Independent and coordinate trafficking of single 
_Drosophila_ germ plasm mRNAs

Shawn C. Little¹², Kristina S. Sinsimer¹, Jack J. Lee¹, Eric F. Wieschaus¹² and Elizabeth R. Gavis¹³

Messenger RNA localization is a conserved mechanism for spatial control of protein synthesis, with key roles in generating cellular and developmental asymmetry. Whereas different transcripts may be targeted to the same subcellular domain, the extent to which their localization is coordinated is unclear. Using quantitative single-molecule imaging, we analysed the assembly of _Drosophila_ germ plasm mRNA granules inherited by nascent germ cells. We find that the germ-cell-destined transcripts _nanos, cyclin B_ and _polar granule component_ travel within the oocyte as ribonucleoprotein particles containing single mRNA molecules but co-assemble into multi-copy heterogeneous granules selectively at the posterior of the oocyte. The stoichiometry and dynamics of assembly indicate a defined stepwise sequence. Our data suggest that co-packaging of these transcripts ensures their effective co-assemble into multi-copy heterogeneous granules selectively at the posterior of the oocyte. The stoichiometry and dynamics of assembly indicate a defined stepwise sequence. Our data suggest that co-packaging of these transcripts ensures their effective co-packaging into multi-copy heterogeneous granules at the posterior of the oocyte.

The _Drosophila_ germ plasm, whose assembly and function rely on localized mRNAs, provides an ideal system to address these questions. This specialized cytoplasm at the posterior of the embryo is necessary and sufficient for induction of the germ cell progenitors, the pole cells⁹. Numerous maternal mRNAs are enriched within the germ plasm and then inherited by the pole cells¹⁰,¹¹, where they direct production of proteins required for specification of germ line fate and for germline development¹²,¹³. Formation of the germ plasm is initiated during mid-oogenesis by the kinesin-dependent transport of _oskar_ (osk) mRNA to the posterior of the oocyte. Osk protein produced from this localized RNA recruits the helicase Vasa (Vas) and other proteins, establishing a posterior domain of germ plasm assembly¹⁴. This initial step is essential for a second wave of mRNA localization during late stages of oogenesis, whereby additional osk as well as numerous other mRNAs including _nanos_ (nos), _polar granule component_ (pgc) and _cyclin B_ (cycB) accumulate at the posterior¹⁵–¹⁸. Live imaging of nos mRNA showed that its localization occurs as a consequence of diffusion within the oocyte cytoplasm and entrapment at the posterior in Vas-containing RNPs known as polar granules¹⁶. Later, during embryogenesis, these granules are segregated to the pole cells as those cells bud from the posterior of the embryo¹⁹. The accumulation of numerous different mRNAs within the germ plasm through a common localization pathway and their ultimate destination in the pole cells begs the question of whether their trafficking is coordinated at any step. Moreover, whether the movement of multiple mRNAs to the posterior pole and ultimately into the pole cells is coordinated through co-packaging into common RNPs or whether they are organized into functionally distinct RNPs has not been investigated. To address these questions, we analysed the mRNA composition of RNPs containing germ plasm mRNAs at different stages of oogenesis and embryogenesis by high-resolution fluorescent _in situ_ hybridization (FISH) and quantitative image analysis. Using nos as a paradigm, we show that localization occurs by the exclusive assembly of RNPs containing single mRNAs into multi-copy granules at the posterior cortex. Co-packaging of nos with _cycB_ and _pgc_ occurs selectively at the posterior, forming heterogeneous polar granules that co-transport germ plasm mRNAs into pole cells. In contrast, osk travels in multi-copy RNPs and is segregated from the other mRNAs at the posterior into compositionally distinct granules that can contain hundreds of _osk_

---

¹Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544, USA. ²Howard Hughes Medical Institute, Princeton University, Princeton, New Jersey 08544, USA.

Correspondence should be addressed to E.R.G. (e-mail: gavis@princeton.edu)

Received 29 August 2014; accepted 20 February 2015; published online 6 April 2015; corrected online 1 February 2016; DOI: 10.1038/ncb3143
transcripts. These granules must be excluded from the pole cells for germline development.

RESULTS

nos mRNAs travel as single-copy particles before posterior localization

To better understand how nos becomes assembled into polar granules, we performed FISH using a method capable of detecting individual mRNA molecules in Drosophila embryos20,21 (Supplementary Video 1). We observed two classes of labelled objects: dim punctae at high density filling the embryo interior and bright objects near the cortical surface at the posterior (Fig. 1a,b). Both classes appear as diffraction-limited point sources (Supplementary Fig. 1a). Visualized in sections near the mid-sagittal plane, the bright objects occupy a cap-shaped volume covering the posterior at a depth up to ~5µm beneath the cortex (Fig. 1b). The posterior cap extends ~25µm from the posterior pole, measured as an arc along the cortical surface (Fig. 1a). Localization of nos is observed as an increase in fluorescent signal close to the posterior pole, resulting from the enrichment of bright objects containing many mRNAs (that is, localized granules) accompanied by a depletion of dim punctae (that is, unlocalized particles) that contain fewer mRNAs (Supplementary Fig. 1b–d).

We quantified the mRNA content of the unlocalized particles by comparing the number of particles detected by FISH with the number of mRNAs measured by absolute quantitative reverse transcription PCR (RT–qPCR). Counting dim punctae in the entire left or right halves of embryos and multiplying by two yields an estimate of 2.0 ± 0.5 × 10⁶ objects per embryo. Our ability to detect most dim objects was confirmed by FISH using nos probes alternating in fluorophore colour along the length of the transcript. Plotting the distribution of fluorescence intensities for detected objects in each channel in two-dimensional (2D) histograms, we found that ≥97% of dim objects detected in one channel also appear in the second channel (Fig. 1c,d), indicating high detection efficiency. The total number of detected particles in the embryo (2.0 ± 0.5 × 10⁶) approximates the total number of nos mRNA molecules per embryo determined by RT–qPCR (2.5 ± 0.4 × 10⁶, Supplementary Fig. 1e). Thus, the average mRNA content of the imaged dim punctae is 1.3 ± 0.5 mRNAs. As only ~4% of nos mRNA is localized22 (also see Methods), unlocalized particles, which constitute the vast majority of nos mRNA in embryos, largely represent single mRNAs.

Given the high density of unlocalized nos particles, we estimate that ~15% of unlocalized transcripts reside within the same imaging volume by random chance (Supplementary Fig. 1f–j and Methods).
Figure 2 nos granule assembly occurs exclusively at the posterior oocyte cortex. (a) Maximum z-series projections spanning 5 μm at the posterior oocyte cortex show the extent of nos localization at four different stages of oogenesis. Scale bars, 10 μm (stage 9, 10a); 20 μm (stage 12, 13). (b) Distributions of nos mRNA content per granule during late oogenesis (also see Supplementary Fig. 2). (c) nos content as a function of distance from the posterior pole. (d) Calibration of intensity distributions of nos-GFP granules in time-lapse videos (solid blue bars; arbitrary units) to FISH granules (red outline, absolute mRNA number). Inset depicts one frame of a nos-GFP time-lapse video compared to a FISH image. (e) An individual granule (arrowhead) containing approximately 15 mRNAs travels ~3 μm in 10 s while maintaining nos content. Frames correspond to times t = 4, 7, 10, 12 and 15 s. Displacement (blue) is calculated as root-mean-square distance from the t = 0 position. Fluctuations in mRNA content (red) centred on ~15 mRNAs result from imaging noise (see also Supplementary Fig. 2).

Co-localization detected in excess of this amount would thus indicate concerted co-packaging of multiple nos mRNAs. To estimate the fraction of unlocalized particles containing >1 nos transcript, we examined the fluorescence intensity distributions in 2D histograms after normalizing to the most frequently observed intensity in each channel. As the number of probes decorating each mRNA molecule is drawn at random from a distribution about the mean number of probes, the two intensity measurements should be uncorrelated for particles representing single mRNAs. We observed a weak correlation with a clear skew towards higher mRNA content along the correlated axis (Fig. 1e,f, cyan) compared with the symmetric distribution along the anticorrelated axis (Fig. 1e,f, red). The distribution along the correlated axis is well matched by a model in which 83% and 15% of detected punctae represent 1 and 2 mRNAs, respectively (Fig. 1f). Thus, the predicted rate of spurious co-localization within the same volume is similar to the observed frequency of punctae containing 2 mRNAs. We conclude that unlocalized nos exists as single mRNAs that are not co-packaged before localization. 

nos localization results from the spatially restricted assembly of multi-copy granules

In 2-colour FISH experiments, normalized intensity values for each fluorophore in granules at the posterior pole are highly correlated, indicating that in contrast to unlocalized nos particles, these granules contain multiple nos transcripts (Supplementary Fig. 1k). Normalization to the intensity of single molecules observed in the
Figure 3 Co-localization of polar granule components begins during late oogenesis. (a–d) Detection of nos, pgc and cycB in nurse cells by FISH. (a,b) Confocal sections of nurse cells at stage 8. nos, pgc and cycB are found in separate particles (also see Supplementary Fig. 3). In some instances, putative sites of nascent transcription cluster to one side of a nurse cell nucleus (arrowheads in b) and this is often associated with enrichment of single mRNAs in the cytoplasm closest to the transcription sites; additional sites are present in adjacent z sections. (c,d) Maximum z-series projections spanning 5 μm at stage 10b near the cortex (c) and near a putative ring canal leading to the oocyte (d) showing filamentous networks enriched for nos and cycB RNP. Blue: DAPI staining. Scale bars, 10 μm. (e–g) Maximum z-series projections over 5 μm of the posterior oocyte cortex. pgc and nos are first detected in posterior granules at stage 10a (e) and are often enriched in the same granule (f, stage 10b; g, stage 13). Scale bars, 5 μm (e,f); 10 μm (g). (h–j) 2D histograms of nos and pgc content in all detected particles at stage 10b (h), stage 12 (i) and stage 13 (j). Heat map indicates relative density of data points. Horizontal red and vertical green lines indicate thresholds separating localized granules and unlocalized particles. For all localized granules detected above the threshold in one channel (to the right of the green line for localized pgc; above the red line for localized nos), percentages indicate the fractions with zero content, low content (primarily unlocalized particles), or high content (corresponding to localized granules) in the second channel (as read from left to right for nos in green; from top to bottom for pgc in red). cycB behaves similarly to nos and pgc (see Supplementary Fig. 3).

The localization of nos occurs during late stages of oogenesis. To determine when and where the transition from single mRNAs to multi-copy granules occurs and the relationship between granule formation and posterior localization, we analysed nos during this period (Fig. 2a). Synthesized by the ovarian nurse cells, nos enters the oocyte en masse when the nurse cells `dump' their contents at the end of stage 10 and becomes distributed throughout the oocyte by diffusion and the concurrent streaming of the oocyte cytoplasm (ooplasm)16. Consistent with previous analysis of nos mRNA labelled using the MS2/MS2 coat protein (MCP) system16, we observed continuous accumulation of nos in granules at the posterior of the oocyte beginning at stage 10 of oogenesis. nos-containing granules increase in both number and mRNA content up until the oocyte reaches maturity at stage 14 (Fig. 2b). Similarly to the early embryo, multi-copy granules were observed only within a depth of ~5 μm from the cortex and only within the localization domain (Supplementary Fig. 2a).

The appearance of localized granules as diffraction-limited objects (Supplementary Fig. 1a) suggests that they correspond to individual higher-order RNP containing many mRNAs. To support the idea that the bright objects represent physically associated mRNA particles and not particles transiently corralled into the same volume, we compared our FISH data to data from live imaging of MS2-tagged nos labelled with MCP–GFP (nos’GFP). Localized nos’GFP granules have a spatial distribution and fluorescence intensities similar to those of nos granules detected by FISH, indicating that they probably represent the same entities (Fig. 2d). Previous live imaging experiments showed that nos’GFP granules undergo rapid, directed movement along microtubules at the posterior cortex13. By performing a chi square fit to match to the right sides of the fluorescence intensity distributions of nos granules detected by FISH and nos’GFP granules in live oocytes, we found that objects undergoing rapid, directed runs over several micrometres at ≥0.5 μm s–1 contain upwards of 10–20 nos mRNAs (Fig. 2d). Throughout each run, the intensity measurement remains unchanged, indicating that the detected mRNAs are constituents of the same granule (Fig. 2e).
and Supplementary Fig. 2b–d and Supplementary Video 2). Taken together, our results indicate that nos localization results from the incorporation of single transcript particles into multi-copy granules exclusively at the posterior cortex.

**Germ plasm mRNAs assemble into common but heterogeneous granules at the posterior**

As numerous mRNAs in addition to nos are localized to the germ plasm and incorporated into pole cells, we reasoned that their localization might be coordinated through co-packaging into the same RNP. To determine whether and when co-packaging occurs, we examined the distributions of two additional germ plasm mRNAs, pgc and cycB, in pairwise combinations with nos. All three mRNAs are expressed at high levels in the nurse cells at mid-oogenesis (Fig. 3a–d). Around stage 10, before the onset of nurse cell dumping, they become enriched on colcemid-sensitive track-like structures, probably microtubules, that are best visualized at the cortical surface (Fig. 3c). Tracks are also detected that extend towards the ring canal junctions connecting nurse cells to the oocyte and to each other (Fig. 3d). Despite their similar distributions, we did not detect co-localization of nos, pgc or cycB mRNAs before posterior localization, in either the nurse cells or bulk ooplasm (Supplementary Fig. 3a–d).

Once they reach the posterior, pgc and cycB mRNAs, like nos, begin to form large granules (Fig. 3e–j). To determine whether nos and pgc can reside within the same polar granule, we calculated the distance in 3D space between nearest neighbours in the two channels. In the 2-colour nos FISH experiments described above, >95% of localized granules detected in one channel possess a partner in the other channel at a distance of <200 nm (<3 pixels in the xy plane). Given a density of ~0.7 granules μm$^{-3}$, the expected co-localization rate due to random chance is ~10% (see Methods). Using 200 nm as the criterion for co-localization, we found that by late oogenesis 33% of nos granules contain pgc, and conversely 62% of localized pgc granules contain nos (Fig. 3j). The frequent overlap of pgc and nos granules suggests that nos and pgc mRNAs are physically linked. Similar results were obtained for nos and cycB (Supplementary Fig. 3e,f). We conclude that different germ plasm mRNAs co-assemble in granules exclusively at the posterior cortex.
Dual mRNA labelling experiments showed that a substantial fraction of localized granules contain only one of two different mRNAs regardless of the copy number of that mRNA (Fig. 3h–j and Supplementary Fig. 3f). In contrast, most granules containing >5 molecules of nos or cycB either alone or combined together exhibit co-localization with granules of Vas, visualized by direct fluorescence of GFP–Vas (Fig. 4). The co-localization of germ plasm mRNAs with GFP–Vas occurs in the same spatial domain where we observe their enrichment, consistent with a model wherein Vas activity at the posterior is required to recruit single-copy mRNA particles into nascent granules. Moreover, a small fraction (6%) of localized GFP–Vas objects lack both nos and cycB (Fig. 4j) but probably contain other posteriorly localized mRNAs. The enrichment of granules completely lacking any given mRNA rules out a simple model of granule assembly by random selection of mRNAs from the unlocalized pool. Such a model predicts that, for granules of a given total size, the fraction containing at least one transcript from a particular gene must always be greater than the fraction containing zero copies of that mRNA (Supplementary Fig. 4). This is not observed in the dual mRNA labelling experiments, where the fraction containing zero copies is notably enriched (Fig. 3c and Supplementary Figs 3f and 4). Instead, our data are consistent with a model in which: transcripts of a given gene are preferentially incorporated into granules through the formation of homotypic mRNA clusters; and homotypic clusters containing different mRNAs associate in higher-order assemblies to form heterogeneous polar granules.

**osk multimerization in the nurse cells and anterior oocyte precedes assembly of large localized granules**

In contrast to other germ plasm mRNAs, posterior localization of osk occurs in two mechanistically distinct phases. During mid-oogenesis, osk is transported to the posterior in RNP complexes containing the double-stranded RNA-binding protein Staufen (Stau), which may link osk to kinesin and is required for osk translational activation and anchoring at the posterior cortex. 24–26. In addition, osk mRNA forms higher-order complexes that may play a role in repression of osk translation and coordination of osk transport. 8,27,28. The osk mRNA content of these complexes, and where and when they form, has not been determined. During late oogenesis, osk is localized along with other germ plasm mRNAs by diffusion and posterior entrapment. 15,29. We investigated the nature of osk RNP complexes during both phases of localization using methods described above for nos to confirm detection of single osk mRNAs (Supplementary Fig. 5a–d) and correspondence between objects observed in live and fixed oocytes (Supplementary Fig. 5e–h).
Figure 6 Dynamics of osk granule assembly. (a–g) Confocal images showing the distribution of osk mRNA throughout oogenesis. Z-series projections are shown in all but d. (a) Germarium (outlined) with expression in presumptive nurse cells and accumulation in presumptive oocytes. (b,c) Stage 4 (b) and stage 6 (c) egg chambers shown at saturating settings to illustrate osk expression in nurse cells and high concentration in oocytes. At all stages, anterior nurse cells consistently exhibit higher levels of osk than posterior nurse cells. (d) Single image plane at stage 7 reveals the transient accumulation of osk in the centre of the oocyte. (e) Stage 10a with strong osk enrichment at the posterior pole in mid-sagittal cross-section. (f,g) Cortical views at stage 12 (f) and stage 14 (g). Scale bars, 10 µm (d,e); 20 µm (f,g). (h) Histograms of cortical osk particle intensities during mid to late oogenesis. (i) osk content as a function of distance from the posterior pole. (j) Normalized distribution of mRNA content of all particles found in confocal z-stacks encompassing the posterior 20% of a stage 13 oocyte (top) and a similarly sized volume in the centre of the oocyte (bottom) illustrating a reduction in the fraction of particles containing 2–4 mRNAs in the posterior domain.

Like nos, cycB and pgc, osk is highly expressed in nurse cells and can be found on track-like structures during mid-oogenesis (Supplementary Fig. 5i). However, osk exhibits several differences from nos. First, whereas nos is found uniformly in all nurse cells as single mRNAs, a large fraction of osk particles in nurse cells contain two mRNAs at levels that are twice as high as expected from random co-localization alone (Fig. 5 and Methods). Second, among nurse cells at the posterior of the cluster, the nurse cell connected exclusively to the oocyte and to no other nurse cells contains less osk overall, as well as a lower ratio of particles containing two osk mRNAs compared with other nurse cells (0.8 versus 0.4 particles µm⁻³; Figs 5 and 6b,c). These observations are explained by a mathematical model in which 2-copy particles are preferentially transported between nurse cells as well as between nurse cells and the oocyte (Supplementary Note), suggesting that the formation of 2-copy particles enhances osk transport to the oocyte. Third, osk particles containing 2–4 mRNAs are heavily enriched in the ooplasm from mid-oogenesis onward and persist in the bulk cytoplasm of the early embryo. Co-localization of osk with Stau–GFP was first detected in the oocyte, coinciding with the formation of osk particles containing >2 mRNAs at the anterior and persisting in localized granules (Fig. 5). These results show that, unlike nos and other germ plasm mRNAs, posterior localization of osk is preceded by the assembly in the oocyte of RNPs containing Stau and multiple osk mRNAs.

We next examined the posterior cortical enrichment of osk once it enters the oocyte. We observed bright granules at the posterior cortex during stages 8 and 9 of oogenesis, but the high density of osk in the ooplasm through stage 8 (Fig. 6a–d) precluded us from determining whether these granules assemble before their arrival at the posterior. By stage 9, osk in the bulk ooplasm is found in 1-
4-copy punctae, with nearly all brighter granules near the posterior cortex (Fig. 6–g), where they grow in both number and mRNA content until the end of oogenesis (stage 14). Like nos, the distribution of osk mRNA content in localized granules is heterogeneous and can be described by a log-normal distribution (Supplementary Fig. 5j,k). Total osk content per granule approaches a maximum of ~250 mRNAs during late oogenesis (Fig. 6h). From stage 10 onward, most (>90%) granules containing 50 or more osk mRNAs are found ≤3 µm beneath the cortex, within 30 µm of the posterior pole as measured along the surface of the cortex (Fig. 6i). Thus, large osk granules reside within the same domain where nos granules subsequently form. Particles containing small numbers (2–4) of osk mRNAs are depleted at the posterior cortex (Fig. 6j), suggesting that the large localized granules are assembled preferentially from the smaller osk RNPs. These results indicate that localization occurs through osk granule assembly in a unique manner distinct from other posteriorly localized mRNAs, with the formation of particles containing 2 osk mRNA molecules in nurse cells, the association of these on entry to the oocyte to form particles containing 4 osk transcripts and, by late oogenesis, the assembly of large complexes that contain hundreds of mRNAs.

osk mRNA is continuously segregated from germ granules

The accumulation of osk at the posterior cortex in multi-copy granules concomitantly with nos and other germ plasm mRNAs during late oogenesis raised the possibility that osk might be incorporated into the same localized granules. We therefore performed dual FISH for nos and osk in oocytes expressing either GFP–Vas or Osk–GFP. Granules of localized osk mRNA inhabit regions devoid of nos, GFP–Vas or Osk–GFP (Fig. 7a–j). Nearest-neighbour distance measurements confirmed that 90% of particles containing nos mRNA co-localize with GFP–Vas or Osk–GFP. In contrast, ≤25% of the detected osk mRNA granules reside within 200 nm of a granule containing nos, GFP–Vas and/or Osk–GFP (Fig. 7k). Rather, localized osk granules show 90% concordance with Stau–GFP (Fig. 5c), suggesting a role for Stau in the late localization of osk in addition to its earlier role in osk transport. These results indicate that osk mRNA is segregated from nos and other germ plasm RNAs by incorporation into compositionally distinct granules.

Localization of osk to germ granules impairs germ cell formation

The segregation of osk from nos is maintained into embryogenesis (Fig. 8a). Before pole cell formation, nos granules dissociate from the cortex and accumulate around the centrosomes of posterior nuclei through dynein-dependent transport on astral microtubules. This trafficking promotes incorporation of nos along with the nuclei into pole cells as they bud from the posterior of the embryo (Fig. 8b,c). These granules retain other germ plasm mRNAs, as evidenced by the continued co-localization with cycB (Fig. 8d). Localized osk granules also dissociate from the cortex but do not accumulate around posterior nuclei or become enriched in pole cells (Fig. 8b). By the onset of cellularization, osk granules are nearly absent from the posterior (Fig. 8c), whereas nos and other germ plasm mRNAs are highly enriched in pole cells (Fig. 8f). To determine whether osk is actively excluded from pole cells, we analysed osk–bcd3’UTR embryos in which osk is mislocalized to the anterior by replacement of the osk 3’ untranslated region (3’UTR) with the bicoid (bcd) 3’UTR mRNA localization signal (Fig. 8g–i). Osk
**Figure 8** osk localization to germ plasm impairs germline development. (a) Confocal section at the cortex of an early (nuclear cycle (n.c.) 2) wild-type (WT) embryo labelled with nos and osk probes, showing the continued segregation of nos and osk. (b) Enrichment of nos on astral microtubules and depletion of large osk granules in a n.c. 9 embryo. (c) nos is highly enriched in pole cells of a blastoderm (n.c. 14) embryo, whereas osk is largely absent. nos is shown at lower contrast, and osk at high contrast, compared with a and b. (d,e) Co-localization of nos and cycB during n.c. 3 (d) and n.c. 11 (e). (f) High-magnification view of nos and cycB granules. (g-i) FISH analysis of osk–bcd3 UTR (OB) embryos with bcd and osk probes. (g) Z-series projection of the anterior cortex shows co-localization of bcd and osk–bcd3 UTR mRNAs in granules at n.c. 3. (h) Granules containing osk are excluded from the vicinity of presumptive ectopic pole cells at n.c. 10. (i) bcd particles are enriched in ectopic pole cells at n.c. 14, which are largely devoid of osk. Most osk granules are confined to a region basal to the blastoderm layer. (j,k) FISH analysis of oskΔi1,2–nos3 UTR embryos (OΔN) with osk and nos probes. Z-series projections of the posterior cortex at n.c. 4 (j) and n.c. 14 (k) show co-localization of oskΔi1,2–nos3 UTR and nos through pole cell formation. Scale bars, 10 μm (a,b-e and g-k shown at same scale as a); 2 μm (f). (l) Left: Anti-Vas immunofluorescence in wild-type (upper) and oskΔi1,2–nos3 UTR (lower) embryos. Right: box plot showing mean (red line), 25th and 75th quartiles (blue boxes), and range (black lines) of Vas-positive pole cell numbers (n=40 wild-type and 59 oskΔi1,2–nos3 UTR embryos). (m,n) Anti-Vas immunostaining reveals a reduction in Vas-positive cells in the gonads of late-stage oskΔi1,2–nos3 UTR embryos.

Protein produced at the anterior of osk–bcd3 UTR embryos results in ectopic germ plasm assembly and the induction of functional pole cells at the anterior before this ectopic pole cell formation, a large fraction of granules at the anterior cortex contain both osk and bcd mRNAs (Fig. 8g). During pole cell formation, we unexpectedly observed the recruitment of granules containing exclusively bcd into regions surrounding prospective anterior pole cell nuclei whereas osk-containing granules are clearly excluded from the same region.
(Fig. 8h). By the end of the blastoderm stage, only bcd is highly enriched in ectopic pole cells even though large osk granules persist at the anterior beyond the time when endogenous osk at the posterior has largely disappeared (Fig. 8i). These results indicate that osk is effectively excluded from germ plasm granules that are segregated to the developing germline.

To determine the functional relevance of this exclusion, we examined the consequence of forcing osk localization to nos-containing polar granules. We generated an osk transgene that lacks features required for osk localization31 and whose 3’UTR is substituted by the nos 3’UTR, which directs posterior localization and incorporation into pole cells32 (UAS-oskΔi1,2-nos3’UTR). oskΔi1,2–nos3’UTR mRNA is highly co-localized with nos in polar granules, resulting in the segregation of osk mRNA to pole cells (Fig. 8j,k). Strikingly, both the amount of Vas in the pole cells and pole cell number are significantly reduced in oskΔi1,2–nos3’UTR embryos compared with control embryos (Fig. 8l). In contrast, no defects were observed in embryos expressing gfp–nos3’UTR mRNA, which is similarly localized to polar granules and incorporated into pole cells (Supplementary Fig. 6). Moreover, gonads of oskΔi1,2–nos3’UTR embryos contain few germ cells (Fig. 8m,n). This decrease is not due simply to overexpression of Osk protein, because embryos bearing 6 copies of a wild-type osk transgene develop an excess of pole cells35. These results indicate, therefore, that the inclusion of osk in germ plasm granules destined for pole cells is detrimental to pole cell induction and germline development.

**DISCUSSION**

Our quantitative analysis of germ plasm-localized mRNAs has revealed several intriguing features about the localization process and the coordinate regulation of their integration into the pole cells. We show that nos, pgc and cycB mRNAs are transported within the oocyte as single mRNAs and are co-packaged into granules specifically at the posterior cortex concomitant with localization. Thus, localization serves not only to concentrate these transcripts at the posterior but also generates large, multi-copy polar granules to coordinate the efficient incorporation of these transcripts into the pole cells.

Polar granules are heterogeneous with respect to both the amount of a particular mRNA and the combination of different mRNAs. Although nos mRNA content of polar granules varies over a large range, there is a tendency towards higher values fitting a log-normal distribution. Log-normal distributions are often associated with exponential growth processes, whereby the rate at which an object grows is proportional to the size of the object4,35. Thus, a log-normal distribution suggests that large granules grow at faster rates compared with small ones as assembly is accelerated through positive feedback. In addition, we find that for granules containing both nos and pgc or nos and cycB, the quantities of the two different mRNAs are correlated, and there is a greater fraction of granules completely lacking one species of mRNA entirely than granules containing just a few copies of that mRNA. Together, these data suggest that for each type of mRNA, cooperative interactions generate homotypic RNPs that then: accelerate the recruitment of additional mRNAs of the same type; and facilitate granule assembly by promoting interactions with similarly sized homotypic clusters of other mRNAs. These results also predict the existence of dedicated molecular pathways, one to form homotypic clusters, and another to assemble homotypic clusters of many different transcripts into higher-order granules. These higher-order granules may form by fusion of smaller homotypic granules and indeed fusion of granules labelled with GFP–Vas is observed by live imaging33. Alternatively, clusters of different mRNAs may grow alongside each other on a predefined granule scaffold. The localization of Caenorhabditis elegans germ granules—P granules—occurs through a phase transition in which soluble RNP components condense at the posterior of the embryo36. It is interesting to consider whether formation of homotypic clusters occurs by a condensation of single transcript RNPs mediated by RNA-binding proteins.

In contrast to other posteriorly localized RNAs, which travel as single molecules, osk forms oligomeric complexes beginning in the nurse cells. Previous studies indicated that reporters containing the 3’UTR can hitch-hike on wild-type osk mRNA by 3’UTR-mediated dimerization8,37. Hitch-hiking is not required for osk transport, but co-packaging may be important for translational repression of osk before localization77,28. Consistent with this, multi-copy RNPs are competent for localization by both kinesin-dependent transport during mid-oogenesis and diffusion/entrapment during late stages of oogenesis.

Previous ISH-immuno-electron microscopy analysis of stage 10 oocytes showed co-localization of osk with Stau, but not with Osk protein in polar granules38 and our results indicate that osk/Stau RNPs are continuously segregated from the germ plasm granules. This physical separation has functional consequences. Whereas co-packaging of nos, pgc and cycB coordinates their transport to posterior nuclei and consequent segregation to the pole cells, osk is specifically excluded. Although we do not know why targeting of Osk to polar granules seems to alter their function, it is clearly detrimental to germline development. It will be interesting to determine whether osk/Stau granules contain other mRNAs whose functions are required specifically during oogenesis or early embryogenesis but must be excluded from pole cells.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Note:** Supplementary Information is available in the online version of the paper.

**ACKNOWLEDGEMENTS**

We thank W. Eagle (Princeton University, USA), P. Lasko (McGill University, Canada), P. Macdonald (University of Texas, Austin, USA) and D. St Johnston (Gordon Institute, UK) for fly stocks and reagents, S. Chatterjee and S. Kyin for technical assistance, and E. Abbaszadeh, S. Blythe and B. He for comments on the manuscript. This work was financially supported by National Institute of Health grant R01GM067758 (E.R.G.) and the Howard Hughes Medical Institute (E.F.W.).

**AUTHOR CONTRIBUTIONS**

S.C.L., K.S.S. and E.R.G. designed the experiments. S.C.L., K.S.S., J.J.L. and E.R.G. performed the experiments S.C.L. and E.R.G. analysed the data. S.C.L., E.R.G. and E.F.W. wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

Published online at http://dx.doi.org/10.1038/ncb3143
Reprints and permissions information is available online at www.nature.com/reprints

1. Pratt, C. A. & Mowry, K. L. Taking a cellular road-trip: mRNA transport and anchoring. *Curr. Opin. Cell Biol.* **25**, 99–106 (2013).
7. Batish, M., van den Bogaard, P., Kramer, F. R. & Tyagi, S. Neuronal mRNAs travel
8. Jambor, H., Brunel, C. & Ephrussi, A. Dimerization of oskar et al.
5. Tubing, F. et al.

2. Marchand, V., Gaspar, I. & Ephrussi, A. An intracellular transmission control protocol: assembly and transport of ribonucleoprotein complexes. Curr. Opin. Cell Biol. 24, 202–210 (2012).
3. Lange, S. et al. Simultaneous transport of different localized mRNA species revealed by live-cell imaging. Traffic 9, 1256–1267 (2008).
4. Amrute-Nayak, M. & Bullock, S. L. Single-molecule assays reveal that RNA localization signals regulate dynein–dynamin copy number on individual transcript cargoes. Nat. Cell Biol. 14, 416–423 (2012).
5. Tubing, F. et al. Dendritically localized transcripts are sorted into distinct ribonucleoprotein particles that display fast directional motility along dendrites of hippocampal neurons. J. Neurosci. 30, 4160–4170 (2010).
6. Mikhail, M., Vendra, G., Doyle, M. & Kiebler, M. A. RNA localization in neurite morphogenesis and synaptic regulation: current evidence and novel approaches. J. Comp. Physiol. 196, 321–334 (2010).
7. Batisch, M., van den Bogaard, P., Kramer, F. R. & Tyagi, S. Neuronal mRNAs travel singly into dendrites. Proc. Natl Acad. Sci. USA 109, 4645–4650 (2012).
8. Jambor, H., Brunel, C. & Ephrussi, A. Dimerization of oskar 3’ UTRs promotes hitchhiking for RNA localization in the Drosophila oocyte. RNA 17, 2049–2057 (2011).
9. Mahowald, A. P. Assembly of the Drosophila germ plasm. Int. Rev. Cytol. 203, 187–213 (2001).
10. Rangan, P. et al. Temporal and spatial control of germ-plasm mRNAs. Curr. Biol. 19, 72–77 (2009).
11. Lecuyer, E. et al. Global analysis of mRNA localization reveals a prominent role in organizing cellular architecture and function. Cell 131, 174–187 (2007).
12. Cieplicki, R. M., Rangan, P. & Lehmann, R. Germ cells are forever. Cell 132, 559–562 (2008).
13. Nakamura, A. & Seydoux, G. Less is more: specification of the germline by assembly and transport of ribonucleoprotein complexes. Traffic 9, 1256–1267 (2008).
14. Becalska, A. N. & Gavis, E. R. Lighting up mRNA localization in Drosophila oogenesis. Development 136, 2493–2503 (2009).
15. Sinsimer, K. S., Jain, R. A., Chatterjee, S. & Gavis, E. R. A late phase of germ plasm accumulation during Drosophila oogenesis requires Lost and Rumpelstiltskin. Development 138, 3431–3440 (2011).
16. Forrest, K. M. & Gavis, E. R. Live imaging of endogenous mRNA reveals a diffusion and entrapment mechanism for nascent mRNA localization in Drosophila. Curr. Biol. 13, 1159–1168 (2003).
17. Dalby, B. & Glover, D. M. 3’ non-translated sequences in Drosophila cyclin B transcripts direct posterior pole accumulation late in oogenesis and peri-nuclear association in syncytial embryos. Development 115, 989–997 (1992).
18. Nakamura, A., Amikura, R., Mukai, M., Kobayashi, S. & Lasko, P. Requirement for a non-coding RNA in Drosophila polar granules for germ cell establishment. Science 274, 2075–2079 (1996).
19. Lert, D. A. & Gavis, E. R. Transport of germ plasm on astral microtubules directs germ cell development in Drosophila. Curr. Biol. 21, 439–448 (2011).
20. Littlet, S. C., Tikhonov, M. & Gregor, T. Precise developmental gene expression arises from globally stochastic transcriptional activity. Cell 154, 789–800 (2013).
21. Petkova, M. D., Little, S. C., Liu, F. & Gregor, T. Maternal origins of developmental reproducibility. Curr. Biol. 24, 1283–1288 (2014).
22. Bergsten, S. E. & Gavis, E. R. Role for mRNA localization in translational activation but not spatial restriction of nanos RNA. Development 126, 659–669 (1999).
23. Sinsimer, K. S., Lee, J. J., Thibierge, S. Y. & Gavis, E. R. Germ plasm anchoring is a dynamic state that requires persistent trafficking. Cell Rep. 5, 1169–1177 (2013).
24. Zimyanin, V. L. et al. In vivo imaging of oskar mRNA transport reveals the mechanism of posterior localization. Cell 134, 843–853 (2008).
25. Mickleman, D. R., Adams, J., Grunert, S. & St Johnston, D. Distinct roles of two conserved Staufen domains in oskar mRNA localization and translation. EMBO J. 19, 1366–1377 (2000).
26. Ronco, C., Gavis, E. R. & Lehmann, R. Localization of oskar RNA regulates oskar translation and requires Oskar protein. Development 121, 2737–2746 (1995).
27. Chekulaeva, M., Hentze, M. W. & Ephrussi, A. Bruno acts as a dual repressor of oskar translation, promoting mRNA oligomerization and formation of silencing particles. Cell 124, 521–533 (2006).
28. Besse, F., Lopez de Quinto, S., Marchand, V., Trucco, A. & Ephrussi, A. Drosophila PTB promotes formation of high-order RNP particles and represses oskar translation. Genes Dev. 23, 195–207 (2009).
29. Glotzer, J. B., Saffrich, R., Glotzer, M. & Ephrussi, A. Cytoplasmic flows localize injected oskar RNA in Drosophila oocytes. Curr. Biol. 7, 326–337 (1997).
30. Ephrussi, A. & Lehmann, R. Induction of germ cell formation by oskar. Nature 358, 387–392 (1992).
31. Ghosh, S., Marchand, V., Gaspar, I. & Ephrussi, A. Control of RNP motility and localization by a splicing-dependent structure in oskar RNA. Nat. Struct. Mol. Biol. 19, 441–449 (2012).
32. Gavis, E. R., Curtis, D. & Lehmann, R. Identification of cis-acting sequences that control nascent RNA localization. Dev. Biol. 176, 36–50 (1996).
33. Smith, J. L., Wilson, J. E. & Macdonald, P. M. Overexpression of oskar directs ectopic activation of nanos and presumptive pole cell formation in Drosophila embryos. Cell 70, 849–859 (1992).
34. Koch, A. L. The logarithm in biology. 1. Mechanisms generating the log-normal distribution exactly. J. Theor. Biol. 12, 276–290 (1966).
35. Limpert, E., Stahel, W. A. & Ablat, M. Log-normal distributions across the sciences: keys and clues. Bioscience 51, 341–352 (2001).
36. Brangwynne, C. P. et al. Germline P granules are liquid droplets that localize by controlled dissolution/condensation. Science 324, 1729–1732 (2009).
37. Hachet, O. & Ephrussi, A. Splicing of oskar activation of nanos in the nucleus is coupled to its cytoplasmic localization. Nature 428, 959–963 (2004).
38. Herpers, B., Xanthakis, D. & Rabouille, C. ISH-IEM: a sensitive method to detect endogenous mRNAs at the ultrastructural level. Nat. Protoc. 5, 678–687 (2010).
METHODS

Fly stocks. The following mutants, mutant combinations and transgenic lines were used: yw^1C1 (ref. 39); osk^204 (ref. 40); vasa^4/vasa^2 (refs 41,42); nos^2 (ref. 43); nos-(m2)^2 (ref. 44); osk-(m2)^2 (ref. 45); hsp83-MCP-GFP (ref. 16); gfp-stain (ref. 46); gfp-vas (ref. 47); gfp-nos (ref. 48); osk-bcSIDUT (ref. 30). UASp-nos^{RUT} was created by standard molecular biology methods and introduced into yw^1C1 animals by P element-mediated transformation. The UASp-gfp–osk^{RUT} transgenic line was kindly provided by W. Eagle. Expression was elicited in heterozygous females using the maternal triple Ga4 driver (MTD–Ga4; ref. 50).

Single-molecule FISH. FISH was performed as previously described using oligonucleotides 20 nucleotides in length and complementary to transcripts nos-RNA (CG5637; 75 probes), cycR-RNA (CG5310; 48 probes), pgr-RNA (CG32885; 48 probes) and osk-RNA (CG10901; 99 probes). Sequences are listed in Supplementary Table 1. Custom oligonucleotides with 3’ amine modification were obtained from Biosearch Technologies, conjugated to either Atto 565 (Sigma 72464) or Atto 633 (Sigma 43429) dye, and purified by HPLC as previously described.

Immunostaining. As controls for goodness of single-particle detection, we compared the densities of punctae in early embryos from wild-type females and from females heterozygous or homozygous for the maternal RNA-null nos^{RUT} allele. As expected, heterozygous embryos contain half as many particles as in wild type (0.39 ± 0.01 versus 0.75 ± 0.02 particles μm^{-3}, n = 4 embryos each). In homozygous nos^{RUT} embryos, the number of particles/volume exceeding the ‘difference of Gaussians’ detection threshold was <1% of the number detected in wild type. Similarly for osk, embryos from females homozygous for the mRNA-null allele osk^{RUT} contain half the number of particles of wild-type embryos (0.24 ± 0.03 versus 0.41 ± 0.03 particles μm^{-3} in wild type, n = 4 embryos each). These results are similar to the twofold reduction in particle number observed for maternally supplied hunchback (hb) mRNA in hb deficiency heterozygotes and support previous findings that the FISH method effectively distinguishes true objects from imaging noise.

Immunostaining. For pole cell counting, anti-Vas immunofluorescence was performed on 1–3-h-old embryos as described previously using 1,2,000 rabbit anti-Vas (gift from P. Lasko) and 1,1,000 Alexa Fluor 568 goat anti-rabbit secondary antibodies (Jackson ImmunoResearch). Imags were taken at 2.5 μm intervals throughout the embryo posterior with a Leica TCS SP5 laser-scanning confocal microscope equipped with GaAsP ‘HyD’ detectors in photon-counting mode, with pixels of 76 × 76 nm and z spacing of 340 nm. For each probe set, laser power was adjusted to optimize separation of signal and noise. The same settings were used repeatedly for all samples treated with a given probe set. For each experiment, image stacks were acquired from 3 to 5 different egg chambers/ovaries or embryos at each developmental stage shown. Each stack contained 20,000–100,000 particles, depending on the volume imaged and the size of each egg chamber/ovary/embryo. Laser power exhibited fluctuations of ∼10% between experiments; however, normalization to single mRNA intensity nullifies any effect in our measurements of absolute mRNA amount. For egg chambers/ovaries, z-series represent approximately half the thickness of the tissue starting from near the interface between the follicle cells and the nurse cells/ovocyte. For embryos, image stacks extend from the cortical surface to near the mid-sagittal plane.

As controls for goodness of single-particle detection, we compared the densities of punctae in early embryos from wild-type females and from females heterozygous or homozygous for the maternal RNA-null nos^{RUT} allele. As expected, heterozygous embryos contain half as many particles as in wild type (0.39 ± 0.01 versus 0.75 ± 0.02 particles μm^{-3}, n = 4 embryos each). In homozygous nos^{RUT} embryos, the number of particles/volume exceeding the ‘difference of Gaussians’ detection threshold was <1% of the number detected in wild type. Similarly for osk, embryos from females homozygous for the mRNA-null allele osk^{RUT} contain half the number of particles of wild-type embryos (0.24 ± 0.03 versus 0.41 ± 0.03 particles μm^{-3} in wild type, n = 4 embryos each). These results are similar to the twofold reduction in particle number observed for maternally supplied hunchback (hb) mRNA in hb deficiency heterozygotes and support previous findings that the FISH method effectively distinguishes true objects from imaging noise.

For all FISH results, the fixation, in situ hybridization, and mounting processes lead to a 5% increase in the right or left halves of 4 embryos (0.9 ± 0.03 versus 0.9 ± 0.04 particles per embryo). From these measurements, the average osk particle contains 2.1 ± 1.1 mRNAs. Previous work has estimated that 18% of osk is found at the posterior pole, a substantially larger fraction than for nos. Examining particle intensity and spatial distributions, we estimate that at least 95% of particles are not localized at the posterior pole. As these must account for 82% of osk mRNA, we find that the average unlocalized particle contains (0.82 × 1.9)/0.95 = 1.8 mRNAs. This value is consistent with the estimated frequency of co-localization of 2 or more osk mRNAs (see below and Supplementary Fig. 5d). In these estimates, 40% of particles contain 1 mRNA, 50% contain 2 mRNAs, and 10% 4 mRNAs. This yields a mean mRNA content per particle (0.4 × 1) + (0.5 × 2) + (0.1 × 4) = 1.8. We conclude that FISH is capable of distinguishing individual osk mRNAs.

For all FISH results, the fixation, in situ hybridization, and mounting processes lead to a 5% increase in the right or left halves of 4 embryos (0.9 ± 0.03 versus 0.9 ± 0.04 particles per embryo). From these measurements, the average osk particle contains 2.1 ± 1.1 mRNAs. Previous work has estimated that 18% of osk is found at the posterior pole, a substantially larger fraction than for nos. Examining particle intensity and spatial distributions, we estimate that at least 95% of particles are not localized at the posterior pole. As these must account for 82% of osk mRNA, we find that the average unlocalized particle contains (0.82 × 1.9)/0.95 = 1.8 mRNAs. This value is consistent with the estimated frequency of co-localization of 2 or more osk mRNAs (see below and Supplementary Fig. 5d). In these estimates, 40% of particles contain 1 mRNA, 50% contain 2 mRNAs, and 10% 4 mRNAs. This yields a mean mRNA content per particle (0.4 × 1) + (0.5 × 2) + (0.1 × 4) = 1.8. We conclude that FISH is capable of distinguishing individual osk mRNAs.

Estimation of spurious co-localization. If individual mRNAs are scattered within a volume at random, then two or more mRNAs may occupy the same confocal imaging volume purely by chance. Such spurious co-localization will result in mis-detection of two mRNAs as a single object with an mRNA content of two mRNAs, erroneously seeming to represent a multi-copy particle. The rate of spurious co-localization depends on the density of mRNAs. To determine whether the observed frequency of nos co-localization exceeds the frequency expected for random chance, we used two methods: first, we developed a simple Poisson model of random co-localization; second, we constructed pair-correlation functions.

For the first method, we developed a simple model of random co-localization to predict the frequency of spurious co-localization. The model requires choosing an effective volume occupied by diffraction-limited spots in confocal microscopy, with the assumption that when random co-localization occurs, two or more mRNAs inhabit the same position within an imaging volume and are always mis-detected as 1 particle (and never correctly detected as 2 particles). In reality, our algorithm fails gradually, rather than abruptly, to discriminate between two closely spaced particles as the distance between them decreases. To bias our estimates towards the hypothesis that packaging does indeed occur for unlocalized nos, we intentionally used small effective volumes and chose high mRNA densities at which we fail to distinguish individual mRNAs.

In the first estimate, we assumed that diffusion-limited spots have a volume of (4/3π) × (0.3 × 0.3 × 0.9 μm) = 0.35 μm^3 where, as observed previously with the same antibodies, the radius along the x axis is about fourfold higher than in the yx plane of the image. At a density of 0.8 particles μm^{-3} of 1.3 mRNA per particle = 1.0 mRNA μm^{-3}, unlocalized nos mRNA occupies, on average, 1.0 mRNA μm^{-3} × 0.35 μm^3 per mRNA = 35% of available volume. With this density, the Poisson distribution was used to predict the fraction of imaging volumes containing n = 0, 1, 2, … mRNAs (Supplementary Fig. 1f), assuming that detected particles contain an integer number of mRNAs. Of the total volumes with n = 0 (that is, for all detected particles), the fractions with n = (1, 2, 3) are p = (0.83, 0.15, 0.02), and the fraction of volumes with n ≥ 4 is negligible (Supplementary Fig. 1f inset). This predicts that about 1 – 0.83 = 17% of detected nos punctae represent more than one mRNA that co-localize by random chance. This prediction is consistent with the distribution of normalized intensities of 55,744 particles in a 2D histogram (Fig. 1e,f): the distribution along the correlated axis can be approximated by the product of two of Gaussians centred on means of 1 and 2 mRNAs with standard deviations of 0.28 and √(680/15) = 0.40 mRNAs, because variances of the intensities of two mRNAs are additive. With these fits, the ratio of the area under the Gaussian with mean = 2 to that with mean = 1 mRNA in Fig. 1f is 20% ± 3% or 9.291 ± 1.932 co-localized particles. This is close to the prediction from the Poisson model of p(n=2)/p(n=1) = 0.51, 0.83 = 18%. Moreover, the predicted probabilities closely recapitulate the measured mean particle content: (1 mRNA × p(n=1)) + (2 mRNA × p(n=2)) + (3 mRNA × p(n=3)) = 0.30 ± 0.10 = 0.42 mRNA per particle, similar to the measured value of 1.3 mRNA per particle. This supports the idea that co-localization of two or more unlocalized nos mRNAs arises by random chance alone.
In the above estimate, mis-detection of 2 mRNAs as 1 occurs when the two centroids are separated by ≤0.6 μm in the xy plane. In reality, the algorithm becomes gradually less successful at discriminating two objects as the distance between centroids decreases. Thus, the degree of purely spurious co-localization might be smaller than estimated above. We can make a second estimate that compensates for this effect by using a smaller volume of a diffusion-limited spot of (4/3)π (0.25 × 0.25 × 0.75 μm) = 0.2 μm³. In this case, the mRNA will occupy 20% of the available volume, leading to a predicted spurious co-localization frequency of 10%. Given our estimate from Fig. 1f that the co-localization of unlocalized nos mRNAs is ~20%, the remaining 10% of co-localization would occur for nonrandom reasons, that is, deliberate packaging of two or three nos mRNAs into the same RNP. However, we note that mRNAs are not randomly scattered with equal probability at all locations throughout the embryo interior. Examination of image stacks shows that a large fraction (possibly as high as 50%) of the embryo is devoid of mRNAs, probably because of the presence of organelles, yolk granules or other structures. This results in more dense crowding of mRNAs than expected (Supplementary Fig. 1g). Thus, for nos, random co-localization of individual mRNAs is in the most parsimonious explanation for the fraction of unlocalized punctae that contain only 1 mRNA.

The same reasoning can be used to assess the likelihood that unlocalized osk mRNAs are found in dimeric complexes: the density of unlocalized osk particles is lower than for nos, at 0.4 μm⁻³. Our imaging and analysis algorithm is capable of distinguishing nearly all particles at this density; osk mRNA is present at a density of 0.4 particles μm⁻³ × 1.8 mRNAs per particle = 0.7 mRNA μm⁻³. At this density, mRNA is expected to occupy 25% of the available volume, and so the predicted rate of co-localization of 2 or more mRNAs by random chance is 12%, fivefold lower than the 60% estimated from observations (Supplementary Fig. 5d). As random co-localization markedly fails to account for the fraction of unlocalized punctae containing >1 mRNA, we conclude that unlocalized osk mRNA is deliberately packaged into higher-order RNP complexes containing 2 or more mRNAs.

For the second method, we constructed pair-correlation functions by measuring the density of nos and pge as a function of distance from a set of ‘reference’ nos particles. In determining nos densities, we considered 18% of detected nos particles to represent 2 mRNAs at the same coordinates. We observed no significant change in density of either nos or pge as a function of distance from reference particles (Supplementary Fig. 1j), as expected if co-localization in bulk cytoplasm occurs by random chance alone.

mRNA content of localized granules. Particle fluorescence intensities and offsets were estimated by three methods: 2D elliptical Gaussian fitting; summation of fluorescence in a circular area with radius of 3 pixels (for amplitude) and in a ring with inner radius 6 pixels and outer radius 9 pixels (for offset); and 3D summation as previously described for sites of zygotic gene expression. The fluorescence intensity value for a single mRNA molecule was estimated by two methods: constructing an average image of an unlocalized single-copy particle and either fitting an elliptical Gaussian to obtain an amplitude and offset or summing fluorescence in two dimensions; analysing unlocalized particles within a volume of cytoplasm in the interior of the oocyte or embryo below the posterior cortex. In this region, particle density decreases approaching the posterior pole (Supplementary Fig. 1b), and particle density and fluorescence are roughly linearly related. The slope of a line fitted to a plot of total intensity as a function of particle number yields fluorescence per particle. Normalization of granule intensity to fluorescence per single-copy particle yields absolute mRNA content per granule. In pairwise comparisons, the three analysis methods yielded mRNA counts that differ by a maximum of 20% for granules localized at the posterior cortex. This measurement error was considerably higher (up to 60%) for granules positioned less than 5 μm from the posterior pole.

Time-lapse videos of oocytes expressing nos-GFP or osk-GFP were obtained as previously described from n = 3 (nos-GFP) or n = 4 (osk-GFP) oocytes. Granules were tracked using semi-automated custom software. Average velocities were calculated for 26 (nos) and 22 (osk) granules. Relative fluorescence intensities were obtained by fitting to a plot of total intensity as a function of particle number yields fluorescence per particle. Histograms of granule intensities were fitted to histograms of FISH intensities of a subset of granules found in a region of the posterior cortex comparable to the region visualized by time-lapse imaging. Histograms of granules observed in time-lapse images do not show a clear threshold separating true particles from imaging noise, so the fit was performed to the right side of the FISH distribution under the assumption that dim particles are not visible in time-lapse images. Fitting was performed to minimize chi square error over a range of bin sizes and minimum and maximum intensity values.

39. Lindsay, D. L. & Zimm, G. G. The Genome of Drosophila Melangaster (Academic Press, 1992).
40. Varozi, N. F. & Ephrussi, A. Oskar anchoring restricts pole plasm formation to the posterior of the Drosophila oocyte. Development 129, 3705–3714 (2002).
41. Lehmann, R. & Nusslein-Volhard, C. The maternal gene nanos has a central role in posterior pattern formation of the Drosophila embryo. Development 112, 679–691 (1991).
42. Schübäck, T. & Wieschaus, E. Maternal-effect mutations altering the anterior-posterior pattern of the Drosophila embryo. Roux Arch. Dev. Biol. 195, 302–317 (1986).
43. Wang, C., Dickinson, L. K. & Lehrmann, R. Genetics of nanos localization in Drosophila. Dev. Dyn. 199, 103–115 (1994).
44. Brechbiel, J. L. & Gavis, E. R. Spatial regulation of nanos is required for its function in dendrite morphogenesis. Curr. Biol. 18, 745–750 (2008).
45. Lin, M. D., Fan, S. J., Hsu, W. S. & Chou, T. B. Drosophila decapping protein 1, Dcp1, is a component of the oskar RNP complex and directs its posterior localization in the oocyte. Dev. Cell 10, 601–613 (2006).
46. Martin, S. G., Leclerc, V., Smith-Litière, K. & St Johnston, D. The identification of novel genes required for Drosophila anteroposterior axis formation in a germline clone screen using GFP-Staufen. Development 130, 4201–4215 (2003).
47. Johnstone, D. & Laslo, P. Interaction with eIF5B is essential for Vasa function during development. Development 131, 4167–4178 (2004).
48. Snee, M. J., Harrison, D., Yan, N. & Macdonald, P. M. A late phase of Oskar accumulation is crucial for posterior patterning of the Drosophila embryo, and is blocked by ectopic expression of Bruno. Differentiation 72, 246–255 (2007).
49. Spradling, A. C. in Drosophila: A Practical Approach (ed. Roberts, D. B.) 175–197 (IRL Press, 1986).
50. Petrella, L. N., Smith-Leiker, T. & Cooley, L. The Ovhts polyprotein is cleaved during oogenesis. Development 134, 703–712 (2007).
51. Raj, A., van den Bogaard, P., Rifkin, S. A., van Oudenaarden, A. & Tyagi, S. Imaging individual mRNA molecules using multiple singly labeled probes. Nat. Methods 5, 877–879 (2008).
52. Little, S. C., Tkacik, G., Kneeland, T. B., Wieschaus, E. F. & Gregor, T. The formation of the Bicoid morphogen gradient requires protein movement from anteriorly localized mRNA. PLoS Biol. 9, e1000596 (2011).
53. Duchow, H. K., Brechbiel, J. L., Chatterjee, S. & Gavis, E. R. The nanos translational control element represses translation in somatic cells by a Bearded box-like motif. Dev. Biol. 282, 207–217 (2005).
54. Wang, C. & Lehrmann, R. Nanos is the localized posterior determinant in Drosophila. Cell 66, 637–647 (1991).
55. Ephrussi, A., Dickinson, L. K. & Lehrmann, R. Oskar organizes the germ plasm and directs localization of the posterior determinant nanos. Cell 66, 37–50 (1991).
Corrigendum: Independent and coordinate trafficking of single *Drosophila* germ plasm mRNAs

Shawn C. Little, Kristina S. Sinsimer, Jack J. Lee, Eric F. Wieschaus and Elizabeth R. Gavis

*Nat. Cell Biol.* **17**, 558–568 (2015); published online 6 April 2015; corrected after print 1 February 2016

In the version of this Article originally published, the sentence ‘13% of GFP–Vas granules contain *nos* mRNAs and 11% contain *cycB*’ in the caption of Fig. 4h–j was incorrect; it should have read ‘69% of GFP–Vas granules contain *nos* and 51% contain *cycB*’. This has been corrected in all online versions of the Article.
Unlocalized nos mRNAs are found as individual molecules, whereas localized granules contain many mRNAs. (a) Imaged particles are diffraction-limited objects. Normalized intensities of 50 nm fluorescent beads (black dashed line), unlocalized nos (green), and polar granules (blue) are plotted. Red: fitted Gaussian distribution. (b) nos particle density decreases upon localization. Blue: normalized fluorescence signal density at n.c. 3. Vertical line: border of the localization domain where fluorescence exceeds that found in the anterior. Red: particles/μm³. (c) Histogram of intensities (arbitrary units, log scale) of all particles found in the left half, posterior third of an embryo. Vertical line separates dim and bright peaks. (d) Fraction of objects >164 A.U. at given distance from the posterior pole. Vertical line demarcates the localization domain, where the majority of bright objects are found. (e) Absolute RT-qPCR yields 2.5 ± 0.8*10⁶ nos molecules per embryo. Blue: dilution series of plasmid DNA template. Red: dilution series of in vitro transcribed mRNA. Inset: dilution of single embryos (n = 6 embryos). (f) Poisson model of random co-localization: probability that imaging volumes contain n = (0,1,2,...) mRNAs when particles occupy 0.35 μm³. Inset: probabilities of particles containing n ≥ 1 mRNA. (g) Non-uniform distribution of nos throughout interior of embryo. Pixels lacking signal (red) cover 52% of the image. (h) In two color labeling, rotation of one image stack by 90° yields a 10% co-localization frequency. (i) Poisson model as in (f) with 0.2 μm³ imaging volumes and particles occupying 60% of available volume. (j) Pair-correlation functions showing density of nos (blue) or pgc (red) particles as a function of distance from 594 reference nos particles. Error bars: standard deviations (SD). Mean densities and SD were calculated within bins of 2 pixel width at intervals of 2 pixels. Inset: p-values from paired sample t-test comparing density in first bin to densities at all other distances. Densities are not significantly different (dashed line: p-value of 0.1). (k) Histogram of intensities of cortical objects after normalization to the most commonly observed intensity. Granules have >4 mRNAs. Inset: in dual-color labeling, normalized data fall on a line of slope = 1, indicating 4% measurement error. (l) Cumulative probability distribution of nos mRNA in granules containing >4 nos mRNAs as a function of distance from the posterior pole. Inset: number of localized granules as a function of distance. (m) Histogram of nos mRNA content in oskA87/+ oocytes (stage 10b). (n) Histogram of nos mRNA content in vasPD/vasD1 oocytes (stage 11-12). (o) Mean nos mRNA content of granules with >4 mRNAs as a function of distance from the posterior pole. Upper inset: variance divided by mean (Fano factor). For a process of random arrival of mRNAs at assembly sites, predicted Fano factor is 1 (red line). Lower inset: fractional SD of mRNA content (SD/mean) as a function of distance.
Supplementary Figure 2 Localization results from the formation of granules that maintain nos content during rapid transit. (a) Upper: Normalized nos fluorescence density as a function of position during late oogenesis. Vertical lines indicate position at which density exceeds that found in the oocyte anterior. Lower: Plot showing fraction of all objects containing >4 mRNAs as a function of position. Nearly all bright granules are found within the localization domain. (b-d) Calibrated mRNA content (red) of four selected highly motile nos particles during rapid displacement (blue). Selected frames for each particle from Supplementary Video 2 are shown. Red arrowheads indicate motile particles.
Supplementary Figure 3 Co-localization of nos, cycB, and pgc. (a-d) nos, cycB, and pgc do not co-localize in nurse cells at stage 8. 2D histograms show fluorescence intensities of cycB channel at positions where nos particles are found (a), nos at cycB positions (b), pgc at nos positions (c), and nos at pgc positions (d). Vertical green and horizontal red lines indicate thresholds separating signal from imaging noise. (e) Time course of cycB and nos localization. Scale bars: 5 μm (upper, middle); 10 μm (lower). (f) 2D histograms of nos and cycB content at stages 10a, 12, and 13. cycB begins assembling into large granules slightly earlier than nos, likely because of high levels of cycB expression at earlier times compared to nos. In contrast to the cycB mRNA that accumulates at late stages of oogenesis, this initial population of localized cycB mRNA shows only weak co-localization with nos. Color indicates relative density of data points, with red showing highest density. Red and green lines indicate thresholds separating localized and unlocalized particles. For all particles surpassing the localized threshold in one channel, the fraction surpassing the localization threshold in the second channel is shown in the upper right quadrant (green: cycB, red: nos). The fraction of localized particles containing 0 mRNAs in the second channel is shown in the upper left (for localized nos) or lower right (for localized cycB) quadrants. Very little nos surpasses the localized threshold at early time points.
Supplementary Figure 4 Assembly by random selection does not explain co-localization. (a) Data fit to a model where *nos* and *cycB* mRNAs compete for integration into granules. Plot shows the fraction of localized granules containing 0 *nos* or *cycB* as a function of the sum of *nos* and *cycB* mRNA content. Total mRNA content for each localized granule was determined by summing *nos* and *cycB*. For granules with a given total content, the fraction containing 0 *nos* (blue) or 0 *cycB* (red) was calculated and plotted as a function of the total. Due to measurement uncertainty, granules can possess apparent non-integer mRNA numbers. Therefore, x-axis values were generated in increments of 0.25 mRNA, using bins of total content spanning ±0.5 mRNAs. Lines indicate best fit curves \((1 - p^n)\) where \(n\) is total mRNA content (i.e., fitting observations to binomial distribution). Fitting yields probabilities of \(p = 0.17\) for incorporation of *nos* mRNA and \(p = 0.08\) for incorporation of *cycB* mRNA into a forming granule. Actual total granule content is likely larger, given the enrichment of many genes in the germ plasm. Thus, these probabilities represent upper bounds. (b–e) Probabilities obtained in (a) were used to predict the distribution of *nos* and *cycB* content as a function of total content (b,d). Predictions were compared to measurements (c,e). Heat maps indicate relative densities. Magenta arrows: discrepancy between model and observation, namely, where data points predicted by model are absent in observations despite the presence of many granules completely lacking one of the two mRNAs. Because the actual total content is likely larger, the apparent relationship between observed content and total content (i.e., the slope of a line fitted to the data clouds in c and e) is likely to be lower. However, the change in slope does not account for the absence of data points (arrows) in the observations. Data falling on the line of slope = 1 are those granules that contain only *nos* (c) or only *cycB* (e); in actuality these granules co-localize with other mRNAs (e.g., *pgc*), and therefore have a higher total content than suggested by these plots.
Supplementary Figure 5 Quantification of osk mRNA in localized granules. (a,b) Confocal section taken near the cortical surface (a) or near the mid-sagittal plane (b) of an early (n.c. 3) embryo (anterior left, dorsal up) labeled with osk probes. Green boxed region in inset of (a) shows area displayed in main panel. Middle and right panels show magnified views of red (upper panels) and yellow (lower panel) boxes. Two contrast levels are used to display the extreme brightness of localized granules compared to unlocalized mRNAs. Scale bars: 20 μm (left panels); 2 μm (magnified views). (c) Absolute RT-qPCR for osk using DNA standard (blue) or in vitro transcribed mRNA standard (red) compared to a dilution series of single embryos (inset). (d) In embryos, unlocalized particles display a heavy bias toward 2-4 mRNA, similar to that seen in oocytes. (e-h) Estimates of osk mRNA content in four motile granules. (e’-h’) Selected frames from time-lapse movies of granules analyzed in (e-h). Red arrowheads indicate granule traced and displayed in e-h. In h’, three granules (arrowheads) converge to the same point, resulting in (h) in apparent increased mRNA content at around 30 s (when particles denoted by green and red arrowheads merge) and 50 s (merging of particle denoted by yellow arrowhead). (i) osk mRNAs are often found on track-like structures along with nos in nurse cells during mid-oogenesis. (j) Log normal distribution of localized granule content. (k) osk content of localized granules in an early embryo. (l) Estimated total accumulated osk in the posterior localization domain shows a dramatic increase during late oogenesis. Time points are estimated from ref. 1 and correspond to stages 7, 8, 9, 10b, 12, and 13.
Supplementary Figure 6 Localization of gfp-nos3'UTR mRNA to polar granules does not affect pole cell formation. (a,b) FISH analysis of gfp-nos3'UTR (GN) embryos with nos and gfp probes. Z-series projections of the posterior cortex at n.c. 3 (a) and n.c. 11 (b) show co-localization of GN and nos. Individual channels are shown at right. Scale bars: 10 µm (a); 5 µm (b). (c) Anti-Vas immunofluorescence in wild-type (upper) and GN (lower) embryos. (d) Box plot showing mean (red line), 25th and 75th quartiles (blue boxes), and range (black lines) of Vas-positive pole cell number (WT, n = 29; GN, n = 30).
Supplementary Video Legends

**Supplementary Video 1** Subsection of a confocal stack of an early (n.c. 3) embryo posterior labeled with nos probes. First section is adjacent to the lateral cortex. Stack is shown twice, followed by magnified views of unlocalized and localized regions. To facilitate visualization of unlocalized punctae, the stack is displayed at high contrast such that localized granules appear saturated. However, no saturated pixels are present in raw images, as illustrated with a single cortical image slice displayed at alternating high and low contrast settings. Z-sections are separated by 340 nm.

**Supplementary Video 2** Time lapse movie of germ plasm RNP particles containing nos\(^*\)GFP at the oocyte posterior. Maximum projection of 5 z-sections spanning a total of 6 μm and a total time of 5 min.

Supplementary Table

**Supplemental Table 1** Oligonucleotide sequences used to design probes for this study.
Model of *osk* mRNA transport in the ovarian cyst

Most *osk* particles in nurse cells represent either 1 or 2 mRNAs and the fraction of particles with higher content is negligible (Fig. 5d). The 15 nurse cells (white circles) and oocyte (red) are connected to each other in a stereotypic manner through ring canals (blue). One nurse cell is connected exclusively to the oocyte:

![Diagram](image)

Expressions describing the change over time in the concentration of 1-copy and 2-copy *osk* particles (hereafter “monomers” and “dimers”) in this cell can be written under the assumptions that 1) *osk* entering the oocyte cannot return to the nurse cell and 2) transport to the oocyte is the only means by which *osk* is lost from nurse cells (i.e. degradation of *osk* in nurse cells is small compared to transport):

\[
\frac{dM_1}{dt} = P + 2D_1k_{off} - 2k_{on}M_1^2 - t_MM_1
\]

\[
\frac{dD_1}{dt} = k_{on}M_1^2 - k_{off}D_1 - t_DD_1
\]

where *M*₁ and *D*₁ represent concentrations of monomers and dimers, *k*₁ is the proportionality constant describing the rate at which dimers are created from monomers in a second-order reaction, *k*₂ is the rate constant for the reaction in which monomers are created from the decay of dimers in a first-order reaction, *t*_M and *t*_D are rates at which monomer and dimers are transported into the oocyte, and *P* is the rate of *osk* production via transcription in the nurse cell.
For this simple system at steady state, the production rate from transcription equals the rate at which osk is lost to the oocyte: \( P = t_M M_1 + 2t_D D_1 \).

A related set of expressions can be written for the two nurse cells that are connected to each other and one of which is connected to the oocyte, using the assumption that \( P, t_M \) and \( t_D \) are the same between all cells:

\[
\frac{dM_2}{dt} = P + 2D_2 k_{off} - 2k_{on}M_2^2 - t_MM_2 + t_MM_3
\]

\[
\frac{dD_2}{dt} = k_{on}M_2^2 - k_{off}D_2 - t-DD_2 + t_DD_3
\]

\[
\frac{dM_3}{dt} = P + 2D_3 k_{off} - 2k_{on}M_3^2 - 2t_MM_3 + t_MM_2
\]

\[
\frac{dD_3}{dt} = k_{on}M_3^2 - k_{off}D_3 - 2t_DD_3 + t_DD_2
\]

Here, \( M_2 \) and \( D_2 \) represent monomer and dimer concentrations in nurse cell 2 that is connected only to nurse cell 3, and \( M_3 \) and \( D_3 \) are concentrations in nurse cell 3 connected to both nurse cell 2 and the oocyte. At steady state, production from both cells equals the rate at which osk is lost from cell 3 to the oocyte, thus \( P = \frac{t_M}{2} M_3 + t_D D_3 \).
A set of 8 expressions describes the next most complicated set of cells:

\[
\begin{align*}
\frac{dM_4}{dt} &= P + 2D_4 k_{off} - 2k_{on} M_4^2 - t_M M_4 + t_M M_7 \\
\frac{dM_5}{dt} &= k_{on} M_4^2 - k_{off} D_4 - t_D D_4 + t_D D_7 \\
\frac{dM_6}{dt} &= P + 2D_5 k_{off} - 2k_{on} M_5^2 - t_M M_5 + t_M M_6 \\
\frac{dM_7}{dt} &= k_{on} M_5^2 - k_{off} D_5 - t_D D_5 + t_D D_6 \\
\frac{dM_8}{dt} &= P + 2D_6 k_{off} - 2k_{on} M_6^2 - 2t_M M_6 + t_M M_5 + t_M M_7 \\
\frac{dM_9}{dt} &= k_{on} M_6^2 - k_{off} D_6 - 2t_D D_6 + t_D D_5 + t_D D_7 \\
\frac{dM_{10}}{dt} &= P + 2D_7 k_{off} - 2k_{on} M_7^2 - 3t_M M_7 + t_M M_4 + t_M M_6 \\
\frac{dM_{11}}{dt} &= k_{on} M_7^2 - k_{off} D_7 - 3t_D D_7 + t_D D_4 + t_D D_6
\end{align*}
\]

At steady state, \( P = \frac{t_M}{4} M_7 + \frac{t_D}{2} D_7 \). Similarly, for the set of 8 interconnected cells, \( P = \frac{t_M}{8} M_{15} + \frac{t_D}{4} D_{15} \), where \( M_{15} \) and \( D_{15} \) are monomer and dimer concentrations in the nurse cell connected to the oocyte in the 8 cell cluster.
With several expressions for $P$, it becomes straightforward to assess the ratio of $t_M$ to $t_D$ using our measurements of monomer and dimer concentrations. For example, using cells 1 and 3 we can write

$$t_M + 2t_D = \frac{t_M}{2} M_3 + t_D D_3$$

which rearranges to

$$\frac{t_M}{t_D} = \frac{2D_3 - 2D_1}{2M_1 - M_3}$$

Five additional expressions can also be written to describe the ratio of $t_M$ to $t_D$:

$$\frac{t_M}{t_D} = \frac{2D_7 - 8D_1}{4M_1 - M_7}$$

$$\frac{t_M}{t_D} = \frac{2D_{15} - 16D_1}{8M_1 - M_{15}}$$

$$\frac{t_M}{t_D} = \frac{2D_7 - 4D_3}{2M_3 - M_7}$$

$$\frac{t_M}{t_D} = \frac{2D_{15} - 8D_3}{4M_3 - M_{15}}$$

$$\frac{t_M}{t_D} = \frac{2D_{15} - 4D_7}{2M_7 - M_{15}}$$

The thickness of the egg chamber does not permit visualization of every nurse cell and precludes us from testing all possible combinations. However, fortuitous orientation of egg chambers allows us to locate nurse cells that are clearly connected to only the oocyte or to >4 other nurse cells (i.e., we can identify cells 1 and 15). The concentrations of monomers and dimers in these cells, and the ratio $t_M/t_D$, are as follows during stages 8, 9 and 10b:
| Stage | $M_1$   | $M_{15}$ | $D_1$   | $D_{15}$ | $t_M/t_D$ |
|-------|---------|----------|---------|----------|-----------|
| 8     | 0.34 / µm3 | 0.58 / µm3 | 0.009 / µm3 | 0.11 / µm3 | 0.04      |
| 9     | 0.33 / µm3 | 0.53 / µm3 | 0.01 / µm3  | 0.09 / µm3  | 0.01      |
| 10b   | 0.32 / µm3 | 0.57 / µm3 | 0.01 / µm3  | 0.10 / µm3  | 0.01      |

The transport rate of monomers is therefore 25 to 100 times slower than dimers.

Note that the preceding assumes that each independent “system” of 1, 2, 4, or 8 interconnected nurse cells achieves its own unique steady state. This seems reasonable, given the similarity observed over time in $M_1$, $M_{15}$, $D_1$ and $D_{15}$, and given that the processes of transcription, dimer formation/dissociation, and transport are very rapid compared to the time needed to complete a given stage of oogenesis. The model also relies on the assumption that osk transcription is the same in all nurse cells. It is not known whether all nurse cells produce osk at the same rate during the times at which these measurements were made. Between stage 4 and 6 of oogenesis, posterior nurse cell ploidy is higher than that of anterior nurse cells, suggesting that expression of osk might be greater in the posterior than in anterior nurse cells. However, contrary to this prediction, we observe that the concentration of osk in posterior nurse cells is noticeably lower than in anterior cells even during these stages (Fig. 6b). At stage 6 and later, ploidy is equal. It is therefore reasonable that the production rates of osk via transcription are similar between all nurse cells.