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The centrosome-nucleus complex and microtubule organization in the *Drosophila* oocyte

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Molecular motors transport the axis-determining mRNAs *oskar*, *bicoid* and *gurken* along microtubules (MTs) in the *Drosophila* oocyte. However, it remains unclear how the underlying MT network is organized and how this transport takes place. We have identified a centriole-containing centrosome close to the oocyte nucleus. Remarkably, the centrosomal components, γ-tubulin and *Drosophila* pericentrin-like protein also strongly accumulate at the periphery of this nucleus. MT polymerization after cold-induced disassembly in wild type and in *gurken* mutants suggests that in the oocyte the centrosome-nucleus complex is an active center of MT polymerization. We further report that the MT network comprises two perpendicular MT subsets that undergo dynamic rearrangements during oogenesis. This MT reorganization parallels the successive steps in localization of *gurken* and *oskar* mRNAs. We propose that in addition to a highly polarized microtubule scaffold specified by the cortex oocyte, the repositioning of the nucleus and its tightly associated centrosome could control MT reorganization and, hence, oocyte polarization.

KEY WORDS: Microtubule organization, Nucleus, Centrosome, Polarity, Oocyte, Oogenesis, *Drosophila*

INTRODUCTION

The microtubule (MT) cytoskeleton of eukaryotic cells is involved in many cellular functions. MTs allow delivery of cellular components to specific intracellular locations by means of MT motors, but how MT arrays contribute to polarized transport remains a central question. The *Drosophila* oocyte provides an excellent model system to study polarized MT-dependent transport, because MTs control the localization of several transcripts encoding axis determinants (for a review, see Palacios and St Johnston, 2001). The ovarian egg chamber consists of a cluster of 16 interconnected germ cells, surrounded by a monolayer of follicular epithelium. During egg chamber formation, one cell of the cluster is singled out to become the oocyte, while its 15 sister cells develop into nurse cells. This process of oocyte specification requires polar transport of oocyte determinants mediated by minus-end directed MT motors (for a review, see Palacios and St Johnston, 2001). MT nucleation is believed to be restricted to the posterior of the oocyte from where MTs extend through the ring canals into the nurse cells. The MT organization in the oocyte is unchanged until mid-oogenesis at the onset of stage 7. Then, a signal emitted from the overlaying follicle cells triggers the reorganization of the MT network and the migration of the nucleus to the anterior. Subsequently, *bicoid* (*bcd*) mRNA is localized to the anterior cortex of the oocyte. *oskar* (*osk*) mRNA is transported to the posterior pole and *gurken* (*grk*) mRNA is localized to an antero-dorsal cap near the oocyte nucleus (reviewed by Riechmann and Ephrussi, 2001). The localization of these axes determining transcripts occurs in several steps and is dependent on MTs, cytoplasmic Dynein and Kinesin I. During the period crucial for mRNA localization (stage 7-9), MTs are distributed in an antero-posterior (AP) gradient, the highest concentration occurring in the anterior part of the oocyte (Theurkauf et al., 1993; Theurkauf et al., 1992; Clark et al., 1997; Micklem et al., 1997). The orientation of MTs within this gradient was determined using minus- and plus-end markers, i.e. Nod-βgal and Kinesin-βgal, respectively (Clark et al., 1994; Clark et al., 1997). However, the use of these markers was not sufficient to explain the successive MT-dependent localization steps of *osk* and *grk* mRNA. Furthermore, the behavior of the microtubule organizing center (MTOC) during oogenesis is complex, and the localization and nature of the MTOC during this period are unknown. Shortly after oocyte specification, nurse cell centrosomes migrate toward the oocyte, where they eventually accumulate at the posterior of the nucleus (Bolivar et al., 2001; Mahowald and Strassheim, 1970). Centrioles reportedly disappear, as they could not be detected after stage 4 (Mahowald and Strassheim, 1970), whereas the PCM is still associated with the oocyte nucleus, where γ-tubulin, Centrosomin and Pericentrin/AKAP450 can be easily detected (Martinez-Campos et al., 2004; Tavosanis and Gonzalez, 2003). From stage 1 to stage 6, MTs are nucleated from a posterior MTOC and at the end of stage 9, PCM components belonging to the γ-TuRC complex have been found at the anterior and lateral cortices (Cha et al., 2002; Schnorrer et al., 2002). However, during stages 7 to 8, the nature of the MTOC remains elusive.

In the present work we have studied the nucleation and the organization of the MT network in the oocyte in order to better understand mRNA transport. We show that the association of a centriole-containing centrosome with the nucleus surrounded by PCM material constitutes an MTOC. Using a new fixation method that allows detection of essential components of the MT network and MTOC, we found that the oocyte contains two different MT populations. The two MT populations evolve differentially during oogenesis, which could constitute a scaffold for differential mRNA transport.

MATERIALS AND METHODS

Fly stocks

w^{118} served as wild-type control and parental stock to generate transgenic flies. The grk^{2B6}, grk^{HK}, grk^{DC} alleles were used for mutant analysis (Neuman-Silberberg and Schuepbach, 1993). MT extremities were detected using either Kin-LacZ or Nod-LacZ (Clark et al., 1997). Germ line clones for *Khc* were generated using $Khc^{7.288}$ *FRT G13* (Januschke et al., 2002). *tubGAl4* is described in Januschke et al. (Januschke et al., 2002). MTs in

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living egg chambers were analyzed using polyubiquitin-GFPS65C-alphaTub84B (Rebollo et al., 2004) and Tau-GFP (Micklem et al., 1997). The last 199 amino acids of the C-terminus of CG6735 (Kawaguchi and Zheng, 2004) were amplified by PCR and cloned into the pUASpmGFP6 vector.

MT detection

MT detection was adapted from a method developed to preserve the cytoarchitecture in cells in which antibody penetration is reduced (Pizon et al., 2002). Ovaries were incubated in BRB80 buffer (80 mmol/l PIPES pH 6.8, 1 mmol/l $MgCl_2$, 1 mmol/l EGTA), containing 1% Triton X100 (BRB-80-T) for 1 hour at 25°C without agitation. Then ovaries were fixed in MeOH at –20°C for 15 minutes and rehydrated for 15 hours at 4°C in PBS 0.1% Tween, then blocked for 1 hour in PBS 0.1% Tween containing 2% (w/v) bovine serum albumin (BSA) before incubation with primary antibody overnight.

Immunohistochemistry and in-situ hybridization

Rabbit Anti-Khc AKIN02-A (Cytoskeleton, Denver, USA) 1:250, monoclonal anti-α-tubulin (DM1A) FITC Conjugate (SIGMA) 1:250, rat Anti-tyrosinated Tubulin YL 1/2 1:200 (Kilmartin et al., 1982), mouse anti-polyglutamylated MTs on centrosomes, ID5 (Beisson et al., 2001). Rabbit anti-γ-tubulin: R46 [γTub37C (Raynaud-Messina et al., 2001)], 1:500 dilution, Rb1011 [γTub37C (Tavosanis and Gonzalez, 2003)] 1:5, R77 [γTub23C (Debec et al., 1995)] 1:500. Mouse anti-β-galactosidase (Promega), 1:200. Rabbit anti-PKCz C20 (Santa Cruz Biotechnology, Inc.), 1:1,000. Anti-Osk and anti-Grk as described in (Januschke et al., 2002). PicoGreen (Molecular Probes) 3 μl/1 ml PBS 0.1% Tween for 45 minutes following RNAse A treatment 0.4 μg/ml for 1 hour before staining. In-situ hybridization was performed using *grk*- and *osk*-specific probes according to Wilkie and Davis (Wilkie and Davis, 2001). Images were taken on a LEICA SP2 AOBS microscope (40× 1.25 NA oil), except for Fig. 2C,D,F, Fig. 3C,E, Fig. 4F-H and Fig. 5E-H, which were taken on a Perkin Elmer Ultra View confocal scanner. Deconvolution was carried out using the HUYGENS 2.6 software and the QMLE algorithm with seven iterations and 3D reconstruction using the AMIRA 2.2 software. Living oocytes were dissected in BRB buffer or M3 insect medium (Sigma). Electron microscopy protocol is available upon request.

MT disassembly and regrowth

Complete depolymerization: ovaries were incubated for 30 minutes in BRB-80-T at 25°C then placed on ice for 30 minutes and fixed. Complete regrowth: ovaries were incubated for 30 minutes in BRB-80-T on ice, then incubated at 25°C for 30 minutes and subsequently fixed. Partial regrowth: ovaries were incubated in BRB-80-T at 25°C for 27, 25, 20 or 15 minutes, placed on ice for 30 minutes and then transferred to 25°C for 3, 5, 10 or 15 minutes, respectively, then fixed and stained for MTs. Colchicine treatment: flies were fed for 15 hours with colchicine (65 μg/ml) and analyzed for MTs.

RESULTS

A centrosome localizes close to the oocyte nucleus during mid-oogenesis

Both the nature and the localization of the MTOC beyond stage 6 of *Drosophila* oogenesis have not yet been clarified. Up to stage 6, γ-tubulin has been shown to closely associate with the nucleus at the posterior of the oocyte. In addition, electron microscopy studies have demonstrated the presence of centrioles close to the oocyte nucleus up to stage 4 (Mahowald and Strassheim, 1970). Thus, until stage 6, the centrosome associates with the nucleus at the posterior of the oocyte. In *Drosophila* females, meiosis takes place in the absence of centrosomes (Mahowald and Strassheim, 1970). It has therefore been speculated that, at stage 6, centrosome organization changes, involving the disappearance of centrioles and the generation of MTs from a diffuse organizing center. To better understand this process, we re-investigated the distribution of γ-tubulin in the oocyte. Before repolarization of the MT cytoskeleton, we found that γTub23C and γTub37C (Sampaio et al., 2001; Sunkel et al., 1995) localize in a layer around the nucleus, with an enrichment at the posterior pole of the oocyte (Fig. 1A). This is in agreement with the location of the MTOC at this stage. After repolarization of the MT cytoskeleton, both γ-tubulin isoforms remain located in a perinuclear manner (Fig. 1B,C). Interestingly, γTub37C, but not γTub23C, labels a small body close to the oocyte nucleus (Fig. 1, compare A,C). In addition, γTub37C and γTub23C also exhibit differential expression patterns in embryos: γTub37C is located with the centrosomes of mitotic cells, whereas γTub23C is not (Tavosanis and Gonzalez, 2003). Thus, γ-tubulin is distributed in close association with the nucleus periphery and possibly on a centrosome-like structure. Pericentrin/AKAP450 is another major component of the centrosome. Green fluorescence protein (GFP) fusion of the C-terminal part of Pericentrin/AKAP450 and its *Drosophila* homolog pericentrin-like protein (D-PLP) have been shown to localize to the centrosomes respectively in cultured human cells (Keryer et al., 2003), *Drosophila* embryos and spermatocytes (Martinez-Campos et al., 2004; Rebollo et al., 2004). Using the UAS/Gal4 system (Brand and Perrimon, 1993), we specifically expressed GFP-cter-D-PLP, in the germline and detected a bright dot in the vicinity of the nucleus before and after nuclear migration (Fig. 1D). GFP-cter-D-PLP was also detected in all germline nuclei, as has been observed previously (Martinez-Campos et al., 2004). From stage 7 onward, the bright dot remained in the immediate vicinity of the oocyte nucleus (<1 μm distance, Table 1). Furthermore, both GFP-cter-D-PLP and γTub37C co-localized to this discrete body, indicating that this structure could correspond to a centrosome (Fig. 1E-G). In G2 centriole, tubulin is highly polyglutamylated (Bobinnec et al., 1998). The ID5 antibody labels basal bodies and centrioles in several species (Beisson et al., 2001). Using this antibody, we detected a dot close to the nucleus throughout oogenesis that remained detectable up to stage 10A (Fig. 1H). This suggests that the dot represents a centriole-containing centrosome. Indeed, using electron microscopy, (Fig. 1I,J), we clearly detected two to possibly four centrioles closely associated with the nucleus in stage 9 oocytes. This demonstrates the existence of centrioles associated with the nucleus at least up to stage 9. We could not unambiguously detect MT fibers emanating from those centrioles. We then analyzed the link between centrosome and nucleus using colchicine. In flies fed with colchicine, MTs in the germline were completely depolymerized, and the oocyte nucleus was mispositioned (Fig. 1L). In the oocyte, we observed that the nucleus and the centrosome were significantly separated, the distance between them increasing during oocyte growth (Fig. 1M,N and Table 1). In a few cases, we noticed that the nucleus could reach the anterior cortex without the centrosome; however, we never observed a centrosome at the anterior without the nucleus (Table 1). We conclude that the close localization of the centriole-containing centrosome to the nucleus depends on MTs.

Two differently orientated MT subsets are present in the oocyte

The structure of the MT network during mid-oogenesis is dynamic. At stage 7, MTs are visible as a mesh at the anterior cortex. Later, at stage 10, MT bundles are observed that promote cytoplasmic streaming (Theurkauf et al., 1993; Theurkauf et al., 1992).

In-between MT distribution has been described as an AP gradient (Micklem et al., 1997). However, high-resolution images of oocyte MTs are lacking. Therefore, we modified for the *Drosophila* egg chamber a protocol frequently used to increase the detection of the MT cytoskeleton in cell culture to characterize MT organization in the oocyte during the crucial period in which *bcd*, *grk* and *osk* mRNAs are localized. We detected MTs throughout oogenesis using

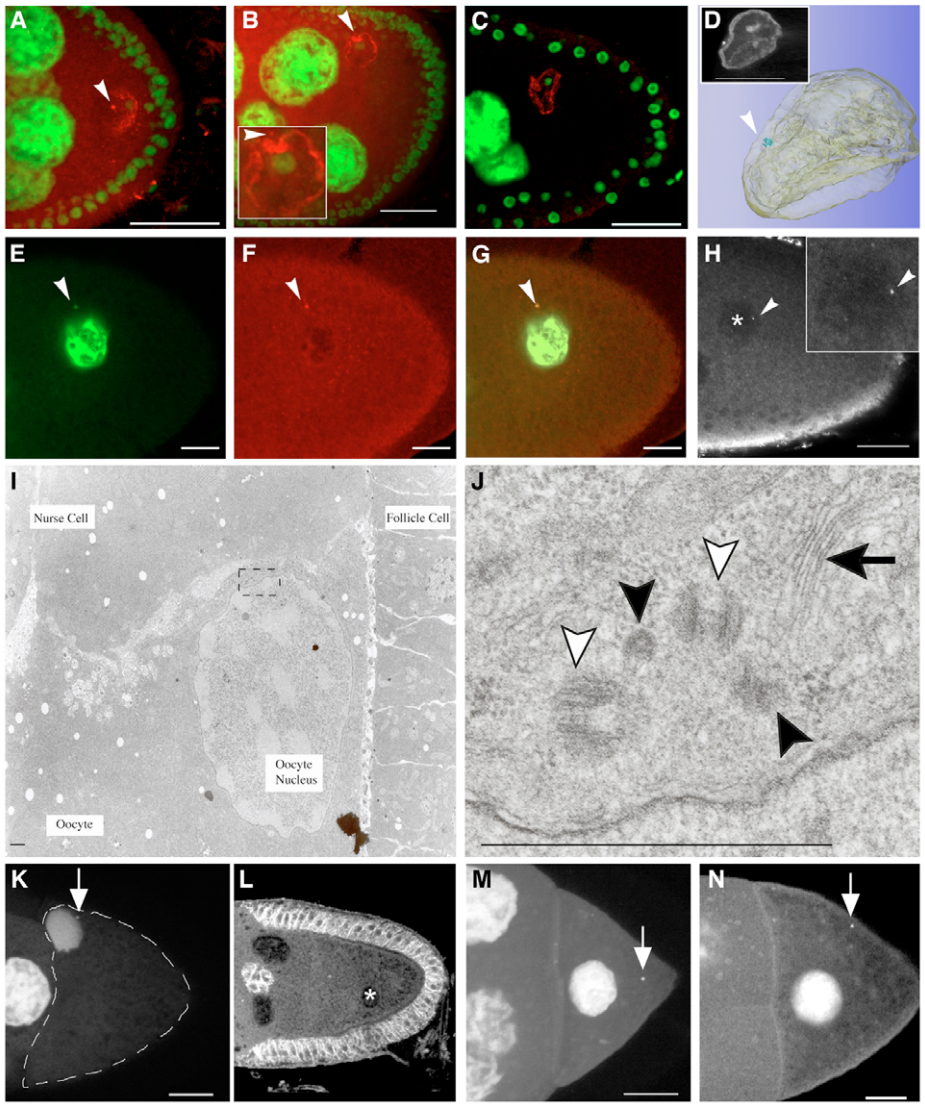

Fig. 1. MTOC material and centrioles localize to the oocyte nucleus during mid-oogenesis. In stage 6 (A) and stage 8 (B), γTub37C, (Rb1011, red) is detected around the oocyte nucleus and as a dot close to it (arrowheads in A,B). (**A-C**) DNA green. (C) γTub23C (R77, red) is detected around the oocyte nucleus at stage 8. A similar distribution is observed at stage 6 (data not shown). (**D-G**) Egg chambers expressing GFP-cter-D-PLP. (D) 3D reconstruction of the oocyte nucleus. D-PLP is detected as a dot (light blue, arrowhead) on the surface of the nucleus (inset, original data). (E-G) D-PLP (single view in E, green) and γTub37C (single view in F, red) co-localize (G) during stage 8 to the same dot close to the nucleus (arrowheads). (**H**) Polyglutamylated MTs (ID5 antibody) are detected as a dot in the vicinity of the nucleus during stage 9 (arrowhead, inset magnification view). (**I**,**J**) Electron micrographs of the anterior dorsal corner of the oocyte; anterior is to the top, dorsal to the right. (I) Low power magnification of the area around the oocyte nucleus. The centrioles are found in the boxed area. (J) At high magnification, two centrioles (white arrowheads) are visible, most probably there are two more (black arrowheads). Golgi cisternae are in the vicinity of the centrioles (arrow). (**K**) Egg chamber expressing D-PLP. The centrosome is found close to the nucleus (arrow; oocyte outline indicated with dashed line). (**L-N**) Flies fed with colchicine. (L) The nucleus is misplaced and anti-Khc staining revealed MTs only in the follicle cells. The centrioles (arrows) are separated from the oocyte nucleus in a stage 8 (M) or stage 9 (N) oocyte expressing D-PLP. Asterisk, oocyte nucleus. Scale bars: 20 μm in A-H,K-N; 1 μm in I,J.

α-Tubulin but also with a Kinesin heavy chain antibody (α-Khc), which revealed the MT array and its complexity in unprecedented definition (Fig. 2A,B). We noticed that the range of detected details was increased and more reproducible with α-Khc antibody. To control the specificity of Khc detection, we generated germline and follicle cell mutant clones homozygous for $khc^{7.288}$. In such mutant cells, no Khc was detected (Fig. 2B inset), indicating that the detection is specific. Labeling with antibodies directed against aromatic C-terminal amino acid residues (Tyr or Phe) of α-tubulin (Badin-Larcon et al., 2004) and against Khc largely overlapped (Fig. 2I,J). This confirmed that the structures revealed by Khc were MTs. We also detected a Khc fraction at the posterior of the oocyte (Fig. 2J), as it has been previously shown (Brendza et al., 2002). That Khc accumulates along MTs may be due to permeabilization before fixation, which could cause rigor binding of Khc to MTs. Such case has been observed for the motor XKCM1 without affecting MT organization itself (Walczak et al., 1996). This detection procedure may also permit the extraction of a soluble pool of Khc and reveal the remaining fraction distributed along the MTs (Hollenbeck, 1989). With our detection procedure, Khc revealed by Kinesin-βgal (Clark et al., 1994) exhibited a more restricted distribution compared with α-Khc antibody (Fig. 3H,I). This is probably due to the substitution of the C-terminal part of Khc by the β-galactosidase in the reporter construct, impairing the recycling of the chimeric Kinesin motor leading to its accumulation exclusively at the posterior. We also show that with our detection method, the MT minus-end marker, Nod-βgal, was detected in the antero-dorsal corner above the oocyte nucleus as well as in the opposite antero-ventral corner (Fig. 3J,K), as it has been reported before (Clark et al., 1997; MacDougall et al., 2003). Moreover, localized determinants such as Osk and Grk were correctly positioned in the oocyte (Fig. 3L,M).

To confirm that our detection method does not alter MT organization, we analyzed MT distribution in follicle cells, which should be sensitive to the extraction procedure, as they are more directly exposed than the oocyte (Fig. 3A-E). MT distribution in different follicle cell types was unchanged, when comparing living and fixed egg chambers. The main body follicle cell MTs seemed unchanged (Fig. 3D,E). Main body follicle cell MTs have been shown to be highly stable, and might therefore reflect the sensitivity of the protocol with limitations (Doerflinger et al., 2003). Nevertheless, stretched follicle cells showed strikingly similar MT patterns in living and fixed conditions as well (Fig. 3B,C). Apical-basal polarity was not affected in follicle cells, as demonstrated by the correct apical localization of atypical protein kinase C (Fig. 3F,G). Importantly, the MT distribution of living egg chambers

Table 1. Analysis of distance from centrosome to nucleus

(A) Distance of centrosome to nucleus in GFP-cter-AKAP450 expressing oocytes in µm ± s.e.m. (*n*)

	Stage 5/6	Stage 7	Stage 8	Stage 9	Stage 10
Wild type	0.84±0.41 (5)	0.19±0.14 (6)	0.28±0.13 (10)	0.32±0.21 (16)	<0.1 (5)
Colchicine*	N.D.†	3.58±1.21 (5)	11.59±1.79 (20)	32.56±5.98 (14)	40.88±7.18 (5)

(B) Position of the centrosome in the oocyte after feeding GFP-cter-AKAP450 expressing flies with colchicine

	‡	§	¶	**	††	‡‡
Cases	109	25	24	3	3	0

*Flies were fed with colchicines.
†Not determined.
‡Nucleus random, centrosome random.
§Nucleus wild-type, centrosome at the posterior.
¶Wild type.
**Nucleus random, centrosome close.
††Nucleus wild type, centrosome at the anterior.
‡‡Nucleus random, centrosome wild type.

expressing GFP-α-Tubulin at stage 7 (Fig. 2C) and stage 9 (Fig. 2F) was similar to the one observed using anti-α-Tubulin and Khc antibodies (Fig. 2D,E,G,H). Therefore it seems that our fixation conditions preserve the wild-type MT organization and that Khc can be suitable to label bulk MTs.

When fixed wild-type oocytes were analyzed by confocal microscopy, MT organization in the oocyte appeared unchanged from stage 2 (data not shown) to stage 6 (Fig. 4B, parts 1-5). With stage 7 (~60%, *n*=11), MT organization was modified and two MT subsets became apparent. This organization was more evident at stage 8 (~69%, *n*=23). A first subset consisted of cortical MTs oriented along the dorso-ventral (DV) axis parallel to the oocyte nurse cell border, and juxtaposed to the lateral cortices, wrapping the oocyte from stage 7 to 9 (Fig. 4C-E, parts 1 and 5). At least some MT bundles of this subset could be traced back to the oocyte nucleus. The DV orientation of MT bundles, depicted as black fibers in the schematic representations (Fig. 4C, part 6; Fig. 4D, part 6; Fig. 4E, part 6), was highly reproducible for all stages and persisted throughout mid-oogenesis.

A second MT subset, depicted as red fibers in the schematic representations (Fig. 4C, part 6; Fig. 4D, part 6; Fig. 4E, part 6), was present in the center of the oocyte. Although there was some variability in the patterns observed, we found that each developmental stage showed a characteristic MT distribution. During stage 6 (~82%, *n*=17), MTs from this subset were cortical and extended from the nucleus at the posterior to the anterior cortex (Fig. 4B, parts 2-4). At stage 7 (~90%, *n*=11), compact bundles of MTs formed a circle-like structure resembling a diaphragm. This subset was formed by long MT bundles that extended (once or more) along the entire cortex (Fig. 4C, parts 2-

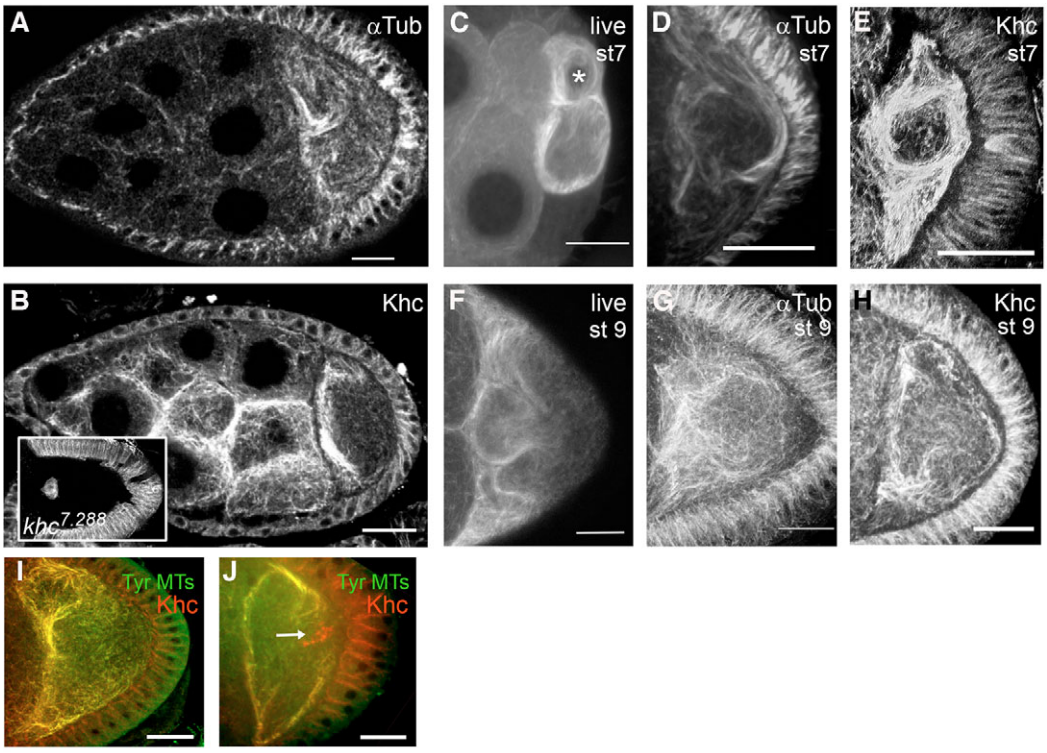

Fig. 2. Comparative analysis of MTs in living and fixed egg chambers. MTs in fixed stage-8 egg chambers detected with anti-α-Tubulin-FITC (**A**) and anti-Khc (**B**) (inset in B specificity of the anti-Khc antibody, a *Khc*$^{7.288}$ mutant germline clone shows no anti-Khc staining in the oocyte). (**C-E**) Stage 7 egg chambers; (**F-H**) stage 9 egg chambers. (C,F) Living egg chambers expressing GFP-α-Tubulin. (D,G) Fixed egg chambers stained with anti-α-Tubulin-FITC. (E,H) Fixed egg chambers stained with anti-Khc. (**I**,**J**) Stage 9 egg chambers, Khc (red) and aromatic c-ter amino acid of αTub (green). The arrow in J points to the posterior Khc pool.

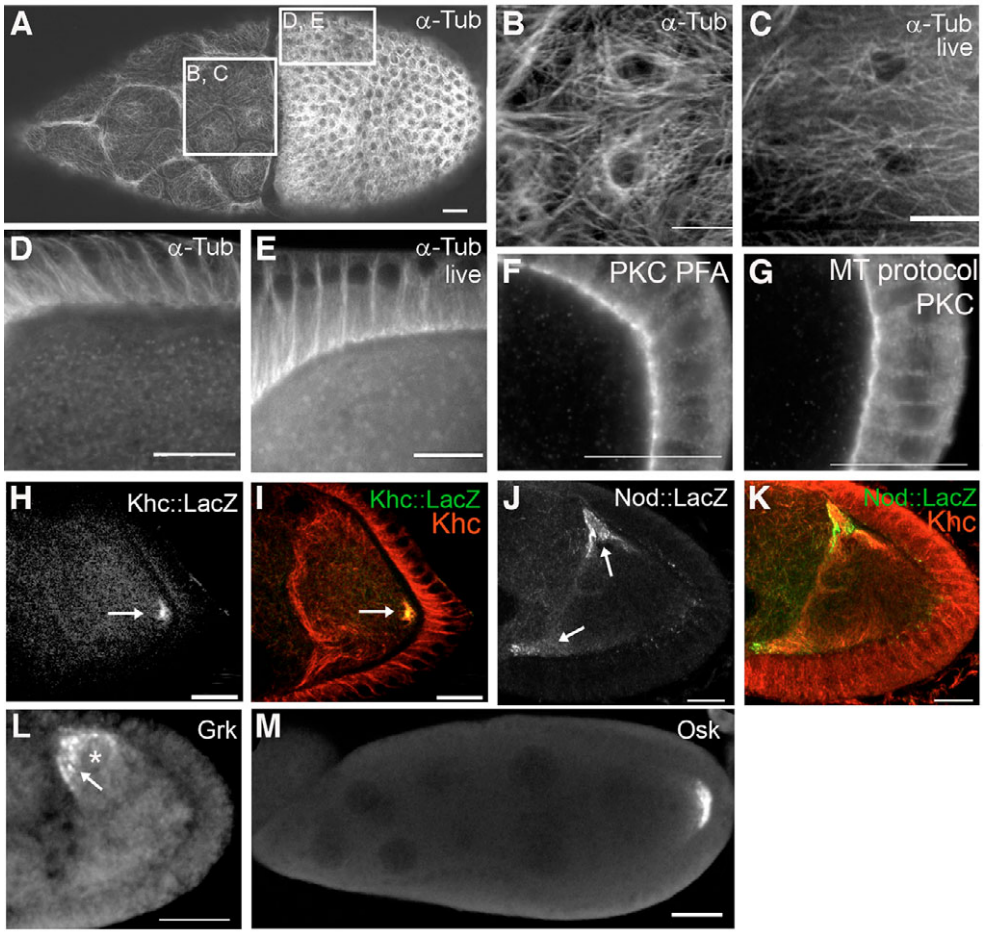

Fig. 3. Extraction procedure is not affecting egg chamber polarity. (**A**) Surface view of a fixed stage-10 egg chamber stained for anti-α-Tubulin-FITC. (**B**,**C**) MTs in stretched follicle cells revealed by anti-α-Tubulin-FITC in fixed egg chamber (B) and by GFP-α-Tubulin in living egg chamber (C). MTs in main body follicle cells revealed by anti-α-Tubulin-FITC in fixed egg chamber (**D**) and by GFP-α-Tubulin in living egg chamber (**E**). (**F**,**G**) Atypical protein kinase C. Posterior follicle cells fixed after a standard procedure using 4% paraformaldehyde (F) or after the extraction protocol (G). (**H**,**I**) Fixed oocyte expressing Kin::LacZ, stained for anti-Khc (red) and anti-β-Gal (green) (arrow, posterior fraction of Khc). (**J**,**K**) Fixed oocyte expressing Nod::LacZ, stained for anti-Khc (red) and anti-β-Gal (green) (Arrows, anterior distribution of Nod). Grk (**L**) and Osk (**M**) protein after the extraction protocol. Scale bar: 20 μm.

4). By stage 8 (~65%, *n*=23), the oocyte had considerably grown and individual MT bundles were therefore easier to track. MT bundles emanated from the anterior and the nucleus to point toward the posterior (Fig. 4D, part 2). MTs extended again along the entire cortex, after which they turned to the central cytoplasm (Fig. 4D, part 3). This, in turn, generated free MT (plus) ends in the center of the oocyte (Fig. 4D, part 4). By stage 9 (~72%, *n*=18), the central MT network was clearly oriented along the oocyte AP axis. One or two thick MT bundles extended from the anterior, pointing toward the posterior pole. These bundles formed a structure resembling a horseshoe, with its open side facing the posterior (Fig. 4E, parts 2-4). Importantly, both subsets could also be detected in living egg chambers, as shown for the DV subset (Fig. 4F,H) and the AP subset (Fig. 4G). Thus, MTs show strong rearrangements throughout mid-oogenesis, which results in two perpendicular MT arrays reflecting the two axes of the oocyte.

The centrosome-nucleus complex is an active center for MT nucleation in the oocyte

We developed an ex-vivo assay to localize MT nucleation sites by dissecting ovaries and placing them on ice for 30 minutes. This treatment resulted in complete depolymerization of MTs (Fig. 5B). When allowed to recover at 25°C for 30 minutes, MT distribution could be re-established to the wild-type situation, in which both the cortical and the central subsets of MTs were detectable (Fig. 5I,J). γ-Tubulin distribution was not affected by cold-induced MT depolymerization (Fig. 5C). When short periods of regrowth were analyzed, MT nucleation appeared limited to the close vicinity of the nucleus and was often asymmetric (Fig. 5D,E), suggesting a centrosome-associated nucleation activity. MT regrowth appeared to be stepwise, as after 15 minutes only the DV cortical subset was established. MTs clustered around the oocyte nucleus and aligned along the cortex in the DV direction (Fig. 5F,G). The cortical location of these fibers was clearly revealed by the presence of Khc-positive dots at either the dorsal or the ventral side (Fig. 5H). This indicates that the DV MT subset is the first to regrow. We repeated the regrowth experiment using colchicine. After the drug was washed out, MT repolymerization was observed at the oocyte nucleus (data not shown). Taken together, these results indicate that, at least with our detection method, the oocyte nucleus and its immediate surroundings have the capacity to nucleate MTs.

The MT network is inverted in *grk* mutants

To test whether the centrosome-nucleus complex could direct the repolarization of the MT network, we analyzed how MTs distribute in *grk* mutant oocytes. In this mutant, the nucleus frequently remains at the posterior of the oocyte due to a failure in the signaling cascade that induces the repolarization of the cytoskeleton (Gonzalez-Reyes et al., 1995; Roth et al., 1995). In *grk* mutant oocytes similar in size to wild-type stage 8, the MT distribution was dramatically affected. Specifically, we found that MT organization appeared completely reversed compared with wild type, in which the nucleus is at the anterior and MT plus-ends are located toward the posterior at stage 8 (Fig. 6A,B versus Fig.

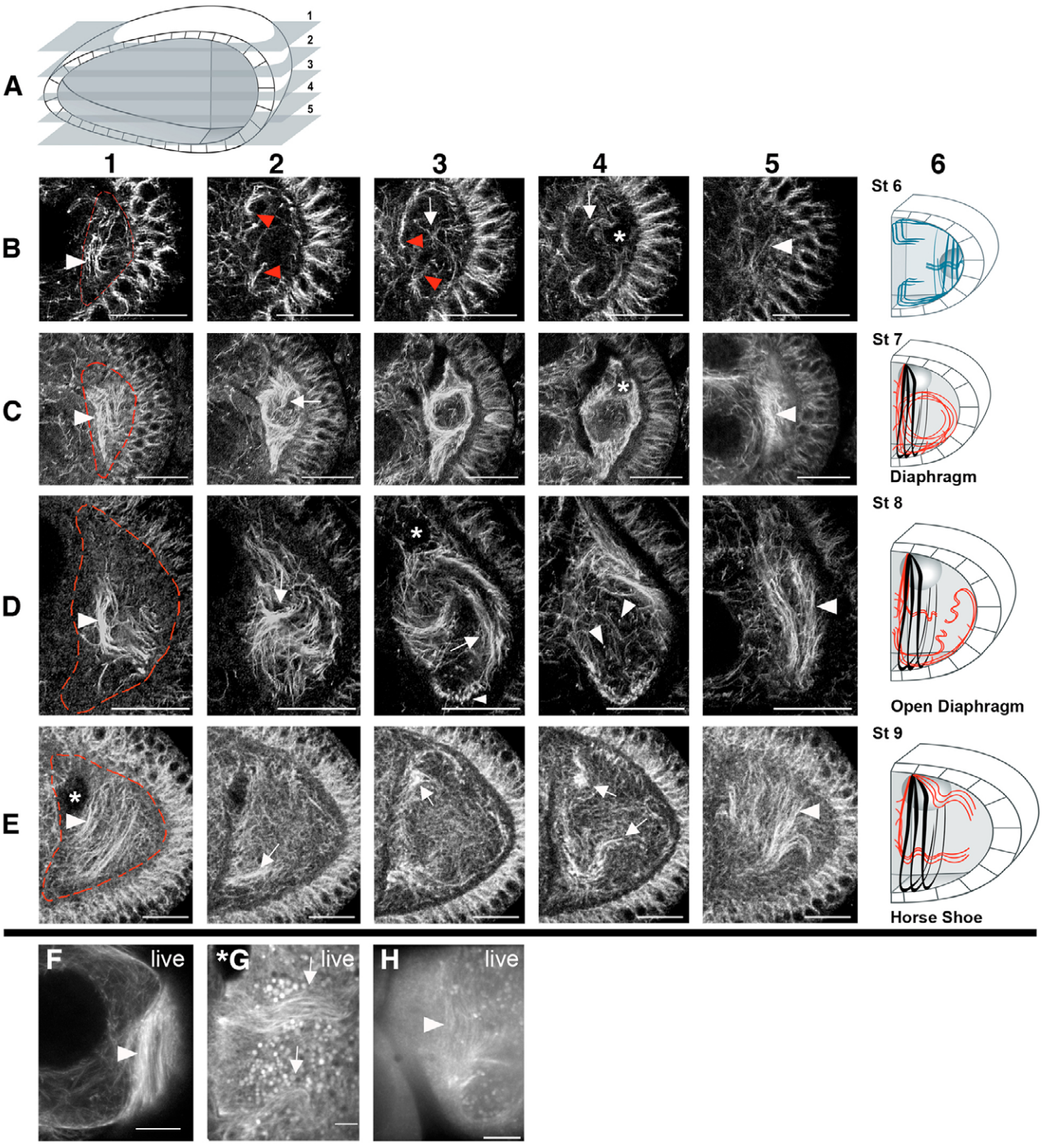

Fig. 4. See next page for legend.

6E,F). In slightly older oocytes, MTs remain stretched out along the cortex from the posterior toward the anterior, where they fold back to the center of the oocyte (Fig. 6B). MT ends in the center are most probably plus-ends, as the pool of Khc (localized at the posterior of wild-type oocytes, Fig. 3I) co-localizes with Kinesin-βGal to the center of the oocyte, between the flanking MT ends (Fig. 6D,G) (Clark et al., 1994). Interestingly, MT distribution in *grk* oocytes was strikingly similar to MTs of wild-type egg chambers before the migration of the oocyte nucleus (Fig. 6E,F). Likewise, the centrosome, as revealed by γ-tubulin, which is found at the posterior of stage 6 wild-type oocytes, stays at the posterior in *grk* mutants (Fig. 6H). Thus, in *grk* mutants, distribution of MT and MTOC seemed similar to their distribution in wild-type stage 6.

Fig. 4. Two different MT subsets coexist inside the oocyte. Optical planes from confocal Z-sectioning of oocytes stained with anti-Khc according to the sectioning experimental setup presented in **A**. The numbers 1-5 represent the position of representative optical sections in a wild-type oocyte (1 represents the most proximal plane close to the cortex, and 5 the most distal plane). (B) Stage 6, (C) stage 7, (D) stage 8 and (E) stage 9 oocytes. Two MT subsets can be distinguished according to orientation and stage-dependent changes. The DV subset: throughout oogenesis, cortical MTs oriented along the DV axis could be observed. At the proximal and distal cortices, MTs running parallel to the DV axis can be seen from stage 7 on (C1, C5; D1, D5; E1, E5; white arrowheads) and to a lesser extent in stage 6 (B1, B5; white arrowheads). The AP subset: (**B**) Before oocyte repolarization at stage 6, MTs extend along the cortex and fold back slightly at the anterior (B2 and B3, red arrowheads). The nucleus is encapsulated by MTs and short fibers point from the nucleus toward the anterior (B3 and B4, arrows). (**C**) By stage 7, long MTs are rolled up in the shape of a 'diaphragm' in the center of the oocyte (C2-C4). MT bundles emanating from the perinuclear region project toward the cortex (C2, arrow). The center of the cytoplasm contains only a few MT fibers. (**D**) In stage 8, the 'diaphragm' has opened and MT bundles project from the anterior in the AP direction (D2, arrow). MTs bundle at the posterior and join the center (D3, arrow). Transverse sections of MTs appear as dots on the ventral side of the oocyte (D3, arrowhead). Free MT ends are found in the center of the oocyte (D4, arrowheads). (**E**) In stage 9, the central MTs originate from the anterior and the oocyte nucleus (E2 and E3, arrows). One or two thick bundles that resemble a 'horseshoe'-like pattern orient in the AP direction (E4, arrows). (B6,C6,D6,E6) Schematic representations of the MT distribution at mid-oogenesis. (B6) MT distribution before repolarization is shown in blue. After repolarization, the invariant DV subset is depicted in black, the dynamic AP subset in red. (C6) Stage 7 ('diaphragm' state). (D6) Stage 8 ('open diaphragm' state). (E6) Stage 9 ('horseshoe' state). (**F-H**) Living egg chambers expressing Tau-GFP. (F) The cortical DV (arrowhead) subset can be detected in living stage 8 oocyte. (G) Stage 10A oocyte, in which two MT bundles in AP orientation can be seen in the center (arrows). (H) Stage 11: thin bundles of MTs can be detected in the DV orientation (arrowhead) close to the cortex. Red dotted lines mark the outline of the oocyte. Asterisk, location of oocyte nucleus. Scale bar: 20 μm.

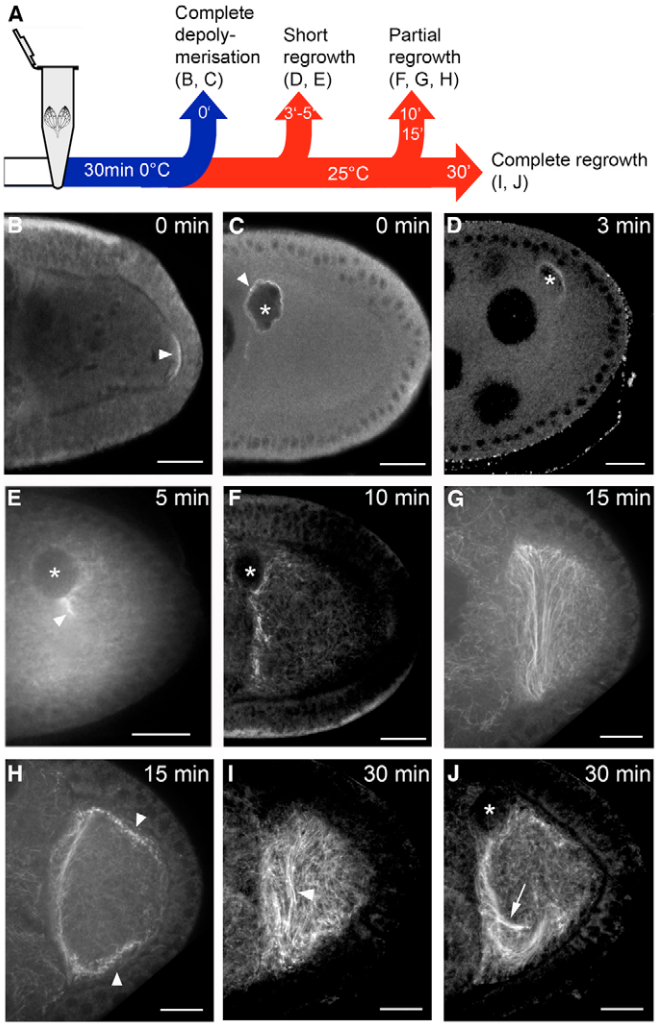

Fig. 5. The nucleus is an active center for MT polymerization. (**A**) Schematic representation of the experimental setup for cold-induced MT disassembly. Complete depolymerization: dissected ovaries were extracted (30 minutes), placed on ice (30 minutes), fixed and analyzed for MTs by anti-Khc. To allow MT regrowth, ovaries were incubated at 25°C for 3-30 minutes. (**B**) Stage 9 oocyte, complete MT depolymerization. Khc was only detectable at the posterior (arrowhead). (**C**) Cold-shock treatment did not alter γTub37C localization (arrowhead). (**D**,**E**) Centers of MT polymerization (arrowhead) were detected in the vicinity of the oocyte nucleus, never at the posterior or at cortical regions after short recovery times. (**F**) MTs appear along the anterior margin after 10 minutes regrowth. (**G**) MT regrowth along the cortex in the DV orientation during intermediate recovery times. (**H**) Optical section taken in the center of the same oocyte as in G. Clouds of Khc positive dots at the ventral and dorsal sides (arrowheads) represent transverse cuts of the cortical MTs. The AP subset in the center was not yet established. (**I**,**J**) Optical section of the same oocyte. Thirty minutes recovery at 25°C was sufficient to regrow MTs to the control situation. Cortical DV subset (I, arrowhead) and, in the center (J), MTs in the AP direction (arrow). Asterisk, oocyte nucleus. Scale bar: 20 μm.

grk mutant oocytes, having mispositioned nuclei, provide an ideal basis to test the MT nucleating capacity of the centrosome-nucleus complex using the cold-shock assay. After cold-shock treatment of *grk* oocytes, we checked for complete MT depolymerization (Fig. 6I). As in the wild type, during the initial period of recovery at 25°C, MT polymerization took only place in the immediate vicinity of the mispositioned oocyte nucleus (Fig. 6J). Therefore, as in wild-type oocytes, MT nucleation is often asymmetric and restricted to the area surrounding the nucleus. This result strengthens the possibility that the centrosome-nucleus complex is an active MTOC.

DISCUSSION

In polarized cells, the MT network is oriented to allow the delivery of cellular components to specific locations. Here we show that in the *Drosophila* oocyte a centriole-containing centrosome is present in close association with the nucleus, which itself is covered by PCM components until late in oogenesis. In addition, we show that MTs can nucleate from this centrosome-nucleus complex. The MTs appear to form two orthogonal MT populations that develop through several steps during mid-oogenesis. We propose that the migration of the nucleus in the oocyte could control the reorganization of the MT network.

The centrosome and the nucleus could constitute an MTOC in the oocyte

In region 2 of the germarium, nurse cell centrosomes migrate toward the oocyte. Later, in region 3, these centriole-containing centrosomes become located as an aggregate between the oocyte

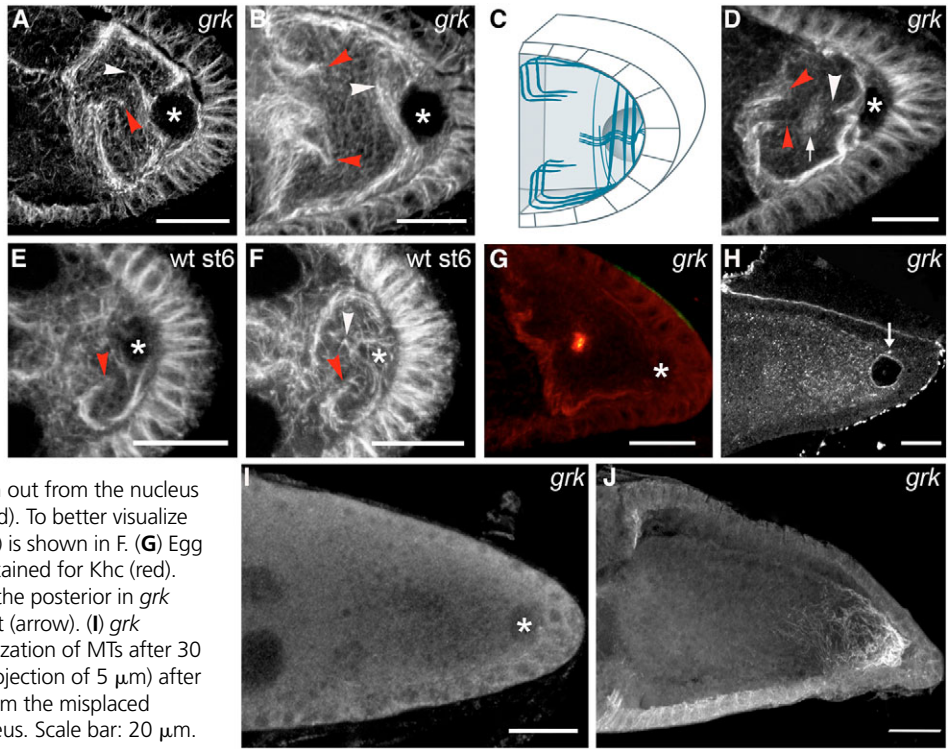

Fig. 6. *grk* mutant oocytes conserve the MT configuration of wild-type stage 6 oocytes. (**A**,**B**) *grk* mutant egg chambers stained for anti-Khc. In oocytes comparable to wild-type stage 8 (A) and stage 9 (B), the nucleus is misplaced to the posterior and surrounded by MTs. (**C**) Schematic representation of the MT network in *grk* oocytes. (**D**) *grk* stage 9 oocyte showing the accumulation of Khc (arrow) between the MT extremities of the long cortical MTs (red arrowheads) and the short central MTs (white arrowhead). (**E**,**F**) Wild-type stage 6 oocytes showing a similar MT distribution. MT bundles project from the nucleus at the posterior along the cortex to the anterior, where they bend back to the center (red arrowheads). Short bundles stretch out from the nucleus into the center of the oocyte (white arrowhead). To better visualize MTs, a projection of 10 optical sections (4 μm) is shown in F. (**G**) Egg chamber expressing Kinesin:βGal (green) co-stained for Khc (red). (**H**) γTub37C (R46) localizes to the nucleus at the posterior in *grk* mutant oocytes and the centrosome close to it (arrow). (**I**) *grk* mutant oocyte, showing complete depolymerization of MTs after 30 minutes cold-shock. (**J**) *grk* mutant oocyte (projection of 5 μm) after a 15-minute recovery at 25°C. MTs regrow from the misplaced nucleus at the posterior. Asterisk, oocyte nucleus. Scale bar: 20 μm.

nucleus and the follicle cell border (Mahowald and Strassheim, 1970; Bolivar et al., 2001). Pericentriolar material closely associated with the oocyte nucleus could be clearly detected until stage 6 with several centrosomal markers, such as γ-tubulin, Centrosomin and D-Tacc (Martinez-Campos et al., 2004; Tavosanis and Gonzalez, 2003). From stage 4 onward, the fate of the centriole cluster had been unknown (Mahowald and Strassheim, 1970). In this study, we show that both γTub37C and γTub23C are localized in a perinuclear manner throughout oogenesis. γTub37C highlights a discrete body close to the nucleus. This body is similarly detected by the centrosomal marker D-PLP and by a specific antibody for polyglutamylated Tubulin, which detects centrioles. Consistent with this, we have detected two to possibly four centrioles in the immediate vicinity of the nucleus in stage 9 oocytes. This result demonstrates that at least until stage 9, a centriole-containing centrosome is present in the oocyte. Currently, we do not know whether they are still present at the onset of meiosis I during stage 13, as it has previously been proposed that the meiotic spindle is achieved without centrosomes (Mahowald and Strassheim, 1970). During skeletal muscle morphogenesis, myotube centrosomes dissociate from their nuclei, centrioles disappear and the centrosomal matrix is redistributed to the nucleus periphery (Tassin et al., 1985). Similarly, during oogenesis, centrioles from nurse cell centrosomes may disappear. However, their pericentriolar material may relocate to the oocyte nucleus periphery. This would explain the specific enrichment of the oocyte nucleus with perinuclear MTOC material. The only centrosome remaining associated with a nucleus is that of the oocyte. Furthermore, the structure of this centrosome remains intact. We conclude that the four centrioles found close to the nucleus in stage 9 may correspond to the initial oocyte centrosome in the duplication phase observed in G2.

Previous studies have shown that in the oocyte, MTs were more abundant at the anterior than at the posterior (Micklem et al., 1997; Theurkauf et al., 1993; Theurkauf et al., 1992) and that MT minus-ends are associated with the cortex (Cha et al., 2002), suggesting that MTs could be nucleated there. With this work, we did not detect MT nucleation at the lateral cortex, and MT disassembly and regrowth experiments suggest that MT nucleating activity is associated with the centrosome-nucleus complex. A lack of sensitivity in our MT detection method is unlikely to explain the absence of nucleation from the oocyte cortex, as cortical MTs could be detected in fixed egg chambers. Still, we cannot exclude that our experimental procedures favor the regrowth from the oocyte nucleus. However, only some MTs can be traced back to the nucleus surrounding (see Movie 1 in the supplementary material), others only to the cortex. In addition, the MT minus-end marker Nod-βgal is detected at both corners of the anterior cortex. Therefore we propose that MTs are nucleated at the centrosome-nucleus complex, but only some of them are attached there; the remaining MTs might be released. Such MTs would be subsequently translocated and captured at cortical sites. MT translocation could involve actin filaments and motor proteins. Interestingly, MT organization in the oocyte is affected when actin organization is impaired (Manseau et al., 1996). Such a mechanism has been observed in different cell types (for a review, see Bornens, 2002). Hence, while MT nucleation is likely to be restricted to the centrosome-nucleus complex, MT minus-ends are not. This would be in accordance with the observation that injected *bcd* mRNA in the oocyte accumulates at lateral cortices (Cha et al., 2001).

We have observed that MT reorganization in the oocyte after stage 6 occurs always at the onset of anterior migration of the nucleus, suggesting an association between the nucleus and the MTOC. In agreement with this, a mutation impairing the anchoring of the nucleus at the anterior cortex induced a change in the MT network (Guichet et al., 2001). Likewise, in *grk* mutant oocytes with the nucleus at the posterior, the MT network seems organized as in wild-type stage 6 before MT reorganization. MT disassembly and regrowth suggest, at least with our detection method, that a MT

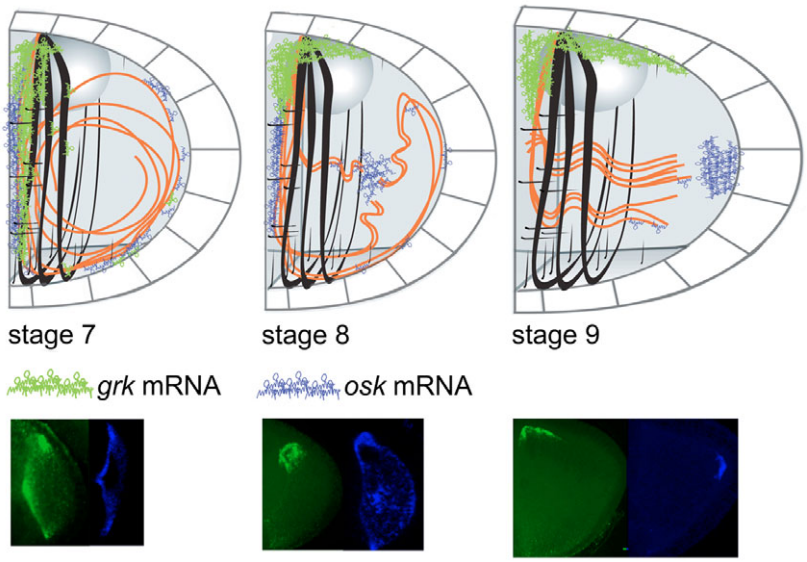

Fig. 7. Correlation of the stage-dependent MT organization and *grk* as well as *osk* mRNA localization from stage 7 through stage 9. *grk* (green) and *osk* (blue) are visualized by fluorescence in-situ hybridization. At stage 7 (left), *grk* and *osk* are found mostly at the anterior cortex. *grk* is rapidly transported toward the oocyte nucleus in the transition from stage 7 to stage 8, exploiting the DV MT subset (black). The DV subset comprises long cortical MT fibers as well as the shorter cortical fibers. *osk* mRNA localization correlates with the successive progression of AP MT subset (red) formation. *osk* is transported from the anterior toward the center following the open diaphragm MTs in the center. By stage 9 (right), upon oocyte growth and complete AP orientation of center MTs, *osk* is transported toward the posterior.

nucleating activity is associated with the centrosome-nucleus complex, both in wild-type and in *grk* mutant oocytes. Interestingly, laser ablation of the nucleus at the anterior of the oocyte inhibited localization of determinants to the posterior, revealing a failure of MT-dependent posterior transport in the absence of the nucleus (Montell et al., 1991). These results are in support of a role for the centrosome-nucleus complex in the nucleation of the MT network necessary for correct polarized transport in the oocyte. Interestingly, MT nucleation from the nuclear envelope, as well as from centrosomes, has been described recently in *Drosophila* spermatocytes (Rebollo et al., 2004).

Some MTOC components have been detected along the lateral cortex (Cha et al., 2002; Schnorrer et al., 2002), however, their MT nucleating activity has not been established. MT regrowth experiments under our experiment condition, argue against such lateral MTOC activity. MT nucleation activity of cortical MTOC components could be repressed, for example by a Ran GTP gradient controlled by the nucleus. This would ensure that centrosomal or non-centrosomal MTOC activity is restricted to the vicinity of the nucleus. Such a mechanism has been reported for *Xenopus* oocytes as well as for somatic cells (Gruss et al., 2001; Keryer et al., 2003).

A complex MT scaffold participates in axis specification

We are able to follow MT organization in the oocyte in high detail: cortical MTs run in parallel to the DV axis throughout oogenesis, and a subset of MTs oriented in the AP direction develops progressively in the center. Using time-lapse images of GFP-Tubulin or Tau-GFP in live oocytes, we were able to reproduce similar MT organization patterns to those observed with fixed samples. The presence of two orthogonal subsets has been proposed earlier (MacDougal et al., 2003) and appears very probably adapted to specify different compartments by facilitating differential mRNA transport (Brendza et al., 2000; Cha et al., 2002; Duncan and Warrior, 2002; Januschke et al., 2002; MacDougall et al., 2003). Indeed, there is a strong correlation between MT redistribution and mRNA localization (Fig. 7). *grk* and *osk* mRNAs show a dynamic and stage-dependent localization pattern (Ephrussi et al., 1991; Neuman-Silberberg and Schuepbach, 1993). Both mRNAs are located at the posterior before nuclear migration and move toward the anterior shortly after nuclear migration and reorganization of the MT cytoskeleton. From stage 7 onward, *grk* and *osk* mRNAs show striking differences in their localization (see cartoon, Fig. 7). During stage 7, *grk* localizes along the anterior margin facing the nurse cell border. During stage 8, *grk* is already restricted to the antero-dorsal corner around the oocyte nucleus (Fig. 7). This transport step is dependent on MT motors (Duncan and Warrior, 2002; Januschke et al., 2002; MacDougall et al., 2003). The DV subset, being fully established at stage 7, is a candidate for such transport. This could further explain why *grk* mRNA localization to the anterio-dorsal cap always precedes *osk* mRNA localization to the posterior. Throughout the subsequent stages, as at least some of the *grk* message arrives from the nurse cells, the DV MT subset is likely to facilitate rapid transport toward the nucleus. Likewise, *osk* mRNA localization to the posterior seems to be coupled to the central MTs. *osk* localization occurs in a step-wise manner and is only completed during stage 9. This correlates with the progressive development of the center MT subset toward an AP orientation. We never observed MT fibers touching the posterior cortex. Thus, *osk* mRNA transport from the posterior-most ending of MTs obtained during stage 9 might rely on other uncharacterized mechanisms.

During stages 7-9, *bcd* mRNA is transported to the oocyte, where it localizes as a ring in the anterior cortex. As it enters the oocyte, *bcd* mRNA encounters MTs necessary for its maintenance organized at the anterior cortex. In *grk* mutant oocytes, *bcd* localizes to the anterior as well as to the posterior pole. The anterior localization of *bcd* suggests the presence of MT minus-ends there. We did not detect MT nucleation activity at the anterior cortex in the cold-shock experiments. Therefore, posteriorly nucleated MTs might also in *grk* mutants be translocated to, and anchored at, the anterior cortex. However, we cannot exclude that we might have missed MT nucleation activity at the cortex due to the experimental setup.

Previous data suggest that MT distribution ranges from cortical enrichment of MTs during stage 7 (Theurkauf et al., 1992), via an intermediate step with MT plus-ends in the center (Palacios and St Johnston, 2002), toward MT bundling, which promotes ooplasmic streaming late in oogenesis (Theurkauf, 1994). The presence of perpendicular MT subsets has been proposed

(MacDougall et al., 2003). Thus, MT organization changes dramatically during mid-oogenesis, but stage-dependent configurations have not yet been established, and high-resolution images of MTs were missing. Our results suggest different stage-dependent MT configurations. This complex oocyte-wide network, in which two MT subsets with a different spatial organization are apparent, could provide a basis for MT motors to organize differential transport.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/1/129/DC1

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