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RESEARCH ARTICLE

Early Asymmetries in Maternal Transcript Distribution Associated With a Cortical Microtubule Network and a Polar Body in the Beetle *Tribolium castaneum*

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The localization of maternal mRNAs during oogenesis plays a central role in axial specification in some insects. Here we describe a polar body–associated asymmetry in maternal transcript distribution in preblastoderm eggs of the beetle *Tribolium castaneum*. Since the position of the polar body marks the future dorsal side of the embryo, we have investigated whether this asymmetry in mRNA distribution plays a role in dorsal-ventral axis specification. Whilst our results suggest polar body–associated transcripts do not play a significant role in specifying the DV axis, at least during early embryogenesis, we do find that the polar body is closely associated with a cortical microtubule network (CMN), which may play a role in the localization of transcripts during oogenesis. Transcripts of the gene *T.c.pangolin* co-localize with the CMN at the time of their anterior localization during oogenesis and their anterior localization is disrupted by the microtubule-depolymerizing agent colcemid. *Developmental Dynamics 239:2875–2887, 2010*.

Key words: *Tribolium*; mRNA localization; anterior-posterior axis; dorsal-ventral axis; microtubule network; polar body; *T.c.pangolin*

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INTRODUCTION

Understanding how asymmetries in morphology and/or function arise is a recurring challenge in the field of developmental biology. mRNA localization is emerging as a powerful means by which polarity is established at the cellular level, for example, in the establishment of axial polarity in oocytes (King et al., 2005; Becalska and Gavis, 2009), in the control of fibroblast migration (Condeelis and Singer, 2005), or in axon guidance in neurons (Martin et al., 2000). Since one mRNA can be translated many times, mRNA localization is an efficient mechanism to localize proteins at high concentrations, with regulation at the translational level facilitating rapid changes in protein levels in response to fluctuations in local conditions.

All animals develop from a single cell, and thus it is not surprising that mRNA localization is also found to play a role in the initial establishment of embryonic axes in a wide variety of species (Singer, 2008; Becalska and Gavis, 2009). Data emerging from studies on a number of insects suggest that the localization of maternal mRNAs plays a prominent role in axis specification within this group of arthropods. In most insects, early zygotic divisions occur without the formation of complete cell membranes, allowing asymmetrically distributed maternal mRNAs, and/or the proteins they encode, to form morphogenic gradients that directly influence developmental fates across a wide field of early cleavage nuclei. Many holometabolous insects (species that undergo a pupal phase during their

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life-history) undergo what is commonly known as "long-germ" embryogenesis. In these species, the cellularization of zygotic nuclei is delayed well into the blastoderm stage, such that broad protein gradients can form from anterior and posterior sources of maternally localized mRNAs resulting in the simultaneous patterning of the entire embryonic anterior-posterior axis during early embryogenesis. Two well-studied examples of long-germ holometabolous insects are the fruitfly *Drosophila melanogaster* and the parasitic wasp *Nasonia vitripennis*. In *Drosophila*, the anterior-posterior (AP) and dorsal-ventral (DV) axes are established via the asymmetric localization of four maternal mRNAs during oogenesis (Becalska and Gavis, 2009): *gurken, bicoid, oskar*, and *nanos*. *gurken* mRNAs encode an EGF signaling pathway ligand of the *tgf*α family and exhibit a spatiotemporally dynamic behaviour during oogenesis, first localizing to the posterior and then later anteriodorsally, playing a central role in the establishment of both the AP and DV axes (MacDougall et al., 2003), while the anterior localization of *bicoid* mRNAs (Clark et al., 2007; Weil et al., 2008) and the posterior localization of *oskar* and *nanos* mRNAs (Wang et al., 1994; Zimyanin et al., 2008) are together essential for correct AP axis specification. The efficient localization of *gurken, bicoid*, and *oskar* mRNAs during oogenesis is dependent on active transport along microtubules mediated by the plus-end-directed motor protein kinesin (for *oskar*) or the minus-end-directed motor protein dynein (for *gurken* and *bicoid*), in addition to transcript, microtubule, and/or motor protein-specific cofactors (Becalska and Gavis, 2009). In *Nasonia*, broad embryonic patterning gradients are also established via the maternal localization of transcripts, although the maternal genes involved in AP axis specification largely differ from those in *Drosophila* (for a discussion on the evolutionary significance of this, see Peel, 2008): *giant* maternal mRNAs are localized to the anterior pole, *caudal* and *nanos* maternal mRNAs are localized to the posterior pole, while *orthodenticle-1* maternal mRNAs are localized to both poles (Lynch et al., 2006; Olesnicky et al., 2006; Brent et al., 2007; Olesnicky and Desplan, 2007). Pharmacological disruption of the oocyte cytoskeleton has shown that anterior mRNA localization of *orthodenticle-1* and *giant* and the posterior localization of *caudal* are microtubule-dependent (Olesnicky and Desplan, 2007). As in *Drosophila*, specification of the *Nasonia* DV axis is also dependent on the asymmetric distribution during oogenesis of mRNAs encoding an EGF signaling pathway ligand of the *tgf*α family (MacDougall et al., 2003; Lynch et al., 2010).

In this report, we focus on maternal transcript asymmetries in the holometabolous beetle *Tribolium castaneum*. *Tribolium* differs developmentally from *Drosophila* and *Nasonia* in two significant ways. First, *Tribolium* is a "short-germ" insect. In short-germ insects, the head and anterior thoracic segments are patterned during the blastoderm stage, while more posterior segments form sequentially at later stages of embryogenesis within an extending and cellularized posterior domain. Second, *Tribolium* females possess telotrophic meroistic ovaries as opposed to the polytrophic meroistic ovaries of *Drosophila* and *Nasonia*. Meroistic ovaries are characterized by the presence of nurse cells, germline-derived cells that do not develop into oocytes, but instead are specialized and devoted to producing patterning molecules and nutrients that are transferred to the oocyte proper (Büning, 1994). Telotrophic meroistic ovaries differ from polytrophic meroistic ovaries in that nurse cells remain in the tropharium, at the anterior end of the ovariole, rather than staying in close proximity to the oocyte during oogenesis (Trauner and Büning, 2007). As they progress down the ovariole, oocytes remain connected to the nurse cells in the tropharium via an extending trophic (nutritive) cord.

It is currently unclear whether maternally localized sources of mRNA play a role in the establishment of the body axes in *Tribolium*, or indeed any other short-germ holometabolous insect. Bucher et al. (2005) have shown that maternal transcripts encoded by the genes *T.c.eagle* and *T.c.pangolin* are localized to the anterior pole in young *Tribolium* embryos, although knockdown by RNA interference suggested that they play no role in anterior patterning. The transcripts of all other maternal genes examined to date are distributed throughout the newly laid *Tribolium* egg, including some transcripts that are maternally localized in other holometabolous species, e.g., *T.c.orthodenticle, T.c.caudal*, and *T.c.tgf*α (Lynch et al., 2006, 2010; Olesnicky et al., 2006; Olesnicky and Desplan, 2007).

In this report, we describe an asymmetry in maternal transcript distribution in *Tribolium* that is associated with a polar body. Polar bodies are the unused cellular byproducts of female meiosis that are developmentally suppressed and/or extruded from the egg (Fischer et al., 2004; Azoury et al., 2009). The position of the polar body is associated with the embryonic DV axis in *Tribolium* (Lynch et al., 2010), so we have investigated whether polar body–associated transcripts might play a role in DV patterning during embryogenesis. Although we find no evidence that polar body–associated transcripts play a role in DV axis specification during early embryogenesis in *Tribolium*, our study reveals that a complex microtubule network exists throughout oogenesis and early embryogenesis that most likely plays a role in the AP localization of maternal transcripts, such as *T.c.pangolin*. We show that *T.c.pangolin* transcripts are associated with this microtubule network during their anterior localization in oogenesis and that a drug that inhibits microtubule polymerization disrupts their anterior localization. Our data provide the foundation for future studies on mRNA localization in *Tribolium*, which, due to its divergent and perhaps ancestral developmental characteristics, may offer important insights into the evolution of mRNA localization mechanisms in insects.

RESULTS

In Situ Hybridization Reveals the Existence of Polar Body–Associated Maternal mRNAs

During studies on pre-blastoderm embryos of *Tribolium castaneum*, we noticed a consistent feature of

alkaline phosphatase in situ hybridization experiments using probes that specifically target maternal transcripts: For each maternally expressed gene examined, we observed a single and discrete patch of staining that was located on one side of pre-blastoderm eggs (Fig. 1). Our sample of maternally inherited transcripts included some encoded by genes previously reported to play a major role in anterior-posterior patterning in *Tribolium*, namely the posterior determinant *T.c.caudal* (Fig. 1A, B) (Schulz et al., 1998; Wolff et al., 1998; Schoppmeier et al., 2009) and the anterior determinants *T.c.hunchback* (Fig. 1C) (Wolff et al., 1995, 1998; Schroder, 2003) and *T.c.orthodenticle-1* (Fig. 1 D) (Schroder, 2003), the maternally localized transcripts of *T.c.eagle* and *T.c.pangolin* (Fig. 1E, F) (Bucher et al., 2005), as well as transcripts of genes encoding components of the JAK/STAT signalling pathway (Fig. 1G–N) (Arbouzova and Zeidler, 2006). We did not observe an equivalent staining in pre-blastoderm embryos following in situ hybridization experiments using sense strand control probes (shown for *T.c.caudal* in Fig. 1B), or probes targeting zygotically expressed genes (data not shown). DAPI co-staining showed that the stained region was centered on the nucleus of a polar body, a condensed spot of DNA that lies in a highly superficial position (unlike the dividing zygotic nuclei that lie deeper within the egg; Fig. 1A′, A″). The polar body DNA revealed by DAPI staining persists at its superficial location until at least the blastoderm stage, at which point it becomes indistinguishable from the DNA of blastoderm nuclei (data not shown).

The polar body was also clearly visible during very early stages in live embryos derived from the transgenic line EFA-nGFP, which ubiquitously express nuclear-localized GFP. We have used this line to show that the polar body has a strong association with the future dorsal side of the egg, i.e., opposite to where the embryonic blastodisc will later condense (Lynch et al., 2010). We, therefore, found the polar body–associated asymmetry in maternal transcript distribution intriguing, and thought it might have a biological significance with respect to dorsal-ventral patterning. We decided to examine polar body–associated transcripts more closely using high-resolution fluorescent in situ hybridization (FISH), before examining their potential function via the mechanical removal of cytoplasm surrounding the polar body.

Maternal Transcripts Localize to the Egg Cortex and Extend Deeper Into the Egg Near the Polar Body

The major advantage of FISH over alkaline phosphatase and other enzymatic in situ hybridization methods is that it allows the position of transcripts to be examined in three dimensions using confocal microscopy, and in relation to cellular features by parallel immunostaining of one or more relevant proteins.

We carried out FISH experiments on pre-blastoderm eggs using probes targeting *T.c.domeless, T.c.orthodenticle-1, T.c.hunchback*, and *T.c.pangolin* transcripts. Signal from maternal transcripts was observed predominantly around the cortex of the egg, and had a characteristic speckled appearance (Fig. 2). This signal was not seen in eggs of the same age that were stained using sense strand control probes and imaged using exactly the same confocal microscope settings (data not shown). We are, therefore, confident that the speckled signal we see predominantly reflects the presence of maternal transcripts, and not unspecific or background fluorescence.

We paid particularly close attention to fluorescence signal emanating from transcripts in the vicinity of the polar body, which was labelled with TOPRO-3 iodide to highlight DNA or by using an acetylated-tubulin antibody that labels polar body–associated microtubules (Fig. 2). Single confocal sections showed that the concentration of transcripts was not noticeably higher around the polar body compared to other regions of the egg cortex (Fig. 2C′). However, projected images of several focal planes recapitulated the apparent higher levels of transcript around the polar body that we had previously noted (shown in Fig. 2 for *T.c.domeless, T.c.orthodenticle-1, T.c.hunchback*, and *T.c.pangolin*). Thus, it became clear that the higher amounts of maternal transcripts that we observe near the polar body reflect the presence of transcripts that extend deeper into the egg, in this region of the egg cortex, rather than higher transcript concentrations in the cytoplasm. This is particularly clear in single sections taken at a 45° angle through the surface of the egg (shown for *T.c.hunchback* in Fig. 2C′) and also in sub-cortical single sections (shown for *T.c.pangolin* in Fig. 2D″).

Maternal Transcripts Associated With the Polar Body Play No Role in Dorsal-Ventral Patterning During Embryogenesis

The fact the polar body has a strong tendency to lie on the future dorsal side of the egg (Lynch et al., 2010) encouraged us to examine the possibility that the abundance of maternal transcripts near the polar body might play a role in dorsal-ventral axis specification. To do this, we took advantage of the EFA-nGFP line, in which the polar body is clearly visible in live embryos for around the first hour of embryogenesis or up until the 4-nuclei stage (see below). We mechanically removed the polar body cytoplasm from young eggs by pricking the cortex close to the polar body with a glass needle. The fluorescent polar body nucleus was often observed to move out of the egg within the flow of surrounding cytoplasm and yolk. Some hours later, blastoderm cells surrounded the wound, and sealed it, at least partly via melanization (see Supp. Fig. S1, which is available online). We then examined the morphology of these embryos up to hatching.

We carried out two sets of control pricks in parallel with the experimental polar body pricks. To control for possible polar body independent effects of removing cytoplasm and yolk from the presumptive dorsal side of the egg, we carried out adjacent pricks targeted to a position midway between the polar body and the farther pole of the egg along the AP axis

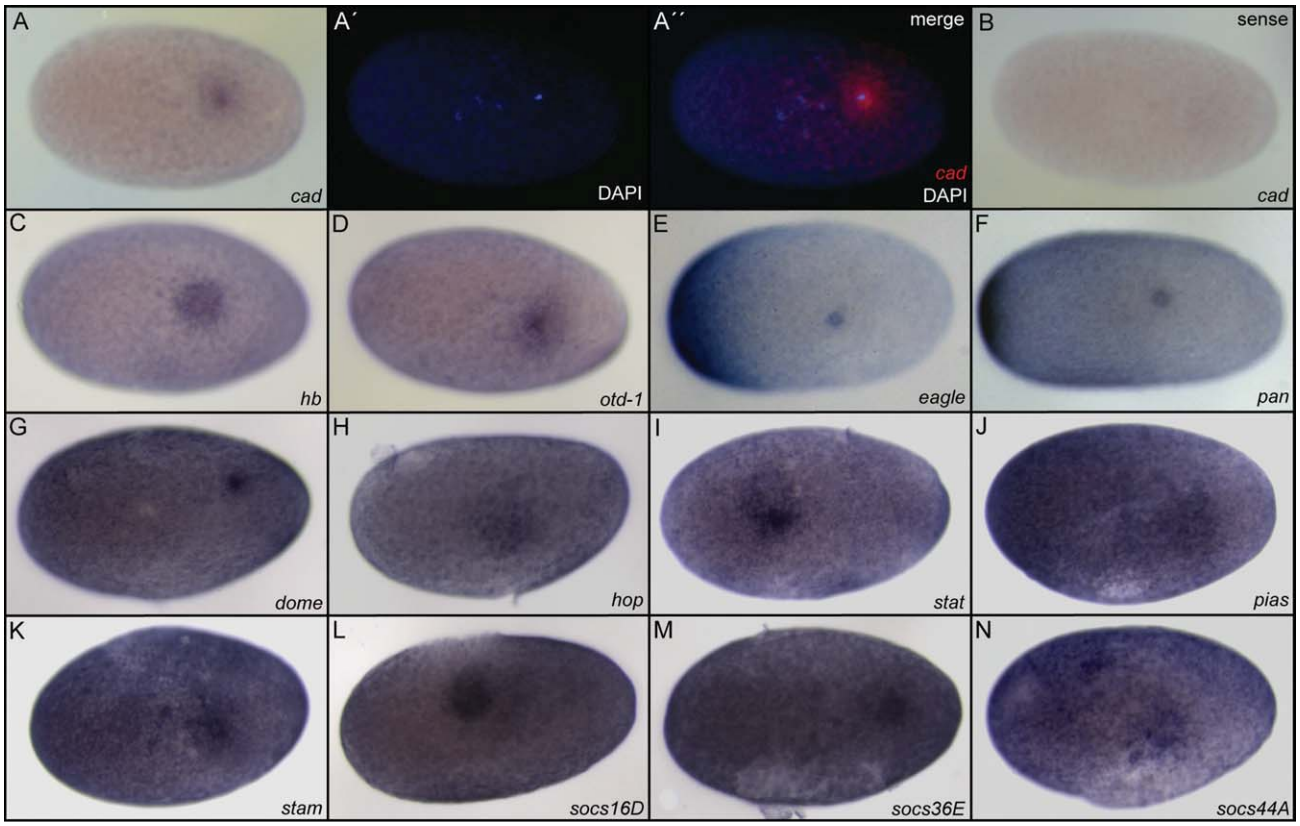


Fig. 1. In situ hybridization reveals an accumulation of maternal transcripts surrounding the polar body in pre-blastoderm embryos. Maternal transcripts examined here include the mRNAs of the anterior-posterior patterning genes *T.c.caudal* (**A**), *T.c.hunchback* (**C**) and *T.c.orthodenticle-1* (**D**); the mRNAs of *T.c.eagle* (**E**) and *T.c.pangolin* (**F**), which are also localized to the anterior; as well as the mRNAs of eight genes encoding components of the JAK/STAT signaling pathway *T.c.domeless* (**G**), *T.c.hopscotch* (**H**), *T.c.stat* (**I**), *T.c.pias* (**J**), *T.c.stam* (**K**); and *T.c.Socs* genes most similar to *Drosophila melanogaster socs16D* (**L**), *socs36E* (**M**), and *socs44A* (**N**). The polar body nucleus was identified using DAPI staining, shown here for *T.c.caudal* (**A**′). **A**″: A merge of DAPI (blue) and inverted alkaline phosphatase (red) channels. **B:** Polar body–specific staining was not seen in sense strand controls. The anterior egg pole is to the left in all panels.

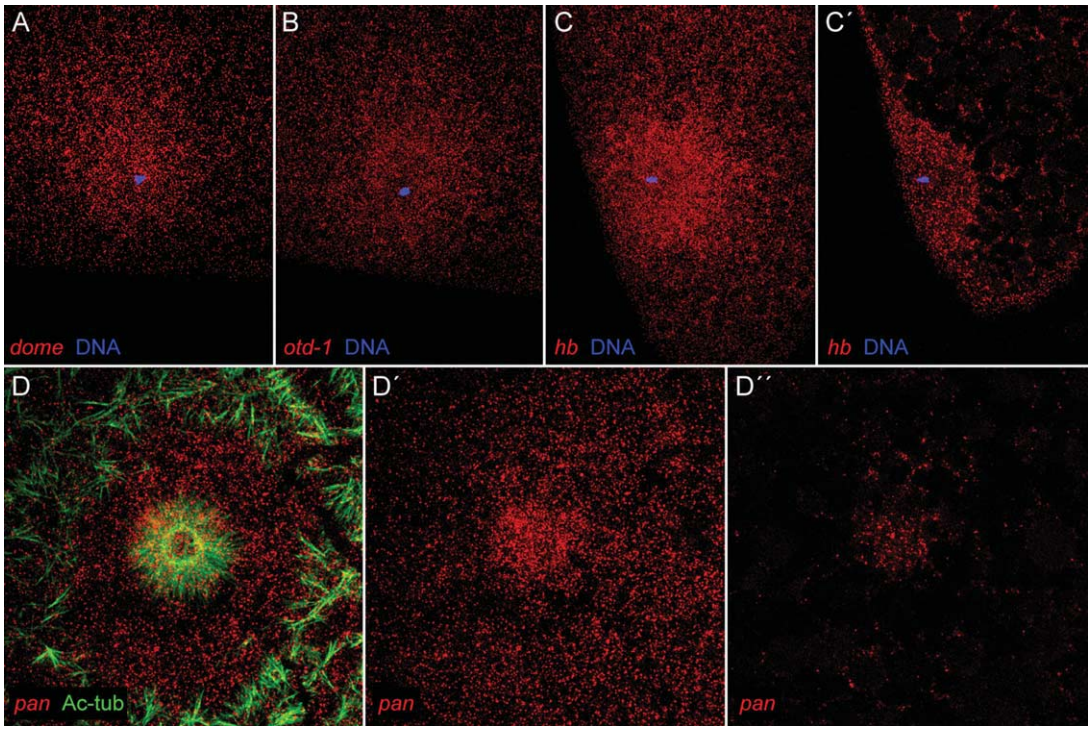
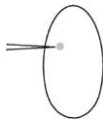
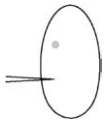
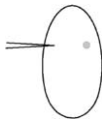


Fig. 2.

TABLE 1. A Description of the Twelve Classes of Embryonic Phenotypes Described Following Polar Body Pricks, Adjacent Control Pricks, and Lateral Control Pricks Carried Out on 0-1-Hr (1-4 Nuclei Stage) Embryos, and Their Relative Frequencies Across the Three Treatment Groups

		Polar body prick		Adjacent Control Prick		Lateral Control Prick	
							
Class of phenotype		No. eggs	%	No. eggs	%	No. eggs	%
1	Egg collapsed from pricking. No development	2	0.8	3	1.2	1	0.4
2	Intact egg after pricking. No development	11	4.3	15	5.8	16	6.2
3	Evidence of blastoderm cells (sometimes few and/or disorganized). No evidence of germ rudiment	10	3.9	8	3.1	3	1.2
4	Evidence of extra-embryonic (serosal) cells. Clear sign of a disorganized germ rudiment (often simply a clump of cells)	18	7.0	15	5.8	17	6.6
5	Development stalled shortly after condensation of the germ disc	3	1.2	0	0	0	0
6	Clear serosal cell layer and a clearly elongated germ band, although abnormal: misshapen, mis-positioned, and/or not fully elongated	31	12.0	30	11.6	36	14.0
7	Development stalled at fully elongated germ band stage (head and tail curled around the egg poles). Germ band usually degenerates	46	17.8	64	24.8	49	19.0
8	Development stalled at later germ band stage: Fully elongated germ band shows clear signs of germ band thickening/limbs. Often degenerates	15	5.8	13	5.0	7	2.7
9	Embryo displays clear signs of incomplete and/or abnormal dorsal closure	3	1.2	7	2.7	8	3.1
10	Late embryo displaying clear abnormalities (in segmental and/or appendage development)	6	2.3	5	1.9	7	2.7
11	Larva fully developed, but fails to break out of its cuticle, probably due to submersion in halocarbon oil (often exhibits contractions)	44	17.1	38	14.7	41	15.9
12	Normal larva successfully hatches	69	26.7	60	23.3	73	28.3
Total number of eggs/embryos		258		258		258	
Total number of embryos that developed normally until at least the extended germ band stage		183	70.9	187	72.5	185	71.7
Total number of embryos that developed into a normal larva		113	43.8	98	38.0	114	44.2

(Table 1, "adjacent control prick" column). To control for any general effects of pricking we carried out lateral control pricks that were targeted at right angles to the polar body, at the same level along the anterior-posterior egg axis (Table 1, "lateral control prick" column).

We found that a significant volume of cytoplasm and yolk could be removed from around the polar body very early in embryogenesis and embryos would still develop into normal larvae. This occurred in 43.8% (n=258) of polar body pricks, which is very similar to the survival rate obtained with adjacent (38.0%, n=258) and lateral (44.2%, n=258) control pricks. The remaining embryos showed a variety of phenotypes that were similar in type and frequency among embryos that were subjected to polar body pricks, or adjacent and lateral control pricks (Table 1 and Supp. Fig. S1). No embryos could be confidently described as exhibiting abnormalities in the establishment of the dorso-ventral axis. Our experiments, therefore, suggest that the embryonic phenotypes observed following polar body pricks derive from damage related to pricking or from an inherent sensitivity of the EFA-nGFP line (see Experimental Procedures section), rather than an effect specific to removing polar body–associated transcripts.

Fig. 2. Fluorescent in situ hybridization (FISH) shows that maternal transcripts are distributed throughout the egg cortex in pre-blastoderm eggs. Projections of confocal images from a number of focal planes recapitulate the impression gained from alkaline phosphatase in situs (see Fig. 1) that higher numbers of *T.c.domeless* (**A**), *T.c.orthodenticle-1* (**B**), *T.c.hunchback* (**C**), and *T.c.pangolin* (**D, D′**) transcripts are found in the vicinity of the polar body. Single confocal sections show that this results from the cytoplasm and associated maternal transcripts extending deeper into the yolk in that area of the egg, and is not due to higher concentrations of maternal transcripts (**C′**: a section through the embryo in C; **D″:** a sub-cortical section through the embryo in D, D′). The polar body was marked using TOPRO-3 iodide, which labels DNA (A–C), or by using an antibody against acetylated-tubulin (D), which labels polar body–associated microtubules, as shown in Figure 3.

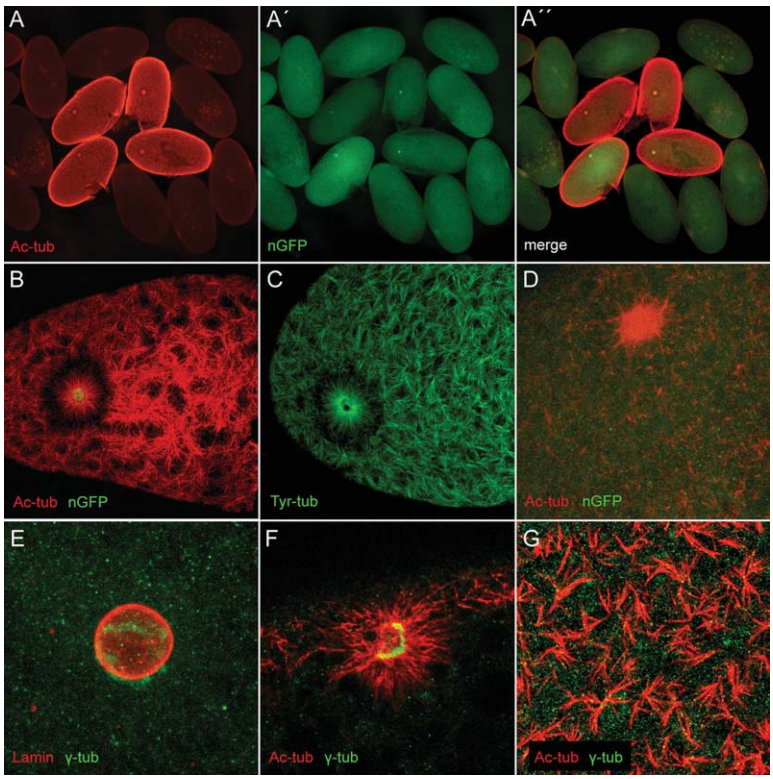


Fig. 3.

Fig. 3. The cortical microtubule network (CMN) during early embryogenesis in *Tribolium*. Antibodies against acetylated tubulin (**A,B**) and tyrosinated tubulin (**C**) reveal a complex cortical microtubule network (CMN) in early embryos, up until the second zygotic division. The CMN disappears at the 4-nuclei stage (**D**) at the same time as the polar body nucleus breaks down, as determined by the loss of nuclear GFP in embryos of the transgenic line EFA-nGFP. A–A″ represent a population of early embryos, the majority of which have already lost the CMN. High levels of gamma tubulin were observed in a ring around the polar body nucleus (shown in **E**; a projection of the polar body with nuclear envelope labeled using an antibody against lamin) at the same level from which polar-body-associated microtubules radiate (shown in **F**; a section through a polar body). Although spots of gamma tubulin were observed throughout the egg cortex, they do not obviously co-localize to the ends of microtubules that compose the CMN (**G**; a projection of a representative area of the CMN).

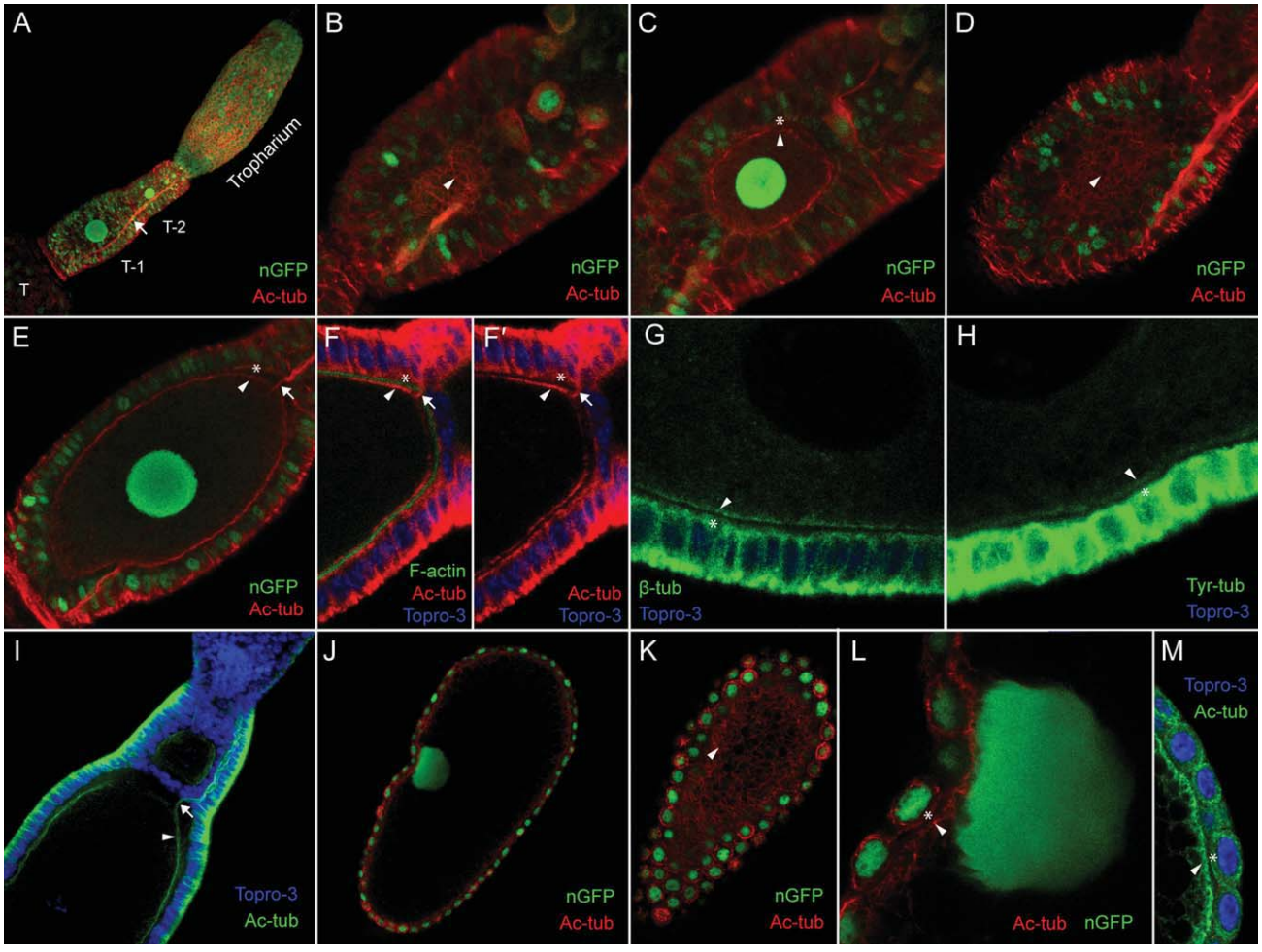


Fig. 4.

The Polar Body Is Associated With a Cortical Microtubule Network (CMN) That Has Its Origins in Early Oogenesis

In an effort to explore the mechanism of mRNA localization in *Tribolium* embryos, we examined the structure of the microtubule cytoskeleton in early embryonic stages. Using an acetylated tubulin antibody to label polymerized microtubules, we observed a complex arrangement of the microtubule cytoskeleton around the polar body, consisting of microtubules radiating out from the polar body nucleus, a halo that contains few stable microtubules, and an intricate network of microtubules covering the entire cortex in very young embryos (Fig. 3A, B). The same pattern of microtubules was observed using a tyrosinated tubulin antibody (Fig. 3C). The polar body–associated microtubules coincide with the area of maternal transcript accumulation, suggesting that these cytoskeletal features might be structuring this area, allowing the cytoplasm—carrying maternal transcripts—to extend deeper into the yolk.

The cortical network appears to be composed of a series of locally organized microtubule asters. Based on the percentage of embryos that exhibit this network in 2-hr egg collections, we estimate that it persists for approximately the first hour of embryogenesis at 28°C. By the second round of zygotic nuclear divisions (the 4-nuclei stage), the star-like polar body cytoskeleton and CMN are no longer detectable by immunostaining (Fig. 3A, D). Close inspection of acetylated tubulin together with nuclear-localized GFP (in the transgenic line EFA-nGFP) shows that the CMN disappears shortly after the polar body nuclear envelope breaks down (Fig. 3A).

To identify microtubule-organizing centres (MTOCs) within these young embryos, we used antibodies against gamma-tubulin and the *Drosophila* centrosomal protein Dgrip (Ferree et al., 2006). We observed that gamma-tubulin is located in a ring around the polar body nuclear envelope, consistent with a role in nucleating the microtubules radiating from the polar body nucleus (Fig 3. E,F). Discrete spots of gamma-tubulin were also detected throughout the cortex, but these were not obviously associated with individual microtubules comprising the network or with the center of microtubule asters (Fig. 3F,G). The antibody against Dgrip was found not to cross-react in *Tribolium*, as determined by its failure to label centrosomes (data not shown).

Next, we decided to examine the origins of the CMN during oogenesis (Fig. 4). We carried out immunostainings for acetylated tubulin and GFP on *Tribolium* ovaries dissected from EFA-nGFP transgenic females (Fig. 4A–E, J–L). We also carried out immunostainings for acetylated tubulin (Fig. 4I, M), beta tubulin (Fig. 4G), and tyrosinated tubulin (Fig. 4H) on ovaries dissected from wildtype females. All three tubulin antibodies revealed a very similar pattern of microtubule organization within ovarioles. All three tubulin antibodies detected a CMN in early oocytes, at the stage when they have left the tropharium but are yet to be encircled by a single layer of follicle cells (Fig. 4B,C), and throughout later stages of oogenesis (Fig. 4D–M). In early and mid-stage oocytes (up to mid-vitellogenesis), the CMN is contiguous with microtubules within the trophic cord that connects the oocytes to the nurse cells (arrows in Fig. 4E,F, I). The microtubules of the oocyte CMN (arrowheads in Fig. 4B–I, K–M) are clearly distinct from the microtubules forming the cytoskeleton of overlying somatic follicle cells (marked by an asterisk in Fig. 4C, E–H, L,M). This is most clear in mid-stage ovaries co-stained for filamentous actin (F-actin); a layer of F-actin clearly separates the oocyte CMN from microtubules forming the cytoskeleton of follicle cells (Fig. 4F). It appears that this F-actin layer lies between the CMN and the oocyte plasma membrane.

The same images also suggest that microtubule organization in the CMN might evolve during the course of oogenesis. Microtubules in the CMN of very young oocytes appear to be arranged predominantly perpendicular to the ovariole axis (Fig. 4B), whereas those in the CMN of later oocytes have a more random orientation (Fig. 4D, K). At no stage during oogenesis do we observe the prominent microtubule asters that are seen within the embryonic CMN (Fig. 3B,C). Due to the difficulty in obtaining and fixing very late oocytes, we cannot be sure that the CMN we observe in early embryos represents the final stages of the CMN that was first established during early oogenesis. However, we believe this is likely to be the case.

T.c.pangolin mRNA Transcripts Localize to the CMN

Our discovery of a CMN in oocytes and early embryos raises the obvious question of whether it plays a role in the anterior localization of maternal transcripts such as *T.c.eagle* and *T.c.pangolin* (Bucher et al., 2005). Using alkaline phosphatase in situ hybridization (Fig. 1E, F) and FISH (Fig. 5A, B) for *T.c.pangolin* on preblastoderm embryos, in combination with immunostaining of acetylated tubulin, we observed that the anterior localization of *T.c.pangolin* transcripts is largely completed by early embryogenesis. *T.c.pangolin* transcripts were observed in a circular

Fig. 4. The cortical microtubule network (CMN) during oogenesis. The CMN exists throughout oogenesis. Ovarioles have been stained for acetylated tubulin (red in **A–F**, **J–L**, and green in **I, M**), beta tubulin (green in **G**), tyrosinated tubulin (green in **H**), GFP (green, in ovarioles from EFA-nGFP transgenic females; A–E, J–L), F-actin (green in F), and TOPRO-3 (blue). A typical *Tribolium castaneum* ovariole is shown in A, with the terminal (T) and two preceding oocytes (T-1, T-2) labeled. An arrow marks the trophic cord. Higher-magnification views of the T-2 and T-1 oocytes are shown in B,C and D,E, respectively, in superficial (B,D) and deep (C,E) focal planes. The CMN (arrowheads) is clearly visible and distinguishable from microtubules comprising the cytoskeleton of overlying follicle cells (asterisk). This is particularly clear in oocytes co-stained for F-actin (F). K represents a superficial section through the CMN and overlying follicular epithelium, and L is a close up of the oocyte nucleus, which appears to contact the CMN in late vitellogenic oocytes. Microtubules within the trophic cord are contiguous with microtubules of the anterior CMN (arrows in E, F, I). The anterior poles of oocytes are towards the right/top-right.

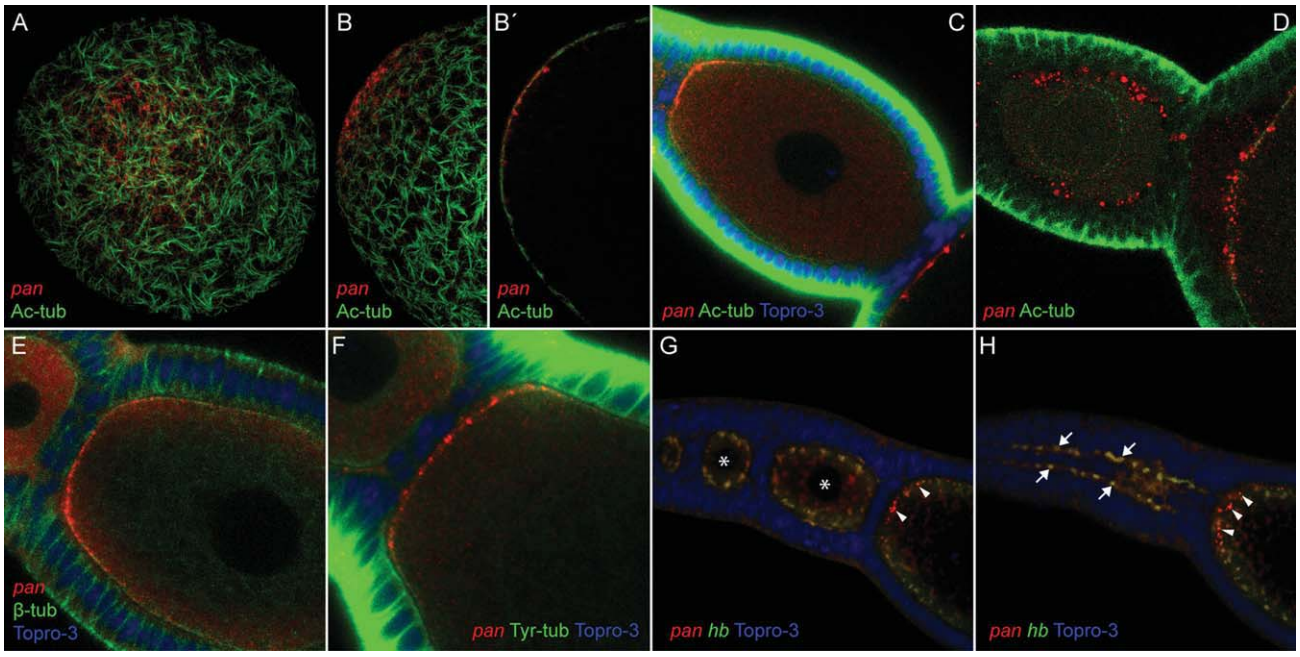


Fig. 5. *T.c.pangolin* mRNA localization during early embryogenesis and oogenesis. **A,B:** *T.c.pangolin* mRNA is localized to the anterior egg pole at the level of the CMN during early embryogenesis; view from the anterior egg pole in A, lateral view in B, single optical section through cortex in B′. **C–H:** *T.c.pangolin* mRNA is localized to the anterior CMN during early vitellogenesis; CMN is viewed using antibodies against acetylated tubulin (C,D), beta tubulin (E), or tyrosinated tubulin (F). In younger, pre-vitellogenic oocytes, *T.c.pangolin* mRNA is not localized within oocytes (G). Double fluorescent in situ hybridization experiments (G,H) show that localized (*T.c.pangolin*) and non-localized (*T.c.hunchback*) maternal transcripts are transported into oocytes together; signal co-localizes in pre-vitellogenic oocytes (G, asterisk) and in the trophic cords (H, arrows). While strong and discrete spots of *T.c.pangolin* signal appear at the anterior in early vitellogenic oocytes (arrowheads in G, H), this is not seen for *T.c.hunchback*, which remains widely distributed in the cortex of expanding vitellogenic oocytes. With both *T.c.pangolin* and *T.c.hunchback* in situ probes, signal was sometimes observed to be punctate in nature (G,H) and sometimes more uniform (E, see also Fig. 6C). Anterior poles of oocytes are towards the left/top-left in all panels.

patch covering the anterior pole of the egg at, or just below, the level of the CMN (Fig. 5A, B, B′) even in the youngest eggs with an intact star-like polar body cytoskeleton and CMN.

To determine whether the CMN might play a role in *T.c.pangolin* mRNA localization during oogenesis, we carried out FISH for *T.c.pangolin* on *Tribolium* ovarioles, in combination with immunostaining for acetylated tubulin, tyrosinated tubulin, or beta tubulin (Fig. 5C–F). In vitellogenic oocytes, we observed strong spots of *T.c.pangolin* signal on the CMN at the anterior cortex (Fig. 5C–F). In contrast, younger, pre-vitellogenic oocytes had signal throughout the oocyte cytoplasm (see Figs. 5G,H and 6C). Our results, therefore, show that *T.c.pangolin* mRNA is associated with the CMN at the anterior cortex during the stages when it becomes localized to the anterior pole.

The strong and highly discrete signal that we observe for *T.c.pangolin* in the anterior of vitellogenic oocytes is not seen for maternal transcripts that are not localized during oogenesis, such as the mRNA of *T.c.hunchback* (Fig. 5G,H). Double fluorescent in situs for *T.c.pangolin* and *T.c.hunchback* show that these transcripts co-localize during their movement along the trophic cords, and in pre-vitellogenic oocytes (Fig. 5G,H). With both probes, this staining appears sometimes uniform (29% ovarioles examined, n=24, Fig. 5E, and similar to oocytes in Fig. 6 C) and sometimes punctate (71% ovarioles examined, n=24, Fig 5G,H) during these stages. It is presently unclear if the difference between uniform and punctate staining represents real variation among ovarioles, temporal variation, or a fixation artifact.

T.c.pangolin mRNA Localization Requires an Intact Oocyte Microtubule Cytoskeleton

Our observation that strong and discrete spots of *T.c.pangolin* signal localize to the anterior oocyte CMN in early vitellogenic oocytes led to us asking whether an intact microtubule cytoskeleton is required for the localization and/or anchoring of *T.c.pangolin* mRNA. To examine this, we fed young female *Tribolium* soft dough made by mixing wholemeal flour with water containing two different concentrations of colcemid (0.1 or 1.0 mg/ml), a chemical compound that inhibits microtubule formation. We then dissected their ovaries after 24 hr of feeding in order to look for defects in oogenesis. Control females were fed flour dough of the same consistency but made using water lacking colcemid.

We found that colcemid feeding caused a range of defects in oogenesis. These included the premature and abnormal asymmetric positioning of the female pronucleus within pre-vitellogenic oocytes (Fig. 6A, D,E), a noticeable reduction in the frequency and extent of *T.c.pangolin* mRNA anterior localization within oocytes (Fig. 6B–E), and in extreme cases abnormalities in the relative size of female

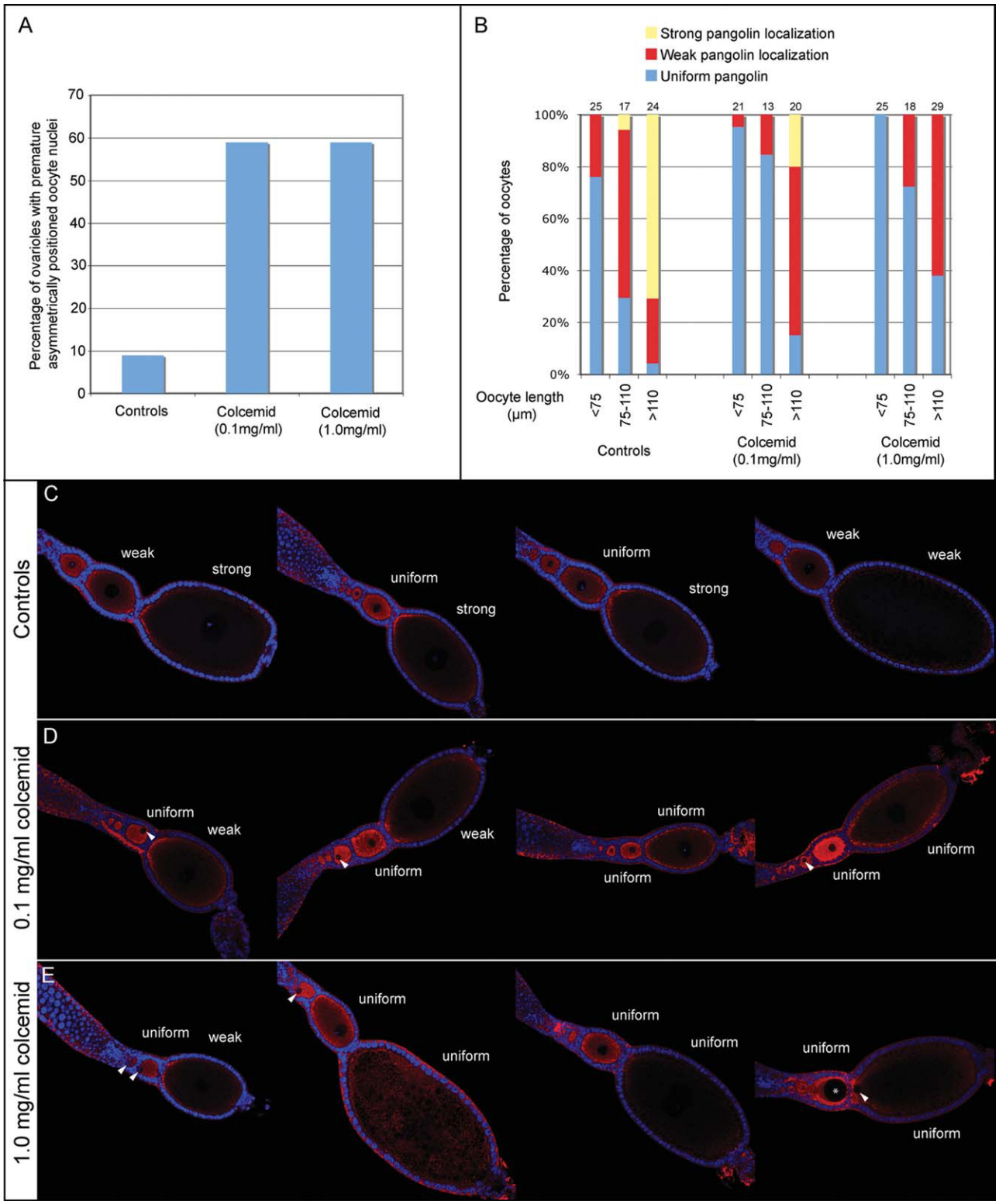


Fig. 6. The effects of colcemid feeding on oogenesis. **A:** Histogram showing the percentage of ovarioles from each colcemid treatment group exhibiting pre-vitellogenic oocytes with prematurely asymmetrically positioned oocyte nuclei. **B:** Histogram showing the effect of one day of colcemid feeding on the frequency and extent of *T.c.pangolin* mRNA localization for oocytes at three consecutive stages of oogenesis: (1) oocyte length <75 μm (previtellogenic and very early vitellogenic stages), (2) 75–110 μm (early vitellogenic stages), and (3) >110 μm (mid-vitellogenic stages). Late vitellogenic stages do not often survive FISH intact, and those that do give unreliable staining patterns (i.e., high background resulting from trapped probe). Numbers above the bars represent the number of oocytes examined for each stage of oogenesis. **C–E:** Examples of ovarioles dissected from control females (C), or from females fed with 0.1 mg/ml colcemid (D) or 1.0 mg/ml colcemid (E), stained with a probe for *T.c.pangolin* mRNA (red) and with TOPRO-3 (blue). Prematurely asymmetrically localized nuclei are marked with arrowheads; an abnormally large oocyte nucleus is marked with an asterisk in E.

pronuclei (Fig. 6E, far-right ovariole). Colcemid feeding also led to a noticeable reduction in female fecundity (around a threefold reduction in egg number laid by females fed the higher colcemid dose), although the vast majority of the eggs laid by colcemid fed females developed and hatched into wildtype larvae, suggesting that eggs exhibiting abnormalities resulting from colcemid feeding cannot be laid.

To assess the effects of colcemid treatment at different stages of oogenesis, we scored oocytes of different maturity (different oocyte length) for whether they exhibit strong *T.c.pangolin* localization (a clear excess of *T.c.pangolin* signal in the anterior and an absence of *T.c.pangolin* signal in the posterior), weak *T.c.pangolin* localization (a gradient of *T.c.pangolin* mRNA across the oocyte, higher in the anterior) or uniform *T.c.pangolin* localization (uniform levels of *T.c.pangolin* signal within the oocyte, or similar peaks of *T.c.pangolin* signal at the anterior and posterior). In the oocytes of control females, both the frequency and extent of *T.c.pangolin* mRNA localization increased during oogenesis (with increasing oocyte length), reflecting the gradual localization of *T.c.pangolin* mRNA during early vitellogenesis (Fig. 6B,C, see also Supp. Fig. S2). A similar stage-dependent trend in levels of *T.c.pangolin* mRNA localization was observed for oocytes from females fed with colcemid. However, oocytes from females fed with colcemid exhibited a clear overall reduction in the frequency and extent of *T.c.pangolin* mRNA localization for any given stage of oocyte maturation. This effect was dose dependent (Fig. 6B, D, E, see also Supp. Fig. S2).

DISCUSSION

We have documented that, in *Tribolium* embryos, maternal mRNAs accumulate at higher levels in the vicinity of the polar body, but this asymmetry does not play a significant role in dorsal-ventral axis specification during embryogenesis. Instead, this accumulation of maternal transcripts may be linked indirectly to the dorsoventral axis through two earlier events: First, the migration of the female pronucleus to the oocyte cortex during mid-oogenesis, which is likely to be a key event in dorso-ventral axis specification (Lynch et al., 2010) (see also Fig. 4J, L); this establishes the dorsal position of the polar body when it is born, during the subsequent meiosis. And second, the CMN that forms at the surface of the egg and helps to localize maternal transcripts during oogenesis. In early embryos, the architecture of the microtubule network appears to be spatially organized around the polar body, creating an area of the egg cortex where the cytoplasm extends deeper into the yolk. Single confocal sections show that maternal transcripts reside in the small spaces between yolk spheres (see Fig. 2C′). This implies that the maternal transcripts of many genes are found predominantly around the egg cortex due to passive displacement of cytoplasm by the yolk spheres that are laid down during vitellogenesis, rather than by a mechanism of active localization to the egg surface. Thus, the polar body may simply provide a larger reservoir of cytoplasm in which maternal transcripts are able to passively reside during early embryogenesis.

The results of our co-localization and colcemid-feeding experiments suggest that the CMN plays a role in the anterior localization and/or anchoring of some maternal transcripts during oogenesis. The strong and discrete signal obtained for *T.c.pangolin* is reminiscent of that from mRNA transport "particles" and/or localized mRNA foci in *Drosophila* oocytes (Weil et al., 2008; Becalska and Gavis, 2009) and may represent similar particles involved in the transport and/or anchoring of *T.c.pangolin* transcripts in *Tribolium*. Indeed, the structure of the CMN we describe here is also interesting in light of recent work on the mechanism of *oskar* mRNA localization in *Drosophila* (Zimyanin et al., 2008). The precise organization of microtubules in *Drosophila* oocytes has long remained unclear and controversial. One reason for this might be a rapid turnover or rearrangement of microtubules in *Drosophila* oocytes (Januschke et al., 2006). Using live imaging of fluorescently labeled transcripts, Zimyanin et al. (2008) demonstrated that *oskar* mRNA particles localize to the posterior via a posteriorly biased random walk, using a microtubule network that is only weakly polarized along the anterior-posterior axis. Our observation that microtubules of the CMN form a network in which they appear to extend randomly in all directions might be consistent with a similar mechanism of mRNA transport operating in *Tribolium*. Perhaps microtubules in the *Tribolium* CMN are not arranged quite as randomly as first impressions might suggest.

The oocyte microtubule cytoskeleton may also have an important role in positioning the oocyte nucleus, which is directly linked to axis specification (Lynch et al., 2010). The movement of the female pronucleus from a posterior position to the future anterodorsal side of the oocyte during *Drosophila* oogenesis is known to be important for anteroposterior as well as dorsoventral patterning, and to be dependent on a reorganization of the oocyte microtubule cytoskeleton (Becalska and Gavis, 2009). Our observation that the *Tribolium* female pronucleus is often prematurely asymmetrically localized in a random position within pre-vitellogenic oocytes following colcemid feeding is consistent with a conserved role for microtubules in the control of female pronucleus positioning during holometabolous insect oogenesis. In *Tribolium*, it would appear that microtubules are required to maintain the female pronucleus in a central position within the oocytes, up until mid-to-late vitellogenesis when the pronucleus migrates to the future dorsal site of the egg (Lynch et al., 2010).

Together, our data provide the foundation for future studies into mechanisms of maternal mRNA localization during oogenesis in the short-germ beetle, *Tribolium*. The next step will be to examine mRNA localization in vivo, via the development of live imaging techniques for *Tribolium* ovarioles, and through the establishment of transgenic lines analogous to those used to study mRNA localization in *Drosophila* (Becalska and Gavis, 2009). It will be interesting to determine whether *Tribolium* and *Drosophila* localize maternal mRNAs using microtubule populations that share organizational similarities and/

or localize maternal mRNAs using homologous cofactors. If so, it would imply that the striking divergence in the genes that establish the anterior-posterior axis within different holometabolous insects has occurred via the recruitment of maternal mRNAs of different genes to a pre-existing mRNA localization microtubule network.

EXPERIMENTAL PROCEDURES

In Situ Hybridization and Antibody Stainings

Pre-blastoderm embryos were fixed in 4% formaldehyde in PBS for 15–30 min, according to established protocols for *Tribolium* (Schinko et al., 2009; Shippy et al., 2009). Ovarioles were dissected and fixed according to Lynch et al. (2010).

Alkaline phosphatase in situ hybridizations were carried out using DIG-labelled RNA probes and NBT/BCIP, according to established protocols (Schinko et al., 2009; Shippy et al., 2009). The proteinase K step was found to be unnecessary on pre-blastoderm eggs and thus omitted. In experiments involving co-immunostaining for acetylated tubulin, all primary and secondary antibodies were added prior to carrying out the alkaline phosphatase reaction.

Fluorescent in situ hybridization (FISH) was carried out according to protocols developed by Kosman et al. (2004). The proteinase K incubation and associated post-fixation were omitted. In combined FISH/immunostainings on ovarioles, the xylene incubation step was also omitted as it led to deformation of late-stage oocytes. The penetration of the anti-Ac-Tub antibody was improved in these experiments by carrying out a 1-hr incubation in 1% Triton-X in PBS, prior to the remaining post-fixation.

RNA probes were DIG or Biotin labeled and prepared according to Kosman et al. (2004) using gene fragments amplified from *Tribolium castaneum* genomic DNA and cloned into the pGEM-T Easy Vector (Promega, Madison, WI). We noticed that Biotin-labelled probes gave weaker signal and failed to detect signal deeper within oocytes when compared to DIG-labelled probes. All fluorescent double in situs were, therefore, carried out using reciprocal probe combinations (i.e., *hunchback*-DIG probe + *pangolin*-BIO probe and *hunchback*-BIO probe + *pangolin*-DIG probe). Gene fragments were amplified using the following primers: *T.c. caudal*: ACAAGTGCGTGTGACCCAAAGT and TAGGCTGACTCTGGTAGGAGG TTT (451-bp fragment), AGACGAG AACGAAGGACAAATATCGGG and ACTGTATGGTTCACACTCACGCAC (590-bp fragment), AGACCCGGACG AAAGACAAATACC and CTCTGCA ACACAACAAGACGTGGA (666-bp fragment); three probes made separately and combined. *T.c. hunchback*: TGGCAATTCGGCGTTTCCCAGA and TGCAAGTGAACGGGTTGTGGAA (1,561-bp fragment). *T.c. orthodenticle-1*: TCCAGCAGCAGCAGAACA AGA and CTGACTGAATCAACC CAATCTAATCACC (1,131-bp fragment). *T.c. T.c.pangolin*: ATCATGGA-CACCACCGAAGAACCT and CTCT TACCCTTCGGCCGAGTATTT (835-bp fragment), TTCGCAGACGCAAG AAGAAGTGCATACGA and TGCACT GAACACAGTCTGGCCCACC (521-bp fragment); two probes made separately and combined. *T.c. domeless*: TTTCGAAGTGGTGGCCTTTGGT and TTATGCCATTGAGAGCTTCGGGTG (1,189-bp fragment). *T.c. hopscotch*: TGTTTCACCGATTGCCAGACACCT and GTAGTCACTATATTTAACGC CACTTTCCCT (1,051-bp fragment), TCGCAGGAGGTTCTGAACAATGAG and TGCGTTAGCAGGTCTTGCGT TT (1,115-bp fragment); two probes made separately and combined. *T.c. stat*: AAAGTGGCCGGTCTTATGCA ACTC and CAACATCTCCTCGGC TTGCTTCTT (1,164-bp fragment). *T.c. stam*: TGCCTGGACCACCACA TTGACTT and TCGACTGCATCACC TTCATCCCTT (1,135-bp fragment). *T.c. pias*: ATGCCACAAAGCAACCA ACGCA and GTCATCATCAGAGTCG GAACACGTCAA (1,116-bp fragment). *T.c. socs16D*: CCGTTTACACA TAGAGGTGGGTCTTC and GGGTTC TTCACGCTCAGAGGTATTT (1,103-bp fragment), CGCACTCGATGTGTC CACAATTTATTCCT and TGCTGT AGGCTCTGCACTTGCTT (1,227-bp fragment); two probes made separately and combined. *T.c. socs36E*: AAT-CAGTCTGACTTCGCTCGGT and GT GAGCACTTCATATTGCGCCT (1,058-bp fragment), ACAACGCCTGTTTG TTGGGT and GGCTCGTATTCTCT TGAATGGAGTTG (912-bp fragment), ACTATATGAGGAGGTGTCACCAAT TAC and CCTCTCCACTCTCACTTT CTGTCT (10,792-bp fragment); three probes made separately and combined. *T.c. socs44A*: TCGGTATTGA CGGTATGGAAGTGG and TTCAA-CATTTGAGCGGACGGAG (1,232-bp fragment).

Protein immunostainings were carried out according to established protocols (Patel, 1994). The following antibodies were used in this study: Mouse anti-acetylated Tubulin (Sigma, St. Louis, MO: T6793, 1:500); Mouse anti-tyrosinated Tubulin antibody (Sigma, Cat. No. T9028, 1:250); Mouse anti-beta Tubulin antibody (Developmental Studies Hybridoma Bank, E7, 1:100); Mouse anti-lamin (Developmental Studies Hybridoma Bank, ADL67.10, 1:40); Rabbit anti-GFP (Minotech, Heraklion, Crete, Greece; Cat. No. 721, 1:250); Rabbit anti-gamma Tubulin (Abcam, Cambridge, MA; ab11317, 1:1,000); Rabbit anti-Dgrip84 (kindly provided by Yixian Zheng; Oegema et al., 1999). F-actin staining was carried out using Phaloidin-FITC (Sigma, Cat. No. P5282, 1:1,000).

Egg-Pricking Experiments

Eggs were collected from the EFA-nGFP transgenic line 171.12 (M. Averof, unpublished data), incubated on white flour for 90 min at 28°C and partially dechorionated by washing in 0.5% bleach for 1 min (2 × 30 sec submersions with sustained rinsing in water before, after, and between submersions). Using a Leica MZ16F epifluorescence stereoscope, three eggs with a clear nGFP-labeled polar body were identified as quickly as possible, transferred to a microscope slide with the polar body positioned at a 45° angle to the objective lens, and left to dry for a few seconds (residual flour acts as a glue), before being submersed in a drop of halocarbon oil. Polar body, adjacent, or lateral control pricks were then carried out on the three eggs using a glass needle attached to a micromanipulator. The pricks were carried out under the stereoscope using the GFP excitation and low-intensity white light, to allow

simultaneous viewing of the polar body (GFP) and the glass needle tip. Pricks were considered successful, and included in the study, if cytoplasm/yolk was observed to exit the egg. This process of pricking three eggs was repeated, alternating polar body, adjacent, and lateral pricks. Equal numbers of polar body, adjacent, and lateral pricks were carried out per collection (usually a total of 27 or 36 eggs). Embryos were then left to develop at 28°C in Petri dishes with damp tissue paper, and imaged at approximately the same time every day for 5 to 6 days. Needles were prepared using aluminosilicate glass capillaries (Harvard Apparatus, Holliston, MA; SM100F-10) pulled on a Sutter P-97 micropipette puller, and the tip was cut manually using a surgical blade.

In a preliminary experiment, 73.7% (n=224) of wildtype embryos developed to larvae following a randomly positioned prick compared with only 32.4% (n=204) of nGFP line embryos undergoing an identical procedure, suggesting that the EFA-nGFP 171.12 line is more sensitive to mechanical manipulation.

Colcemid Feeding Experiments

A soft dough was produced by mixing wholemeal flour with water, or with water supplemented with 0.1 or 1.0 mg/ml of colcemid (Sigma, Cat. No. D-7385). Two hundred recently eclosed females were starved for 2 days and then fed soft dough without colcemid, soft dough supplemented with 0.1 mg/ml colcemid, or soft dough supplemented with 1.0 mg/ml colcemid. The ratio of wholemeal flour to liquid (water ± colcemid) comprising the dough was the same across the three treatment groups. Females were fed at room temperature. After 1 and/or 2 days feeding, ovaries were dissected, fixed, and stained for *T.c.pangolin* mRNA using the FISH techniques described above.

Image Capture

Embryos that had undergone alkaline phosphatase in situ hybridization and fluorescent immunostainings were imaged on a Leica MZ16F epifluorescence stereoscope using a Leica DFC300FX camera. Embryos that had undergone FISH and fluorescent immunostainings were imaged on a Leica SP2 laser scanning confocal microscope.

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