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Lighting up mRNA localization in *Drosophila* oogenesis

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The asymmetric localization of four maternal mRNAs – *gurken*, *bicoid*, *oskar* and *nanos* – in the *Drosophila* oocyte is essential for the development of the embryonic body axes. Fluorescent imaging methods are now being used to visualize these mRNAs in living tissue, allowing dynamic analysis of their behaviors throughout the process of localization. This review summarizes recent findings from such studies that provide new insight into the elaborate cellular mechanisms that are used to transport mRNAs to different regions of the oocyte and to maintain their localized distributions during oogenesis.

Introduction

mRNA localization has emerged as an important mechanism for generating asymmetric protein distributions that promote morphological and functional cell polarization during development. A recent in situ hybridization screen uncovered over 1500 transcripts with distinct subcellular localizations in early *Drosophila* embryos, hinting at diverse roles for mRNA localization in regulating cell physiology (Lécuyer et al., 2007). In contrast to direct protein targeting, mRNA localization provides an effective way in which to create high local protein concentrations, as each transcript can be translated many times. The coupling of translational activity to mRNA localization prevents premature or ectopic protein synthesis that might be deleterious to the cell. In addition, an existing pool of localized mRNA allows for precise temporal control of local protein synthesis in response to external stimuli.

The first localized mRNAs were discovered in ascidian eggs over 20 years ago (Jeffery et al., 1983), and localized transcripts have since been found in many other cell types, including fibroblasts and neurons, as well as in plant cells and fungi (see Box 1). mRNA localization is particularly important during early development, when many organisms rely on proteins synthesized from maternal mRNAs to guide development until the onset of zygotic transcription. As these mRNAs are present in the egg at fertilization, when and where their protein products are expressed must be regulated post-transcriptionally. Accordingly, numerous maternal mRNAs have been shown to be asymmetrically localized in oocytes and eggs from a broad spectrum of organisms, including echinoderms, ascidians, insects, amphibians and fish (Kloc and Etkin, 2005; Palacios and St Johnston, 2001). These mRNAs direct the local synthesis of proteins essential for embryonic germ layer specification, axis formation and germline determination.

Although ascidian and *Xenopus* eggs provided the first model systems for studying localized transcripts, the *Drosophila* oocyte has become a major workhorse for both functional and mechanistic analysis of mRNA localization owing to the availability of powerful genetic and transgenic tools. Intensive analysis of four mRNAs, *gurken* (*grk*), *bicoid* (*bcd*), *oskar* (*osk*) and *nanos* (*nos*), whose localizations to different regions of the oocyte are essential to specify the anteroposterior (AP) and dorsoventral (DV) body axes of the *Drosophila* embryo, has provided insight into the role of mRNA localization in setting up developmental asymmetries, as well as into the mechanisms that mediate mRNA localization.

Localization is a multistep process that requires recognition of cis-acting sequences – localization signals – within mRNAs by localization factors, packaging of these RNA-protein complexes (designated hereafter as RNPs) into transport particles, trafficking of these particles within the cytoplasm and, finally, anchoring of mRNAs at their target destination. RNP assembly may begin in the nucleus, with subsequent remodeling occurring in the cytoplasm to recruit additional factors that confer specificity to the transport process. In many cases, mechanisms that maintain translational silencing during transport particle formation and transit are superimposed. We refer the reader to recent reviews for detailed coverage of these molecular events (Gavis et al., 2007; Kugler and Lasko, 2009; Lewis and Mowry, 2007).

Much of the framework for modeling mRNA localization pathways derives from invaluable biochemical, genetic and histological studies. However, these approaches provide only static snapshots of a continuous event. In this review, we discuss how the application of powerful new imaging technologies to the analysis of *grk*, *bcd*, *osk* and *nos* mRNAs in *Drosophila* oocytes has provided a window into the highly dynamic features of mRNA trafficking by visualizing the movements of RNP particles and the paths that they follow. We summarize recent findings that reveal unanticipated complexities in transport and anchoring mechanisms and in their cellular requirements.

Role of mRNA localization in the establishment of embryonic body axes

Axial polarity of the *Drosophila* embryo arises from asymmetries set up during oogenesis by a series of mRNA localization events (Fig. 1; see Box 2 for an overview of *Drosophila* oogenesis). Establishment of both the AP and DV axes depends on signaling from the oocyte to subsets of follicle cells via Grk, a transforming growth factor α-like ligand (González-Reyes et al., 1995; Neuman-Silberberg and Schüpbach, 1993). To achieve this, *grk* mRNA is localized first to the posterior of the oocyte, where Grk signaling to the overlying follicle cells triggers a reorganization of the oocyte cytoskeleton. This enables the subsequent localization of *bcd* and *osk* mRNAs to the anterior and posterior poles of the oocyte, respectively, and the dorsal anterior localization of *grk* itself (González-Reyes et al., 1995). There, Grk signaling initiates a cascade of events that will ultimately generate the embryonic DV axis.

Osk protein, translated upon posterior localization of *osk* mRNA, initiates the assembly of the germ plasm, a specialized cytoplasm that is maintained at the posterior pole into embryogenesis and contains determinants, including *nos* mRNA, that are necessary for germline and abdominal development (Lehmann and Nusslein-Volhard, 1986). *nos* mRNA accumulates at the posterior of the oocyte only at late stages of oogenesis, and this accumulation is dependent on Osk and germ plasm assembly (Wang et al., 1994). Although *nos* is translated upon its posterior localization, Nos protein has no known function in

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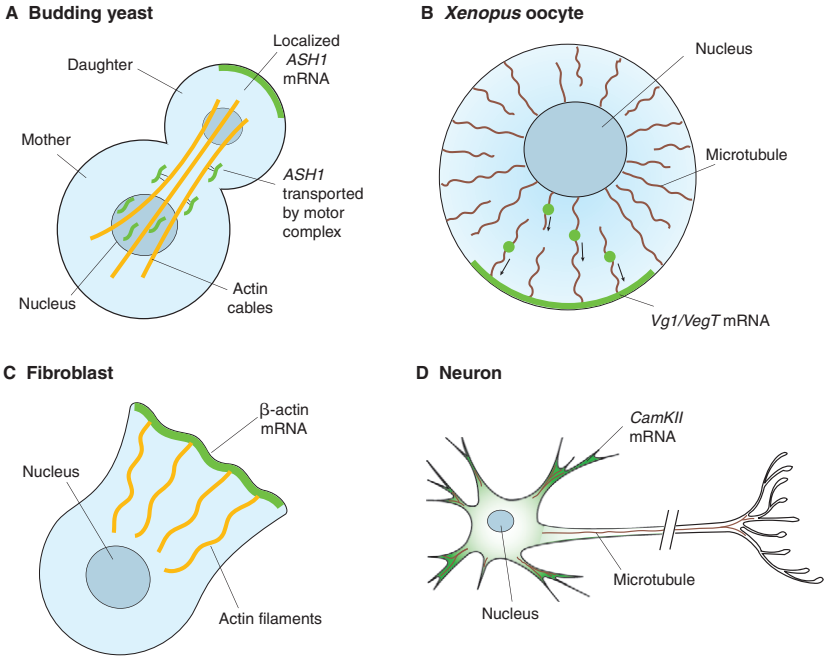
Box 1. Key examples of localized mRNAs



In budding yeast, localization of *ASH1* mRNA to the bud tip by myosin-mediated transport on actin cables (see A in figure) targets Ash1p to the daughter cell, where it is required to repress mating type switching (reviewed by Gonsalvez et al., 2005). Thus, mating type switching occurs only in the mother cell, thereby ensuring that both yeast mating types are present in the population. Germ layer specification in *Xenopus* embryos relies on localized mRNAs, including *Vg1* and *VegT* (reviewed by King et al., 2005) (see B in figure), which are transported to the vegetal pole of the oocyte by kinesin motors and anchored to the cortex by an actin-dependent mechanism. After fertilization, *Vg1* and *VegT* RNAs are inherited by the embryonic vegetal cells, where Vg1 protein, a TGFβ homolog, participates in mesoderm specification, while VegT, a transcription factor, regulates endoderm specification and mesoderm induction. mRNA localization plays an important role in the polarization of somatic cells, such as fibroblasts and neurons (see C,D in figure). Localization of β-actin mRNA to the leading edge of migrating fibroblasts provides a high local concentration of actin monomers that drives assembly of the actin filaments needed for forward movement. Similarly, β-actin mRNA localization to growth cones in developing axons promotes the motility required for axon guidance (reviewed by Condeelis and Singer, 2005). Dendritic localization of RNAs like calcium/calmodulin-dependent protein kinase IIα (CaMKIIα) mRNA in hippocampal neurons facilitates a rapid response to synaptic activity in the form of local protein translation and contributes to learning and memory-related synaptic plasticity (reviewed by Martin et al., 2000).

the oocyte (Forrest et al., 2004). Similarly, Bcd, whose synthesis is repressed during oogenesis, functions only after fertilization (Driever and Nüsslein-Volhard, 1988). Localization of these mRNAs during oogenesis, however, endows the newly fertilized embryo with sources for the production of opposing protein gradients that establish the AP body axis. Bcd, acting both as a transcriptional activator and a translational repressor, determines the head and thoracic regions, while Nos, a translational repressor, determines the abdomen (reviewed by Thompson et al., 2007). In addition, localized *nos* mRNA is incorporated into the germ cells as they form at the posterior of the embryo, supplying the Nos protein that is necessary for germline development (Gavis et al., 2008).

Genetic manipulations that disrupt localization or cause the mislocalization of *grk*, *bcd*, *osk* or *nos* during oogenesis provide compelling evidence that the specific subcellular distributions of these mRNAs are essential for their function in axial and germline development. For example, mutations that abolish *osk* or *nos* localization produce embryos that lack an abdomen (Ephrussi et al., 1991; Wang et al., 1994). These embryos also exhibit the respective defects in germ cell formation and function that are characteristic of *osk* and *nos* mutants (Ephrussi and Lehmann, 1991; Gavis et al., 2008). Conversely, the targeting of *osk* or *nos* to both poles of the oocyte results in embryos in which the anterior structures are replaced by mirror image duplications of posterior structures (Ephrussi and Lehmann, 1992; Gavis and Lehmann, 1992). Similarly, failure to localize *grk* mRNA leads to DV polarity defects (Neuman-Silberberg and Schüpbach, 1993). The ability to test the significance of mRNA localization to developmental gene function has been a major contribution of the *Drosophila* system.

Live fluorescent visualization methods

In situ hybridization experiments have elucidated mRNA localization patterns and, combined with genetic, cell biological and biochemical manipulations, have been used to demonstrate that the mRNA localization process requires cis-acting signals, trans-acting factors, and cytoskeletal components. However, in situ hybridization can only detect the steady state accumulation of mRNAs. Thus, to understand how mRNAs travel to their destinations and to investigate the roles played by localization factors and cytoskeletal elements in executing this process, we need to be able to visualize mRNA localization as it occurs in real time. A further challenge in the analysis of *Drosophila* oocyte mRNAs arises from the increasing impenetrability of oocytes to molecular probes as the vitelline membrane and egg shell are laid

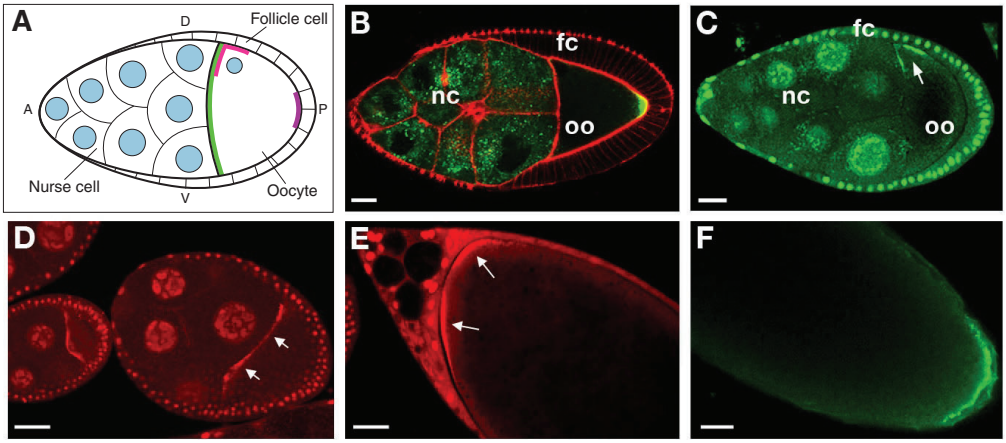


Fig. 1. Localized distributions of *grk*, *bcd*, *osk* and *nos* mRNAs. (**A**) Schematic showing *grk* (pink), *bcd* (green) and *osk* (purple) mRNA localization in mid-oogenesis (stage 9). *nos* mRNA is not yet localized at this stage. The anteroposterior (AP) and dorsoventral (DV) axes are indicated. (**B**) GFP-Stau (green), as proxy for *osk* mRNA, at the posterior pole of the oocyte (oo) during mid-oogenesis. GFP-Stau is also detected in the nurse cell (nc) cytoplasm. The actin cytoskeleton is highlighted in red with phalloidin. fc, follicle cells. Orientation is the same as in A. (**C-F**) Visualization of endogenous mRNAs using the MS2 system: (C) *grk* and (D) *bcd* during mid-oogenesis; (E) *bcd* and (F) *nos* in late oocytes. Owing to the promoter used, the MCP-GFP and MCP-RFP fusion proteins are expressed in both the nurse cells and follicle cells, whereas the MS2-tagged mRNAs are produced only in the nurse cells. MCP-GFP/RFP that is not bound to mRNA enters both the nurse cell and follicle cell nuclei. Scale bars: 20 μm. Image in B was modified, with permission, from Huynh et al. (Huynh et al., 2004); image in C was modified, with permission, from Jaramillo et al. (Jaramillo et al., 2008); images in D and E are reproduced, with permission, from Weil et al. (Weil et al., 2006). Image in F is courtesy of K. Sinsimer (Princeton University, Princeton, NJ, USA). *bcd*, *bicoid*; *grk*, *gurken*; GFP, green fluorescent protein; MCP, MS2 coat protein; *nos*, *nanos*; *osk*, *oskar*; RFP, red fluorescent protein.

down. The difficulty of detecting localization patterns at later stages of oogenesis by in situ hybridization left events that occur during this sizeable temporal window largely unexplored. The development of live-imaging methods using fluorescently tagged RNAs and proteins has recently overcome these limitations to the analysis of mRNA localization dynamics. At lower resolution, time-lapse imaging has provided integrated spatiotemporal information about RNA distributions during the course of oogenesis. Imaging at higher spatial and temporal resolution has permitted detailed characterization of the kinetics and paths of individual RNA particles. The application of techniques like fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) has further facilitated a dynamic analysis of localization events. These methods assess the mobility of fluorescent molecules, by monitoring either the restoration of fluorescence to a photobleached area of a cell caused by the movement of fluorescently labeled molecules from surrounding unbleached regions (FRAP) or the depletion of fluorescently labeled molecules from an unbleached region after repetitive photobleaching of an adjacent area (FLIP).

Visualization of RNA localization in living cells was first accomplished in oligodendrocytes by injection of *in vitro* transcribed myelin basic protein RNA labeled with fluorescein (Aigner et al., 1993). Similarly, injection of fluorescently labeled transcripts has been a successful strategy for investigating the localization of *grk* and *bcd* RNAs in *Drosophila* oocytes, with the relatively large size of the egg chamber making it possible to perform injections into both the nurse cells and oocyte (Cha et al., 2001; Clark et al., 2007; Delanoue et al., 2007; MacDougall et al., 2003; Mische et al., 2007) (Fig. 2A). The ability to control the site and time at which the fluorescent RNA is introduced enables the investigator to perform pulse-chase type experiments, making this method particularly advantageous for the dissection of multi-step RNA localization pathways. Although caution must be taken to discern artifacts that result from damage to the egg chamber during injection, a more difficult issue to address is whether the behavior of RNA injected directly into the cytoplasm recapitulates the behavior of native mRNA that has first been processed in the nucleus. Additionally, the benefit of being able to introduce large quantities of RNA that facilitate detection must be weighed against the possibility that the introduction of nonphysiological amounts of exogenous RNA could result in its aggregation or could saturate the localization machinery.

The recent development of genetically encoded fluorescent tagging methods has enabled the direct visualization of endogenous transcripts (Fig. 2B). Pioneered in yeast for *ASH1* mRNA (Bertrand et al., 1998), this approach involves tethering a fluorescent protein, such as green or red fluorescent protein (GFP or RFP) to mRNA. This is usually accomplished by fusing GFP (or another fluorescent protein) to the bacteriophage MS2 coat protein (MCP) and, in parallel, inserting multiple stem-loop binding sites for the coat protein into the transcript of interest (Fig. 2B). When the MCP-GFP fusion protein and MS2-tagged RNA are co-expressed, binding of the MCP domain to its cognate stem-loops generates fluorescently tagged mRNA in vivo. The system was adapted for transgenic use in *Drosophila* to investigate *nos* mRNA localization in late oocytes (Forrest and Gavis, 2003), and has been subsequently applied to *grk, bcd* and *osk* (Jaramillo et al., 2008; Weil et al., 2006; Zimyanin et al., 2008) (Fig. 1C-F), as well as to several neuronal mRNAs (Ashraf et al., 2006; Brechbiel and Gavis, 2008; Estes et al., 2008). A major strength of this system is the production of fluorescent transcripts, at or near physiological levels, that have been subject to endogenous processing steps. The method has been particularly advantageous in the *Drosophila* ovary, where localization pathways are often active over many hours and at developmental stages that are not readily penetrated by other detection methods. Moreover, the analysis of steady-state localization afforded by this method provides a complement to the investigation of transient events following RNA

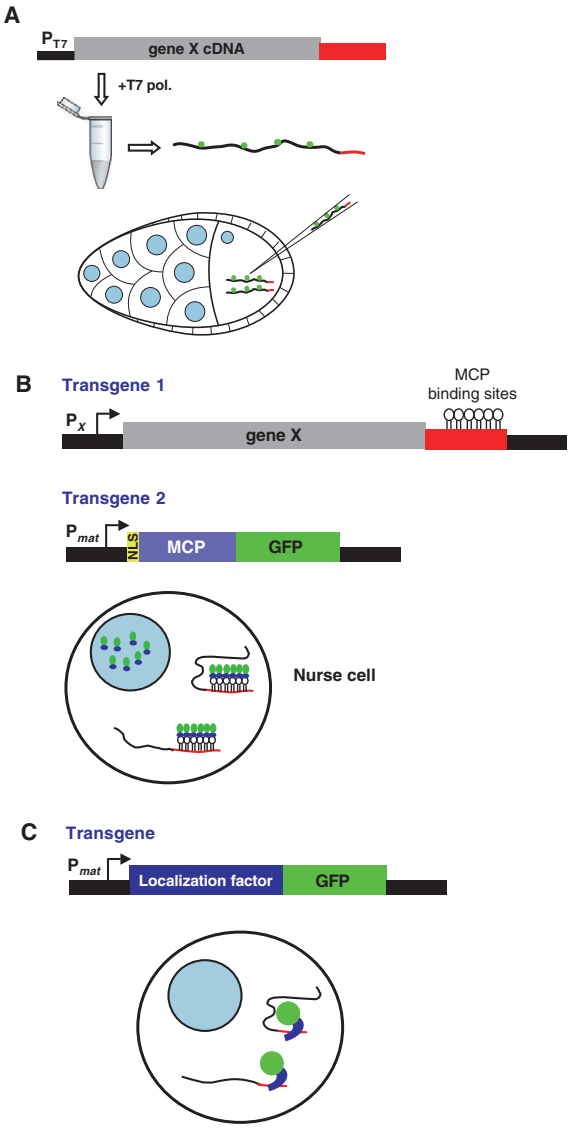


Fig. 2. Fluorescent labeling methods. (**A**) A construct designed for in vitro transcription of the gene of interest from a bacteriophage promoter (T7 in this example), with the coding region shown in gray and the 3′UTR containing the RNA localization signal in red. Transcription of this construct by T7 polymerase in the presence of a fluorophore-coupled nucleotide produces fluorescently labeled RNA for injection into cultured egg chambers. RNA can be injected directly into the oocyte as illustrated or into the nurse cells. (**B**) In vivo labeling of endogenous mRNA by the MS2 system. This strategy requires two components: a transgene (transgene 1) that encodes the target RNA with an insertion of tandem copies of the stem-loop binding site for the bacteriophage MS2 coat protein (MCP), shown here in the 3′ UTR, usually under the control of its own promoter (P_x); and a transgene (transgene 2) that encodes a fluorescent protein fused to MCP (GFP is shown here) under the control of a maternally active promoter (P_{mat}). Transgenic fly lines for each component are crossed together to generate females that express both the tagged RNA and the MCP-GFP protein in their ovarian nurse cells. When the two transgenes are thus coexpressed, the binding of MCP to its recognition motif labels the RNA with GFP. The nuclear localization signal (NLS) in the MCP-GFP fusion protein retains excess unbound protein in the nucleus, reducing cytoplasmic background. Fluorescently labeled mRNA enters the oocyte from the nurse cells (not shown). (**C**) Transgenic expression of GFP-tagged localization factors. A transgene encoding a localization factor fused to a fluorescent protein (e.g. GFP) under the control of its own or a maternally active promoter. Expression of the transgene in the nurse cells of transgenic females will result in production of the fusion protein in the nurse cells. Colocalization of the fusion protein with the target RNA could occur in the nurse cells or oocyte (not shown), through direct RNA-protein interaction (as shown) or through their co-assembly into a larger RNP.

injection and has been essential for the dissection of anchoring mechanisms. Of potential concern with this approach, however, is the possibility that the inserted MS2 stem-loops will destabilize a transcript or impair its ability to interact with localization factors. Moreover, binding of the transcript by multiple copies of MCP-GFP could compromise transport particle assembly or function. Comparison of RNA distributions achieved using this method with those detected by in situ hybridization, together with genetic rescue experiments, is therefore essential to confirm that the tagged transcripts faithfully reproduce the behavior of the native mRNA.

In an alternative approach to the direct detection of mRNA in vivo, GFP-tagged localization factors have been used as proxies for their target mRNAs (Fig. 2C). Because it relies on the identification of appropriate factors and a prior knowledge of coordinated mRNA-protein transport, this method is less widely applicable. In addition, a single localization factor might provide only a partial readout of an mRNA's travels if its association with the RNP is limited to particular steps in the localization pathway. Moreover, as individual localization factors are increasingly implicated in the transport of multiple different mRNAs, sometimes to different locations, discriminating which of the fluorescent RNPs contain the mRNA of interest could be difficult. Nonetheless, the need for only a single transgene and the ease of detecting the tagged protein have made two such protein fusions, GFP-Exu and GFP-Stau, choice tools for monitoring the transport of *bcd* and *osk* mRNAs (Mische et al., 2007; Theurkauf and Hazelrigg, 1998; Zimyanin et al., 2008) (Fig. 1B). Genetic requirements for Exuperentia (Exu) and Staufen (Stau) function in *bcd* and *osk* mRNA localization, together with colocalization studies, have implicated Exu and Stau as components of *bcd* and *osk* RNPs (reviewed by Kugler and Lasko, 2009; St Johnston, 2005). However, whether Exu is specific for *bcd* or can also contribute to *osk* RNPs is not yet clear. Because Stau functions in *osk* localization during midoogenesis and in *bcd* localization only at late stages of oogenesis, GFP-Stau can serve as an independent proxy for each mRNA in the oocyte.

RNA localization dynamics in *Drosophila* oogenesis

Recent analyses of RNA localization dynamics during *Drosophila* oogenesis, made possible by new imaging technologies, have led to revised models for the localization of four key mRNAs. Below, we discuss findings from these studies that have provided new insights into the mechanisms that mediate the transport of these mRNAs from the ovarian nurse cells into the oocyte, their localization within the oocyte, and their anchoring at targeted regions of the oocyte cortex.

Transport from nurse cells to oocyte

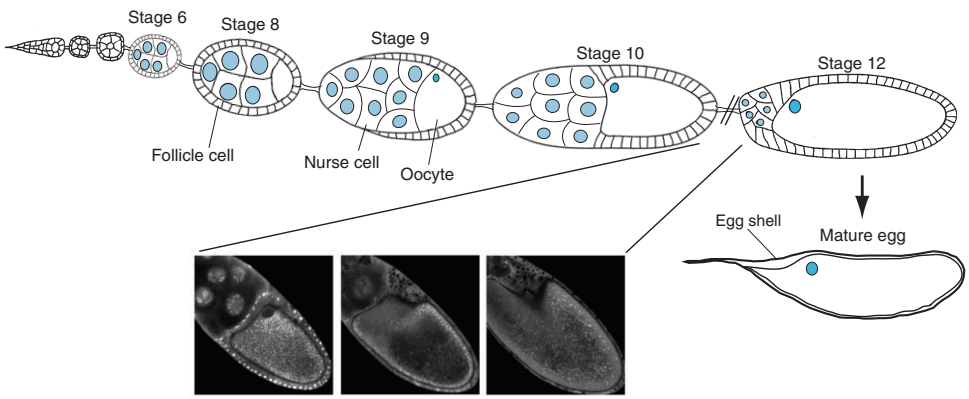
Maternal mRNAs, proteins and even organelles produced in the ovarian nurse cells must be transferred into the oocyte for the production of the egg and early embryo. Until stage 10 of oogenesis,

when the nurse cells contract and extrude or 'dump' their contents into the oocyte, the transport of cargoes from the nurse cells to the oocyte is continuous and selective. This intercellular transport occurs via the ring canals that connect the nurse cells to the oocyte and to each other (Box 2). During the first half of oogenesis, microtubules that extend from a microtubule-organizing center (MTOC) at the posterior of the oocyte through the ring canals into the nurse cells could provide a direct route to the oocyte (Theurkauf et al., 1992). However, this microtubule 'highway' is decommissioned at stage 7, when Grk-dependent signaling events between the oocyte and overlying follicle cells trigger the disassembly of the posterior MTOC and the reorganization of the microtubule cytoskeleton. Live-imaging studies of *bcd* and *grk,* using injected fluorescent transcripts or GFP-Exu as a localization factor proxy for *bcd* mRNA, have shed light on the mechanism of nurse cell-to-oocyte transport at mid-stages of oogenesis, as well as on its cytoskeletal basis (Cha et al., 2001; Clark et al., 2007; Mische et al., 2007; Theurkauf and Hazelrigg, 1998).

In the nurse cells, injected *bcd* and *grk* transcripts and GFP-Exu each form particles that move rapidly and on linear tracks toward ring canals at the nurse cell-oocyte boundary, where they accumulate (Cha et al., 2001; Clark et al., 2007; Mische et al., 2007; Theurkauf and Hazelrigg, 1998) (see Movie 2 in the supplementary material; Fig. 3A). During stages 8-9, microtubules appear to be concentrated at these ring canals in arrays that project into the nurse cell cytoplasm, suggesting a possible path for mRNA transport. Indeed, treatment of oocytes with microtubule-depolymerizing drugs prevents the directional movement of GFP-Exu, and of *bcd* and *grk* RNA. To test whether the observed motility reflects active transport, Mische et al. and Clark et al. compromised dynein function genetically (Mische et al., 2007; Clark et al., 2007). The resulting decrease in motility of both GFP-Exu and *grk* RNA particles in nurse cells indicates that oocyte-destined mRNAs share a common dynein-dependent transport pathway for translocation to the ring canals. How RNA particles encounter the ring canal-associated microtubules is not yet clear. In addition to directed movement, GFP-Exu particles exhibit randomly oriented microtubule-dependent movement throughout the nurse cell cytoplasm (Mische et al., 2007; Theurkauf and Hazelrigg, 1998). These particles could therefore encounter ring canal-associated microtubules through a microtubule-dependent random walk. By contrast, particles of injected *grk* RNA distant from the ring canals appear to follow cytoplasmic flows (Clark et al., 2007). Random movements caused by these flows might facilitate chance encounters with ring canal-associated microtubules.

GFP-Exu and *grk* particles that accumulate at the ring canal subsequently pass through into the oocyte. Movement through the ring canals is slower than movement towards them, however, suggesting that transport to and through the ring canals may occur by two distinct mechanisms. Intriguingly, the size and shape of GFP-Exu particles change as they enter the oocyte (Mische et al., 2007). This might reflect a remodeling of the RNP to exchange localization factors required in the nurse cells for oocyte-specific factors or to sort co-transported mRNAs that are destined for different locations within the oocyte. The formation of a *bcd*-Exu RNP in the nurse cells is not required for transport of injected *bcd* RNA to the oocyte, but is essential for the anterior localization of *bcd* in the oocyte (see below) (Cha et al., 2001). Thus, it is an intriguing possibility that Exu coordinates a remodeling event that triggers the recognition of *bcd*-containing particles by the oocyte transport machinery.

Box 2. *Drosophila* oogenesis



The *Drosophila* ovary is composed of 14-16 ovarioles, each of which contains a series of developing egg chambers (reviewed by Spradling, 1993) (see figure). Each egg chamber is composed of an oocyte and its 15 sister nurse cells, which comprise a 16-cell cyst, surrounded by a somatic follicular epithelium. The germ cell cyst arises from a stem cell-derived cystoblast that undergoes four synchronous divisions. Incomplete cytokinesis during these divisions leaves the 16 cells interconnected by actin-rich cytoplasmic bridges referred to as ring canals. One of these cells differentiates as the oocyte and enters meiosis, while the remainder become polyploid nurse cells.

The egg chamber develops through 14 morphologically distinct stages to give rise to a mature oocyte. Throughout oogenesis, the nurse cells produce maternal RNAs, proteins and organelles that are delivered to the developing oocyte via the ring canals. This supply of maternal factors by the nurse cells is critical to development of the oocyte and the future embryo as the oocyte nucleus is largely transcriptionally quiescent. At the end of stage 10, when the nurse cell cluster and the oocyte are similar in volume, the nurse cells contract to extrude or 'dump' their contents into the oocyte and are subsequently eliminated by apoptosis (see inset in figure). Vigorous streaming of the oocyte cytoplasm (ooplasmic streaming), which accompanies nurse cell dumping, mixes the incoming nurse cell cytoplasm with the ooplasm (see Movie 1 in the supplementary material). The follicle cells, which migrate to enclose the oocyte, secrete both the vitelline membrane and the chorion or egg shell to protect the maturing egg.

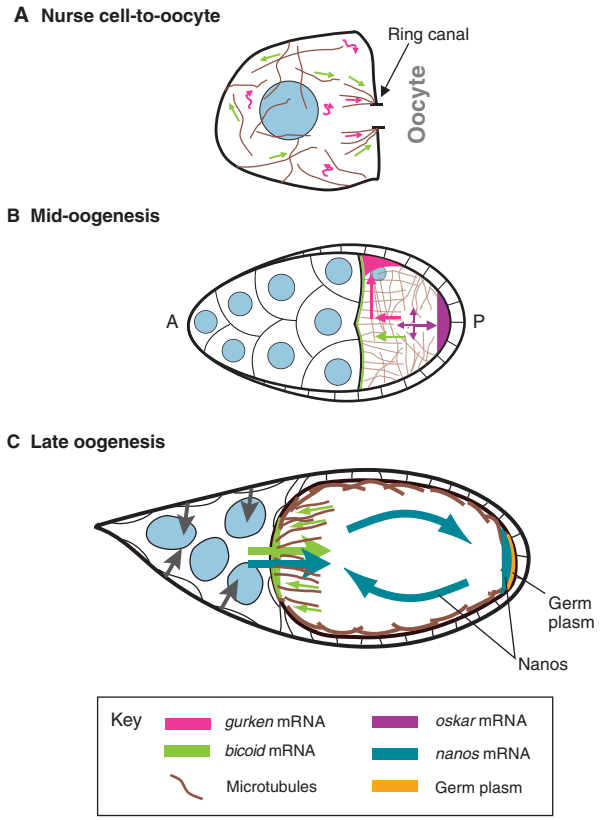


Fig. 3. Models for mRNA localization. In all panels, microtubules are shown in brown, nurse cell and follicle cell nuclei in blue. (**A**) Movements of *grk* and *bcd* mRNAs within the nurse cells during mid-oogenesis. Straight arrows indicate directed movement on microtubules, squiggly arrows indicate movement of *grk* with cytoplasmic flows. (**B**) Microtubule-dependent transport of *grk*, *bcd* and *osk* mRNAs within the oocyte during mid-oogenesis. The oocyte nucleus is shown in gray. Colored arrows show the directions of RNA movements. (**C**) Localization of *bcd* and *nos* at late stages of oogenesis. Contraction of the nurse cells for dumping is indicated by gray arrows pointing inward; entry of *bcd* and *nos* into the oocyte is indicated by large straight arrows. Small green arrows depict transport of *bcd* on anterior microtubules, curved dark green arrows depict diffusion of *nos* facilitated by ooplasmic streaming.

Transport during mid-oogenesis

After entering the oocyte, mRNAs must be translocated to specific cortical regions. Because RNPs enter the oocyte at its anterior end, anterior localization could occur simply by them being 'captured' through interactions with cytoskeletal or membrane proteins at the anterior cortex. By contrast, posterior localization requires that RNPs traverse distances of 50 μm or more. The anterior localization of *bcd* and the posterior localization of *osk* mRNA are both disrupted by the treatment of egg chambers with microtubule-depolymerizing drugs (Pokrywka and Stephenson, 1991; Pokrywka and Stephenson, 1995). These early experiments, which were performed using in situ hybridization to detect end-stage localization, could not distinguish whether microtubules act directly or indirectly. Nonetheless, they suggested that the oocyte possesses microtubule-based machinery for the delivery of mRNAs to different cortical destinations.

Reorganization of the oocyte microtubule cytoskeleton during mid-oogenesis results in an apparent AP gradient of microtubules (Theurkauf et al., 1992). A longstanding model proposed that these microtubules are polarized along the AP axis, with minus ends nucleated at the anterior cortex and plus ends projected towards the posterior. Accordingly, whether an RNP particle is transported to the anterior or posterior pole would depend on whether it associates with dynein or kinesin motor proteins. This model is based on a number of observations. First, partial microtubule depolymerization leaves short microtubules associated with the anterior cortex (Theurkauf et al., 1992). Because microtubules depolymerize from the plus end, this result suggests that microtubules are nucleated at the anterior cortex. Furthermore, a fusion between the motor domain of kinesin and β-galactosidase (kinesin-β-gal) that localizes to the plus ends of microtubules in neurons (Giniger et al., 1993) accumulates at the posterior of mid-stage oocytes (Clark et al., 1994). Consistent with the proposed polarity of the microtubule cytoskeleton, genetic studies subsequently implicated the plus-end-directed motor kinesin I in the localization of *osk* mRNA to the posterior pole (Brendza et al., 2000) and the minus-end-directed motor dynein in the localization of *bcd* mRNA to the anterior (Duncan and Warrior, 2002; Januschke et al., 2002).

This simple model was first called into question by the live-imaging studies of Cha et al. (Cha et al., 2001), who compared the behavior of fluorescent *bcd* RNA transported to the oocyte following its injection into nurse cells with the behavior of *bcd* RNA injected directly into the oocyte. Whereas *bcd* transcripts injected into the nurse cells accumulate specifically at the anterior oocyte cortex, those injected directly into the oocyte accumulate at the nearest cortex and this nonpolarized transport depends on Exu and microtubules. Subsequent work by Mische et al. (Mische et al., 2007) showed that the nonpolarized cortical transport of *bcd* RNA injected into the oocyte is mediated by dynein. The ability of the injected RNA to be transported by dynein to any part of the cortex suggests that microtubule minus ends are not restricted to the anterior cortex and that microtubule organization alone is not sufficient to dictate asymmetric RNA localization. Further support for this conclusion comes from high-resolution imaging of *bcd* RNP particles formed after injection of *bcd* RNA directly into the oocyte. These particles move with linear trajectories but in random directions near the oocyte anterior (Mische et al., 2007).

How then is *bcd* directed to the anterior cortex? Fluorescent *bcd* RNA does accumulate selectively at the anterior cortex if it is injected first into nurse cells, then withdrawn and injected into a naïve oocyte (Cha et al., 2001). By contrast, localization occurs in a nonpolar manner if *bcd* is injected first into nurse cells from *exu* mutant egg chambers. This result places a prior requirement for Exu in the nurse cells for the subsequent localization of *bcd* mRNA in the oocyte. Thus, although Exu present in the oocyte is sufficient for the nonpolar localization of injected *bcd* RNA, the Exu-dependent assembly of *bcd* RNPs in the nurse cells is crucial for targeting to the anterior cortex. Components of this RNP might promote dynein-mediated transport on a select subpopulation of microtubules nucleated from the anterior cortex or might prevent transport on microtubules nucleated at the lateral cortex (Cha et al., 2002) (Fig. 3B). Distinguishing which, if either, of these scenarios is correct will be facilitated by the analysis of genetically encoded fluorescent *bcd* mRNA. Intriguingly, a recent analysis of *Vg1* mRNA transport in *Xenopus* oocytes suggests that kinesin-bound *Vg1* RNPs may use a distinct subpopulation of microtubules that has the plus ends oriented towards the vegetal cortex (Messitt et al., 2008).

A different way to achieve net polarized transport during mid-oogenesis has come to light from a dynamic analysis of *osk* localization (Zimyanin et al., 2008). This study tracked the movement of *osk* RNP particles during midstages of oogenesis by using either the MS2 system to label *osk* mRNA directly with GFP or by using GFP-Stau as a proxy for *osk* transport particles. Live imaging revealed rapid, directed movements of both GFP-Stau and *osk* RNP particles throughout the oocyte, consistent with kinesin-dependent transport (see Movie 3 in the supplementary material). Surprisingly, however, *osk* particles move with similar average velocities in all directions, showing only a slight posterior bias. As the velocity of all rapidly moving *osk* particles, regardless of their direction and position in the oocyte, is decreased in kinesin I mutants with reduced motor speed but not in dynein mutants, it is unlikely that differential transport by opposing motor proteins leads to the observed bias. Rather, Zimyanin et al. (Zimyanin et al., 2008) proposed that kinesin transports *osk* on randomly oriented microtubules, but that a slight polarization in oocyte microtubule organization leads to a small excess of posteriorly directed transport events (Fig. 3B). Over the relatively long period that *osk* is being localized (6-10 hours), this weak bias could suffice to greatly enrich *osk* mRNA at the posterior of the oocyte. Importantly, the interpretation of microtubule orientation based on the behavior of *osk* RNPs fits the existing data. Assuming a slight posterior bias of microtubule plus ends, plus-end markers, such as kinesin–β-gal, would be expected to accumulate at the oocyte posterior by a biased random walk, similarly to *osk*. In addition, the observed accumulation of injected *bcd* RNA at the nearest cortex (Cha et al., 2002) and the dynein-dependent movement of *bcd* RNA particles in multiple directions (Mische et al., 2007) is consistent with a largely random distribution of minus ends.

Intriguingly, the posterior bias in *osk* movement is reversed in mutants for several *osk* localization factors, including *Tropomyosin II* (*TmII*; *Tm1* – FlyBase), *barentsz* (*btz*) and *mago nashi* (*mago*). Previous in situ hybridization experiments have shown that these mutants accumulate *osk* mRNA at the anterior of the oocyte, instead of at the posterior as in wild-type oocytes (Erdélyi et al., 1995; Newmark and Boswell, 1994; van Eeden et al., 2001). A plausible explanation is that these factors are required to uncouple *osk* RNP particles from the dynein motors that mediate nurse cell-to-oocyte transport so that they can associate with kinesin in the oocyte, although aberrant microtubule organization could be a contributing factor in *mago* mutant egg chambers (Micklem et al., 1997).

During mid-oogenesis, *grk* mRNA displays a spatiotemporally dynamic distribution that first became evident from in situ hybridization experiments. In early oocytes, *grk* accumulates at the posterior pole; following microtubule reorganization, *grk* accumulates transiently along the anterior oocyte cortex, then becomes restricted to the dorsal anterior region during stages 8-9 (see Fig. 1) (Neuman-Silberberg and Schüpbach, 1993). To distinguish whether these distributions arise by distinct localization mechanisms or whether they are interdependent, MacDougall et al. (MacDougall et al., 2003) tracked individual RNP particles formed after the injection of fluorescent *grk* RNA into oocytes. By varying the site of injection and the time at which particles were imaged after injection, they showed that *grk* particles follow two distinct paths in sequence, the first directed toward the anterior cortex and the second directed dorsally along the anterior cortex (Fig. 3B). Both steps can be inhibited by microtubule depolymerization and injection of anti-dynein heavy chain (dhc) antibodies that block dynein function (MacDougall et al., 2003). Further evidence for dynein-dependent transport of *grk* RNA on microtubules is provided by high-resolution ultrastructural studies that combine in situ hybridization and immunoelectron microscopy (ISH-EM). This method detects clusters of injected *grk* transcripts assembled in nonmembranous electron dense structures that probably represent transport particles (Delanoue et al., 2007). Most of these particles reside within 100 nm of the nearest microtubule and colocalize with dynein, consistent with *grk* transport particles in transit.

Whether *grk* and *bcd* transcripts are transported to the anterior cortex on the same set of microtubules is not known. However, the complex trajectory followed by *grk* RNP particles indicates that, unlike *bcd*, they must be able to recognize two different microtubule subpopulations. Several studies have reported evidence for a network of microtubules associated with the dorsally located oocyte nucleus that might mediate transport of *grk* from the anterior cortex to its dorsal anterior position (Januschke et al., 2006; MacDougall et al., 2003). Presumably, components specific to *grk* or *bcd* RNPs regulate the choice of microtubules by their associated dynein motors, possibly by recognizing chemical modifications or microtubule-associated proteins (MAPS) that are present on different subsets of microtubules. The *grk* mRNA localization factor Squid (Sqd) is a candidate for such a specificity factor. In situ hybridization to *grk* mRNA first showed that *grk* accumulates at the oocyte anterior but does not shift anterodorsally in *sqd* mutants (Neuman-Silberberg and Schüpbach, 1993). Subsequently, MacDougall et al. (MacDougall et al., 2003) found reduced dorsal anteriorly directed movement and increased ventrally directed movement of injected *grk* particles in *sqd* mutant egg chambers. This effect on the directionality of *grk* RNP particles implicates Sqd in selective microtubule utilization.

Localization during late stages of oogenesis

The fluorescent tagging of endogenous mRNA using the MS2 system has been instrumental in the investigation of mRNA localization during late stages of oogenesis, when molecular probes have limited access to the egg because of the presence of the vitelline membrane and the egg shell. The use of genetically encoded fluorescent *nos* and *bcd* mRNAs led to the discovery of two mechanistically distinct, late-acting localization pathways that operate simultaneously at late stages of oogenesis to position these RNAs at opposite poles of the oocyte (Forrest and Gavis, 2003; Weil et al., 2006; Weil et al., 2008).

nos represents a class of mRNAs, including *cyclin B*, *germ cell-less* and *polar granule component*, that is deposited in the oocyte by nurse cell dumping and localizes to the posterior late in oogenesis (Dalby and Glover, 1992; Jongens et al., 1992; Nakamura et al., 1996; Wang et al., 1994). Time-lapse imaging of endogenous fluorescent *nos* mRNA showed a steady accumulation of *nos* at the posterior pole of the oocyte following the onset of nurse cell dumping (Forrest and Gavis, 2003). At the posterior, *nos* colocalizes with the germ plasm component Vasa (Vas) in large particles, consistent with the known genetic requirement for germ plasm in *nos* localization. In contrast to the localization of *grk*, *bcd* and *osk* mRNA during mid-oogenesis, the posterior localization of *nos* is reduced but not abolished by complete depolymerization of the microtubule cytoskeleton, providing evidence against microtubule-dependent transport. The reduction in *nos* localization, however, points to the role of another microtubule-dependent process called ooplasmic streaming. Immediately prior to nurse cell dumping, oocyte microtubules reorganize to form parallel arrays beneath the oocyte cortex. These cortical microtubules mediate a vigorous kinesin-dependent flow of oocyte cytoplasm that mixes the incoming contents of the nurse cells with the ooplasm (Gutzeit and Koppa, 1982; Palacios and St Johnston, 2002; Theurkauf, 1994) (see Movie 1 in the supplementary material). Because microtubule

depolymerization blocks ooplasmic streaming but not nurse cell dumping, the effect on *nos* localization supports a model whereby *nos* RNPs diffuse throughout the oocyte but become trapped at the posterior by association with the germ plasm (Fig. 3C). Ooplasmic streaming thus enhances localization by facilitating encounters between *nos* RNPs and the germ plasm (Forrest and Gavis, 2003). Supporting this model, high-resolution imaging of fluorescent *nos* mRNA has subsequently detected individual *nos* mRNA particles moving in concert with yolk granules during ooplasmic streaming, consistent with passive transport (Weil et al., 2008) (see Movie 4 in the supplementary material).

Time-lapse imaging of genetically encoded fluorescent *bcd* mRNA during the transition from mid to late stages of oogenesis showed that *bcd* continues to accumulate at the anterior during nurse cell dumping. This late localization actually accounts for the majority of *bcd* mRNA that is present at the anterior of the embryo (Weil et al., 2006). Differing genetic requirements suggest that the initial and late phases of *bcd* localization use mechanistically distinct pathways. Mutations in *stau* and *swallow* (*swa*) selectively disrupt the late phase of *bcd* localization, and particles containing both *bcd* mRNA and Stau are detected only at these stages (St Johnston et al., 1989; Weil et al., 2006; Weil et al., 2008). Although *exu* is essential for *bcd* localization during both mid and late oogenesis, Exu phosphorylation is selectively required during the initial period (Riechmann and Ephrussi, 2004). Differences in *bcd* RNP assembly and composition might adapt *bcd* to the cellular machinery operating at different stages.

In contrast to *nos*, transport of *bcd* mRNA is both microtubule and dynein dependent. Live imaging of the microtubule marker Tau-GFP revealed a subpopulation of microtubules that project from the anterior cortex, distinct from the cortical microtubules that drive ooplasmic streaming (Fig. 3C). By co-visualizing microtubules labeled with Tau-GFP together with fluorescent *bcd* mRNA, Weil et al. (Weil et al., 2008) demonstrated directly that *bcd* RNP particles travel on these microtubule tracks (see Movie 5 in the supplementary material). The anterior microtubules are probably nucleated by a MTOC formed at the anterior cortex at the onset of nurse cell dumping (Schnorrer et al., 2002), suggesting that a specific reorganization of microtubules at the anterior cortex is responsible for maintaining an anterior transport pathway, while the majority of microtubules are reorganized for ooplasmic streaming. Surprisingly, Weil et al. (Weil et al., 2006) found that localization of *bcd* mRNA during nurse cell dumping can also be disrupted by actin depolymerization. This actin dependence proved to be indirect, however, because of a role for cortical actin in anchoring the anterior microtubules (Fig. 4A).

The finding that maximal *bcd* mRNA accumulation occurs after nurse cell dumping suggests that other mRNAs localized during mid-oogenesis could continue to be localized at late stages. Although endogenous *osk* mRNA has not yet been examined, injected *osk* transcripts accumulate at the posterior of late oocytes, facilitated by ooplasmic streaming (Glotzer et al., 1997). Thus, molecular asymmetries established through the polarized transport of mRNAs during mid-oogenesis might be maintained and reinforced by the subsequent localization of additional transcripts provided to the oocyte during nurse cell dumping.

Anchoring of mRNAs at the oocyte cortex

The effectiveness of a localization pathway depends on the ability to maintain transcripts at the target destination. The term ‘anchoring’ is commonly used to describe this end point of localization, implying a stable association of mRNA with a component of the cellular architecture. For mRNAs like *osk* and *nos*, anchoring is requisite for translation (Gavis and Lehmann, 1994; Markussen et al., 1995; Rongo et al., 1995). Transport and anchoring phases of localization pathways have been difficult to separate by traditional static imaging methods that visualize cumulative events. By permitting the investigation of the steady-state behavior of RNAs at the oocyte cortex, live-imaging methods have revealed an unexpected diversity of mechanisms for maintaining transcript localization.

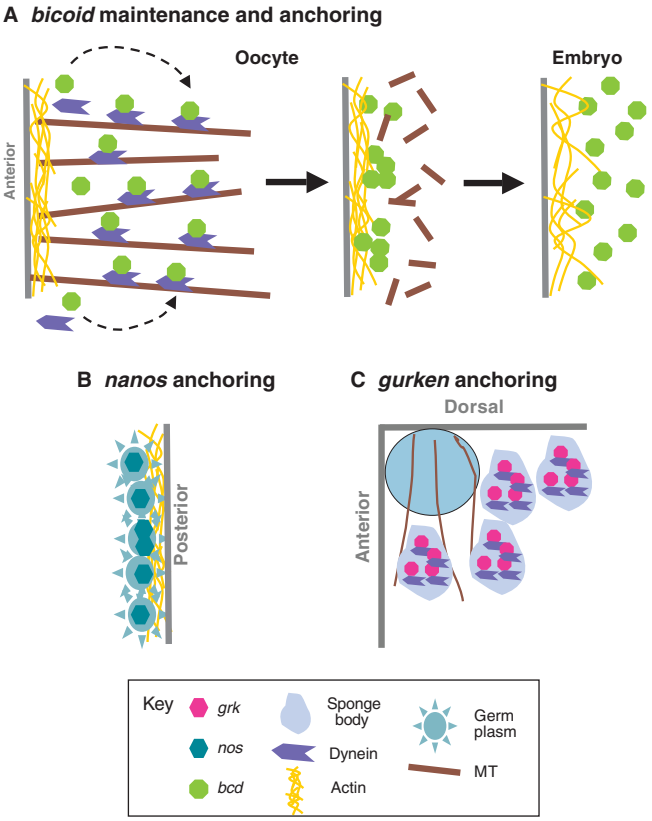


Fig. 4. Maintenance of localized mRNAs. (**A**) Maintenance of *bcd* mRNA at the anterior cortex of late oocytes by continual active transport on microtubules (MT), transition to a static actin-dependent anchoring mechanism at the end of oogenesis, and release from the tight cortical anchor at fertilization. (**B**) Stable, actin-dependent anchoring of *nos* mRNA and germ plasm (including *osk*) in late oocytes. (**C**) Dynein-dependent anchoring of *grk* mRNA in sponge bodies during mid-oogenesis. The oocyte nucleus is depicted as a blue circle.

Live imaging of both GFP-tagged *nos* mRNA and Vas-GFP has provided evidence for the retention of posteriorly localized mRNAs via actin-dependent anchoring of the germ plasm (Fig. 4B). Following treatment of late oocytes with cytochalasin D to depolymerize actin filaments, *nos* mRNA and Vas-GFP that have previously accumulated at the posterior cortex are released coincidently and swept away by the force of ooplasmic streaming (Forrest and Gavis, 2003). The dependence of *nos* localization on the germ plasm and the coincident release of *nos* and Vas suggests that *nos* mRNA is anchored to the cortical actin cytoskeleton via its association with the germ plasm. FRAP experiments, in which RFP-tagged *nos* RNA at the posterior cortex of a late (stage 13) oocyte was irreversibly photobleached, showed no fluorescence recovery by exchange with unbleached RFP-tagged *nos* mRNA from the nearby cytoplasm, confirming that this cortical association is highly stable (Weil et al., 2006).

An actin-dependent posterior anchoring system could be established through a feedback loop that initiates with *osk* mRNA localization itself. Upon localization to the posterior during mid-

oogenesis, *osk* mRNA is translated to produce two protein isoforms, Long Osk and Short Osk (Markussen et al., 1995; Rongo et al., 1995). Long Osk is required in turn to maintain *osk* mRNA and Short Osk localization at the posterior, whereas Short Osk recruits Vas and other germ plasm components (Breitwieser et al., 1996; Vanzo and Ephrussi, 2002). Both isoforms appear to be involved in the organization of long F-actin projections at the posterior cortex (Vanzo et al., 2007). Whether this actin organization is required for the posterior anchoring of *osk* is not known, but a role for actin is consistent with the partial release of *osk* mRNA from the posterior cortex observed after the treatment of egg chambers with actin-depolymerizing drugs (Cha et al., 2002). An interplay between actin, *osk* mRNA, germ plasm, and *nos* mRNA anchoring is further supported by the ability of mutations in the cortical actin binding protein Moesin to cause the delocalization *osk* mRNA, Vas protein and *nos* mRNA, along with detachment of F-actin from the posterior cortex (Jankovics et al., 2002; Polesello et al., 2002).

In contrast to posteriorly localized mRNAs, *bcd* mRNA retention at the anterior of the oocyte requires microtubules. By performing in situ hybridization on paraffin sections, Pokrykwa and Stephenson (Pokrykwa and Stephenson, 1991) found that treatment of stage 10 egg chambers with microtubule-depolymerizing drugs disrupts *bcd* localization. A role for microtubules in maintaining anterior *bcd* mRNA localization during stages 10-12 was confirmed by live-imaging experiments, which showed that *bcd* RNP particles detach from the anterior cortex upon treatment of cultured oocytes with microtubule-depolymerizing drugs (Weil et al., 2006). Surprisingly, however, microtubules do not serve to anchor *bcd* to the anterior cortex. Rather, FRAP and FLIP analysis revealed that the association of *bcd* mRNA with the anterior cortex is dynamic, even after nurse cell dumping and ooplasmic streaming are complete and *bcd* accumulation appears maximal. High-resolution imaging during this time period showed dynein-dependent translocation of *bcd* particles, with the majority directed towards or along the anterior cortex (Weil et al., 2006; Weil et al., 2008) (see Movie 4 in the supplementary material). Together, these results indicate that *bcd* mRNA does not become stably anchored during these stages and that steady-state localization requires continual active transport (Fig. 4A).

The ability to monitor the behavior of *bcd* particles as oogenesis progresses towards fertilization has uncovered additional temporal complexity in the *bcd* mRNA localization pathway. Such a temporal analysis of *bcd* mRNA particle dynamics revealed that *bcd* mRNA shifts from continuous active transport to stable actin-dependent anchoring at the end of oogenesis, with *bcd* RNPs coalescing in large foci in association with the cortical actin cytoskeleton (Weil et al., 2008) (Fig. 4A). Furthermore, through a combination of in vitro manipulations and mutational analysis, Weil et al. (Weil et al., 2008) showed that calcium signaling events that occur prior to fertilization trigger the dissipation of these foci throughout the anterior of the egg, probably through an effect on the actin cytoskeleton (Fig. 4A). Intriguingly, a similar mechanism appears to release *Xenopus Vg1* mRNA from its actin tether during fertilization (Yisraeli et al., 1990). Although it is unclear why a stable anchoring system is not established until late in oogenesis, the transition to actin-dependent anchoring could ensure the integrity of *bcd* mRNA localization, like that of *nos*, during periods when eggs are held dormant in the female prior to fertilization.

Like *bcd*, *grk* mRNA also undergoes a shift from dynamic maintenance to stable cortical association, but in this case the transition coincides with the shift from anterior to dorsal anterior *grk* localization. FRAP experiments performed on both injected and endogenous fluorescent *grk* RNA showed fluorescence recovery of *grk* at the anterior cortex of the oocyte, but not at its dorsal anterior position (Delanoue et al., 2007; Jaramillo et al., 2008). Thus, *grk* mRNA is statically anchored only after it becomes dorsally restricted and not during its transient localization at the anterior cortex. Surprisingly, however, dorsal anterior anchoring of *grk* is compromised by the injection of antibodies to dhc (Delanoue et al., 2007). To further investigate this dynein-dependent anchoring mechanism, Delanoue et al. (Delanoue et al., 2007) turned to ISH-EM. By this method, injected and endogenous *grk* transcripts are detected, together with dynein heavy chain and Sqd, at the dorsal anterior region in large cytoplasmic structures that resemble previously described sponge bodies (Fig. 4C). Hypomorphic mutations in *dhc* disrupt these sponge bodies and cause the dispersal of *grk* RNA. Dynein therefore plays two biochemically distinct roles in *grk* localization: first as a motor for transport and then as an anchor via an as yet unknown function in sponge body integrity. Sponge bodies form in *sqd* mutants, but when *grk* RNA is injected into the mutant oocytes, it fails to enter the sponge bodies and remains in transport particles (Delanoue et al., 2007). Inhibition of Sqd function by antibodies causes the detachment of anchored *grk* RNA; ultrastructural analysis showed that *grk* resides in transport particles rather than in sponge bodies when Sqd is inhibited. This accumulation of transport particles outside of the sponge bodies suggests an additional role for Sqd in the remodeling of transport particles to anchoring complexes following their transport to the dorsal anterior corner.

Conclusions

The ability to visualize mRNA localization events in real time and to monitor the behavior of RNA particles at high temporal and spatial resolution has advanced and expanded our understanding of the cellular mechanisms used to transport mRNAs to different destinations and to maintain their localized distributions. Live-imaging studies have also provided new insight into the functional organization of the oocyte cytoskeleton and its use by mRNA transport machinery.

Detailed dynamic analysis has revealed intriguing similarities, as well as differences, in behavior among localized mRNAs. The use of a common dynein-mediated transport pathway in nurse cells raises the possibility that oocyte-destined mRNAs like *grk*, *bcd* and *osk* might be packaged together into the same nurse cell transport particle. Remodeling of these particles upon their arrival in the oocyte would then segregate different component mRNAs into distinct oocyte transport particles coupled to the appropriate motors for translocation within the oocyte. Such a co-transport model can be tested either by co-injection of RNAs labeled with different fluorophores or by using different RNA stemloop and cognate protein pairs to label two different endogenous transcripts simultaneously. Co-transport of different mRNAs to the yeast bud tip has recently been observed using the latter method (Lange et al., 2008).

In another direction, co-visualization of fluorescently labeled mRNAs and proteins will provide insight into the temporal regulation of RNP assembly and the remodeling events that alter the composition of RNP particles at different stages of the localization process. Given the growing number of localization factors reported to regulate multiple mRNAs, determining how these proteins contribute to the localization of each target mRNA will be facilitated by the ability to follow differentially labeled mRNA-protein pairs. For example, Stau coassembles with *osk* mRNA transport particles during mid-oogenesis and with *bcd* particles in late oocytes and in

early embryos, consistent with the genetic requirements for *stau* in *osk* and *bcd* mRNA localization at these stages (Weil et al., 2006; Weil et al., 2008; Zimyanin et al., 2008).

One remaining gap in our knowledge stems from the inability to follow the entire path of an individual RNP particle en route to its destination, owing to the tendency of particles to move out of the plane of focus and to the photobleaching that occurs during long periods of imaging at high resolution. The continuing generation of brighter and more photostable fluorophores together with the development of new microscopes that allow rapid imaging in four dimensions hold the promise for another great leap forward.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/15/2493/DC1

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