Development 134, 1955-1965 (2007) doi:10.1242/dev.02832

A Dynein-dependent shortcut rapidly delivers axis determination transcripts into the *Drosophila* oocyte

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The primary axes of *Drosophila* are set up by the localization of transcripts within the oocyte. These mRNAs originate in the nurse cells, but how they move into the oocyte remains poorly understood. Here, we study the path and mechanism of movement of *gurken* RNA within the nurse cells and towards and through ring canals connecting them to the oocyte. *gurken* transcripts, but not control transcripts, recruit the cytoplasmic Dynein-associated co-factors Bicaudal D (BicD) and Egalitarian in the nurse cells. *gurken* RNA requires BicD and Dynein for its transport towards the ring canals, where it accumulates before moving into the oocyte. Our results suggest that *bicoid* and *oskar* transcripts are also delivered to the oocyte by the same mechanism, which is distinct from cytoplasmic flow. We propose that Dynein-mediated transport of specific RNAs along specialized networks of microtubules increases the efficiency of their delivery, over the flow of general cytoplasmic components, into the oocyte.

KEY WORDS: *Drosophila*, Oogenesis, Oocyte, Nurse cells, Intracellular mRNA localization, Microtubules, Actin, Cytoplasmic Dynein, Bicaudal D, Egalitarian, Ring canals

INTRODUCTION

In *Drosophila*, early embryonic development is supported by maternal deposition of the contents of the oocyte into the fertilized egg (Spradling, 1993). The oocyte nucleus is transcriptionally inactive during most of oogenesis (King and Burnett, 1959). Consequently, the majority of the contents of the growing oocyte originates from the polyploid nurse cells, with the exception of yolk granules and some specific signals that are secreted from the overlying follicle cells (Spradling, 1993). Such long-distance transport of cellular components, including mRNA, between interconnected cells or within very large cells is a common and important process in many biological systems (Pollard and Earnshaw, 2002). Nurse cell-to-oocyte transport occurs through actin-rich ring canals and can be divided into four distinct phases (Mahajan-Miklos and Cooley, 1994). First, in early oogenesis, oocyte specification in the germarium depends on the selective transport of Bicaudal D (BicD) and Egalitarian (Egl) (Mach and Lehmann, 1997; Suter and Steward, 1991), which, together with microtubules (MTs), are required for oocyte specification (Koch and Spitzer, 1983; Theurkauf et al., 1993). Second, during a period of 2 days in mid-oogenesis, from stages 2 to 7, both the nurse cells and the oocyte grow at the same rate (Spradling, 1993), and a variety of important transcripts that encode axis specification factors are transported into the oocyte. During the third phase, at stages 8-10A of oogenesis, this process continues, except the relative size of the oocyte increases in relation to the nurse cells because of the transport of yolk from the follicle cells and cytoplasm from the nurse cells. The fourth and last transport phase involves the 'dumping' of the entire nurse cell contents into the oocyte at stage 10B, leading to the doubling of the oocyte volume within 30 minutes (Mahajan-Miklos and Cooley, 1994).

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Accepted 12 February 2007

It has been proposed that MTs provide the tracks along which minus-end-directed motors, such as Dynein, could transport proteins, organelles and mRNAs into the oocyte (Theurkauf et al., 1993). This hypothesis is supported by the fact that strong mutations in components of the Dynein complex, including Dhc, Lis-1, BicD and Egl, result in egg chambers devoid of oocytes (Gepner et al., 1996; Liu et al., 1999; McGrail and Hays, 1997; Swan et al., 1999). However, no direct evidence for these proposals has been obtained and the exact mechanism by which these proteins affect this process is unclear. Moreover, the specificity of a putative Dynein-dependent nurse cell-to-oocyte transport system for different cargoes, such as organelles and mRNAs, remains elusive, as does the exact path of transport.

Key transcripts that must be transported from the nurse cells to the oocyte during mid- to late-oogenesis include: *gurken* (*grk*) mRNA, which encodes a TGFα homologue responsible for setting up the primary axes (Gonzalez-Reyes et al., 1995; Neuman-Silberberg and Schüpbach, 1993); *bicoid* (*bcd*) mRNA, encoding the anterior morphogen (Driever and Nüsslein-Volhard, 1988); and *oskar* (*osk*) mRNA, encoding a key factor responsible for posterior patterning and germ cell determination (Ephrussi et al., 1991). Once in the oocyte, the minus-end-directed MT-dependent molecular motor, cytoplasmic Dynein (Dynein), is required for the localization of *grk* (MacDougall et al., 2003) and *bcd* (Januschke et al., 2002b) mRNA. By contrast, the plus-end-directed molecular motor Kinesin I is required for *osk* mRNA localization to the posterior of the oocyte. The transport of each of these mRNAs into the oocyte has been suggested to involve the same mechanism as that of apical transport of pair-rule transcripts in the blastoderm embryo (Bullock and Ish-Horowicz, 2001), which was previously shown to involve MTs (Lall et al., 1999) and Dynein (Wilkie and Davis, 2001). The former study showed the signal-dependent nurse cell-to-oocyte transport of ectopically expressed pair-rule transcripts, which are ordinarily not expressed during oogenesis. Taking these observations together, they suggest the existence of a similar Dynein-dependent process that could mediate nurse cell-to-oocyte mRNA transport during oogenesis. However, the role of Dynein in the movement of transcripts, such as *bcd*, *osk* and *grk* mRNA, that are expressed in oogenesis, remains unclear.

The transport of *bcd* and osk mRNA into the oocyte has been known for some time to be MT dependent (Pokrywka and Stephenson, 1991). Furthermore, *bcd* mRNA forms particles that move into the oocyte at speeds suggestive of an active-transport process (Cha et al., 2001). Exu, a factor required genetically for *bcd* mRNA localization (St Johnston et al., 1989), was shown to be required within the nurse cells for injected *bcd* RNA to localize correctly once in the oocyte (Cha et al., 2001) and to move as particles from the nurse cells to the oocyte (Theurkauf and Hazelrigg, 1998). However, the role of motor proteins in nurse cell-to-oocyte transport was not addressed directly, although a Dynein-binding protein, Swallow (Schnorrer et al., 2000), and the Dynein co-factor Dynactin (Januschke et al., 2002a) were shown to be required for *bcd* mRNA localization in the oocyte. Thus, although the requirement for motor proteins in mRNA localization in the oocyte has been demonstrated conclusively, the mechanism and path of the nurse cell-to-oocyte transport of specific mRNAs remains poorly studied. It is also not known whether mRNAs are simply swept along by the flow of cytoplasm from nurse cell to oocyte. Although some selectivity of cytoplasmic transport has been observed (Bohrmann and Biber, 1994), it is also possible that cellular components are transported into the oocyte via the pressure created by the synthesis of cytoplasmic components in the nurse cells.

Here, we define the path of movement and mechanism of *grk* mRNA transport from nurse cells to oocyte. By injecting fluorescently labelled *grk* RNA into nurse cells and assaying its movement, we show that *grk* RNA is first transported rapidly and directly towards the ring canals, accumulating in a discrete zone in front of the ring canals, followed by its rate-limiting transport through the ring canals. We show that this transport is MT-, BicD- and Dynein-dependent, and that *grk* RNA recruits the Dynein-associated proteins Egl and BicD in the nurse cells. We show further that *bcd* and *osk* RNA are likely to be transported by a similar mechanism. We propose that Dynein-dependent transport of *grk*, *bcd* and *osk* transcripts towards the ring canal allows the more-efficient transport of key axis specification transcripts than would the slower transport of cytoplasmic components, which occurs throughout much of oogenesis.

MATERIALS AND METHODS

Fly strains

Stocks were raised on standard cornmeal-agar medium at 25°C. The strains used include: wild type (*Oregon-R*, *OrR*); *Tau-GFP* (D. St Johnston, Wellcome Trust/Cancer Research UK Gurdon Institute, Cambridge, UK), to visualize MTs; *w;p*[*UASp-GFP-actin*] driven by 'triple driver' (*w;pCOGGal4VP16; NGT40; nosGal4VP16*) (L. Cooley, Yale University Medical School, New Haven, CT) to visualize actin; and dhc^{6-6}/dhc^{6-12} trans-heterozygotes (T. Hays, University of Minnesota, Minneapolis, MN), which are female-sterile. To examine the role of BicD in *grk* RNA nurse cell-to-oocyte transport in mid-oogenesis, *w; Df(2L)TW119/CyO; P [w+hsBic-D]-94* and *w;* $BicD^{r8}$*/CyO* (B. Suter, University of Bern, Bern, Switzerland) were crossed to generate *w; Df(2L)TW119/* $BicD^{r8}$*; P [w+hsBic-D]-94/+ (Bic-*D^{mom}*)* flies, as described by Swan and Suter (Swan and Suter, 1996). For controls, siblings of the cross (*w, Df(2L)TW119/CyO* or *w,* $BicD^{r8}$*/CyO; P [w+hsBic-D]-94/+*) and *OrR* flies were used. To confirm the absence of BicD protein in *Bic-*D^{mom} ovaries, each fly had one ovary dissected and fixed for BicD immunostaining, whereas the other ovary was used for the in vivo study of *grk* RNA nurse cell-to-oocyte transport.

RNA in situ hybridization

In situ hybridization was performed as previously described (MacDougall et al., 2003; MacDougall et al., 2001). Digoxigenin anti-sense RNA probes were transcribed using full-length *grk* (G. Schupbach, Princeton University, Princeton, NJ), *osk* (A. Ephrussi, EMBL, Heidelberg, Germany) and *bcd* (C. Nusslein-Volhard, Max Planck Institute, Tübingen, Germany) cDNAs. In each experiment, at least 40 ovarioles (stage 2-9) and ten egg chambers (stage 10A and older) were examined from *OrR* or dhc^{6-6}/dhc^{6-12} females.

Synthesis of fluorescent and biotinylated RNA

RNAs were in vitro transcribed using T3, T7 and SP6 polymerases and UTP-AlexaFluor546, UTP-AlexaFluor488, UTP-Cy3 or UTP-Biotin as previously described (Wilkie and Davis, 2001). Plasmids templates were: full-length *grk* (G. Schupbach); *bcd* (C. Nusslein-Volhard); *K10* 3′ UTR (S. Bullock, MRC LMB, Cambridge, UK); *I factor* (also known as *I-element*; V. Van de Bor, University of Nice, Nice, France); and a 3 kb fragment of *lacZ* amplified from pG5-L-G3 (Thio et al., 2000) and cloned into pGEM-T.

Injection of RNAs and inhibitors

Ovaries were dissected in Series 95 halocarbon oil (KMZ Chemicals) and egg chambers were adhered to coverslips using tungsten needles. Fluorescently labelled RNA (250-500 ng/μl) was injected into nurse cell cytoplasm using Femtotip needles (Eppendorf). At least two batches of RNA were injected for each experiment, on separate occasions. Colcemid was co-injected with fluorescently labelled RNA at 100 μg/ml into *Tau-GFP* egg chambers. To study the requirement of MTs in *grk* RNA ring canal accumulation, colcemid (100 μg/ml) was pre-injected over each *Tau-GFP* egg chamber followed by *grk* RNA injection into the nurse cells 10 minutes later. AlexaFluor568-coupled Phalloidin was injected at a concentration of 4 units/ml (0.13 μM) into wild-type nurse cells to visualize ring canals and to facilitate imaging of cytoplasmic movements at the same plane of focus as a ring canal.

Immunostaining of injected egg chambers

Biotinylated RNAs were injected into wild-type nurse cells and fixed after 15-20 minutes. Egg chambers were fixed for 20 minutes by placing 3.7% formaldehyde in PBT (0.1% Tween) over the halocarbon oil. All subsequent staining steps were performed in a watchglass under a dissecting microscope to avoid losing the injected egg chambers during the washes. Biotin-labelled RNA was detected with Avidin-conjugated AlexaFluor488 or AlexaFluor546 (Molecular Probes). BicD and Egl were visualized with anti-BicD (B. Suter) and anti-Egl (R. Lehmann, New York University School of Medicine, NY), which were used at dilutions of 1/20 and 1/4000, respectively. Secondary antibodies were AlexaFluor488- and AlexaFluor568-coupled (Molecular Probes). In all recruitment experiments, at least three egg chambers were analyzed for each condition. Alexa568-coupled Phalloidin (1/50) was used to visualize actin.

Four-dimensional imaging and deconvolution

Fixed egg chambers were mounted in Vectashield (Vector Laboratories). Imaging was performed on a wide-field DeltaVision microscope (Applied Precision, Olympus IX70 microscope, Coolsnap HQ camera from Roper). Images were acquired with a 20×/0.75NA or 100×/1.35NA objective lens and subsequently deconvolved (Davis, 2000) using Sedat/Agard algorithms with Applied Precision software. Several egg chambers were imaged in parallel depending on the time resolution needed. For high-power imaging of MTs, *Tau-GFP* and *Tau-GFP/+;* dhc^{6-6}/dhc^{6-12} egg chambers were sectioned through the ring canal at 1 μm intervals with a 100×/1.35NA objective. Live imaging of RNA particles at 100×/1.35NA was performed at 1 second intervals for approximately 3 minutes, after which time photobleaching prevented the identification of particles for tracking.

Particle tracking

RNA particles were manually tracked with SoftWoRx (Applied Precision) and Metamorph (Universal Imaging Corporation) software as previously described (MacDougall et al., 2003). Nurse cell cytoplasmic movements near the ring canals were analyzed as for RNA particles, at 100×/1.35NA and 1 second time intervals, by differential interference contrast (DIC) (n=4 egg chambers). Trails of RNA or cytoplasmic particles were generated using SoftWoRx. Speeds of particle movements are shown as average speeds±standard error (s.e.). It was difficult to estimate the proportion of

RNA particles that move in directed tracks, because stationary particles are difficult to distinguish from autofluorescent cytoplasmic bodies. By contrast, rapidly moving RNA particles of a similar intensity of fluorescence are easily identified.

RESULTS

Injected *grk* RNA moves from the nurse cells through ring canals into the oocyte and localizes correctly

To investigate the mechanism by which *grk* moves from the nurse cells to the oocyte, we established an injection assay based on previous assays described for *bcd* RNA in the nurse cells (Cha et al., 2001) and *grk* RNA in the oocyte (MacDougall et al., 2003). Full-length *grk* RNA was injected into the nurse cells at different stages of oogenesis. At all stages tested, ranging from 6 to 10A, injected *grk* RNA was able to move into the oocyte from the nurse cells and then localize correctly within the oocyte (Fig. 1A,C,E, and see Movies 1, 2, and Table S1 in the supplementary material) in a manner similar to the endogenous transcript (Fig. 1B,D,F). At early stages, before the reorganization of MTs and the migration of the oocyte nucleus, injected *grk* RNA localized correctly in a posterior crescent near to the oocyte nucleus (Fig. 1A,B), where MT minus ends are thought to be concentrated (Micklem et al., 1997). By contrast, when *grk* RNA was injected into the nurse cells of stage 8-10A egg chambers (Fig. 1C-F), it became localized in a dorso-anterior crescent near the oocyte nucleus in a very similar pattern to its localization when injected into the oocyte, and to the endogenous transcript at the same stage of oogenesis (MacDougall et al., 2003). We found that the efficiency of transport of injected *grk* RNA into the oocyte was higher in the earlier stages and that some injected RNA remained in the nurse cells, within the time scale of the experiment, which was limited, by egg chamber survival in halocarbon oil, to 1-2 hours.

To determine whether the movement of injected *grk* transcript from the nurse cells to the oocyte is specific, we injected a number of other transcripts, including control RNAs. We first injected *bcd* RNA, which had previously been shown to move efficiently into the oocyte (Cha et al., 2001). *bcd* RNA injected into the nurse cells moved into the oocyte at an equivalent rate to *grk* RNA (Fig. 1G,H and see Table S1 in the supplementary material) and localized to the anterior of the oocyte, in the same pattern as the endogenous transcript. We then injected fluorescently labelled *lacZ* RNA and found that these control transcripts failed to accumulate substantially in the oocyte, and that the small amount of RNA that entered the oocyte was evenly distributed in the cytoplasm (Fig. 1I and see Table S1 in the supplementary material). We conclude that injected *grk* and *bcd* RNA are specifically transported efficiently from the nurse cells into the oocyte.

To elucidate the path and details of the movement of *grk* and *bcd* RNA from the nurse cells, we studied the movement at higher magnification and time resolution. We found that, in nurse cells, injected *grk* RNA forms particles that move in directed paths to a specific location near the oocyte, which appeared to be ring canals (Fig. 2A and see Movie 3 in the supplementary material). By contrast, injected *lacZ* RNA did not accumulate in similar locations. To test directly whether *grk* RNA localized at ring canals, we fixed the injected egg chambers 20 minutes after the injection of Biotin-labelled *grk* RNA and stained them with AlexaFluor488-Phalloidin to co-visualize the ring canals with RNA. We found that, in 88% of egg chambers (n=8), *grk* RNA accumulated at a region in front of the ring canals (Fig. 3A, and see Movie 4 and Table S2 in the supplementary material). By contrast, injected *lacZ* RNA failed to accumulate in this manner (n=12).

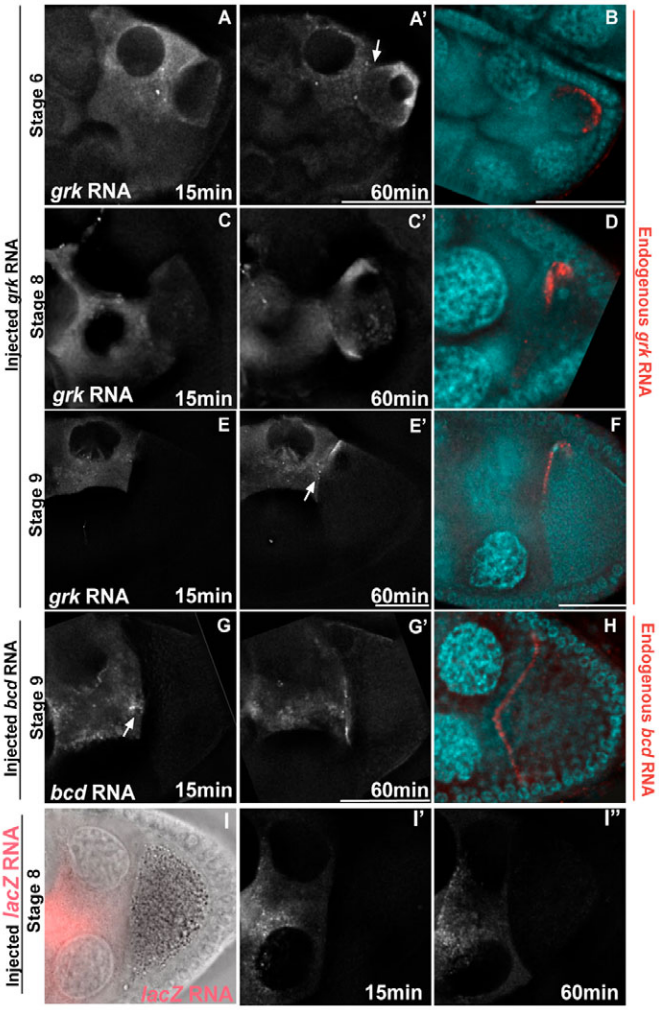


Fig. 1. Injected *grk* RNA moves from the nurse cells into the oocyte and localizes specifically, in the same way as the endogenous transcript. (**A**,**C**,**E**,**G**) *grk* RNA (A,C,E) and *bcd* RNA (G) localization at 15 minutes after injection; (**A′**,**C′**,**E′**,**G′**) at 60 minutes after injection. (**B**,**D**,**F**,**H**) In situ hybridization showing endogenous *grk* mRNA (B,D,F) and *bcd* mRNA (H) in equivalent-stage egg chambers (RNA in red, DNA in cyan). (A) Stage 7 (also see Movie 1 in the supplementary material); (C) young stage 8; (E,G) stage 9 (also see Movie 2 in the supplementary material) egg chambers. *grk* and *bcd* RNA injected into nurse cells is transported into the oocyte and localizes in the same way as the endogenous mRNAs. (A′,E′,G) Arrows indicate sites of apparent ring canal accumulation. (**I**) *lacZ* RNA (pink) was injected into the nurse cell, as shown by the outline of the egg chamber (grey). (**I′**,**I″**) At 15 (I′) and 60 (I″) minutes after injection. Injected *lacZ* RNA accumulates in the nurse cells, with little movement into the oocyte and no localization. In all panels, anterior is to the left and dorsal is to the top. Scale bars: 40 μm.

To test whether the accumulation of *grk* RNA at the ring canals is a consequence of directed movement towards this region of the nurse cells, we co-visualized injected *grk* RNA with Actin-GFP in living egg chambers. We found that *grk* RNA particles moved in directed paths, rather than randomly, towards the ring canals, which they accumulated in front of before passing through (Fig. 3C-E). These paths are most easily appreciated when viewing the time lapse movies (Movies 3, 4 in the supplementary material). The movement towards and through ring canals occurred at different speeds (Fig.

3), suggesting that they involve distinct mechanisms of RNA transport. These speed differences are consistent with previous observations of Exu-GFP particles (Theurkauf and Hazelrigg, 1998). We found that *K10* (MacDougall et al., 2003) and *I* factor (also known as *I-element* – FlyBase) (Van De Bor et al., 2005) mRNA behave similarly when injected into a nurse cell (see Fig. S1A-C and Tables S1, S2 in the supplementary material).

We then determined whether the movement of *grk* and *bcd* RNA within the nurse cells was specific and could explain their more-efficient accumulation in the oocyte compared with control RNA. To address this issue, we injected *lacZ* RNA. We found that, although *lacZ* RNA formed some particles that move within the nurse cells, fewer particles than with *grk* RNA injection were observed moving directionally towards the ring canals (Fig. 2B). Most significantly, *lacZ* RNA did not concentrate at the ring canal in any of the egg chambers examined, resulting in a more homogenous distribution than injected *grk* RNA (Fig. 2B, Fig. 3B and see Table S2 in the supplementary material). We conclude that, within nurse cells, *grk* and *bcd* transcripts are specifically and actively transported towards ring canals that link these cells to the oocyte. We interpret the accumulation of *grk* RNA in front of the ring canals as indicating that movement through the ring canals is a rate-limiting step.

grk mRNA transport within nurse cells is Dynein and MT dependent

To determine whether *grk* mRNA transport within nurse cells and its accumulation at the ring canals requires MTs, we injected *grk* RNA into egg chambers expressing transgenic Tau-GFP (Micklem et al., 1997), which decorates the MTs (Fig. 4B,C). We found that MTs are organized with a focus of high concentration at the ring canal (Fig. 4A) at the region where injected *grk* RNA accumulates (Fig. 4B,C). We then co-injected the MT depolymerizing drug Colcemid with fluorescent *grk* RNA and found that the accumulation near the ring canals and the movement into the oocyte was inhibited rapidly (Fig. 4D,E). Tau-GFP imaging showed that, 10 minutes after Colcemid injection, MTs were mostly depolymerized (Fig. 4D). We found that MT depolymerization also abolished the directionality of *grk*-particle movement towards the ring canals (Fig. 4F,G). We conclude that the specific movement of *grk* RNA particles in the nurse cells along straight paths to the ring canals is MT dependent.

To test whether cytoplasmic Dynein, the major minus-end MT motor in the cell, is required for *grk* RNA movement towards the ring canals, we injected *grk* RNA into hypomorphic allelic combinations of Dynein heavy chain (Dhc64C), the large force-generating ATPase subunit of the Dynein motor. These viable mutant combinations do not show dorso-ventral polarity defects, but the females are sterile (McGrail and Hays, 1997). We found that, in $dhc^{6\text{-}6}/dhc^{6\text{-}12}$ mutant egg chambers, injected fluorescent *grk* RNA moved towards the ring canals in a directed fashion, but at slower rates than in wild type (n=14). Injected *grk* RNA also moved into the oocyte and localized, but, again, at a slower rate (Fig. 5A-D and see Movies 5, 6 in the supplementary material). To determine whether endogenous *grk* mRNA transport and/or localization is impaired in *dhc* hypomorphic mutants, we carried out in situ hybridization. Endogenous *grk* mRNA was weakly localized in the oocyte of early (stage 2-7) *dhc* mutant egg chambers (Fig. 5E-H). To test whether the MT distribution was

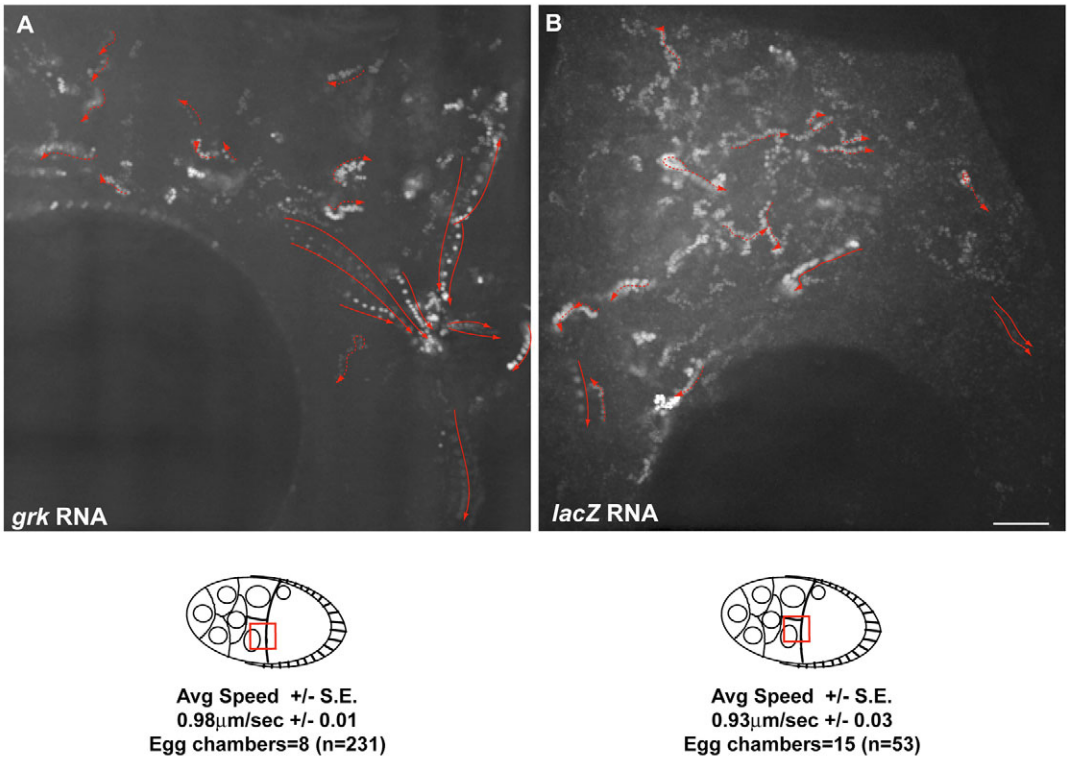
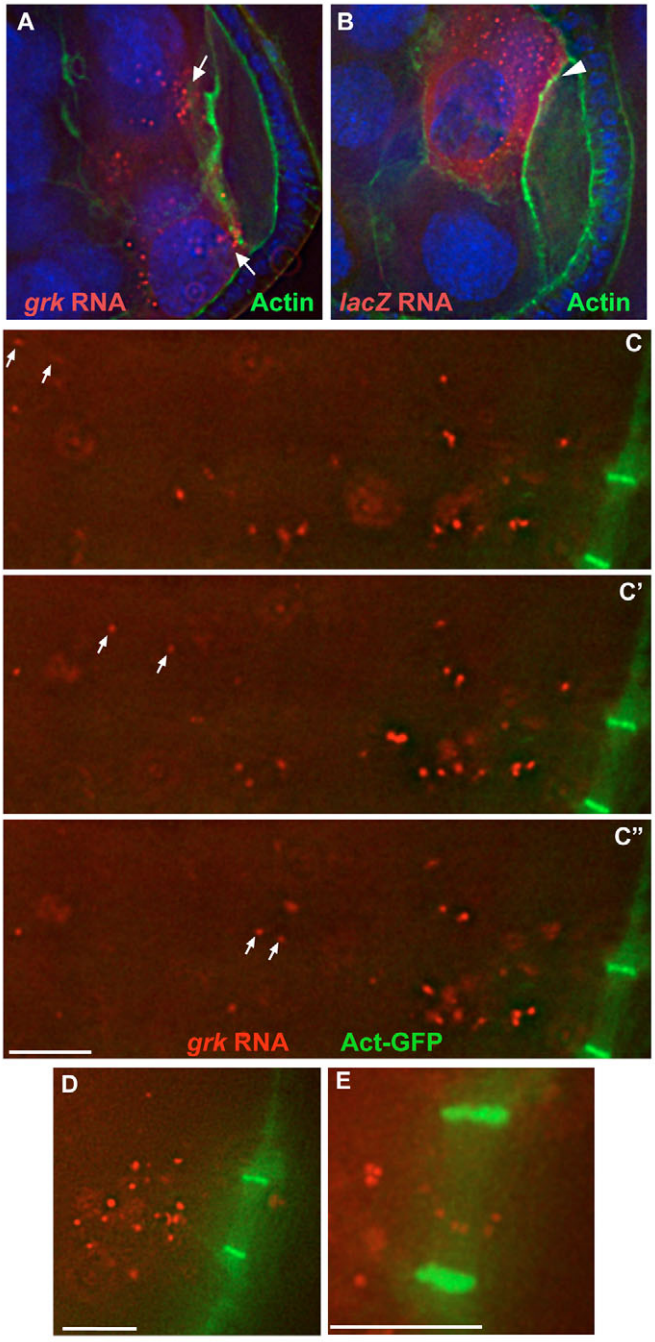


Fig. 2. *grk* RNA particles specifically accumulate at apparent ring canals. (**A**,**B**) Trails of *grk* RNA (also see Movie 3 in the supplementary material) and *lacZ* RNA injections into wild-type nurse cells. Schematics on the left represent the site of injection (red outline) and show the average speed±standard error of the mean (S.E.), the number of egg chambers injected and the total number of particles tracked for analysis (n). Trails at 1 second intervals were superimposed (1 minute duration). Red arrows indicate the direction of RNA particle movement; broken lines show those particles which seem to follow nurse cell cytoplasmic streaming, and were thus not tracked for analysis. Anterior is to the left and dorsal is to the top. Scale bar: 5 μm.



RNA Particle Movements	Toward the ring canal	Through the ring canal
Avg speed, +/- S.E. n = number of egg chambers, p = number of particles	1.45 μm/sec, +/- 0.087 n=3, p=14	0.25 μm/sec, +/- 0.036 n=3, p=7

Fig. 3. *grk* RNA particles move in directed paths towards ring canals. (**A**,**B**) Injected and subsequently fixed egg chambers show that *grk* RNA (A) but not *lacZ* RNA (B) accumulates at the ring canal (arrows). (B) Arrowhead points to a ring canal itself. (**C**) Frames from a time-lapse movie of a nurse cell injected with *grk* RNA (red) in an Actin-GFP (green) egg chamber (also see Movie 4 in the supplementary material). Panels correspond to successive time points, at 5 second intervals. Arrows indicate two *grk* RNA particles moving on the same path towards a ring canal that is connected to the oocyte. (**D**) *grk* RNA (red) accumulation at the ring canal at approximately 3 minutes after injection. (**E**) *grk* RNA particle (red) moving through a ring canal shown as a trail, successive time-points (at 5 second intervals) being superimposed on each other. Notice the difference in distances between RNA particles moving through the ring canal (E) and those moving towards ring canals (C). At the bottom of the figure is a table showing the corresponding *grk* RNA particle statistics. In all panels, the oocyte is to the right. (C-E) Red is *grk* RNA; green is Actin-GFP. Scale bar: 5 μm.

affected in *dhc* mutants, we imaged the distribution of MTs in living nurse cells of $dhc^{6\text{-}6}/dhc^{6\text{-}12}$ mutant egg chambers, using Tau-GFP, and found that it was indistinguishable from wild-type controls (see Fig. S2 in the supplementary material), consistent with our previous work in the oocyte (MacDougall et al., 2001). We conclude that the transport of *grk* RNA within the nurse cells towards ring canals and from the nurse cell to the oocyte is Dynein dependent.

To test whether other localized transcripts require Dynein for their transport from the nurse cells to the oocyte, we carried out in situ hybridization with anti-sense probes against *bcd* and *osk*. We found that, in *dhc* mutants from stage 10 onwards, more *bcd* mRNA accumulated in the nurse cells than in wild type (see Fig. S3A,B in the supplementary material). In addition, *osk* mRNA was also less 'focused' at the posterior of the oocyte in early stage (2-7) $dhc^{6\text{-}6}/dhc^{6\text{-}12}$ mutants (Fig. 5K). From stage 10A onwards, both *bcd* and *osk* mRNA showed sites of nurse cell cytoplasmic accumulation in $dhc^{6\text{-}6}/dhc^{6\text{-}12}$ mutants that were not evident in wild-type egg chambers (Fig. 5J,L).

These results could be explained by either a defect in nurse cell-to-oocyte transport or by a defect in RNA localization within the oocyte. To distinguish between these possibilities and determine directly whether the movement of *grk* RNA towards the ring canals is Dynein dependent, we studied the movement of RNA particles at high resolution in $dhc^{6\text{-}6}/dhc^{6\text{-}12}$ mutant egg chambers. We found that the speed of movement of *grk* RNA particles within the nurse cells towards the ring canals was substantially reduced (Fig. 6A,B). We conclude that the transport of *grk* RNA within the nurse cells towards the ring canals joining them to the oocyte occurs by Dynein along MTs and that Dynein is also required for the efficient transport of *bcd* and *osk* RNA from the nurse cell to the oocyte.

grk and *bcd* RNA recruits BicD and Egl within the nurse cells, and BicD is required for the accumulation of *grk* RNA in front of the ring canal

To investigate the basis of the specificity of *grk* transport compared with that of control *lacZ*, we first determined whether these RNAs recruit the Dynein co-factors BicD and Egl upon injection into the nurse cells. BicD and Egl have previously been shown to be required for oocyte specification (Mach and Lehmann, 1997) and for the efficient Dynein-dependent transport of mRNA in the embryo (Bullock and Ish-Horowicz, 2001). We injected biotinylated *grk* and *bcd* RNA into wild-type egg chambers, fixed them after 20 minutes and detected BicD and Egl protein. Our results show that both BicD and Egl were recruited by injected *grk* and *bcd* RNA at the site of injection in the nurse cell cytoplasm and at the ring canals, whereas injected *lacZ* RNA failed to recruit BicD and Egl (Fig. 7A-D and data not shown, respectively). To test whether BicD is required for *grk* RNA transport to the ring canals, we injected *grk* RNA into nurse cells of *BicD* mutant egg chambers. We used the $BicD^{mom}$ mutant combination, in which the early lethality of a strong allele of *BicD* is rescued with the early expression of *BicD* from the heat shock

promoter (Swan and Suter, 1996). In this way, later-stage egg chambers that lack BicD protein are obtained (Fig. 7E,H). We injected fluorescent *grk* RNA into the nurse cells of *BicD*^{mom} egg chambers and found that the RNA failed to accumulate in front of the ring canals, as it does in controls (Fig. 7F,G,I,J) (n=10). We conclude that BicD, and probably also Egl, is required for *grk* RNA to move along directed paths within the nurse cells towards the ring canals and that *lacZ* RNA is not able to move along these paths because it fails to recruit BicD and Egl.

grk RNA-particle movements are distinct from the general flow of cytoplasm from nurse cell to oocyte

There are considerable cytoplasmic flows in the oocyte and nurse cells. Therefore, we tested whether the movements of *grk* RNA that we observed could be caused indirectly by cytoplasmic flow. To address this, we imaged the movement of cytoplasm in the nurse cells using differential interference contrast (DIC), as previously described (Bohrmann and Biber, 1994), and plotted trails of nurse

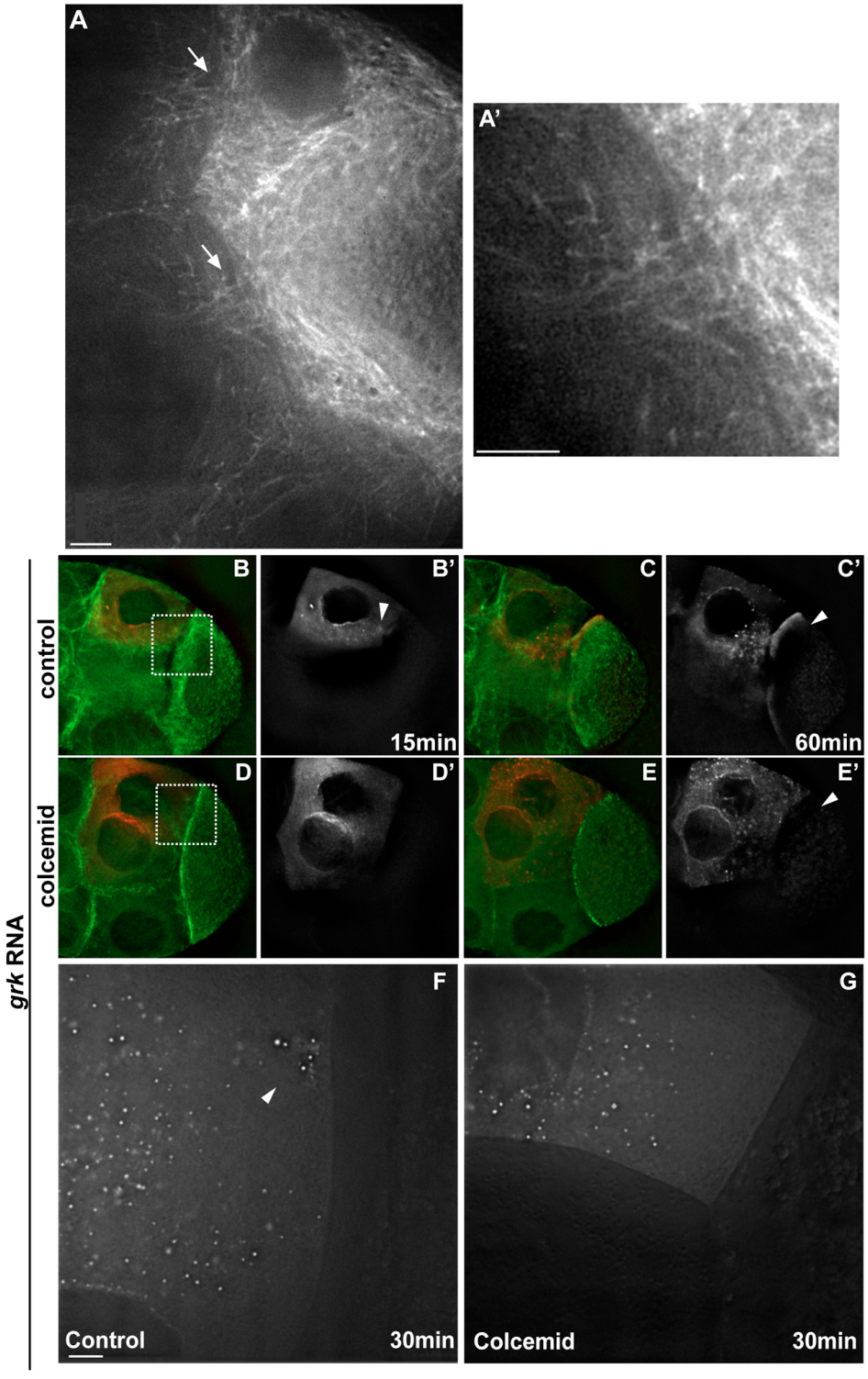


Fig. 4. Microtubules are required for *grk* RNA movement towards the ring canals and for transport into the oocyte. (**A**) Tau-GFP egg chambers show an enrichment of microtubules (MTs) at the ring canals (arrows). (**A'**) Higher-magnification of ring canal in A. (**B-E**) Tau-GFP (green) living egg chambers injected with *grk* RNA (red) at 15 (B,D) and 60 (C,E) minutes after injection. (B,C) *grk* control; (D,E) *grk*-colcemid co-injection. (**B'**,**C'**,**D'**,**E'**) Only injected RNA is shown, at the corresponding time points. (**F**,**G**) Higher-magnification of boxed regions shown in B and D. Control (F) and Colcemid-treated (G) egg chambers at 30 minutes after injection with *grk* RNA. Notice how, in the absence of MTs, *grk* RNA particles fail to accumulate at the ring canals as they do in controls (F, arrowhead). In all panels, anterior is to the left and dorsal is to the top. Scale bar: 5 μm.

cell cytoplasmic movements. Our results show that the path and direction of cytoplasmic flow in the nurse cells is significantly different to that of the injected *grk* RNA (see Fig. S4 in the supplementary material). We found that cytoplasmic particles did not move in directed paths towards the ring canals. By contrast, *grk*, and probably *bcd* and *osk*, RNAs are specifically transported by Dynein along direct paths that lead to ring canals, and are therefore transported more efficiently into the oocyte. We propose that these distinct routes to the ring canals are along a subset of MTs that we observe as a particularly high concentration of MTs near the ring canals (Fig. 4A and see Fig. S2 in the supplementary material).

DISCUSSION

Within nurse cells, we have identified a new path of Dynein-dependent transport to the ring canals that link the nurse cells to the oocyte. We show that *grk* RNA moves along this route, and our data suggest that *bcd* and *osk* transcripts also follow the same path. This intracellular shortcut requires BicD and is distinct from the route taken by general cytoplasmic components and control RNAs, which move into the oocyte less effectively. Our data suggest that the distinction between RNA components that follow this direct path and those that do not is the ability to recruit the Dynein-associated co-factors BicD and Egl. We propose that Dynein-dependent transport of *grk*, *bcd* and *osk* transcripts towards the ring canals follows a MT network, which is distinct from other networks in the nurse cells (see Fig. S5 in the supplementary material).

The movement of macromolecules, including RNA, within and between cells is an important process in the biology of most organisms. Although diffusion is, in principle, fast enough to distribute macromolecules over relatively large cellular distances, active transport can provide more-efficient, directed movements to specific locations as well as overcome effective diffusion barriers

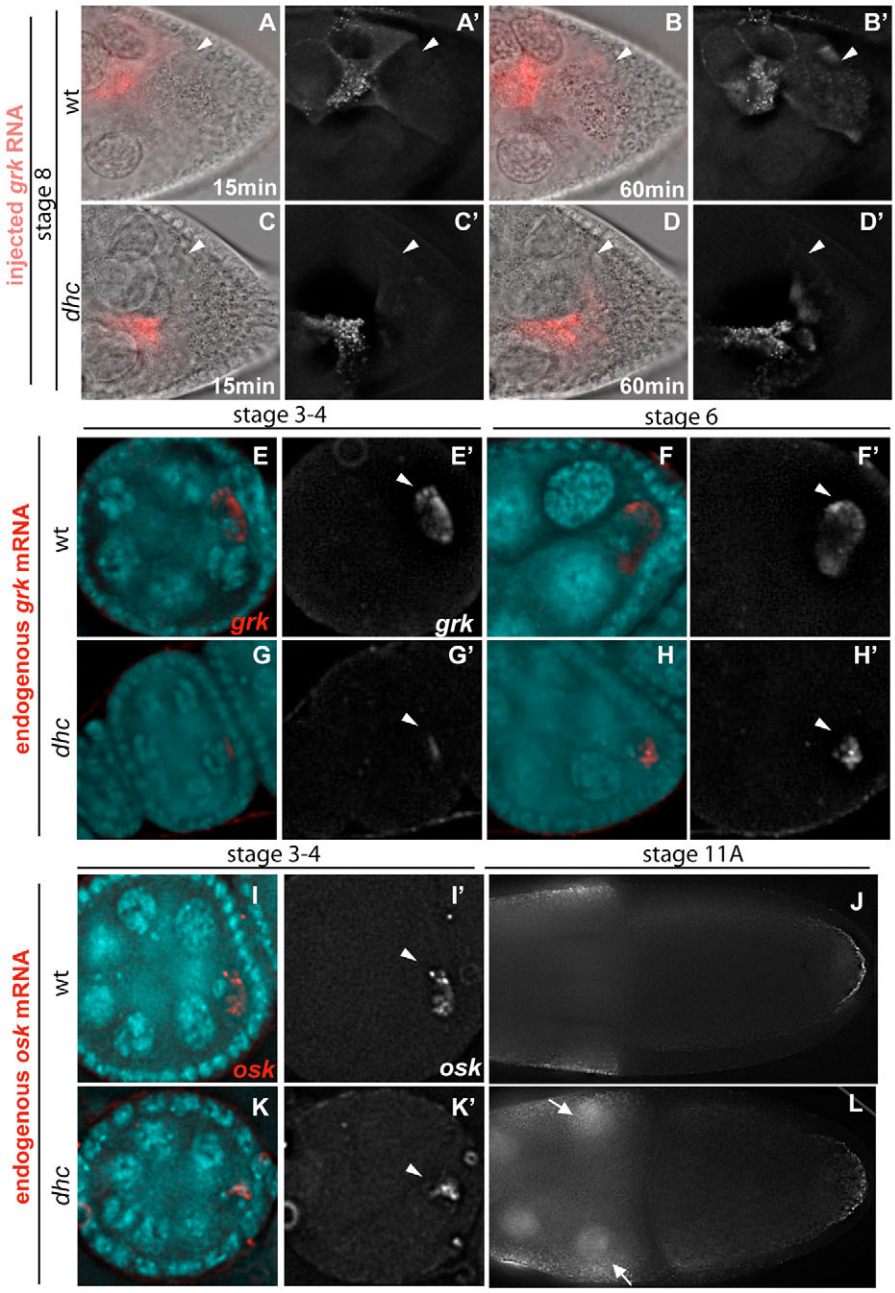


Fig. 5. Dynein is required for the efficient transport of *grk* RNA into the oocyte and for its localization. (**A-D**) Wild type (A,B; wt) and *dhc* mutant (C,D; *dhc*) stage-8 egg chambers injected with *grk* RNA (pink) in the nurse cells. Notice the decrease in *grk* RNA transport and localization in the oocyte in *dhc* mutants (compare B′ with D′) (also see Movie 5 in the supplementary material). (A-D) Grey is brightfield illumination showing the outline of the nuclei and oocyte. Arrowheads indicate the position of the oocyte nucleus. (**A′**,**B′**,**C′**,**D′**) Injected *grk* RNA only. (**E-L**) *grk* (E-H) and *osk* (I-L) mRNA in situ hybridization in wild-type (E,F and I,J; wt) and *dhc* mutant (G,H and K,L; *dhc*) egg chambers. (E,G,I,K) Stage 3-4 egg chambers; (F,H) stage 6 egg chambers; (J,L) stage 11A egg chambers. Notice the reduction in *grk* and *osk* mRNA signal and localization in the oocyte of *dhc* mutants (arrowheads; compare E′ with G′ and F′ with H′ for *grk* mRNA, and I′ with K′ for *osk* mRNA). (L) In late-stage egg chambers, cytoplasmic foci of *osk* mRNA on the periphery of *dhc* mutant egg chambers is observed (arrows). (E-L) Cyan is DNA; red is mRNA; black and white panels show mRNA only. In all panels, anterior is to the left and dorsal is to the top.

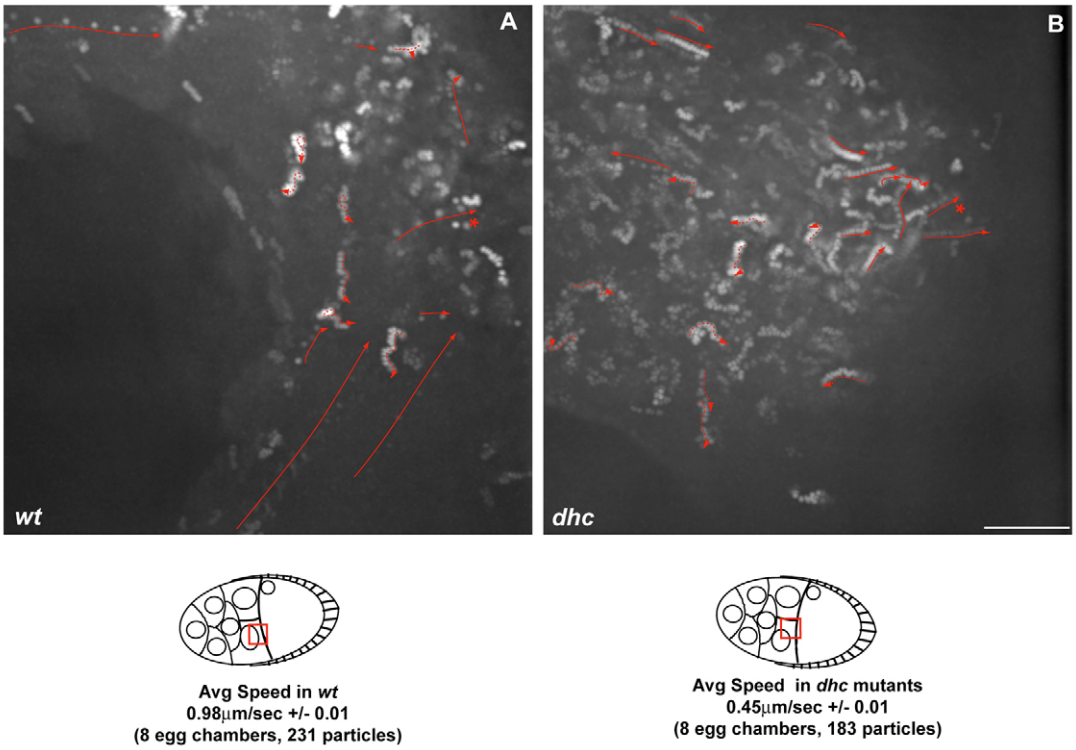


Fig. 6. Dynein is required for the efficient transport of *grk* RNA particles towards ring canals. (**A**,**B**) Trails of *grk* RNA injected into wild-type (A; wt) and *dhc* mutant (B) nurse cells. Schematics on the left represent the site of injection (red outline) and show the average speed±standard error of the mean, the number of egg chambers injected and the total number of particles tracked for analysis. Trails at 1 second intervals were superimposed (0.5 minute duration). Red arrows indicate the direction of RNA-particle movement; broken lines show those particles that seem to follow nurse cell cytoplasmic streaming, and were thus not tracked for analysis. A decrease in the speed of *grk* RNA particles in *dhc* mutants is observed by the differences in distance travelled (*) (also see Movie 6 in the supplementary material). Anterior is to the left and dorsal is to the top. Scale bar: 5 μm in B for A,B.

provided by cytoskeletal networks in the cell (Luby-Phelps, 2000). Moreover, diffusion of mRNA within a dense cytoplasm is very slow, because transcripts are decorated with many proteins to form very large ribonucleoprotein particles with a large hydration volume (Shav-Tal et al., 2004). It has therefore been proposed that all mRNAs, both localized and unlocalized, are associated with molecular motors to varying degrees to allow them to be rapidly distributed within the cytoplasm, and the presence of a localization signal increases the efficiency of association with the motors (Fusco et al., 2003) and perhaps even influences the activity of the motors and their co-factors (Bullock et al., 2003). Our study highlights that the presence of localization signals and recruitment of specific motor co-factors affects the choice of intracellular routes adopted by such cargo in the oocyte.

We were not able to determine the proportion of *grk* RNA particles that move compared with ones that were stationary because it is hard to distinguish stationary particles from autofluorescence. By contrast, rapidly moving RNA particles of the same intensity are easy to distinguish from background. By showing directly that Dynein is required for the transport of axis specification transcripts from the nurse cells to the oocyte, our work explains previous work on this topic that did not directly address the mechanism of transport from the nurse cells into the oocyte (see Introduction). Our results also explain why the movement of *bcd* and *osk* mRNA into the oocyte is MT dependent (Pokrywka and Stephenson, 1991), and why pair-rule transcripts, which are transported in the blastoderm embryo in a MT-dependent manner (Lall et al., 1999), by Dynein (Wilkie and Davis, 2001), are also transported into the oocyte when exogenously expressed in the nurse cells (Bullock and Ish-Horowicz, 2001). We suggest that nurse cell-to-oocyte transport is likely to be a fairly promiscuous transport system that can deliver any transcript that has the capacity for transport by the Dynein motor complex along MTs to their minus ends. It is therefore likely that the Dynein-dependent shortcut is deployed by many other transcripts that are localized in the oocyte during mid-oogenesis, such as *orb*, *K10* and nanos (*nos*). In fact, given that the oocyte nucleus is largely transcriptionally inactive, it is possible that up to 10% of all transcripts thought to be localized in the oocyte (Dubowy and Macdonald, 1998) could first be transported by the same Dynein-dependent mechanism into the oocyte.

The Dynein-dependent transport route we have uncovered within the nurse cells is likely to allow transcripts encoding axis specification determinants to be delivered rapidly at key times in oogenesis. In particular, cytoplasmic transport during stages 5-8 is likely to be relatively slow and non-specific, so delivery of transcripts from the nurse cell nuclei to the oocyte cytoplasm is likely to be very slow, if it involves an undirected diffusion-based process. Certainly, *osk* and *bcd* mRNA (Chekulaeva et al., 2006; Ferrandon et al., 1997; Tekotte and Davis, 2006; Wagner et al., 2001) and other transcripts are thought to form large multimeric complexes in the nurse cells, so are unlikely to be easily dispersed within the cytoplasm by free diffusion. *osk* and *grk* are transported into the oocyte at the same stages of oogenesis, and both require Bruno (also known as Arrest – FlyBase) (Castagnetti et al., 2000;

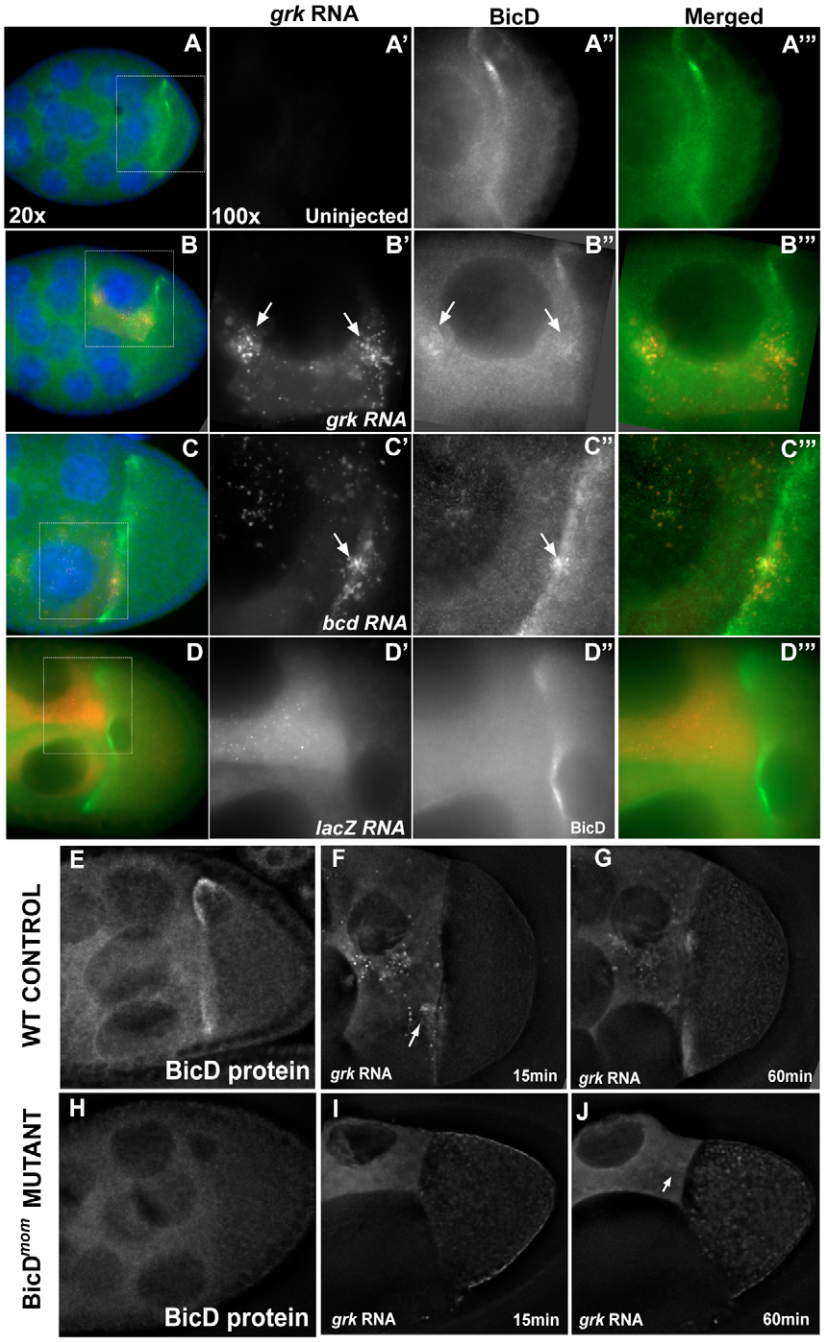


Fig. 7. BicD protein is recruited by *grk* and *bcd* RNA and is necessary for *grk* RNA accumulation at ring canals. (**A-D**) Stage 8-9 egg chambers injected with RNA (red); BicD protein (green) and DNA (blue) are also shown (acquired at 20×). Outlined region represents the area shown (acquired at 100×) in the panels to the right (RNA, BicD and merged). (**A-A‴**) Uninjected egg chamber showing the BicD localization pattern around the oocyte nucleus. (**B-C‴**) Injected *grk* RNA (B′) and *bcd* RNA (C′) colocalize (arrows) with BicD (B″ and C″, respectively). (**D-D‴**) Injected *lacZ* RNA forms particles that do not accumulate (D′) and that do not colocalize with BicD (D″). (**E**,**H**) Immunofluorescence showing BicD localization in wild-type controls (E) and *BicD*^{mom} (H) egg chambers. Notice the absence of BicD protein in *BicD*^{mom} egg chambers (H). (**F**,**G**,**I**,**J**) Wild-type control (F,G) and *BicD*^{mom} (I,J) egg chambers injected with *grk* RNA at 15 (F,I) and 60 (G,J) minutes after injection. In the absence (H-J) of BicD protein, *grk* RNA ring canal accumulation (arrow) and localization in the oocyte is strongly reduced. In all panels, anterior is to the left and dorsal is to the top (except D, dorsal side where the oocyte nucleus is).

Filardo and Ephrussi, 2003; Kim-Ha et al., 1995) and Hrp48 (also known as Hrb27C – FlyBase) (Goodrich et al., 2004; Huynh et al., 2004; Norvell et al., 2005; Yano et al., 2004); however, it is unclear whether they are transported within the same complexes into the oocyte. At stage 10B, the mechanism we have described is not required, because the rapid dumping of all of the cytoplasmic contents of the nurse cells into the oocyte occurs. However, by stage 10B, most of the major patterning transcripts have probably been localized in the oocyte.

Our work does not address directly the speed of passive diffusion of RNA into the oocyte or the mechanism of cytoplasmic flow and dumping in stage 10B. Although Kinesin 1 is required for cytoplasmic movements within the oocyte (Palacios and Johnston, 2002), it is not required for the general growth of the oocyte or for the presence of mRNAs in the oocyte (Brendza et al., 2000). These observations suggest that Kinesin 1 is not important for cytoplasmic transport or for specific mRNA transport into the oocyte.

The existence of a specific intracellular route for the transport of transcripts in nurse cells adds to existing evidence that there are various minus-end destinations to which different cargos are delivered by Dynein within the same cell. For example, within the oocyte, *bcd* RNA is transported to the cortex if injected into the oocyte, but to the anterior, after transport into the oocyte, following injection into the nurse cells (Cha et al., 2001). *grk* RNA is transported in two steps, both of which depend on Dynein. The second step is towards the oocyte nucleus, and is unique to *grk* and *I factor* RNA (Van De Bor et al., 2005), but is not shared with *bcd* and *K10* transcripts (MacDougall et al., 2003), despite the fact that

all of these transcripts are probably being transported by Dynein. There are, therefore, likely to be several distinct MT routes along which Dynein can transport cargos within egg chambers. In neurons, choices between distinct MT routes are made by Kinesin-dependent vesicle transport depending on the presence of a specific neurotransmitter-receptor-interacting protein, GRIP1 (Setou et al., 2002). How Dynein chooses between distinct MT networks is less clear, but could be based on distinct isoforms of the motor complex, on distinct kinds of MTs with different tubulin isoforms, or on their decoration with different MT-associated proteins. In addition, there is evidence that cargos can influence the behaviour of their motor (Bullock et al., 2003), raising the interesting possibility that cargos could also influence the choice of MT route adopted by their motors. Our work suggests that the presence of BicD and Egl could also influence the choice of MT route adopted by motors. Future work, including new approaches for co-visualizing MTs and RNAs in living cells, will be required to distinguish between all of these possible ways of selecting intracellular routes. Whatever the basis of such distinct routes, they are likely to exist for various kinds of molecular motors and to be functionally important for a wide range of tissues and cargos.

We thank Nina MacDougall for originally establishing the injection assay for *grk* RNA movement from the nurse cell to the oocyte. We are grateful to Simon Bullock, David Finnegan, Catherine Rabouille, Hildegard Tekotte and Georgia Vendra for their incisive comments on the manuscript. This work was supported by a Wellcome Trust Senior Research Fellowship (067413) to I.D., Edinburgh University Studentship to A.C. and a Marie Curie Postdoctoral Fellowship to C.M.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/10/1955/DC1

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Table S1. Statistics for RNA injections in wild-type nurse cells (20× lens)

RNA	Egg chamber stage	% nurse cell to oocyte transport (*n*)	% oocyte localization (*n*)
grk	6-7	100 (18)	67 (18)
	8-9	100 (30)	60 (30)
	10	100 (7)	43 (7)
bcd	8-9	100 (41)	76 (41)
K10	8-9	100 (21)	66 (21)
I factor	8-9	100 (14)	92 (14)
lacZ	6-7	25 (8)	0 (8)
	8-9	33 (18)	0 (18)
grk and Colcemid	8-9	0 (12)	0 (12)
bcd and Colcemid	8-9	0 (14)	0 (14)

Statistics show the percentage of injected egg chambers in which nurse cell to oocyte transport and oocyte localization for the different RNAs occurs. In brackets is the number of egg chambers injected.

Table S2. Statistics for RNA ring canal accumulation in living (20× lens) and fixed (biotin RNA) egg chambers

RNA	Egg chamber stage	% ring canal accumulation (*n*)
grk	6-7	44 (18)
	8-9	67 (30)
	10	57 (7)
bcd	8-9	66 (41)
K10	8-9	76 (21)
I factor	8-9	85 (14)
lacZ	6-7	0 (8)
	8-9	0 (18)
Biotin *grk*	8-9	88 (8)
Biotin *lacZ*	8-9	0 (12)

Statistics show the percentage of injected egg chambers in which different RNAs accumulate at the ring canals. Analysis was carried out for injection in wild-type living egg chambers, and fixed egg chambers stained with actin to verify ring canal accumulation (biotin RNAs). In brackets is the number of egg chambers injected.