accumulation could simply reflect a delay in localization due to the presence of excess osk. We therefore expressed osk*GFP in females heterozygous for an osk RNA null allele, oskA87 (Jenny et al., 2006), to achieve an approximately wild-type level of osk RNA (see Fig. S4A-D in the supplementary material).

Fluorescence intensity quantification shows that in oskA87 heterozygotes the amount of osk*GFP at the posterior of cortex triples by the end of nurse cell dumping (see Fig. S4E in the supplementary material). This enhancement of osk*GFP localization probably reflects the alleviation of competition between transgenic osk*GFP mRNA and wild-type osk mRNA for a limiting posterior anchor.

To confirm the dynamic behavior of osk at the posterior, we performed fluorescence recovery after photobleaching (FRAP) experiments on early stage 12 wild-type oocytes that were still undergoing opolasmic streaming. The fluorescence from osk*GFP was irreversibly inactivated in a small, three-dimensional region of interest (ROI) at the posterior cortex by high-intensity illumination, and fluorescence recovery in the ROI volume was monitored over time (Fig. 4E-H). Notably, fluorescence in the ROI increases steadily throughout the recovery time course, even surpassing the initial fluorescence levels in two of the three oocytes monitored (Fig. 4R, data not shown). These data, together with the results from our time-lapse imaging studies, provide strong evidence that osk*GFP continues to accumulate at the posterior cortex throughout the period of opolasmic streaming. We cannot, however, rule out the possibility that local reorganization of RNPs anchored in adjacent regions of the cortex might, in part, contribute to the recovery of osk*GFP.

The second phase of osk accumulation fails to occur in lost rump mutants

Consistent with the in situ hybridization results, osk*GFP localization is indistinguishable between lost rump and wild-type egg chambers at stages 9 and 10 (data not shown). By contrast, imaging of stage 12 and 13 egg chambers reveals a dramatic decrease in the amount of osk*GFP at the posterior of the double mutants relative to wild type (Fig. 4I-L). Moreover, time-lapse imaging of individual egg chambers shows that, on average, the amount of osk*GFP at the posterior cortex decreases by 77% between stages 10B and 12 (n=5; Fig. 4Q,Q'). This is in stark contrast to the 45% increase in posteriorly localized osk*GFP observed in wild-type oocytes (Fig. 4Q,Q'). Thus, not only is the second phase of osk localization attenuated in lost rump oocytes, but osk*GFP localized during mid-oogenesis is not maintained at later stages of oogenesis despite the presence of an intact actin cytoskeleton (Fig. 4I-L,Q,Q'; see Fig. S3G-H in the supplementary material). Furthermore, quantification of Osk levels in stage 12 and 13 egg chambers shows a 30% decrease in Osk in lost rump relative to wild type (Fig. 3F). The mRNA localization defect, resulting in diminished production of Osk throughout the remainder of oogenesis, explains the loss of localized osk mRNA and Osk protein observed in lost rump embryos (Fig. 3B,C).

Despite reduced accumulation of osk*GFP at the posterior of stage 12 lost rump oocytes, we were able to perform FRAP analysis. osk*GFP fluorescence shows substantial recovery in the three-dimensional ROI, but unlike the wild-type case, the
initial fluorescence levels are low and are not exceeded (Fig. 4M-P,S). This recovery, together with the net loss of osk*GFP from the posterior cortex between stages 10B and 12, could be explained by dissociation of the majority of osk that was localized during mid-oogenesis from the cortex with a small amount of new accumulation during dumping. Alternatively, partial repopulation of the bleached region could result from local reorganization of the remaining germ plasm at the posterior cortex in the absence of new osk accumulation. Although we cannot yet distinguish whether one or both of these possibilities applies, results from both the time-lapse imaging and FRAP analyses demonstrate a requirement for lost and rump in the late phase accumulation and maintenance of osk at the posterior cortex.

In lost rump embryos, not only is the amount of localized osk and nos reduced, but both RNAs occupy a much smaller region of the posterior cortex than they do in wild-type embryos (Fig. 3A,B). We calculated the total volume occupied by osk*GFP at the posterior of wild-type and lost rump oocytes in staged egg chambers using identical conditions for imaging and quantification for all samples (see Materials and methods). At stage 10A, osk*GFP occupies the same mean volume in wild-type or lost rump oocytes (Fig. 4T). However, by late stage 12/early stage 13, the mean volume of osk*GFP at the posterior in lost rump oocytes is 63% of its mean volume in wild-type oocytes (Fig. 4U). Taken together, these data suggest that the failure to accumulate additional osk mRNA during late stages of oogenesis results in failure of the germ plasm to expand in volume proportionate to the

Fig. 4. Continued accumulation of germ plasm during late stages of oogenesis. (A-D) Time-lapse confocal projections of the posterior of a live Drosophila oocyte expressing osk*GFP. The time course captures the localization of osk*GFP starting prior to the onset of nurse cell dumping, stage 10B (A), and ending after nurse cell dumping is complete, stage 12 (D). Elapsed time (t) is indicated; posterior is up. (E-H) Fluorescence recovery after photobleaching (FRAP) experiment performed on osk*GFP at the posterior of a wild-type stage 12 oocyte. The photobleached region of interest (ROI) is indicated by the rectangle. The zero time point corresponds to the completion of photobleaching in the ROI. Time elapsed in the FRAP recovery period is indicated; posterior is up. (I-L) Time-lapse confocal projections of osk*GFP at the posterior of a live lost rump oocyte from stage 10B (I) to stage 12 (L). Elapsed time is indicated; posterior is up. (M-P) FRAP experiment performed on osk*GFP at the posterior of a stage 12 lost rump oocyte. The photobleached ROI is indicated by the rectangle. The zero time point corresponds to the completion of photobleaching in the ROI. Time elapsed in the FRAP recovery period is indicated; posterior is up. (Q) The total amount of posterior osk*GFP fluorescence in time-lapse confocal z-stack images was quantified for six wild-type and five lost rump oocytes (see Materials and methods) and the net change in fluorescence intensity between the initial time point (equivalent to panels A or I, labeled as t0) and final time point (equivalent to panels D or L, labeled as t3) was plotted for each oocyte (Q). Plot of the average net change in fluorescence intensity ± s.e.m. for each genotype is shown in Q’. (R,S) Quantification of the FRAP experiments from the wild-type (R) or lost rump (S) oocytes shown in E-H and M-P. Similar results were observed for two additional wild-type and three additional lost rump stage 12 egg chambers. (T,U) Quantification of the volume (µm³) occupied by osk*GFP at the posterior of individual wild-type (solid circles) and lost rump (open circles) oocytes at stage 10A (T) and stage 12/13 (U). The mean value for the volume of osk*GFP fluorescence (horizontal bars) in lost rump oocytes is not significantly different from wild type at stage 10A but is significantly smaller by stage 12/13 (P<0.0086).
size of the growing oocyte and, consequently, only a small disc of localized germ plasm is observed in the embryo. Examination of nos*GFP in stage 14 oocytes showed that nos localization is affected in parallel to osk, leading to the reduction of localized nos mRNA and Nos protein in the embryo (see Fig. S5 in the supplementary material, data not shown).

A late phase of Vas accumulation
To determine whether protein components of the germ plasm also continue to accumulate late in oogenesis, we performed similar time-lapse imaging of GFP-Vas. Localization of which during mid-oogenesis has been well studied, we have now identified a second, previously uncharacterized phase of accumulation. The concomitant accumulation of Vas at the posterior during this period indicates that this phase of Vas localization leads to germ plasm amplification. This late phase of osk localization specifically requires Rump, which binds to osk in vivo, like the interaction in vitro, does not depend on Lost. Taken together with previous observations (Jain and Gavis, 2008), these results suggest that Rump recognizes mRNAs designated for posterior localization and recruits Lost to these RNP complexes to mediate localization at late stages of oogenesis.

DISCUSSION
Posterior localization of osk underlies the formation of germ plasm at the posterior of the oocyte and, ultimately, the development of the germ line and abdomen of the animal. Whereas osk localization during mid-oogenesis has been well studied, we have now identified a second, previously uncharacterized phase of osk localization that occurs late in oogenesis, beginning with nurse cell dumping. The concomitant accumulation of Vas at the posterior during this period indicates that this phase of osk localization leads to germ plasm amplification. This late phase of osk localization specifically requires Rump, which binds to osk, and Lost, which interacts with Rump. In lost rump embryos, the reduction of germ plasm at the posterior leads to fewer germ cells and abdominal segmentation defects. Thus, the localization of osk during mid-oogenesis is not sufficient to ensure wild-type germline and abdominal development. Rather, the second phase of osk localization and germ plasm accumulation is crucial for embryogenesis. Our results are consistent with results from a previous study showing that continued production of Osk during late stages of oogenesis is essential for abdomen formation (Snee et al., 2007) and they reveal the mechanism behind this accumulation.
The failure of osk to accumulate at the posterior of lost rump mutants at late stages of oogenesis could result from the failure of osk to reach to the oocyte posterior altogether or the failure to become entrapped and anchored there. Nurse cell dumping and ooplasmic streaming occur normally in lost rump mutants (see Fig. S3E,F in the supplementary material), suggesting that the defect does not lie in the ability of osk to reach the posterior cortex. The lack of localized osk in embryos from mutants such as staufen, which disrupt osk localization during mid-oogenesis, or osk mis-sense mutants, which lack functional Osk protein and fail to maintain osk at the posterior after stage 9, suggests that the late phase of osk localization depends on the prior localization and translation of Osk and recruitment of germ plasm components during mid-oogenesis (Ephrussi et al., 1991; Kim-Ha et al., 1991; St Johnston et al., 1991). Consistent with this evidence, Osk is required for posterior accumulation of synthetic osk mRNA following injection into stage 11 oocytes (Glotzer et al., 1997). Thus, Rump and Lost probably mediate the interaction of osk with pre-existing Osk or other germ plasm proteins, and/or the actin cytoskeleton. Eliminating both Rump and Lost produces a more severe RNA localization defect than eliminating either one but does not completely abolish localization. Partially redundant contributions by RNA-binding factors like Lost that make that RNP assembly and function robust to perturbation.

Not only is the late phase of osk localization affected in lost rump mutants, but osk that had accumulated at the posterior of the oocyte during mid-oogenesis is de-localized. Although difficult to quantify, we also notice a transient decrease in osk at the posterior in wild-type oocytes immediately following the onset of nurse cell dumping (see Fig. 4A-C). One possible explanation is that the Osk-dependent anchoring mechanism established during mid-oogenesis cannot withstand the force of ooplasmic streaming and is, therefore, unable to maintain osk at the posterior cortex. Retention of both previously anchored and newly arriving osk at the cortex would, therefore, require the transition to a more robust anchoring system, through the activity of Rump and Lost. Once established, this anchor would support the continued accumulation of germ plasm through the remainder of oogenesis.

Because nos localization depends on the germ plasm, the nos localization defect observed in lost rump oocytes and embryos could be secondary to the osk localization defect. However, several pieces of evidence suggest that lost and rump might also regulate nos localization in parallel. Rump and Lost were both isolated by co-purification with the nos +2' localization element. Rump binds to a specific sequence motif in the nos +2' localization element, and mutation of these sequences or elimination of Rump compromises +2' element localization function independently of osk localization. Finally, nos localization is frequently more diffuse in lost mutants whereas osk is largely unaffected. As an attractive hypothesis, nos might be transported to or anchored at the posterior together with osk, as part of the same RNP. Incorporation of nos and osk into this RNP and/or the ability to interact with Osk or other anchoring factors would then depend on the interaction of Lost and Rump with each mRNA. Alternatively, Lost and Rump might contribute to the assembly of an independent nos RNP that is competent to associate with the germ plasm at the posterior pole. Development of methods to image osk and nos simultaneously during late oogenesis will be crucial to distinguish between these possibilities.

We have previously shown that a late-acting localization pathway is responsible for the majority of anteriorly localized bcd in the embryo (Weil et al., 2006). The late phase of bcd localization is genetically distinct from bcd localization during mid-oogenesis but does not depend on Rump or Lost, indicating that these proteins are specific for posterior localization at late stages. Taken together, our results indicate that mRNA localization pathways functioning during late stages of oogenesis amplify localized mRNA distributions generated during mid-oogenesis to endow the embryo with the requisite concentrations of determinant mRNAs and germ plasm components needed for body axis and germline development. Our work suggests that localization factors such as Lost and Rump adapt mRNAs for utilization of multiple, mechanistically distinct localization pathways necessitated by dramatic changes in ovarian physiology and the oocyte cytoskeleton during oogenesis.
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