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Genome-wide analysis demonstrates conserved localization of messenger RNAs to mitotic microtubules

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RNA localization is of critical importance in many fundamental cell biological and developmental processes by regulating the spatial control of gene expression. To investigate how spindle-localized RNAs might influence mitosis, we comprehensively surveyed all messenger RNAs (mRNAs) that bound to microtubules during metaphase in both *Xenopus laevis* egg extracts and mitotic human cell extracts. We identify conserved classes of mRNAs that are enriched on microtubules in both human and *X. laevis*. Active mitotic translation occurs on *X. laevis* meiotic spindles, and a subset of microtubule-bound mRNAs (MT-mRNAs) associate with polyribosomes. Although many MT-mRNAs associate with polyribosomes, we find that active translation is not required for mRNA localization to mitotic microtubules. Our results represent the first genome-wide survey of mRNAs localized to a specific cytoskeletal component and suggest that microtubule localization of specific mRNAs is likely to function in mitotic regulation and mRNA segregation during cell division.

Introduction

Localization of mRNAs within cells functions to spatially restrict gene expression, which is critical for cell fate determination and proper body patterning during embryogenesis (St Johnston, 2005). Localized mRNA translation is also important for many processes in differentiated somatic cells (Kislauskis et al., 1997; Adereth et al., 2005; Huttelmaier et al., 2005) and is used extensively in neuronal cells to restrict the expression of specific proteins to synapses (Kiebler and Bassell, 2006). In *Xenopus laevis* and *Drosophila melanogaster*, mRNAs coding for proteins involved in mitotic progression are enriched on the mitotic spindle (Raff et al., 1990; Groisman et al., 2000). Furthermore, cytological and biochemical studies of *X. laevis* egg extract spindles and sea urchin microtubules have shown that ribosomes are tightly associated with mitotic microtubules, suggesting that localized translation might be a key regulator of mitosis (Suprenant, 1993; Liska et al., 2004; Mitchison et al., 2004). However, the extent of mRNA targeting to specific subcellular structures, such as the spindle, has not been comprehensively investigated and its underlying mechanisms and functions are poorly understood.

To gain insight into the role of localized mRNAs during mitosis, we comprehensively identified mRNAs that localize to microtubules in meiotic *X. laevis* egg extracts and mitotic human cell extracts using Affymetrix microarrays. We found that a specific and conserved group of mRNAs, termed microtubule-bound mRNA (MT-mRNA), is enriched on microtubules. We observed that only a subset of MT-mRNAs was associated with microtubule-bound polyribosomes, indicating that mitotic spindles contain both translationally active and inactive mRNAs. Furthermore, we found that active translation is not required for targeting of endogenous or exogenous mRNAs to mitotic microtubules. We propose that localization of specific mRNAs to the spindle is a conserved mechanism for enhancing protein localization and for segregation of translationally inactive mRNAs.

Results and discussion

To address whether mRNA localization contributes to cell division, we sought to identify mRNAs that localize to microtubules using extracts prepared from *X. laevis* eggs, which are naturally arrested in metaphase of meiosis II by cytostatic factor (CSF) activity. To isolate mRNAs that are targeted to the spindle apparatus, we prepared a small-scale cDNA library from MT-RNAs from *Xenopus tropicalis* egg extracts (Brown et al., 2007) and

Figure 1. **Specific mRNAs are enriched on *X. laevis* meiotic microtubules.** mRNA from total *X. laevis* egg extract or from purified taxol-stabilized microtubules was hybridized to Affymetrix microarrays. The ratio of the signal of each microtubule-associated mRNA to its signal in total extract was calculated. (A) Histogram of the ratios of all mRNAs present at detectable levels ($Log_2 > 5$) in egg extracts. Note that the vast majority of mRNAs are underrepresented on microtubules. (B) Various mRNAs from A that were identified as being enriched on microtubules and a control mRNA were transcribed in vitro, labeled with Alexa 488, and added to *X. laevis* extract spindles. Identified MT-mRNAs localized to spindles, whereas a control mRNA did not. (C) 100 spindles from 3 different extracts were examined to quantify labeled mRNA localization. Error bars represent standard deviation. Bar, 10 μm.
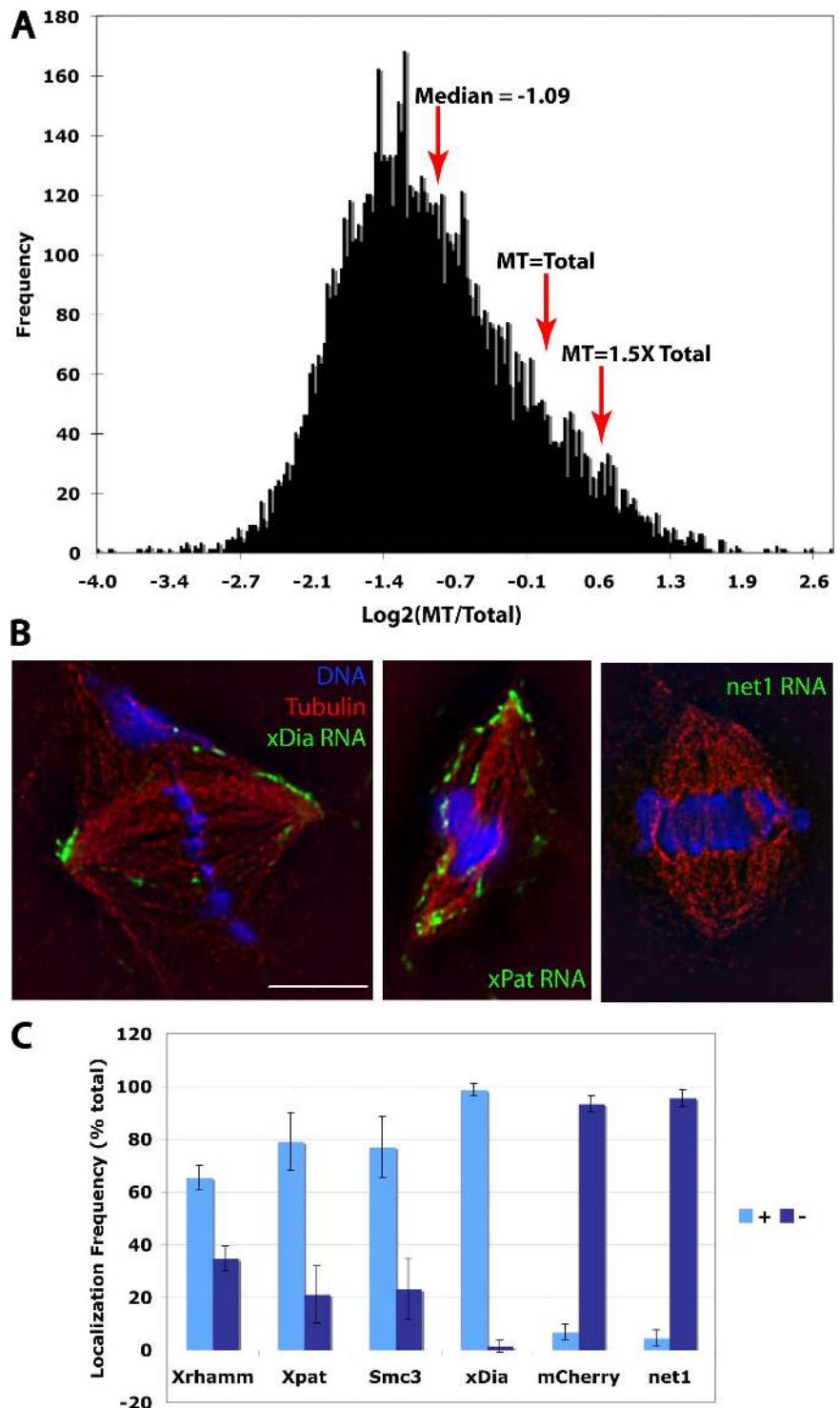
identified ∼50 mRNAs and 50 ribosomal RNAs (rRNAs) by sequence analysis. *X. tropicalis* was used because its sequenced genome facilitated comprehensive identification of cloned RNAs. To test whether the mRNAs identified were enriched on meiotic microtubules, we evaluated a subset of randomly chosen MT-mRNAs from the library by comparing their levels in total extract to the amount in the microtubule-bound fraction using semiquantitative RT-PCR (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200705163/DC1). 8 out of 10 mRNAs

tested were enriched on microtubules compared with total extract, suggesting that specific mRNAs are targeted to microtubules during mitosis.

To comprehensively identify mRNAs that associate with meiotic microtubules, we took advantage of the available Affymetrix *X. laevis* microarrays. We purified taxol-stabilized microtubules from metaphase-arrested *X. laevis* egg extracts by centrifugation through a glycerol cushion and observed that ∼2–5% of the total mass of the RNA in the extract cofractionated
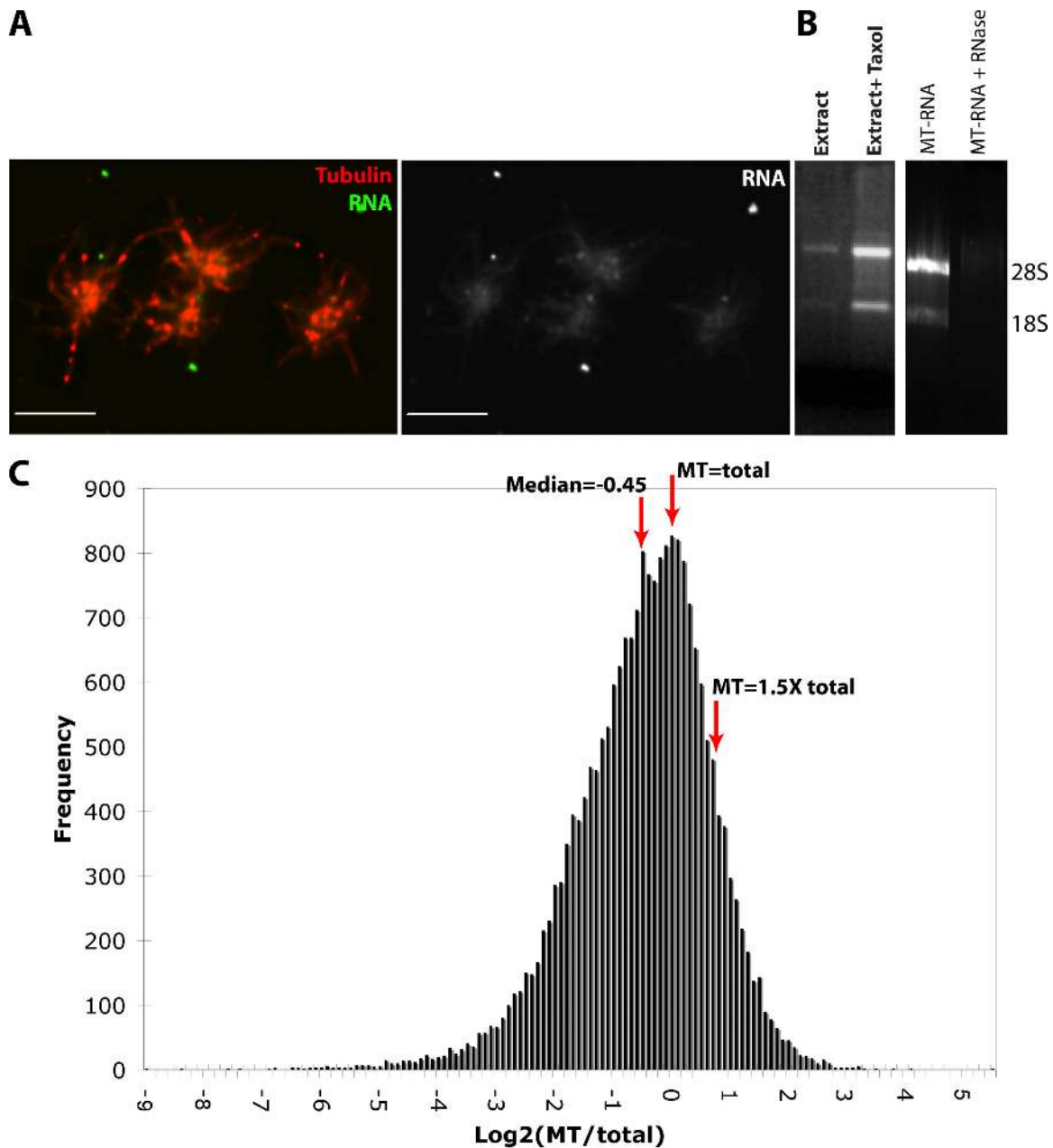
Figure 2. **Specific mRNAs are localized to microtubules in mitotic human extracts.** (A) Mitotic extracts were prepared from synchronized human cells, and microtubule asters induced by the addition of taxol were fixed and spun onto coverslips and stained for RNA (using SYTO RNASelect) and tubulin. RNA was localized to microtubule asters in a granular staining pattern. (B) Asters from this extract were pelleted through a glycerol cushion and isolated RNA was run on an agarose gel and stained with ethidium bromide. rRNA and mRNA pelleted more efficiently from extracts when microtubules were polymerized. Pelleted nucleic acid diminished upon treatment with RNaseA. (C) MT-mRNA and total mRNA were hybridized to Affymetrix microarrays as described in Fig. 1. As seen with *X. laevis,* specific mRNAs were enriched on mitotic human microtubules, whereas the vast majority of mRNAs were not. Bars, 5 μm.

with microtubules (unpublished data). Copurification of RNA with microtubules was specific to pelleted microtubules, as no RNA pelleted without inducing microtubule polymerization (Blower et al., 2005; unpublished data), and similar amounts of RNA cofractionated with microtubules regardless of how microtubule polymerization was induced (taxol, DMSO, RanQ69L, or sperm DNA; unpublished data). We then prepared cRNA from microtubule-associated RNAs and total-extract RNAs, hybridized the cRNA to Affymetrix microarrays, and compared the relative abundance of mRNAs on microtubules to total extract.

Extract mRNAs exhibited a continuum of binding to microtubules, with ∼5% of mRNAs >1.5-fold enriched on microtubules compared with total extract (Fig. 1 A). We found the cyclin B mRNA, which was previously reported to localize to the mitotic spindle (Groisman et al., 2000), several developmentally regulated mRNAs that are targeted to the animal or vegetal cortex of the *X. laevis* egg (Kloc et al., 2002; Table S2, available at http://www.jcb.org/cgi/content/full/jcb.200705163/DC1), and several mRNAs enriched on microtubules that were also identified in our small-scale library (e.g., centromere-associated

Figure 3. **Sites of active translation are located on *X. laevis* meiotic spindles.** (A) Spindles with replicated DNA and centrosomes were formed in *X. laevis* egg extracts and labeled with the fluorescent puromycin derivative F2PMe for 30 min. Spindles were fixed, spun onto coverslips, and stained for the ribosomal subunit S6. Fluorescent puromycin was found along spindle microtubules and concentrated near spindle poles where it colocalized with S6. (B) Increasing amounts of biotinylated lysine tRNA were added to mitotic extracts for 1 h and spindles were isolated and blotted for incorporation of biotinylated lysine using streptavidin. Left, streptavidin blot; right, same samples stained with Coomassie blue as a loading control. Asterisks indicate endogenous biotinylated proteins. Molecular masses in kilodaltons are indicated. Bars, 5 μm.
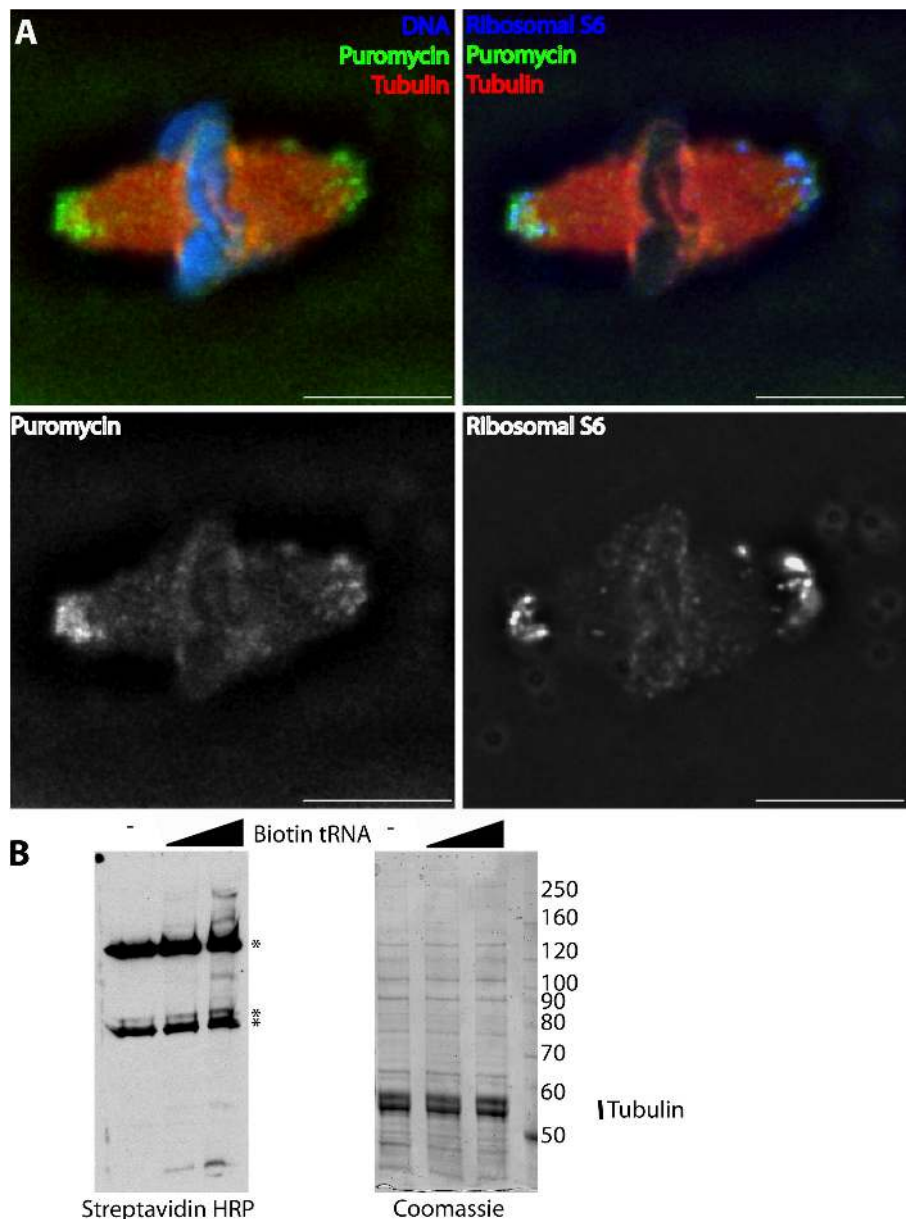
protein E [CENP-E] and dynein). The known MT-mRNA cyclin B was ~1.5-fold enriched on microtubules, suggesting that even low levels of enrichment on microtubules might be biologically relevant. These findings demonstrate that our genome-wide approach accurately identifies microtubule-associated mRNAs.

Analysis of a subset of the enriched MT-mRNAs using the Affymetrix Netaffx analysis package demonstrated that mRNAs annotated to function in mitosis, DNA metabolism (replication, repair, and topology), and germ cell and body axis determination were overrepresented on mitotic microtubules (Table S5, available at http://www.jcb.org/cgi/content/full/jcb.200705163/DC1). Furthermore, examination of the 3′ UTRs of MT-mRNAs revealed that the cytoplasmic polyadenylation element (CPE) was present in 7% of MT-mRNAs, which represents a 4.1-fold enrichment compared with all mRNAs in the database. These results are consistent with and extend previous findings that ribosomes and specific mRNAs are tightly associated with polymerized

microtubules in early sea urchin embryos (Suprenant et al., 1989; Suprenant, 1993; Hamill et al., 1994) and they demonstrate that particular classes of mRNAs are targeted to microtubules during cell division.

To determine if the MT-mRNAs identified by microarray analysis localize to mitotic spindles, we transcribed and fluorescently labeled four different MT-mRNAs (Xpat, Xrhamm, Smc3, and xDia) and two control mRNAs (net1 and mCherry) in vitro, added labeled mRNAs to preformed meiotic spindles in *X. laevis* egg extracts, and assayed the localization of the added mRNA 15 min later. All of the MT-mRNAs tested localized to a high proportion of the spindles, whereas the controls did not (Fig. 1, B and C), demonstrating that our microarray analysis accurately identified mRNAs that can localize to the spindle.

We next asked whether localization of specific mRNAs to microtubules during mitosis is conserved among different cell types and species. Synchronized HeLa cells were used to
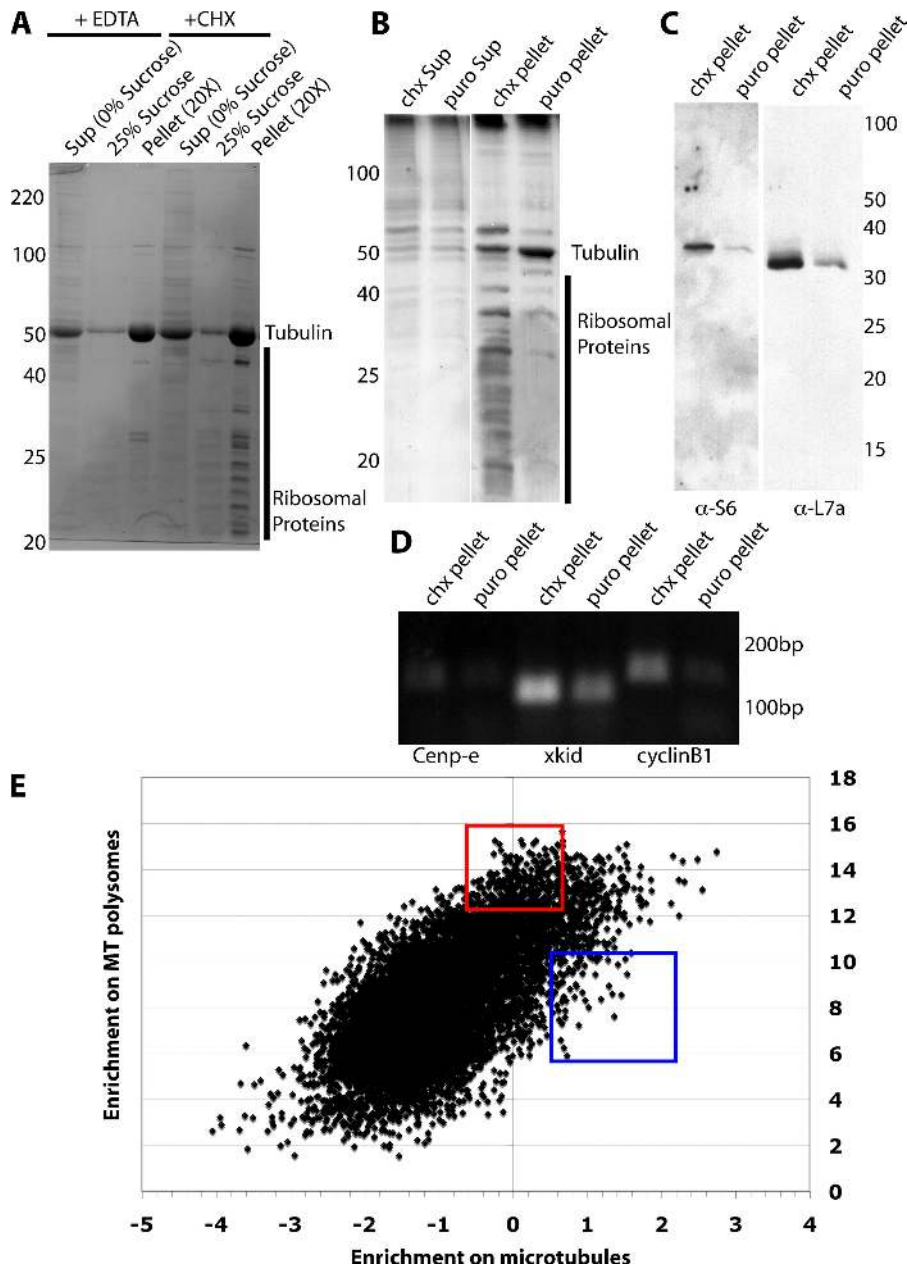
Figure 4. **Polyribosomal mRNAs are associated with *X. laevis* meiotic microtubules.** Taxol-stabilized microtubules were purified from crude *X. laevis* CSF extract by centrifugation through a glycerol cushion, and then resuspended in polysome buffer containing either cycloheximide (CHX) and Mg++ EDTA or puromycin. The resulting resuspension was pelleted through a sucrose cushion (see Fig. 5 A for scheme) and analyzed for protein and RNA content. (A) Ribosomal proteins and RNA only pelleted from the microtubule preparations when polysomes were stabilized with cycloheximide and Mg++. (B) Microtubules were isolated in the presence of either cycloheximide or puromycin. Polysomes were then pelleted through an additional sucrose cushion to isolate microtubule-associated polyribosomes. Ribosomal proteins and RNA only sediment in the presence of cycloheximide, demonstrating that actively translating polyribosomes are present on mitotic microtubules. (C) Protein samples from B were blotted for the ribosomal proteins S6 and L7a, demonstrating reduced levels of ribosomal protein sediment from microtubule preparations in the presence of puromycin. Molecular masses in kilodaltons are indicated. (D) RNA from the polysomal pellets in B and C was assayed by RT-PCR for the presence of MT-mRNAs cyclin B1, Cenp-E, and Xkid. Reduced levels of each were found in the polysomal pellet in the presence of puromycin. (E) PolyA prepared from taxol-stabilized microtubules was hybridized to Affymetrix microarrays. Scatterplot for all mRNAs on the microarray comparing the enrichment of each mRNA on microtubules (from Fig. 1 A, on the x axis) to the enrichment of that mRNA on microtubule-associated polysomes. mRNAs with high y-axis values are likely to be locally translated on the spindle. The red box highlights mRNAs found on polysomes but not dramatically enriched on microtubules, whereas the blue box highlights the converse.

prepare mitotic extracts that assemble radial microtubule asters when treated with taxol, reproducing many aspects of mitotic microtubule organization (Gaglio et al., 1995; Mack and Compton, 2001). RNA localized in a granular pattern on taxol microtubule asters in HeLa extracts, and both rRNA and mRNA specifically copurified with microtubules (Fig. 2, A and B). To identify MT-mRNAs in mitotic HeLa extracts, we hybridized MT-RNA–derived cRNAs to Affymetrix microarrays and compared the abundance of mRNAs in the microtubule sample to the total extract. Similar to our results with *X. laevis,* we observed that there was a continuum of mRNA binding to microtubules, with ~10% of all mRNAs enriched 1.5-fold or more on mitotic microtubules (Fig. 2 C), and that mRNAs encoding proteins involved in various aspects of mitosis and DNA metabolism (replication, repair, and topology) were overrepresented in the MT-mRNA fraction (Table S5). The CPE was also overrepresented in the UTRs of MT-mRNAs with 5.7% of them possessing this element, which is a 4.1-fold enrichment compared with all mRNAs (Table S3, available at http://www.jcb.org/cgi/content/full/jcb.200705163/DC1).

X. laevis oocytes contain both polyadenylated and deadenylated mRNAs (Mendez and Richter, 2001), and lengthening of the poly-A tail is a major mechanism of translational control during early development and is also important in cell cycle control. The cyclin B mRNA contains a CPE that regulates its cytoplasmic polyadenylation via the CPEB protein in developing *X. laevis* embryos (Groisman et al., 2002). In addition, the CPEB protein targets the cyclin B mRNA to microtubules, which leads to an enrichment of the cyclin B protein on the mitotic spindle (Groisman et al., 2000). However, most MT-mRNAs do not contain a CPE, suggesting that there are additional pathways that mediate mRNA targeting to the mitotic spindle.
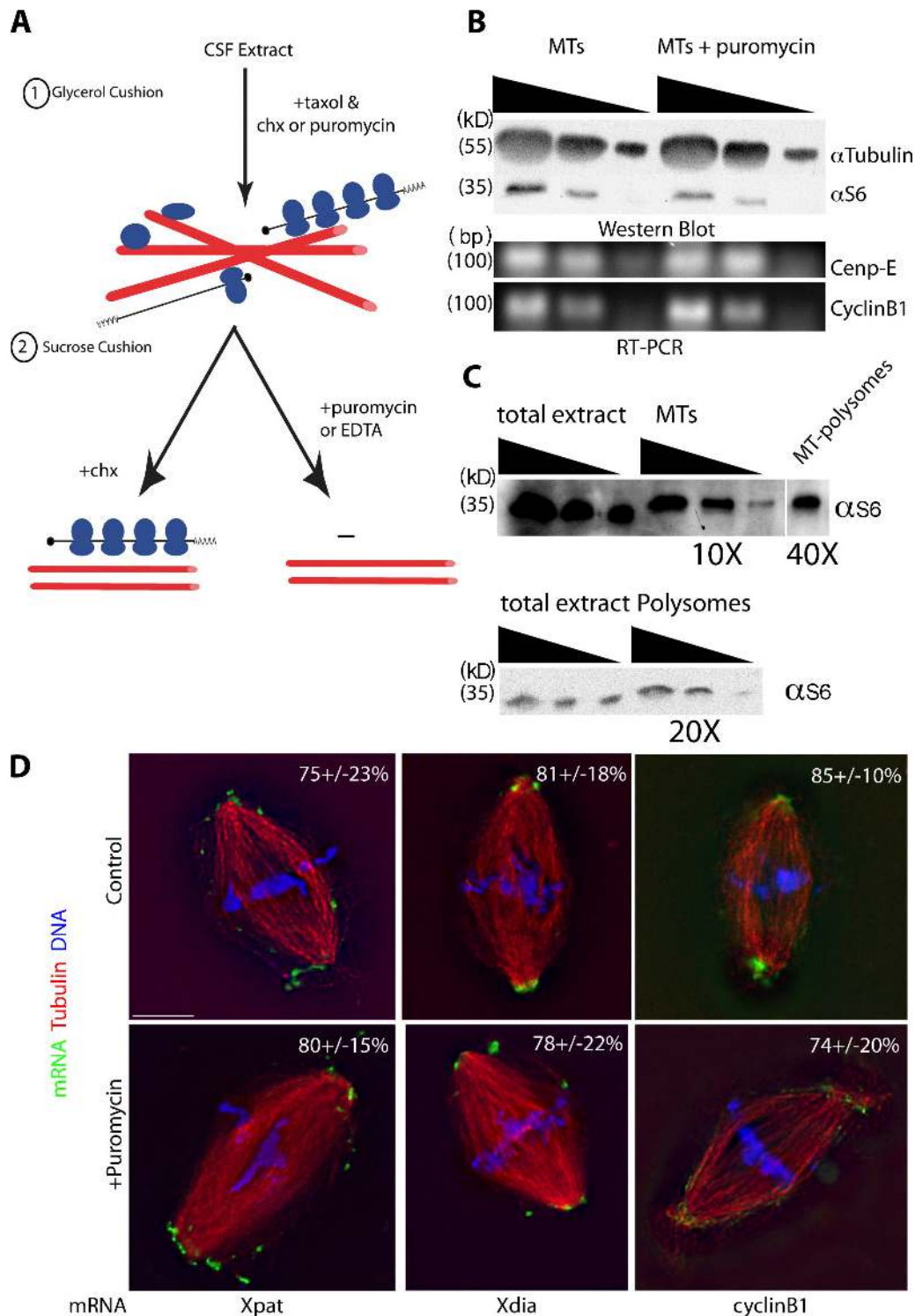
Figure 5. **Translation is not required for mRNA localization to microtubules.** (A) Schematic representation of the experimental scheme used to purify microtubule-associated polyribosomes. In step one, CSF extract is incubated with cycloheximide or puromycin to stabilize or dissociate polysomes, and then microtubules are polymerized by the addition of taxol and pelleted through a glycerol cushion. In step two, ribosome dissociation from microtubules is promoted by adding moderate salt and the resulting mixture is sedimented through a sucrose cushion. Ribosomes still pellet in the presence of cycloheximide, but not in the presence of puromycin or EDTA, indicating that polyribosomes are associated with mitotic microtubules. (B) Taxol microtubules were purified from *X. laevis* CSF extracts (A, step 1) in the presence or absence of puromycin and assayed for the presence of ribosomal S6 protein or tubulin and the CENP-E and cyclin B1 mRNAs. No difference in the levels of ribosomal S6 or the cyclin B1 or CENP-E mRNAs was observed in the presence of puromycin, demonstrating that targeting of ribosomes and mRNAs to microtubules is translation independent. (C) Polysomes were pelleted from taxol-stabilized microtubules (A, step 2, +chx) or from CSF extract (bottom), and ribosomal protein levels were compared with total extract to estimate the relative levels of ribosomes engaged in translation and their enrichment on microtubules. A dilution series of each fraction (10, 5, and 1) was run on SDS-PAGE and blotted for ribosomal S6. The relative loading levels are listed below the blots. We estimate that ~10% of ribosomes in CSF extract are engaged in polysomes, whereas ~15% of ribosomes present on microtubules are engaged in polysomes. (D) In vitro–synthesized Alexa 488–labeled mRNAs for cyclin B1, Xpat, and

The targeting to microtubules of mRNAs involved in mitosis and DNA metabolism suggested that spindle mRNAs might regulate cell cycle events through localized translation. To directly test whether local translation is occurring on the *X. laevis* meiotic spindle, we took advantage of a series of recently developed analogues of the protein synthesis inhibitor puromycin that label sites of active mRNA translation in cultured cells (Starck et al., 2004). We added fluorescent puromycin derivatives to *X. laevis* egg extract reactions containing replicated sperm chromosomes as they were induced to enter metaphase and fixed the spindles at various time points. Fluorescent puromycin formed spots all along spindle microtubules and concentrated near the spindle poles (Fig. 3 A). To confirm that the puromycin spots were sites of active protein synthesis (and not truncated nascent peptides dissociated from ribosomes), we costained spindles with antibodies against the ribosomal protein S6. We found extensive colocalization between the fluorescent puromycin and ribosomal protein S6 (Fig. 3 A), which confirms that local protein synthesis occurs on the meiotic spindle in *X. laevis* egg extracts and is consistent with previous electron microscopy data showing a tight association of ribosomes with spindle microtubules in *X. laevis* egg extracts (Liska et al., 2004; Mitchison et al., 2004). To test whether newly synthesized proteins were incorporated into meiotic spindles, we added biotinylated lysine tRNA to extract reactions, purified spindles through a glycerol cushion, separated the proteins by gel electrophoresis, and tested for incorporation of biotin into newly synthesized proteins using a streptavidin blot. We found that biotinylated lysine was incorporated into many spindle-associated proteins, demonstrating that mitotic translation contributes to the protein content of the mitotic apparatus (Fig. 3 B).

To determine which MT-mRNAs were locally translated on microtubules during mitosis, we purified mRNAs that associated with microtubule-bound polyribosomes. Both rRNA and ribosomal proteins pelleted from microtubule preparations in the presence of cycloheximide and Mg^{2+}, conditions that stabilize polysomes, but not in the presence of EDTA or puromycin, which dissociate polyribosomes (Fig. 4, A–D), confirming that our protocol efficiently isolated microtubule-bound polysomes. To identify the MT-mRNAs that are translated during mitosis, we hybridized the microtubule polysomal mRNAs to Affymetrix microarrays. The levels of MT-mRNAs associated with polyribosomes varied considerably, suggesting that some MT-mRNAs are translated locally on the spindle, whereas others are translationally inactive (Fig. 4 E). Many mRNAs highly represented on microtubule-bound polyribosomes were only marginally enriched on microtubules (e.g., many of the mitotic cyclins; Fig. 4 E, red box), indicating that these mRNAs might not be highly enriched on microtubules but their translation may be spatially restricted to microtubules. Several mRNAs annotated to have a role in development or body patterning that were enriched on microtubules were not highly represented on MT polysomes.

This may indicate that these mRNAs are on the spindle as passive cargo, potentially in a form similar to a P-granule, raising the possibility that targeting of inactive mRNAs to the mitotic spindle may serve as a mechanism for their segregation during cell division.

The observation that many MT-mRNAs are associated with polyribosomes suggests that active translation might be required for targeting some mRNAs to microtubules. In this scenario, as mRNAs are translated into protein the short peptides produced would interact with components of the mitotic apparatus, such as chromosomes or microtubules, thereby localizing polysomes to the spindle. An alternative hypothesis is that mRNAs are actively transported to microtubules in a translation-independent manner, where they can interact with spindle-bound ribosomes and undergo translation. To distinguish between these two possible targeting mechanisms, we examined the effects of the translation inhibitor puromycin on mRNA localization to mitotic microtubules. Because puromycin is a tRNA analogue that blocks peptide bond formation, causing premature chain termination and mRNA release from the ribosome (Azzam and Algranati, 1973), it should disrupt mRNA targeting to microtubules if translation is required. Addition of puromycin to *X. laevis* egg extracts had no effect on the copurification of two endogenous actively translating mRNAs with microtubules (Fig. 5 B). To determine if ribosomes associate with microtubules in a translation-dependent manner, we examined the proportion of spindle-associated ribosomal protein that copurified with polysomes. We found that ~10–15% of microtubule-bound ribosomal protein copurified with polysomes, which is comparable to the amount of ribosomal protein that copurifies with polysomes in total extract (Fig. 5 C), demonstrating that microtubules bind both translationally active and inactive ribosomes. These data demonstrate that translation is not required for the localization of endogenous mRNAs or ribosomes to mitotic microtubules.

To determine if translation inhibition affected the spindle localization of exogenously added mRNAs, we inhibited mitotic translation for 45 min with puromycin, and then added Alexa 488–labeled mRNAs and assayed localization after 15 min. We analyzed mRNAs from three classes: cyclin B1, which is tightly associated with microtubule-bound polysomes and whose protein product is known to associate with the mitotic apparatus; Xpat, which is highly represented on microtubule-bound polysomes but whose protein product is not known to associate with the mitotic apparatus; and Xdia, which is enriched on microtubules but is not significantly represented on polysomes and whose protein product is not known to interact with the mitotic apparatus. Translation inhibition did not impair the localization of any of these three mRNAs to the mitotic spindle, demonstrating that translation is not a mechanism of their localization to mitotic microtubules (Fig. 5 D). Translation inhibition caused subtle morphological changes in localization pattern of the mRNA on

Xdia were added to mitotic spindles formed in the presence of puromycin and assayed for spindle localization. Localization of each mRNA was scored in 100 spindles in 2 different extracts and the percentage with localized mRNAs is listed for each mRNA. Standard error is presented after each mean. Bar, 10 μm.

the spindle, suggesting that although translation does not regulate spindle mRNA targeting, it might affect localization within subdomains of the spindle.

In conclusion, we have found that a specific subset of mRNAs is enriched on mitotic microtubules in both *X. laevis* and humans (Table S4, available at http://www.jcb.org/cgi/content/full/jcb.200705163/DC1). Mechanisms of mRNA localization to subcellular structures are thought to occur through the use of zipcode sequences in the 3' UTR (Kislauskis and Singer, 1992), which are recognized by trans-acting factors that mediate intracellular targeting. There are a few well-documented cases of mRNA localization through a zipcode, but it remains unclear how many mRNAs find their subcellular locations. Our identification of a significant fraction of mRNAs in both *X. laevis* and human mitotic extracts as microtubule targeted during mitosis suggests that this is a conserved and widely used mechanism for enhancing protein localization to regulate mitotic events and delivering translationally inactive mRNAs to daughter cells. We speculate that asymmetric targeting of developmentally regulated mRNAs to one spindle pole could be a mechanism by which organismal asymmetry is established early in *X. laevis* development, and it is worth noting that asymmetric localization of specific mRNAs to one mitotic centrosome during early mollusc development has been reported (Lambert and Nagy, 2002). The next challenge is to identify the cis-acting sequences and trans-acting factors that mediate mRNA localization to microtubules and to understand how translation of specific MT-mRNAs is controlled in space and time, both within the context of the mitotic cell cycle and during development.

Materials and methods

MT-RNA purification

X. laevis egg extracts. X. laevis egg extracts were prepared exactly as previously described (Desai et al., 1999). Microtubule polymerization was induced in 100 μl CSF extract by the addition of taxol to 10 μM. Microtubules were allowed to polymerize for 15 min at 20°C. Extract was then diluted with 1 ml BRB80 (80 mM Pipes, pH 6.8, 1 mM MgCl$_2$, and 1 mM EGTA) + 30% glycerol + 0.1% Triton X-100. Diluted extract was layered over a 10-ml cushion of BRB80 + 60% glycerol. Samples were then centrifuged for 10 min at 17,000 g. The supernatant was aspirated and the interface was washed twice with water. The microtubule pellet was dissolved in RLT solution (RNeasy kit; QIAGEN) and the RNA was purified using the RNeasy kit. The typical yield of microtubule-associated RNA was ~5% of the total RNA present in the extract. Total RNA was purified from untreated CSF extract using the RNeasy kit.

HeLa S100 extract. Mitotic HeLa S100 was prepared from HeLa cells that were synchronized using 2 mM thymidine for 24 h, followed by release into unsupplemented media for 6 h and a 14-h treatment with 10 μM nocodazole, essentially as previously described (Gaglio et al., 1995). Microtubule polymerization was induced by the addition of taxol to the extract to 100 μM. Extracts were incubated at 33°C for 45 min. Extracts were diluted and centrifuged as described for *X. laevis* extracts. RNA was purified from the microtubule pellet or total extract using the RNeasy kit.

For cytological analysis of HeLa mitotic extract asters, asters were formed as described and fixed in solution with 1 ml BRB80 + 0.1% glutaraldehyde for 5 min at 33°C. Samples were then centrifuged onto coverslips as described for *X. laevis* egg extract reactions and fixed to the coverslip by a brief treatment with −20°C methanol. Asters were then stained with SYTOX RNA select (Invitrogen) as described by the manufacturer.

Polysome-associated RNA purification

Purification of polysome RNA was performed according to a modification of Fritz and Sheets (2001). Cycloheximide or puromycin was added to the extract at a concentration of 0.1 mg/ml and incubated for 45 min, and then microtubules were polymerized and isolated as described in the preceding paragraph. The resulting microtubule pellets were resuspended in 2.5 ml PB (20 mM Tris, pH 7.5, 300 mM KCl, 0.5% NP-40, and 2 mM MgCl$_2$ [where indicated]) supplemented with cycloheximide or puromycin and incubated on ice for 15 min. The solubilized microtubule pellet was then layered over a 2.5-ml cushion of PB + 25% sucrose ± cycloheximide or puromycin and centrifuged at 200,000 g for 2 h in a rotor (SW55Ti; Beckman Coulter). Polysome-associated RNA was purified from the pellet using the RNeasy kit and used for microarray analysis or RT-PCR. For protein analysis, polysome pellets were dissolved in 1× SDS-PAGE loading buffer, separated on a 12% gel, and either silver stained or blotted for the ribosomal proteins S6 or L7a (Cell Signaling Technology). For RT-PCR analysis of pelleting of MT-mRNAs, RNA isolated from MT or polysome pellets was reverse transcribed using an oligo-dT primer and SuperscriptIII reverse transcriptase. A dilution series (1:10, 1:100, and 1:1,000) of the resulting product was used as input for 25 cycles of PCR.

Microarrays and data analysis

5 μg each of microtubule-associated RNA or total extract RNA was used as a template for the one-cycle cRNA synthesis kit (Affymetrix). cRNA was hybridized to *X. laevis* or human U133 Plus2 arrays. For each experiment, RNA was prepared from two to three different extract samples.

Microarrays were normalized using the Affymetrix RT controls and all data points with a Log$_2$ <5 were eliminated. The ratio of the signal of a given message on microtubules to total extract was compared using the Log$_2$ of the normalized data. Statistical significance of differences between data was compared using a two-tailed Student's *t* test. The correlation between two trials of polysome-associated mRNAs from two different extracts was 0.91, indicating good reproducibility.

All microarray data was deposited in the ArrayExpress database (www.ebi.ac.uk/arrayexpress/) and is available under accession no. E-MEXP-1320.

mRNAs that were 1.5-fold enriched on microtubules with a p-value <0.05 were analyzed manually for the presence of known RNA sequence elements using the UTR database (www.ba.itb.cnr.it/srs7bin/cgi-bin/wgetz?-page+top).

mRNAs enriched as described by the criteria in the preceding paragraph were used as input to Netaffx analysis software package (Affymetrix) to generate gene ontology (GO) graphs. The GO graphing package calculates whether a particular functional class of genes is overrepresented in the designated subset of genes as compared with the representation of this class of genes in all genes with GO annotations. P-values of <0.05 are statistically overrepresented.

X. laevis extract cytology

1,000 sperm nuclei per microliter were added to CSF-arrested egg extracts that were cycled through interphase by the addition of calcium to 0.6 mM and by a 90-min incubation at 21°C. Extracts were driven back into mitosis by the addition of an equal volume of CSF-arrested extract containing either a buffer control or 100 μg/ml puromycin. For the addition of fluorescent puromycin, F2PMe (provided by S. Starck and R. Roberts, California Institute of Technology, Pasadena, CA) was added to extracts at a concentration of 10 μM.

Cytological analysis of RNA localization

All cDNA clones were obtained from Open Biosystems (clone numbers are listed in parentheses). Full-length clones of XRHAMM (3402708), XDia (4684250), Xpat (5155227), Smc3 (6865177), cyclin B1 (4683804), and mCherry were amplified by PCR and cloned into pCR2.1 downstream of the T7 promoter, and plasmids were verified by sequencing. Plasmids were linearized by KpnI digestion and used to produce Alexa 488–labeled RNA (0.5 μl of 1 mM Alexa 488 UTP [Invitrogen] was included in each 10-μl reaction) using the mMessage Machine T7 in vitro transcription kit (Ambion). The same kit was used to produce RNA encoding net1 (8949944; an mRNA that was dramatically underrepresented on microtubules) directly from cDNA clones. RNAs were precipitated using lithium chloride and resuspended in RNase-free water. Labeled RNA was stored at −80°C until use.

To assess localization of RNAs in *X. laevis* extract spindles, spindles were formed around replicated sperm DNA and 0.02 pmol of labeled RNA was added to the preformed spindles. In extracts where translation had been inhibited, puromycin was added to 100 μg/ml as extracts were cycled into mitosis, and the extract was incubated in the presence of puromycin for 45 min before RNA localization was assessed. RNA was allowed to bind to spindles for 15 min and then structures were diluted into

BRB80 + 4% formaldehyde, 0.1% glutaraldehyde, and 0.5% Triton X-100, incubated for 5 min at room temperature, and centrifuged onto coverslips and fixed in −20°C methanol for 5 s.

To quantify RNA localization to spindles, localization of each RNA to spindles was counted for 100 structures by visual assessment. This was repeated for three independent extract experiments.

Image acquisition
Images were acquired using a microscope (BX61; Olympus) with 60×/1.42NA or 40×/1.2NA objectives and a charge-coupled device camera (ORCA; Hamamatsu) controlled by Slidebook software (3i). All coverslips were mounted in Vectashield containing 1 µg/ml DAPI. Tubulin was visualized by including rodamine-labeled tubulin in all extract reactions at a concentration of 200 nM. All images were acquired at room temperature (∼21°C). Images in Figs. 1–5 were acquired as stacks of images spaced 0.25 µm in the z axis. Images were deconvolved using a constrained iterative algorithm using Slidebook.

Translation analysis
Transcend biotinylated lysine tRNA (Promega) was added to the extract at a 1:10 or 1:20 dilution as the extract entered mitosis. Spindles were pelleted through a 40% glycerol cushion, resuspended in 1× SDS sample buffer, and analyzed for incorporation of biotin by Western blotting with streptavidin HRP (Jackson ImmunoResearch Laboratories).

Online supplemental material
Fig. S1 shows that various MT-mRNAs are enriched on meiotic microtubules in X. tropicalis extracts. Table S1 contains all the microarray data for mRNAs enriched on mitotic microtubules and microtubule-associated polyribosomes in X. laevis. Table S2 contains the relative microtubule enrichment for a group of mRNAs that are localized during X. laevis development. Table S3 contains all the microarray data for mRNAs enriched on mitotic microtubules in HeLa extracts. Table S4 contains a list of genes conserved between X. laevis and HeLa cells, and their relative enrichment on microtubules. Table S5 contains a list of all the classes of genes that are overrepresented on mitotic microtubules in both X. laevis and HeLa cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200705163/DC1.

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