Tum/RacGAP50C provides a critical link between anaphase microtubules and the assembly of the contractile ring in *Drosophila melanogaster*

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Accepted 17 August 2005
Journal of Cell Science 118, 5381-5392 Published by The Company of Biologists 2005
doi:10.1242/jcs.02652

Summary

A central question in understanding cytokinesis is how the cleavage plane is positioned. Although the positioning signal is likely to be transmitted via the anaphase microtubule array to the cell cortex, exactly how the microtubule array determines the site of contractile ring formation remains unresolved. By analysing *tum/RacGAP50C* mutant *Drosophila* embryos we show that cells lacking Tum do not form furrows and fail to localise the key cytokinetic components Pebble (a RhoGEF), Aurora B kinase, Diaphanous, Pav-KLP and Anillin. The GAP activity of Tum is required for cytokinesis: in its absence cytokinesis fails early even though Tum is present on microtubules at the cell equator where the furrow should form. Disruption of the Pebble-interacting domain leaves Tum localised to the cell equator on cortically associated microtubules, again with no evidence of furrowing. These data support a model in which Tum/RacGAP, via its interaction with Pbl, provides a critical link between the anaphase microtubule spindle and cytokinetic furrow formation in *Drosophila* cells.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/118/22/5381/DC1

Key words: Cytokinesis, RacGAP, *Drosophila*, Rho-GTPase signalling, Centralspindlin, GTPase-activating protein

Introduction

After chromosomes have moved to the poles of the mitotic spindle in a dividing cell, a complex set of cellular events, cytokinesis, divides the single cell into two (Glotzer, 2001; Guertin et al., 2002). This process involves a coordinated rearrangement of cytoskeletal components. The metaphase microtubule spindle is remodelled into the central spindle (or spindle midzone), astral microtubules, originating at the centrosomes, grow to contact the cell cortex, and an actomyosin-based contractile ring assembles where the cell will ultimately cleave.

In recent years many proteins involved in cytokinesis have been identified (Giansanti et al., 2004; Glotzer, 2005; Skop et al., 2004), and we are beginning to understand the relationship between many of the components, at least in simple eukaryotes (Wu et al., 2003). Members of the Rho subfamily of small G proteins are central regulators of interphase actin organisation (Etienne-Manneville and Hall, 2002) so it is not surprising that they are involved in the formation and function of the contractile ring. Rho1 is necessary for cytokinesis (Mabuchi et al., 1993; Yoshizaki et al., 2004), at least in some cell types, and a RhoGEF (Pebble in *Drosophila melanogaster*, Let-21 in *Caenorhabditis elegans* and ECT-2 in mammalian systems) is present at the earliest stages of furrow formation and is required for cytokinesis. Pebble (Pbl) has been hypothesised to activate Rho (Prokopenko et al., 1999; Tatsumoto et al., 1999), which, through one or several downstream effectors (such as Diaphanous, Citron kinase and/or Rho kinase), promotes assembly of the contractile ring.

The relationship between the microtubule spindle and the actin-based furrow underlies many of the central unresolved questions of cytokinesis: how is the furrow positioned and how is its formation and function coordinated with other aspects of mitosis (Burgess and Chang, 2005; D'Avino et al., 2005)? Although microtubules are essential for furrow initiation (Hamaguchi, 1975), exactly which microtubules are involved (astral versus central spindle) and the nature of the signal(s) that microtubules deliver (inhibitory or stimulatory) remain the subject of discussion and experimentation (Glotzer, 2004). Signalling between the cortex and astral microtubules has been implicated in furrow positioning for many years (Rappaport, 1986), and compelling data have also emerged indicating that the central spindle can provide a signal for furrow positioning (Gatti et al., 2000; Giansanti et al., 1998).

The central spindle forms during the metaphase-anaphase transition with the bundling of interdigitating inter-polar microtubules and perhaps newly polymerised microtubules (Shu et al., 1995). These microtubules bundle during anaphase and telophase and remain as the midbody when cell division is completed. Two key proteins necessary for successful cytokinesis have been localised to the central spindle: a kinesin-like protein (ZEN-4 in *C. elegans*, Pavarotti or Pav-

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KLP in *D. melanogaster*, and CHO/MKLP1 and MKLP2 in mammals) and a Rho-family GTPase-activating protein (CYK-4 in *C. elegans*, Tumbleweed, abbreviated as Tum and formerly known as RacGAP50C, in *Drosophila* and MgcRacGAP in mammals). Together these proteins form a complex called centralspindlin, which is implicated in assembly of the central spindle (Mishima et al., 2002). Loss of either protein in all organisms examined so far results in loss of the central spindle and failure of cytokinesis (Adams et al., 1998; Kuriyama et al., 2002; Matuliene and Kuriyama, 2002; Mishima et al., 2002; Powers et al., 1998; Raich et al., 1998; Somers and Saint, 2003), although the point of cytokinesis failure seems to vary from organism to organism (Glotzer, 2003; Saint and Somers, 2003).

We recently reported an interaction between the Tum and Pbl proteins, providing a potential link between components of the microtubule spindle and regulation of the actin cortex (Somers and Saint, 2003). We proposed that during anaphase, Pav-KLP-mediated positioning of Tum at the plus ends of cortically associated, interdigitating microtubules results in Pbl localisation and activation at the cell equator, inducing furrow formation.

Support for a role for cortically associated microtubules in furrow induction has come from recent studies of *orbit/mast* mutations in *Drosophila* spermatocytes (D'Avino et al., 2005). This study demonstrated that astral microtubules contact the equatorial cortex, initiating cytokinesis and then become incorporated in the central spindle as 'peripheral central spindle microtubules'.

Our proposal provides molecular detail for models which postulate that regions of microtubule overlap specify furrow position (Alsop and Zhang, 2003; Gatt et al., 2005; Rappaport, 1986). To date, however, the analysis of Tum that has provided evidence for this model has come from in vitro analyses of RNAi-treated S2 cultured cells. Here we use recently generated loss-of-function *tum* alleles (Jones and Bejsovec, 2005) to confirm, in vivo, two predictions of our model. We find that Tum is necessary for the assembly of all other components of the cytokinetic apparatus that we have tested, and that the Tum-Pebble interaction appears to be critical for successful cytokinesis. In addition we show that in the absence of Tum GAP activity, embryonic epidermal cells fail to begin furrowing despite normal localisation of Tum at the cortical midzone, demonstrating a need for GAP activity early in cytokinesis.

Materials and Methods

DNA constructs

A PCR-amplified 5× myc tag was added to the 3′ end of a full-length *tum* cDNA, creating the plasmid that we will simply refer to as *UAS-tum* throughout this paper. This clone was used for all of the Tum constructions described here. *UAS-tum* expression in flies rescues the cytokinetic defect at the cellular level (see Results).

We used PCR amplification and site-directed mutagenesis to delete the first 66 amino acids and introduce an N-terminal methionine to create a construct that encodes a deletion protein lacking the Pav-KLP-interacting region (*UAS-tum*$^{\Delta Pav}$). *UAS-tum*$^{\Delta Pbl}$, a construct deleted for sequences encoding the Pbl-interacting domain (amino acids 66-102) and *UAS-tum*$^{\Delta EIE}$ and *UAS-tum*$^{\Delta YRL}$, two deletions within the GAP domain (amino acids 404-406 for ΔEIE and 416-418 for ΔYRL), were similarly made. All of these substitutions were verified by sequencing and introduced into pUASt vectors for GAL4-induced expression in *Drosophila* embryos (Brand and Perrimon, 1993).

Fixation, immunofluorescence and microscopy

Fly embryos were collected and fixed using standard methods (Karr and Alberts, 1986; Rothwell, 2000; Theurkauf, 1992). For transgene expression studies, overnight egg-lays were done at 18°C to minimise potential dominant-negative effects from overexpression of the mutant transgenes. In the embryonic analysis, co-staining with Tum antibodies was often included to determine the amount of remaining Tum on a cell-by-cell basis.

Antibodies used in this paper include rabbit anti-Pavarotti, and rabbit anti-Aurora B, both from D. Glover (University of Cambridge, Cambridge, UK), rabbit anti-Anillin from C. Field (Harvard University, Boston, MA) and rabbit anti-Diaphanous from S. Wasserman (University of Calfornia, San Diego, CA). Rabbit anti-lacZ (Rockland) and rat anti-myc (JAC6, Abcam) were preabsorbed against wild-type embryos before use to eliminate non-specific background staining. The mouse anti-lacZ monoclonal antibody developed by J. Sanes and the mouse anti-myc monoclonal antibody developed by J. M. Bishop were obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHHD and maintained at the University of Iowa, USA. Mouse anti-tubulin clone B-5-1-2 (Sigma), rat anti-tubulin (cloneYL1/2, Abcam) and sheep anti-tubulin (Cytoskeleton) were used to visualise microtubules. When necessary, embryos were stained with Rhodamine or Cy5-phalloidin (Sigma and Molecular Probes, respectively) and bisbenzimide (Hoechst 33258; 10 μg/ml). Secondary antibodies used were 'highly cross-absorbed' varieties available from Molecular Probes and Jackson Immunochemicals labelled with Alexa-488, Alexa-568, FITC, TMRITC or Cy5.

The rat anti-Tum used was made against the same antigen (a full-length Tum-GST fusion) previously used for our rabbit antibody (Somers and Saint, 2003) and IgG purified using a HiTrap Protein G Sepharose column (Amersham). It recognises a single band on western blots of S2 cells (data not shown) and early embryos, and produces the same immunofluorescence localisation pattern seen with the rabbit antisera previously described (Somers and Saint, 2003).

Embryos were viewed on a Deltavision system (Applied Precision) mounted on an Olympus IX70 inverted microscope using an Olympus 100× PlanApo (n.a. 1.4) lens. Images were collected with a Photometrics CH350 CCD camera using Softworx acquisition software (Applied Precision), post-acquisition manipulation (deconvolution, three-dimensional rotation) was done with Softworx, and final images were assembled using Adobe Photoshop. Images are maximum intensity projections representing 1 μm sections unless otherwise stated.

Fly stocks

tum^{DH15} and *tum*^{AR2} were isolated in a genetic screen for modifiers of a weak *wingless* (*wg*) mutant phenotype (Jones and Bejsovec, 2005). Gal4 drivers used in this work (*prdGAL4* and *enGAL4*) and all of the GFP balancers used in this study were obtained from the Bloomington *Drosophila* Stock Centre. L. Luo (Stanford University, Stanford, CA) provided the *tum*^{1} and *tum*^{347} alleles. The *Tum* RNAi flies have been previously described as RacGAP50C^{RNAi} (Somers and Saint, 2003) and the *UAS::pblGFP* carrying flies were made by P. Smibert (Australian National University, Canberra, Australia). *Df(2R)Exel7128* is a deletion generated by recombination between defined P element insertions in the *tum* region (Parks et al., 2004). It removes all of the *tum* coding region and 15 other neighbouring transcripts.

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In situ hybridisation

In situ hybridisation was performed as previously described (Smallhorn et al., 2004) without proteinase K treatment of the embryos. After the alkaline phosphatase colour development, embryos were washed and processed for immunofluorescent localisation of the myc-labelled transgene and lacZ (balancer chromosome) stripes.

Results

tum is essential for embryonic development

The tum^{DH15} and tum^{AR2} alleles used in this study contain premature stop codons: Arg195 to a stop codon and Trp470 to a stop codon, respectively (Jones and Besjovec, 2005). Although both of these mutations potentially could produce truncated proteins (Fig. 1A) we could detect none by immunofluorescence analysis of mutant embryos or by western analysis of heterozygous or homozygous mutant embryos (data not shown). Both mutations are embryonic lethal when homozygous, as are tum^{DH15}/tum^{AR2} trans-heterozygotes, and they do not complement other *tum* alleles recently described (Goldstein et al., 2005). Wing phenotypes associated with expression of a *tum* RNAi construct (Somers and Saint, 2003) and of a GAP-deficient *tum* transgene (Sotillos and Campuzano, 2000) were both enhanced in a *tum* heterozygous mutant background (data not shown), and the mutant cellular phenotype could be rescued by a *UAS-tum* transgene (see below), all indicating that the phenotypes are the result of mutations in the *tum* gene.

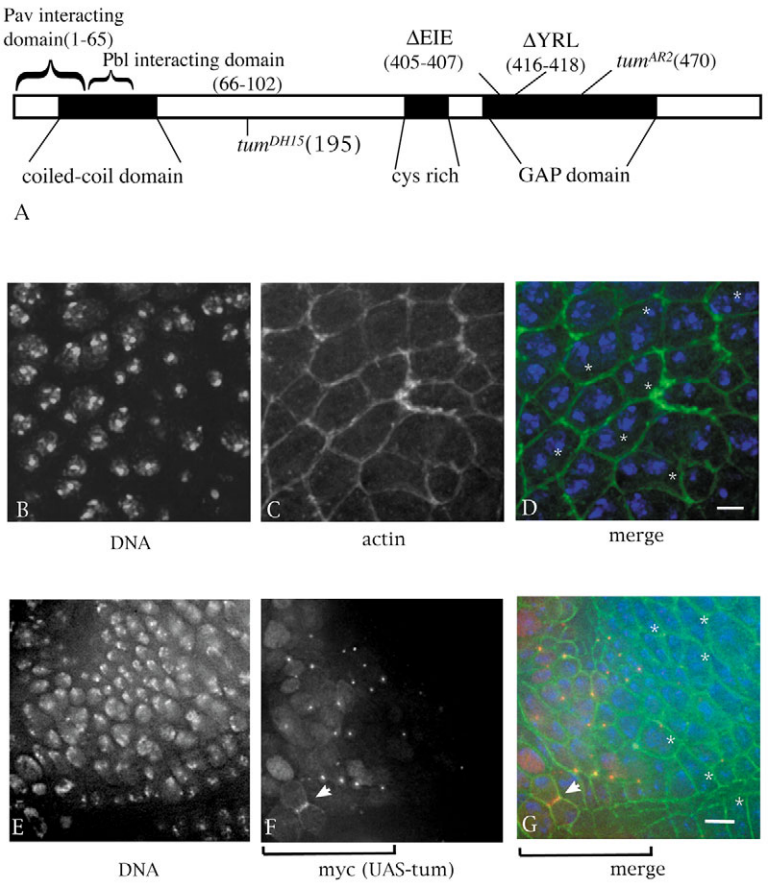

Fig. 1. Cytokinesis fails in the embryonic epithelium of *tum* mutant embryos. (A) Diagram of the Tum protein showing the location of the two premature stop codon mutations in the tum^{DH15} and tum^{AR2} alleles, the Pbl- and Pav-interacting regions deleted in the $tum^{\Delta Pav}$ and $tum^{\Delta Pbl}$ deletions, and the amino acids deleted in the $tum^{\Delta EIE}$ and $tum^{\Delta YRL}$ deletions. Numbers indicate the amino acids deleted in the transgenes or altered to stop codons in the mutants. (B-D) Binucleate cells accumulate in the tum^{DH15} mutant epithelium. DNA (B, and blue in D) and F-actin (C and green in D). Examples of binucleate cells in a stage 11 mutant embryo are indicated with asterisks in D. (E-G) Expression of *UAS-tum* rescues the cytokinetic defect of tum^{DH15} embryos. DNA (E and blue in G), Tum (F and red in G) and F-actin (green in G). Tum was expressed in mutant embryos using the *prdGal4* driver. The bracket below panels F and G indicates the approximate position of the *prd* stripe. Tum is located in midbody remnants and at the cytokinetic furrow of a telophase cell (F and G, arrow). Binucleate cells are abundant in non-expressing stripes of cells (some marked by asterisks in G). Bar, 5 μm.

Because of the known role of Tum in cultured cell cytokinesis, we first looked for mitotic defects in embryos by visualising the actin, tubulin and DNA of embryos that had undergone the 15th and 16th mitotic divisions. Binucleate cells were found in the embryonic epithelium of homozygous mutant embryos from stage 11 (extended germ band) onward (Fig. 1B-D), whereas no binucleate cells were found in $tum^{DH15}/CyO\text{-}P\{en1\}wg^{en11}$ heterozygous siblings (Table 1). tum^{DH15} homozygous embryos, embryos homozygous for a small deletion including the *tum* gene (*Df(2R)Exel7128*) and $tum^{DH15}/Df(2R)Exel7128$ trans-heterozygous embryos had similar frequencies of binucleate cells indicating that tum^{DH15} is probably a null allele (Table 1). However, the tum^{AR2} allele showed marginally fewer binucleate cells (Table 1) suggesting that the partial GAP domain present provides sufficient activity for cytokinesis in some cells, or that the maternal product may persist at a slightly higher level in the presence of this mutated protein. As a result, only tum^{DH15} homozygous embryos were used in the experiments reported here. We used *prd-GAL4*-driven expression (Brand and Perrimon, 1993) of *UAS-tum* in alternating stripes to verify that the observed phenotype was caused by loss of Tum. *Prd-Gal4* expression mirrors that of the *prd* gene (Yoffe et al., 1995) driving expression of transgenes during germ band extension, at a time when maternal supplies of Tum are diminishing in the *tum* mutant embryos (Jones and Bejsovec, 2005). The transgene protein should supplement the maternal stores of Tum and rescue cytokinesis, which would otherwise fail. When expressed in *tum* mutant embryos, Tum rescued the binucleate phenotype (Fig. 1E-G, Table 2). Stripes of binucleate cells (Fig. 1G, asterisks) lacking Tum expression alternated with mononucleate cells with Tum-containing midbodies in the stripes that expressed the protein (Fig. 1E-G). The localisation of UAS-driven Tum was indistinguishable from normal Tum during cytokinesis (see below), localising to the central spindle and at the ends of cortical microtubules at anaphase, and concentrating in the midbody as cytokinesis neared completion (Fig. 1G, arrowhead).

The prominent feature of the *tum* mutant embryos was a failure of cell division and it is this phenotype

Table 1. Frequency of binucleate cells in *tum* mutant embryos

Genotype	Fraction binucleate* (mean ± s.e.m.)	$n^{\dagger}$
$tum^{DH15}/CyO\text{-}P\{en1\}wg^{en11}$	0	719 (5)
tum^{DH15}/tum^{DH15}	0.91±0.04	537 (6)
$tum^{DH15}/Df(2R)Exel7128$	0.88±0.03	538 (5)
$Df(2R)Exel7128/Df(2R)Exel7128$	0.94±0.02	440 (5)
tum^{DH15}/tum^{AR2}	0.70±0.12	674 (6)
tum^{AR2}/tum^{AR2}	0.74±0.08	641 (5)
$tum^{AR2}/Df(2R)Exel7128$	0.71±0.11	662 (7)

*Three 40×40 μm areas were photographed in late/post germ-band-retraction-stage embryos and the fraction of binucleate cells determined and averaged for each embryo. The means and the s.e.m. were calculated from these averages.

†Number of cells scored (number of embryos).

Table 2. Effect of transgene expression, driven by a *prd-GAL4* driver, on the frequency of binucleate cells in tum^{DH15} embryos

Transgene	n*	Expression stripe† (mean ± s.e.m.)	Non-expression stripe† (mean ± s.e.m.)
UAS-tum	566 (5)	0.24±0.06	0.84±0.04
$UAS\text{-}tum^{\Delta EIE}$	539 (5)	0.97±0.02	0.95±0.03
$UAS\text{-}tum^{\Delta YRL}$	644 (4)	0.92±0.06	0.93±0.05
$UAS\text{-}tum^{\Delta Pbl}$	635 (7)	0.91±0.06	0.90±0.09
$UAS\text{-}tum^{\Delta Pav}$	617 (6)	0.96±0.03	0.96±0.03
UAS::pblGFP	575 (5)	0.97±0.02	0.97±0.05

*Number of cells scored (number of embryos).

†Three 40×40 μm areas were photographed in late/post germ-band-retraction-stage embryos, including an expressing stripe and a non-expressing neighbouring stripe and the fraction of binucleate cells determined and averaged for each embryo. The means and the s.e.m. were calculated from these averages.

that is the focus of this report. Several major morphogenetic events occur, including germ band retraction, head involution and dorsal closure, although we did note transient loss of tissue integrity during germ band retraction in some embryos (data not shown). Although we did not study late-stage *tum* mutant embryos extensively, all showed defects in nervous system and muscle organisation (data not shown).

Cytokinesis fails in *tum* mutant embryos

We next conducted a detailed examination of cells undergoing mitosis in mutant embryos during the 15th and 16th divisions. In normal dividing cells, Tum is diffuse at prometaphase (Fig. 2B, asterisks) but is detectable on midzone microtubules during anaphase (Fig. 2B, arrowhead) and in rings in early telophase cells (Fig. 2B, arrow). Midbody remnants litter areas of recent mitotic activity in the embryo (Fig. 2D, arrows). Mitosis in homozygous mutant embryos was found to be indistinguishable from heterozygous sibling embryos up until mid-to-late stage 11 (extended germ band), when aberrant telophase figures and a few binucleate cells were observed in the dividing epithelial cells of some of the embryos (Fig. 1B-D). Older embryos (stage 12) had binucleate cells (data not shown) and aberrant telophase figures in the epithelium (Fig. 2G, asterisks), and lacked midzone or midbody localised Tum protein (Fig. 2F). In many cases we observed that cells in which maternally derived Tum was no longer evident showed

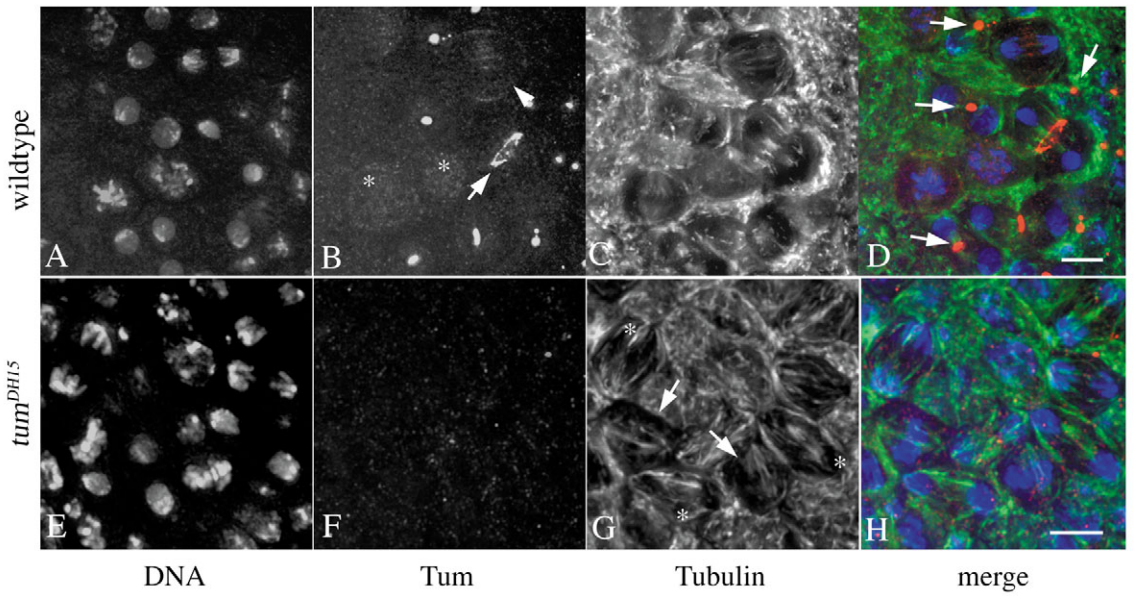

Fig. 2. Anaphase microtubule reorganization fails in *tum* mutant cells. (A-D) Tum localisation in wild-type dividing cells. DNA (A and blue in D), Tum (B and red in D) and microtubules (C and green in D). Prometaphase cells show diffuse Tum staining (asterisks in B). An anaphase cell (arrowhead, B) has Tum localised at the cortex and in microtubule bundles. A telophase cell (arrow, B) has partially completed cytokinesis and has a ring of Tum. Residual midbodies mark the positions of previous divisions (arrows, D). This is a merged Z-stack representing a total 4-μm-thick slice. (E-H) Tum localisation in dividing cells of the tum^{DH15} mutant at stage 12. Tum alone (F) and merged (H, in red) with microtubules in green and DNA in blue. Tum is not detectable in embryos at this time (F). Metaphase cells (arrows, G) appear normal, whereas anaphase and telophase cells (asterisks, G) have a variety of aberrant microtubule bundles. This is a merged Z-stack representing a total 1-μm-thick slice. Bar, 5 μm.

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no evidence of furrowing at a time in late anaphase when wild type cells are constricting. However, some cells with residual maternal Tum progress further in cytokinesis than others, an effect that we attribute to variable perdurance of the Tum maternal protein. Such variability is also seen with the *pbl* zygotic cycle 14 phenotype (Echard and O'Farrell, 2003).

We did not detect obvious defects in the F-actin (Fig. 1C) or microtubule cytoskeletons of mutant cells in interphase, and we observed normal-looking prometaphase and metaphase spindles (arrows in Fig. 2G) adjacent to binucleate cells and aberrant telophase cells (Fig. 2G, asterisks). We conclude that Tum is not involved in these earlier stages of mitosis, but the many examples of aberrant microtubule organisation seen in anaphase and telophase cells indicate that cell division is failing after chromosome separation. These cells had variable microtubule morphologies, ranging from relatively few detectable microtubules with no apparent central spindle, to multiple microtubule bundles (Fig. 2H).

Localisation of key cytokinetic components is disrupted in *tum* mutants

Our earlier studies in S2 cells suggested that Tum is required for early events in contractile ring formation or function. To examine this in embryos, we used immunofluorescence to localise proteins required for cytokinesis in *tum* mutant embryos. We examined Aurora B (AurB), a regulatory kinase; Diaphanous (Dia), a potential immediate downstream target of Rho signalling; Pbl, which binds to Tum; Anillin, an actin binding protein known to localise to the furrow early in some cell types; and Pav-KLP, the kinesin partner of Tum in the centralspindlin complex.

Aurora B

AurB is a chromosomal passenger protein, one of several proteins that initially localise to centromeres at prometaphase and metaphase, and then move to the central spindle and midzone cortical microtubules at anaphase (Adams et al., 2001). MgcRacGAP is a substrate for Aurora B, with multiple phosphorylation events controlling GTPase substrate selection (Minoshima et al., 2003) and binding to another cytokinesis factor, PRC1 (Ban et al., 2004).

In both wild-type and tum^{DH15} embryos, AurB correctly localised to its centromeric position at prometaphase and metaphase (Fig. 3A,B, arrowheads). In normal anaphase cells, AurB is found on the central spindle and on cortical-microtubule sites (Fig. 3A, arrow). In *tum* mutants, in the absence of detectable Tum, AurB was delocalised at anaphase (Fig. 3B, arrow), and never concentrated on the midzone cortex. We conclude that Tum is necessary for the correct localisation of AurB, both in the central spindle and on cortical microtubules, but not for metaphase centromere association.

Diaphanous

Diaphanous (Dia) is a formin homology protein known to be involved in cytokinesis (Castrillon and Wasserman, 1994; Tominaga et al., 2000). Formin homology proteins act as regulators of actin polymerisation in yeast (Pruyne et al., 2002) and as mediators of small-GTPase-microtubule regulation (Ishizaki et al., 2001). In wild-type embryos (Fig. 3C) Dia was found to be uniformly cortical throughout most of the cell cycle. At late anaphase and telophase Dia became more concentrated at the furrow (Fig. 3C, arrowheads). Mutant epithelial cells with no detectable Tum did not show concentration of Dia at the equatorial cortex between the telophase nuclei, the area where a furrow would be expected (Fig. 3D, arrowhead). From these observations, we conclude that Tum is required for Dia localisation, consistent with an early role for Tum in cytokinesis.

Pebble

Because we lack an antibody useful for detecting Pbl in immunofluorescent labelling, we used a GFP antibody to follow the behaviour of a Pbl-GFP fusion (Pbl-GFP) whose expression was driven by a *prd-GAL4* driver in *tum* mutant embryos. In these embryos, Pbl-GFP was expressed in stripes in the odd-numbered segments of the embryo. Its expression did not alter the binucleate phenotype of *tum* mutant embryos (see below, Table 2) but did rescue the *pbl* cytokinetic phenotype when expressed in *pbl* mutant embryos (data not shown) suggesting that it is a reliable indicator of Pbl location.

Like wild-type Pbl (Prokopenko et al., 2000), Pbl-GFP was found to be nuclear during interphase, but at prometaphase it became cortically localised (data not shown). At anaphase it localised to the cortex, occasionally being found at the ends of microtubules (Fig. 3E, arrowhead), and became increasingly concentrated at the cytokinetic furrow as furrowing progressed (Fig. 3F, arrowhead). During anaphase Pbl-GFP could be seen in association with Tum on microtubules at the equatorial cortex (Fig. 3E,F). If Pbl were localised to the equator independently of Tum we would have expected to see some evidence of its concentration at the equator in t*um* mutants. In contrast to this prediction however, Pbl-GFP showed no obvious concentration at the presumptive furrow in *tum* mutants, (Fig. 3G, arrowheads), supporting the idea that Pbl localisation is either directly or indirectly the result of Tum presence and/or activity at the cell equator.

Anillin

Anillin is an actin- and septin-interacting protein (Field and Alberts, 1995; Kinoshita et al., 2002) known to be essential for cytokinesis in vertebrate and fly-tissue-cultured cells (Oegema et al., 2000; Somma et al., 2002). It is also one of the earliest proteins to localise to the furrow site during spermatocyte cytokinesis (Hime et al., 1996; Giansanti et al., 1999). A protein (mid1p) with limited homology to Anillin is involved in furrow positioning in *Saccharomyces pombe* (Paoletti and Chang, 2000). In our analysis, Anillin was found in the nucleus at interphase, and then dispersed at prometaphase to the cortex as particles or granules, concentrating at the furrow at anaphase (Fig. 3H). In tum^{DH15} mutant embryos, Anillin failed to concentrate at the cell equator, remaining distributed around the cortex of anaphase and telophase cells (Fig. 3I, arrow). We conclude that Anillin does not concentrate at the presumptive furrow site in the absence of Tum. Consistent with the observed absence of furrowing, none of the markers of furrow formation were found to localise to the equatorial cortex in cells lacking Tum.

Loss of Tum results in the loss of Pav-KLP in the ectoderm

Pavarotti (Pav-KLP) is the kinesin-like protein partner of Tum in the *Drosophila* equivalent of the *C. elegans* centralspindlin complex (Mishima et al., 2002). It is required for the bundling of microtubules during anaphase and for formation of the central spindle in *Drosophila* (Adams et al., 1998; Goshima and Vale, 2003).

In both early embryos and S2 cells, Pav-KLP localisation is coincident with Tum (Somers and Saint, 2003). In wild-type embryos, Pav-KLP can be readily detected in the spindle midzone of anaphase and telophase cells (Fig. 3J, arrow), at the ends of microtubules at the cell equator and in midbody remnants. Specific localisation of Pav-KLP was not observed in dividing epithelial cells deficient in Tum from stages 11-12 (Fig. 3K) and, in general, mutant embryos at this stage had much lower levels of Pav-KLP staining suggesting that Pav-KLP may be unstable in the absence of Tum.

If Pav-KLP abundance is dependent on the presence of Tum, then expression of Tum in *prd-GAL4* stripes in *tum* mutant embryos should restore or enhance Pav-KLP expression in those stripes. Indeed, Pav-KLP expression and localisation during the mitotic cycle was restored in the stripes of mutant cells expressing *UAS-tum*

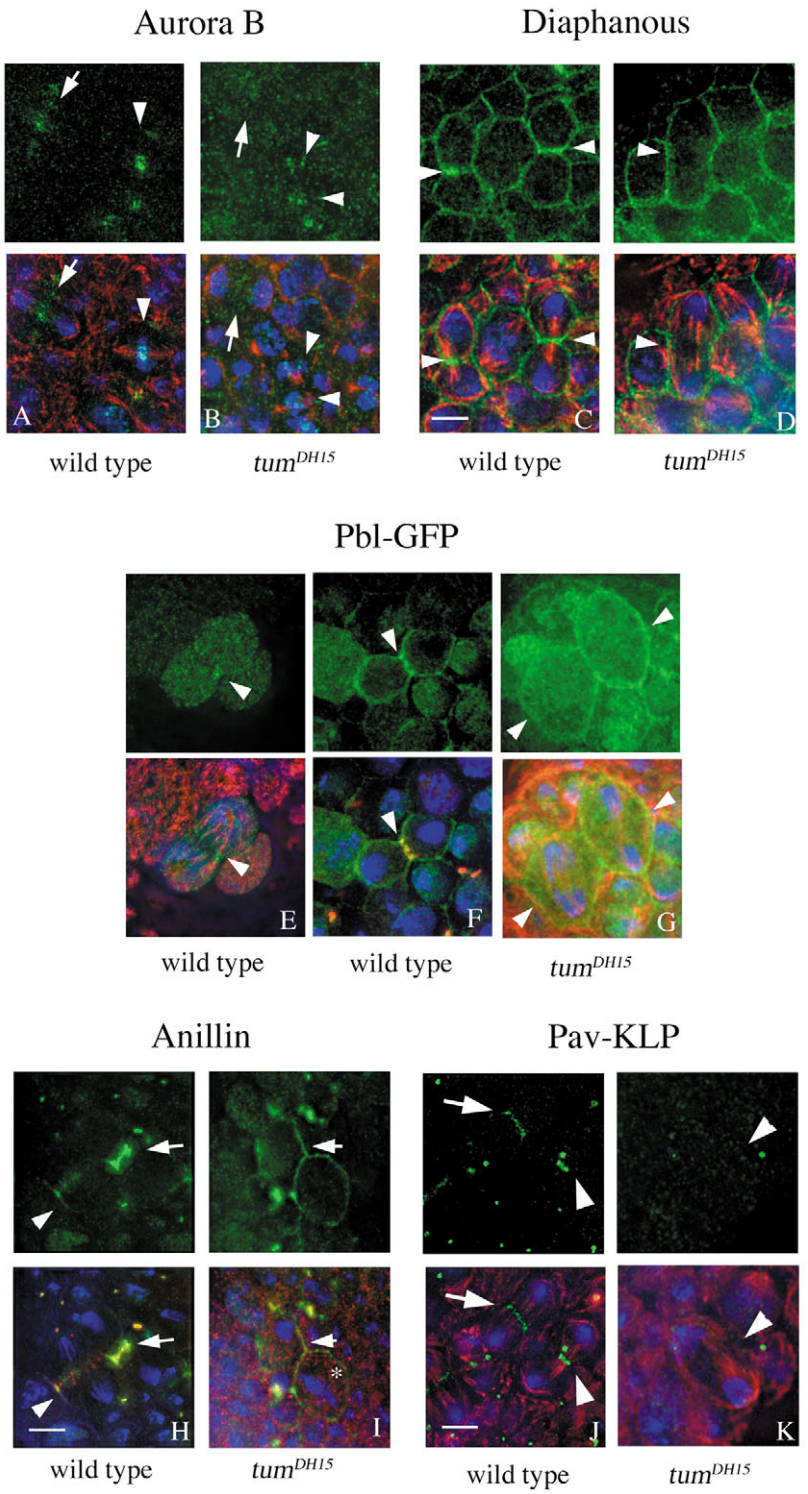

Fig. 3. Loss of Tum results in mislocalisation of other cytokinetic furrow components in mutant embryos. (A,B) The localisation of AurB (green) is shown alone in the upper panels, and merged with the microtubule (red) and DNA (blue) images in the lower panels in wild-type embryos (A) and *tum*^{DH15} embryos (B). AurB is associated with the chromosomes of metaphase cells (arrowheads, A and B) in mutant and wild-type embryos. At anaphase AurB moves to the central spindle and cortex in wild-type cells (arrow, A) but is not localised correctly in mutant cells (arrow, B). (C,D) The localisation of Diaphanous (green) is shown alone in the upper panels, and merged with the microtubule (red) and DNA (blue) images in the lower panels in wild-type (C) and *tum*^{DH15} (D) embryos. Diaphanous is cortical throughout the cell cycle, but concentrates at the midzone cortex of wild-type telophase cells (arrowheads, C). In *tum*^{DH15} mutants, Diaphanous does not concentrate in the midzone cortex (arrowhead, D). (E,F,G) The localisation of Pbl-GFP (green) is shown alone in the upper panels and merged with the microtubule (red E, G), Tum (red, F) and DNA (blue) images in the lower panels in wild-type (E,F) and *tum*^{DH15} (G) embryos. At anaphase, Pbl-GFP is concentrated in the midzone cortex in normal cells (arrowheads, E,F) but remains cortical throughout mitosis in *tum*^{DH15} mutant cells (arrowheads, G). (H,I) The localisation of Anillin (green) is shown alone in the upper panels, and merged with Tum (red) and DNA (blue) in the lower panels in wild-type (H) and *tum*^{DH15} (I) embryos. Anillin concentrates at the midzone at anaphase (arrowhead, H) and concentrates in the furrow as cells undergo cytokinesis (arrow, H). In *tum* mutant cells, Anillin is cortical in metaphase (asterisk, I) and does not concentrate in the midzone cortex during telophase (arrow, I). (J,K) The localisation of Pav-KLP (green) is shown alone in the upper panels, and merged with the microtubule (red) and DNA (blue) images in the lower panels in wild type (J) and *tum*^{DH15} (K) embryos. In wild-type embryos, Pav-KLP concentrates at the furrow at anaphase (arrow, J) and becomes concentrated in the midbody as cytokinesis progresses (arrowhead, J). In *tum* mutant embryos, no Pav-KLP is detected in anaphase or telophase cells (arrowhead, K). Bar, 5 μm. Grayscale images for all color channels can be viewed in supplementary material Fig. S3.

(Fig. 4A-F) with Pav-KLP detectable in equatorial cortical sites (Fig. 4C, arrowhead) and in midbody remnants coincident with the Tum protein (Fig. 4C). The Pav-KLP/Tum interaction appeared to be necessary for this rescue, as a Tum variant lacking the Pav-KLP-interacting domain did not rescue Pav-KLP stability (see below, Fig. 4M-O).

Binding of Tum protein to Pav-KLP could stabilise it or protect it from degradation. Alternatively, Tum could be working indirectly, for example, by enhancing Pav-KLP transcription or translation. To test for transcriptional regulation, we used in situ hybridisation (supplementary material Fig. S1) to look for changes in *pav* mRNA levels in embryos expressing *UAS-tum* in stripes of *prdGAL4*-driven expression. Unlike the Pav-KLP protein level, no change in *pav* mRNA level was observed in Tum-expressing stripes (supplementary material Fig. S1, 41 myc-striped embryos analysed). We conclude that Tum is required for Pav translation or stability in the ectoderm.

GAP-deficient Tum does not rescue cytokinesis in *tum* mutant ectoderm

As an initial step in defining the roles of the protein domains of Tum, we expressed two different forms of Tum with deletions within the GAP domain (amino acids 404-406, *UAS-tum*$^{\Delta EIE}$ and 416-418, *UAS-tum*$^{\Delta YRL}$, Fig. 1A). The equivalent mutations eliminate in vitro GAP activity in α-chimerin GAP (Ahmed et al., 1994) and Cdc42GAP (Leonard et al., 1998), while retaining GTPase binding and other biochemical attributes (see Discussion). When expressed during wing development in *Drosophila* the deletions affect both cytokinesis and vein differentiation (Sotillos and Campuzano, 2000).

Both Tum$^{\Delta EIE}$ and Tum$^{\Delta YRL}$ localised primarily to the interphase nucleus (Fig. 5A,F) and preserved Pav-KLP in stripes of *prd*-driven expression (Fig. 4G-L,) but neither GAP-deficient Tum protein rescued cytokinesis in *tum* mutant embryos (Fig. 5, Table 2). The mitotic localisation of the two proteins differed somewhat. Tum$^{\Delta EIE}$ protein was not seen associated with microtubules nor localised to the cortical midzone at any time during the mitotic cycle (Fig. 5B-E). Tum$^{\Delta YRL}$ protein was most often seen at the ends of microtubules associated with the cortical midzone beginning at early (Fig. 5F, arrowhead) anaphase and continuing throughout nuclear reformation at telophase (Fig. 5G-J). Many late-telophase and interphase binucleate cells have remnants of this equatorial protein persisting in a pattern reminiscent of mid-anaphase in wild-type cells (compare Fig.

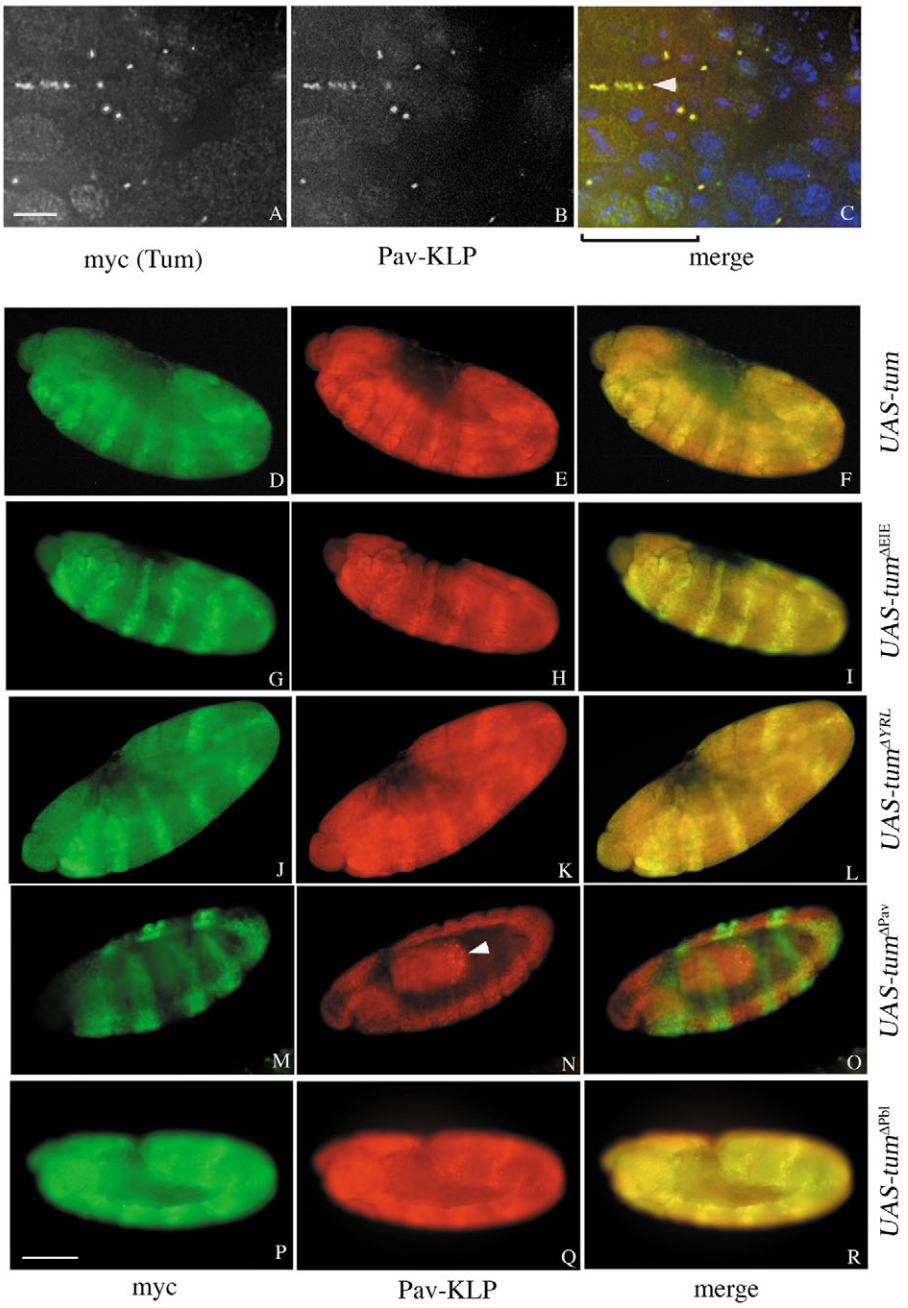

Fig. 4. Elevated Pav-KLP protein levels occur in stripes of cells expressing all but the *UAS-tum*$^{\Delta Pav}$ transgene. In these experiments transgene-derived Tum localisation was detected with a myc antibody (shown in green), and Pav-KLP is shown in red. (A-C) The arrowhead in C indicates two anaphase cells with Pav-KLP and Tum colocalising at the furrow sites in a *prd-GAL4*-induced expression stripe. Midbody remnants containing both proteins are also evident. In panel C DNA staining is blue and the bracket marks the approximate boundary of the *prd* expression stripe. Pav-KLP is detected in stripes of mutant cells expressing Tum (D-F), Tum$^{\Delta EIE}$ (G-I), Tum$^{\Delta YRL}$ (J-L), Tum$^{\Delta Pbl}$ (P-R), but not Tum$^{\Delta Pav}$ (M-O). The Pav antibody detects sequestered Pav-KLP protein in the germ line (arrowhead, N) of Tum$^{\Delta Pav}$-expressing embryos but detects no Pav-KLP in the stripes of Tum$^{\Delta Pav}$ expression. Bar, 5 μm (A-C); 100 μm (D-R).

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2D with Fig. 5J). These results demonstrate that GAP activity is necessary for cytokinesis in the ectoderm and suggest that GAP activity is first needed when Tum reaches the cell equator.

Occasionally (4 out of 56 post-anaphase cells), Tum$^{\Delta YRL}$ protein is seen as rings trapped in the cortex of recently divided cells (supplementary material Fig. S2), suggesting that sufficient wild-type Tum was present in these cells to partially complete cytokinesis. This phenotype is reminiscent of the phenotypes seen in other organisms depleted of their Tum homologs (see Discussion) and suggests that there is a second, later critical phase when Tum GAP activity is needed.

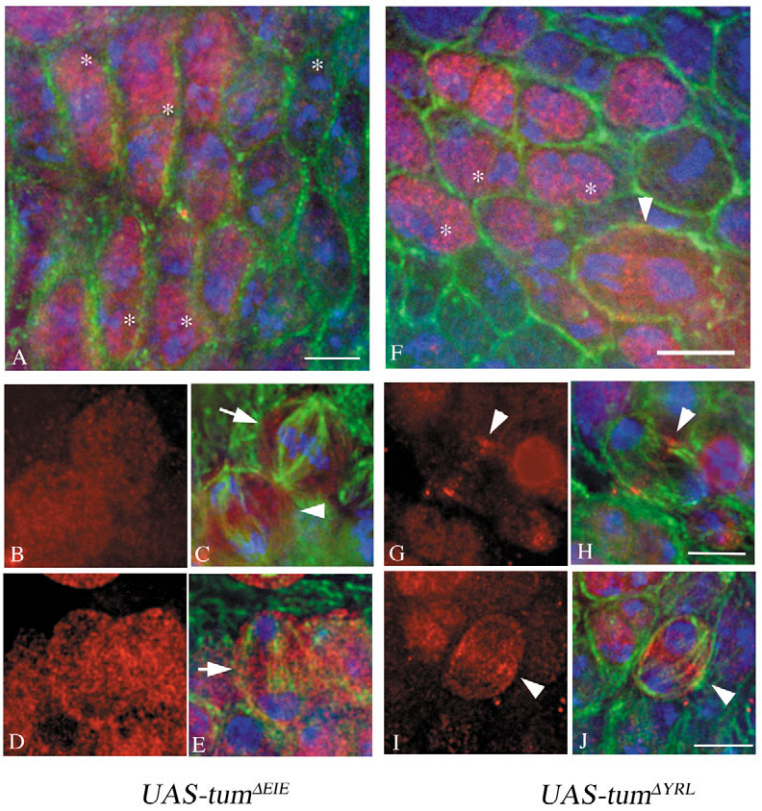

Fig. 5. Transgene deletions demonstrate that a functional GAP domain is required for Tum cytokinetic function. Tum transgenes with GAP domain deletions *UAS-tum$^{\Delta EIE}$* (A-E) and *UAS-tum$^{\Delta YRL}$* (F-L), were expressed in *prd* stripes in *tum^{DH15}* embryos. In all panels, anti-myc antibody shows the transgene localisation in red, tubulin (or F-actin, panel F) localisation is shown in green and DNA in blue. (A-E) When *UAS-tum$^{\Delta EIE}$* is expressed in *tum^{DH15}* embryos, it fails to localise correctly or rescue cytokinesis. Binucleate cells (asterisks, A) are readily detected in stripes of *UAS-tum$^{\Delta EIE}$*-expressing cells, whereas midbody remnants are rarely found. Cells in the mitotic cycle in these stripes have diffuse Tum$^{\Delta EIE}$ protein at metaphase (arrow, C), anaphase (arrowhead, C) and telophase (arrow, E). (F-J) When *UAS-tum$^{\Delta YRL}$* is expressed in *tum^{DH15}* embryos, it localises to the cortex equator, but also fails to rescue cytokinesis. Binucleate cells (asterisks, F) are readily detected in stripes of *UAS-tum$^{\Delta YRL}$*-expressing cells, whereas midbody remnants are rarely found. Tum$^{\Delta YRL}$ localises to the ends of microtubules at the cell equator at anaphase (arrowhead, F) and this localisation does not change throughout telophase or the subsequent interphase (arrowheads, G,H,I,J). Bar, 5 μm. Grayscale images for all color channels can be viewed in supplementary material Fig. S4.

Interaction between Tum and Pbl is required for Tum cytokinetic function

The Pav-KLP-interacting and Pbl-interacting domains of Tum map to amino acids 1-65 and 66-103, respectively (Somers and Saint, 2003). We generated transgenes of *tum* lacking these regions (called *UAS-tum$^{\Delta Pav}$* and *UAS-tum$^{\Delta Pbl}$*, Fig. 1A) and expressed them under the control of the *prdGAL4* driver.

Tum$^{\Delta Pav}$ failed to rescue cytokinesis in *tum^{DH15}* mutant flies (Fig. 6A-E). Tum$^{\Delta Pav}$ protein was found predominantly in the nucleus at interphase (Fig. 6A, asterisk) and was distributed diffusely in the cytoplasm, with cortical enrichment, at prometaphase and metaphase (Fig. 6B-E). This cortical localisation continued into anaphase and telophase, behaviour not seen in the other Tum protein variants (Fig. 6D). Tum$^{\Delta Pav}$ protein was never associated with microtubules, nor did it specifically concentrate at the midzone of the cortex during mitosis. This reconfirms the role of the MgcRacGAP N-terminus in microtubule binding (Hirose et al., 2001) via its association with Pav-KLP. Tum$^{\Delta Pav}$ expression could not preserve stripes of Pav-KLP in *tum* mutant embryos (Fig. 4M-O), supporting the idea that Pav-KLP translation is linked to the presence of Tum or that Pav-KLP is more stable when complexed with Tum.

Expression of Tum$^{\Delta Pbl}$ also failed to rescue cytokinesis (Fig. 6F-K, Table 2). The mutant protein was found in the midpoint of small microtubule bundles often associated with the cortex (Fig. 6F,H,I) corresponding to the equator between the daughter cells, but central spindles were absent or poorly organised in these cells (Fig. 6F). Pav-KLP was readily detectable in stripes of Tum$^{\Delta Pbl}$ expression (Fig. 4P-R), indicating that Tum$^{\Delta Pbl}$ could still interact with Pav-KLP and that the aberrant bundling did not result from loss of the centralspindlin complex.

This bundling of equatorial microtubules in mutant cells was observed only in cells expressing the Tum$^{\Delta Pbl}$ deletion, suggesting that these cells can accomplish some aspects of anaphase microtubule reorganisation and mark the equator of the dividing cell with localised centralspindlin, but that furrowing does not proceed without interaction between Tum and Pbl.

Discussion

Here we have provided a detailed analysis of cytokinesis in *Drosophila tum* mutant embryos. In these embryos, maternal *tum* mRNA products become limiting during cycles 15 and 16. The interphase arrays of microtubules and F-actin in epithelial cells appear unaffected in *tum* mutant cells at this stage, but many of the cycle 16 and cycle 17 divisions fail.

Tum is required for central spindle assembly and localisation of cytokinesis factors

Cells lacking detectable Tum progress through the mitotic cycle, successfully assembling a metaphase spindle and undergoing anaphase A and B. However, they fail to form or maintain a distinct central spindle

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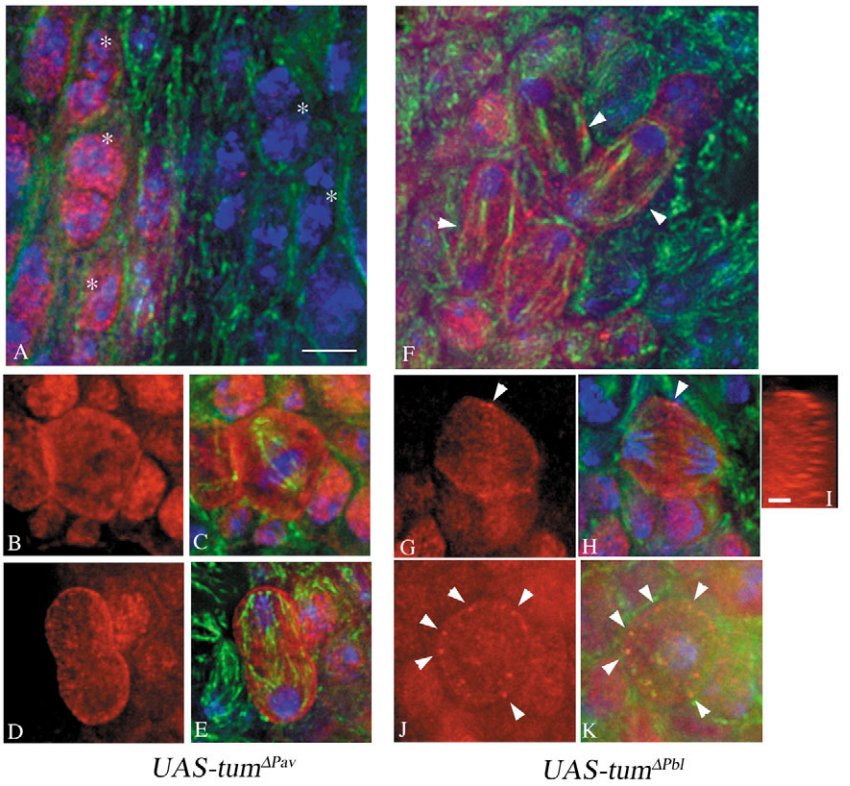

Fig. 6. Transgene deletions demonstrate that interaction with Pbl and Pav-KLP are required for Tum cytokinetic function. Tum transgenes with a Pav-KLP-interacting domain deletion (*UAS-tum*$^{\Delta Pav}$, A-E) or Pbl-interacting domain deletion (*UAS-tum*$^{\Delta Pbl}$, F-K) were expressed in *prd* stripes in *tum*^{DH15} embryos. In all panels, anti-myc antibody shows the transgene localisation in red, tubulin localisation in green and DNA in blue. (A-E) Tum$^{\Delta Pav}$ protein does not localise with microtubules or rescue cytokinesis. Binucleate cells are readily detected in both expressing and non-expressing stripes of these embryos (A, asterisks) but midbody remnants are not seen. This protein is stable and abundant, but remains diffuse and cortical throughout the mitotic cycle (metaphase, B and C; telophase, D and E). (F-K) Cortex-associated microtubule bundles form in cells expressing Tum$^{\Delta Pbl}$ but cytokinesis fails. Tum$^{\Delta Pbl}$ protein can bind to microtubules and locates at the spindle midzone at anaphase (arrowheads, F,G,H). Rotation of panel H reveals that much of this protein is localised in discrete foci on the cortex (I). Occasional post-metaphase cells viewed along the spindle axis confirm this cortical localisation (arrowheads, J,K). Late-anaphase and telophase cells show partial bundling of microtubules (F) but rarely show evidence of incomplete furrowing. Bar, 5 μm (A-K); 3 μm (I). Grayscale images for all color channels can be viewed in supplementary material Fig. S5.

or establish a cytokinetic furrow. Telophase cells possessed variable numbers of microtubule bundles, but these were rarely organised into a central spindle. This phenotype is similar to that seen in cells depleted of the Tum binding partner, Pav-KLP (Adams et al., 1998; Goshima and Vale, 2003).

Significantly, and consistent with the absence of furrowing, none of the other cytokinetic components that we have checked to date, AurB, Dia, Anillin, Pbl and Pav-KLP, localise correctly in the absence of Tum. These observations show that Tum is required at the very earliest stages of furrow formation, consistent with suggestions that the central spindle and/or bundled midzone microtubules direct the earliest events of cytokinesis (Alsop and Zhang, 2003; Gatti et al., 2000; Somers and Saint, 2003).

The Tum-Pbl interaction appears to be necessary for cytokinesis

Our original model (Saint and Somers, 2003; Somers and Saint, 2003) proposed that Tum interacted with Pbl at the cell equator and initiated changes in Rho activity, culminating in formation of the furrow. When the Pbl-interaction-domain deletion protein, Tum$^{\Delta Pbl}$, is the only Tum protein present, cells proceed to a point in cytokinesis where assembled centralspindlin complexes are found at the cortex, bridging bundles of microtubules from opposite poles, but they do not proceed beyond this stage, suggesting that the Pbl-Tum interaction becomes critical at this time. This observation satisfies one important prediction of our model, that disruption of the Tum-Pebble interaction would prevent cytokinesis but not affect earlier centralspindlin-based microtubule localisation, supporting the proposal that the interaction between Tum and Pbl is the bridge between the anaphase mitotic spindle and contractile ring assembly.

The role of Tum GAP activity and the Tum-Pbl interaction in microtubule organisation

Microtubule function in *tum* mutant cells is perturbed at anaphase, leading to the loss or disruption of the central spindle. It has been suggested that central spindle microtubules are unstable in the absence of bundling (Mastronarde et al., 1993) so the concomitant loss of Pav-KLP with Tum could explain this loss of microtubule organisation. However we have also observed cases in cells overexpressing mutant forms of Tum, in which central spindle organisation is disrupted even in the presence of apparently stable centralspindlin complexes. If Tum does not interact with Pbl, or if GAP activity is compromised, central spindles fail to form or are unstable, even though the modified Tum is still delivered to the ends of microtubules associated with the cell cortex. Equatorial-cortex-associated microtubule bundles and misdirected non-cortical bundles form, but normal central spindles are rarely seen in these cells even though Tum$^{\Delta Pbl}$ apparently can bind Pav-KLP. Loss of the central spindle has also been observed in S2 cells depleted of Pbl by RNAi (Somma et al., 2002) and in spermatocyte-specific *pbl* alleles (Giansanti et al., 2004).

Pbl and Tum may exert these effects on central spindle microtubule organisation by directly affecting the bundling activity and higher order structure of centralspindlin. Alternatively, they may act indirectly, influencing centralspindlin function by regulating the local concentration of active GTPases, a possibility that is supported by the failure of GAP-specific mutations to form or maintain a central

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spindle. However, we cannot rule out the possibility that the Tum$^{\Delta Pbl}$, Tum$^{\Delta EIE}$ and Tum$^{\Delta YRL}$ constructs delete critical amino acid residues required for centralspindlin functions that are unrelated to GTPase regulation.

Although the central spindle is perturbed in the absence of a Tum-Pbl interaction or if Tum GAP activity is altered, a subpopulation of microtubules still contacts the cell cortex at the equator and both Tum$^{\Delta Pbl}$ and Tum$^{\Delta YRL}$ can accumulate there, highlighting one difference between this microtubule population and the central spindle. Specific subpopulations of microtubules are beginning to be identified during anaphase (Inoue et al., 2004) and there is some evidence that a subpopulation of microtubules are stabilised at the equatorial cortex (Canman et al., 2003). Although a number of studies have provided links between Rho family GTPases and the stabilisation of microtubule cortex (Ishizaki et al., 2001; Kohno et al., 1996) and microtubule-kinetochore interactions in mammalian cells (Yasuda et al., 2004), the presence of Tum$^{\Delta YRL}$ at the ends of microtubules at the cell equator indicates that if Tum plays a role in the stabilisation of these microtubules at the furrow site it can do so in the absence of GAP activity.

Tum GAP activity is necessary for cytokinesis in ectoderm cells

Experiments with two different GAP deletions demonstrate that GAP activity is required for cytokinesis in the ectoderm. The ΔEIE deletion removes three amino acids at the end of the 'A helix', which positions the catalytic, or finger loop containing the essential arginine (Arg417 in Tum). In vitro analysis of a similar deletion in n-chimaerin eliminates all GAP activity but results in a protein with higher affinity for GTPase than a wild-type protein (Ahmed et al., 1994). An equivalent deletion in Cdc42GAP (Leonard et al., 1998) also lacks GAP activity but, by several biochemical measures, has similar stability and structure to the wild-type protein. The YRL deletion removes the essential catalytic arginine and the two amino acids around it; in n-chimaerin a similar deletion eliminates all GAP activity (Ahmed et al., 1994). The localisation of these two proteins during mitosis in *tum* mutant cells differs. The EIE deletion seems to affect the behaviour of Tum more severely, resulting in diffuse localisation of the protein during anaphase, whereas Tum$^{\Delta YRL}$ protein reaches the cell equator at anaphase but no cytokinetic constriction occurs. Irrespective of these differences, neither GAP-deficient form of Tum was capable of rescuing the *tum* cytokinetic defect.

These results differ from a recent analysis of Tum function in the larval nervous system (Goldstein et al., 2005), where Arg417 substituted Tum was able to rescue cytokinesis in larval neuroblasts. A different balance of cytokinetic mechanisms that contributes to cytokinesis (D'Avino et al., 2005) may have evolved to meet the special requirements for asymmetric cell divisions in the CNS or cell divisions in an epithelial sheet. It is interesting to note, however, that in vitro studies in which the GAP ΔEIE or ΔYRL triplet mutations were compared to single arginine substitution mutants, such as the one used in the study by Goldstein et al. (Goldstein et al., 2005), the single arginine mutations always have significant GTPase-activating ability remaining (Graham et al., 1999). Indeed structural and biochemical studies of several GAP domains have emphasised that stabilisation of the switch 1 and switch 2 loops of bound GTPase by the GAP protein contributes significantly to GAP activity (Graham et al., 1999; Nassar et al., 1998; Rittinger et al., 1997), suggesting the possibility that Arg417-substituted Tum may still have sufficient GAP activity to support cytokinesis, at least in some cell types.

Conserved and divergent functions of tum and its orthologs

Understanding the role of Tum and its orthologs, Cyk-4 and MgcRacGAP, in cytokinesis is complicated by conflicting evidence from different experimental systems (D'Avino et al., 2005; Severson et al., 2000; Somers and Saint, 2003). In contrast to our observations, *C. elegans* eggs and mammalian tissue culture cells in which centralspindlin members are depleted, initiate cytokinesis, but the furrow regresses (Kurz et al., 2002; Matuliene and Kuriyama, 2002; Powers et al., 1998; Raich et al., 1998; Severson et al., 2000). The predominant aberrant phenotype produced by expression of GAP deletion mutants in our experiments was a cell arrested before a furrow was evident, demonstrating that Tum is needed early in furrow formation. We also observed some apparent late-stage cytokinesis failures in embryos expressing Tum$^{\Delta YRL}$ like those seen in mammalian cells, indicating that there is a second critical phase for Tum GAP activity in cytokinesis. We suggest that late-stage defects may reveal an ancestral function of Tum and its homologs, with an earlier cytokinetic function adopted in *Drosophila melanogaster*. There is a growing consensus (Canman and Wells, 2004; Glotzer, 2004; Murata-Hori and Wang, 2002; Severson et al., 2000) that some of the differing observations between cell types and species reflect real differences in the way cells perform cytokinesis, rather than differences in the efficacy of RNAi, the perdurance of proteins, redundancy of protein functions or other experimental variables, but this issue remains unresolved.

Mammalian Tum, MgcRacGAP, exhibits several functions not seen in our current study. Expression of a MgcRacGAP GAP-defective mutant protein in mammalian cells affects chromosome attachment to the spindle at prometaphase via regulation of Cdc42, generating cells arrested in prometaphase or cells with micronuclei (Oceguera-Yanez et al., 2005). We have not seen micronucleation in this study, but we have seen multipolar anaphase cells in *tum* mutants, indicating that Tum-deficient cells that have failed cytokinesis can transit the cell cycle and successfully enter anaphase again, suggesting that Tum does not have a critical role in prometaphase in these cells.

Expression of a MgcRacGAP GAP-defective protein also affects the cell cortex, causing blebbing during anaphase in mammalian cells (Lee et al., 2004). Blebbing in both *Dictylostelium* and mammalian cells is suppressed by substrate attachment (Cunningham, 1995; Gerald et al., 1998) and we suggest that this phenotype, if it exists in *Drosophila* embryos, could be suppressed in cells in an epithelial sheet. All previous studies of Tum function in flies have noted non-cytokinetic functions revealed as aberrations of wingless signalling, EGFR signalling and axon migration, some of which might be indirect results of perturbation of cortical organisation.

We have shown that Tum protein is required for the localisation of all cytokinetic components that we have tested

so far, including Pbl and Dia, two components that mark the earliest events in contractile ring formation. Disruption of the Pbl-interacting domain of Tum leaves centralspindlin at the cell equator, on cortically associated, bundled microtubules that are unable to induce furrowing, supporting our model for Tum-directed positioning of Pbl and, consequently, the cytokinetic furrow in *Drosophila* cells. A Tum protein with a defective GAP domain also arrives at the equatorial cortex at the ends of microtubules and remains there, but furrows do not form, demonstrating that Tum GAP activity is required at this early stage of cytokinesis. These results demonstrate the critical role Tum plays in initiation of cytokinetic furrowing in *Drosophila* cells and provides further evidence for the importance of the Tum-Pbl interaction in this process.

The authors thank Ann Goldstein and Liqun Luo for discussions and members of the Saint Lab for their helpful reading of the manuscript. We would also like to thank Peter Smibert for the *UASPbl::GFP* construct. This work was supported by the Australian Research Council Special Research Centres scheme and the Australian National University to R.S. and by National Institutes of Health grant GM59068 to A.B.

References

Adams, R. R., Tavares, A. A., Salzberg, A., Bellen, H. J. and Glover, D. M. (1998). pavarotti encodes a kinesin-like protein required to organize the central spindle and contractile ring for cytokinesis. *Genes Dev.* **12**, 1483-1494.

Adams, R. R., Maiato, H., Earnshaw, W. C. and Carmena, M. (2001). Essential roles of Drosophila inner centromere protein (INCENP) and aurora B in histone H3 phosphorylation, metaphase chromosome alignment, kinetochore disjunction, and chromosome segregation. *J. Cell Biol.* **153**, 865-880.

Ahmed, S., Lee, J., Wen, L. P., Zhao, Z., Ho, J., Best, A., Kozma, R. and Lim, L. (1994). Breakpoint cluster region gene product-related domain of n-chimaerin. Discrimination between Rac-binding and GTPase-activating residues by mutational analysis. *J. Biol. Chem.* **269**, 17642-17648.

Alsop, G. B. and Zhang, D. (2003). Microtubules are the only structural constituent of the spindle apparatus required for induction of cell cleavage. *J. Cell Biol.* **162**, 383-390.

Ban, R., Irino, Y., Fukami, K. and Tanaka, H. (2004). Human mitotic spindle-associated protein PRC1 inhibits MgcRacGAP activity toward Cdc42 during the metaphase. *J. Biol. Chem.* **279**, 16394-16402.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Burgess, D. R. and Chang, F. (2005). Site selection for the cleavage furrow at cytokinesis. *Trends Cell Biol.* **15**, 156-162.

Canman, J. C. and Wells, W. A. (2004). Rappaport furrows on our minds: the ASCB cytokinesis meeting Burlington, VT July 22-25, 2004. *J. Cell Biol.* **166**, 943-948.

Canman, J. C., Cameron, L. A., Maddox, P. S., Straight, A., Tirnauer, J. S., Mitchison, T. J., Fang, G., Kapoor, T. M. and Salmon, E. D. (2003). Determining the position of the cell division plane. *Nature* **424**, 1074-1078.

Castrillon, D. H. and Wasserman, S. A. (1994). Diaphanous is required for cytokinesis in Drosophila and shares domains of similarity with the products of the limb deformity gene. *Development* **120**, 3367-3377.

Cunningham, C. C. (1995). Actin polymerization and intracellular solvent flow in cell surface blebbing. *J. Cell Biol.* **129**, 1589-1599.

D'Avino, P. P., Savoian, M. S. and Glover, D. M. (2005). Cleavage furrow formation and ingression during animal cytokinesis: a microtubule legacy. *J. Cell Sci.* **118**, 1549-1558.

Echard, A. and O'Farrell, P. H. (2003). The degradation of two mitotic cyclins contributes to the timing of cytokinesis. *Curr. Biol.* **13**, 373-383.

Etienne-Manneville, S. and Hall, A. (2002). Rho GTPases in cell biology. *Nature* **420**, 629-635.

Field, C. M. and Alberts, B. M. (1995). Anillin, a contractile ring protein that cycles from the nucleus to the cell cortex. *J. Cell Biol.* **131**, 165-178.

Gatt, M. K., Savoian, M. S., Riparbelli, M. G., Massarelli, C., Callaini, G. and Glover, D. M. (2005). Klp67A destabilises pre-anaphase microtubules but subsequently is required to stabilise the central spindle. *J. Cell Sci.* **118**, 2671-2682.

Gatti, M., Giansanti, M. G. and Bonaccorsi, S. (2000). Relationships between the central spindle and the contractile ring during cytokinesis in animal cells. *Microsc. Res. Tech.* **49**, 202-208.

Gerald, N., Dai, J., Ting-Beall, H. P. and De Lozanne, A. (1998). A role for Dictyostelium racE in cortical tension and cleavage furrow progression. *J. Cell Biol.* **141**, 483-492.

Giansanti, M. G., Bonaccorsi, S., Williams, B., Williams, E. V., Santolamazza, C., Goldberg, M. L. and Gatti, M. (1998). Cooperative interactions between the central spindle and the contractile ring during Drosophila cytokinesis. *Genes Dev.* **12**, 396-410.

Giansanti, M. G., Bonaccorsi, S. and Gatti, M. (1999). The role of anillin in meiotic cytokinesis of Drosophila males. *J. Cell Sci.* **112**, 2323-2334.

Giansanti, M. G., Farkas, R. M., Bonaccorsi, S., Lindsley, D. L., Wakimoto, B. T., Fuller, M. T. and Gatti, M. (2004). Genetic dissection of meiotic cytokinesis in Drosophila males. *Mol. Biol. Cell* **15**, 2509-2522.

Glotzer, M. (2001). Animal cell cytokinesis. *Annu. Rev. Cell Dev. Biol.* **17**, 351-386.

Glotzer, M. (2003). Cytokinesis: progress on all fronts. *Curr. Opin. Cell Biol.* **15**, 684-690.

Glotzer, M. (2004). Cleavage furrow positioning. *J. Cell Biol.* **164**, 347-351.

Glotzer, M. (2005). The molecular requirements for cytokinesis. *Science* **307**, 1735-1739.

Goldstein, A. Y., Jan, Y. N. and Luo, L. (2005). Function and regulation of Tumbleweed (RacGAP50C) in neuroblast proliferation and neuronal morphogenesis. *Proc. Natl. Acad. Sci. USA* **102**, 3834-3839.

Goshima, G. and Vale, R. D. (2003). The roles of microtubule-based motor proteins in mitosis: comprehensive RNAi analysis in the Drosophila S2 cell line. *J. Cell Biol.* **162**, 1003-1016.

Graham, D. L., Eccleston, J. F. and Lowe, P. N. (1999). The conserved arginine in rho-GTPase-activating protein is essential for efficient catalysis but not for complex formation with Rho.GDP and aluminum fluoride. *Biochemistry* **38**, 985-991.

Guertin, D. A., Trautmann, S. and McCollum, D. (2002). Cytokinesis in eukaryotes. *Microbiol. Mol. Biol. Rev.* **66**, 155-178.

Hamaguchi, Y. (1975). Microionjection of colchicine into sea urchin eggs. *Dev. Growth Diff.* **17**, 111-117.

Hime, G. R., Brill, J. A. and Fuller, M. T. (1996). Assembly of ring canals in the male germ line from structural components of the contractile ring. *J. Cell Sci.* **109**, 2779-2788.

Hirose, K., Kawashima, T., Iwamoto, I., Nosaka, T. and Kitamura, T. (2001). MgcRacGAP is involved in cytokinesis through associating with mitotic spindle and midbody. *J. Biol. Chem.* **276**, 5821-5828.

Inoue, Y. H., Savoian, M. S., Suzuki, T., Mathe, E., Yamamoto, M. T. and Glover, D. M. (2004). Mutations in orbit/mast reveal that the central spindle is comprised of two microtubule populations, those that initiate cleavage and those that propagate furrow ingression. *J. Cell Biol.* **166**, 49-60.

Ishizaki, T., Morishima, Y., Okamoto, M., Furuyashiki, T., Kato, T. and Narumiya, S. (2001). Coordination of microtubules and the actin cytoskeleton by the Rho effector mDia1. *Nat. Cell Biol.* **3**, 8-14.

Jones, W. M. and Bejsovec, A. (2005). RacGap50C negatively regulates wingless pathway activity during Drosophila embryonic development. *Genetics* **169**, 2075-2086.

Karr, T. L. and Alberts, B. M. (1986). Organization of the cytoskeleton in early Drosophila embryos. *J. Cell Biol.* **102**, 1494-1509.

Kinoshita, M., Field, C. M., Coughlin, M. L., Straight, A. F. and Mitchison, T. J. (2002). Self- and actin-templated assembly of Mammalian septins. *Dev. Cell* **3**, 791-802.

Kohno, H., Tanaka, K., Mino, A., Umikawa, M., Imamura, H., Fujiwara, T., Fujita, Y., Hotta, K., Qadota, H., Watanabe, T. et al. (1996). Bni1p implicated in cytoskeletal control is a putative target of Rho1p small GTP binding protein in Saccharomyces cerevisiae. *EMBO J.* **15**, 6060-6068.

Kuriyama, R., Gustus, C., Terada, Y., Uetake, Y. and Matuliene, J. (2002). CHO1, a mammalian kinesin-like protein, interacts with F-actin and is involved in the terminal phase of cytokinesis. *J. Cell Biol.* **156**, 783-790.

Kurz, T., Pintard, L., Willis, J. H., Hamill, D. R., Gonczy, P., Peter, M. and Bowerman, B. (2002). Cytoskeletal regulation by the Nedd8 ubiquitin-like protein modification pathway. *Science* **295**, 1294-1298.

Lee, J. S., Kamijo, K., Ohara, N., Kitamura, T. and Miki, T. (2004). MgcRacGAP regulates cortical activity through RhoA during cytokinesis. *Exp. Cell Res.* **293**, 275-282.

Leonard, D. A., Lin, R., Cerione, R. A. and Manor, D. (1998). Biochemical

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studies of the mechanism of action of the Cdc42-GTPase-activating protein. *J. Biol. Chem.* **273**, 16210-16215.

Mabuchi, I., Hamaguchi, Y., Fujimoto, H., Morii, N., Mishima, M. and Narumiya, S. (1993). A rho-like protein is involved in the organisation of the contractile ring in dividing sand dollar eggs. *Zygote* **1**, 325-331.

Mastronarde, D. N., McDonald, K. L., Ding, R. and McIntosh, J. R. (1993). Interpolar spindle microtubules in PTK cells. *J. Cell Biol.* **123**, 1475-1489.

Matuliene, J. and Kuriyama, R. (2002). Kinesin-like protein CHO1 is required for the formation of midbody matrix and the completion of cytokinesis in mammalian cells. *Mol. Biol. Cell* **13**, 1832-1845.

Minoshima, Y., Kawashima, T., Hirose, K., Tonozuka, Y., Kawajiri, A., Bao, Y. C., Deng, X., Tatsuka, M., Narumiya, S., May, W. S., Jr et al. (2003). Phosphorylation by aurora B converts MgcRacGAP to a RhoGAP during cytokinesis. *Dev. Cell* **4**, 549-560.

Mishima, M., Kaitna, S. and Glotzer, M. (2002). Central spindle assembly and cytokinesis require a kinesin-like protein/RhoGAP complex with microtubule bundling activity. *Dev. Cell* **2**, 41-54.

Murata-Hori, M. and Wang, Y. L. (2002). Both midzone and astral microtubules are involved in the delivery of cytokinesis signals: insights from the mobility of aurora B. *J. Cell Biol.* **159**, 45-53.

Nassar, N., Hoffman, G. R., Manor, D., Clardy, J. C. and Cerione, R. A. (1998). Structures of Cdc42 bound to the active and catalytically compromised forms of Cdc42GAP. *Nat. Struct. Biol.* **5**, 1047-1052.

Oceguera-Yanez, F., Kimura, K., Yasuda, S., Higashida, C., Kitamura, T., Hiraoka, Y., Haraguchi, T. and Narumiya, S. (2005). Ect2 and MgcRacGAP regulate the activation and function of Cdc42 in mitosis. *J. Cell Biol.* **168**, 221-232.

Oegema, K., Savoian, M. S., Mitchison, T. J. and Field, C. M. (2000). Functional analysis of a human homologue of the Drosophila actin binding protein anillin suggests a role in cytokinesis. *J. Cell Biol.* **150**, 539-552.

Paoletti, A. and Chang, F. (2000). Analysis of mid1p, a protein required for placement of the cell division site, reveals a link between the nucleus and the cell surface in fission yeast. *Mol. Biol. Cell* **11**, 2757-2773.

Parks, A. L., Cook, K. R., Belvin, M., Dompe, N. A., Fawcett, R., Huppert, K., Tan, L. R., Winter, C. G., Bogart, K. P., Deal, J. E. et al. (2004). Systematic generation of high-resolution deletion coverage of the Drosophila melanogaster genome. *Nat. Genet.* **36**, 288-292.

Powers, J., Bossinger, O., Rose, D., Strome, S. and Saxton, W. (1998). A nematode kinesin required for cleavage furrow advancement. *Curr. Biol.* **8**, 1133-1136.

Prokopenko, S. N., Brumby, A., O'Keefe, L., Prior, L., He, Y., Saint, R. and Bellen, H. J. (1999). A putative exchange factor for Rho1 GTPase is required for initiation of cytokinesis in Drosophila. *Genes Dev.* **13**, 2301-2314.

Prokopenko, S. N., Saint, R. and Bellen, H. J. (2000). Tissue distribution of PEBBLE RNA and pebble protein during Drosophila embryonic development. *Mech. Dev.* **90**, 269-273.

Pruyne, D., Evangelista, M., Yang, C., Bi, E., Zigmond, S., Bretscher, A. and Boone, C. (2002). Role of formins in actin assembly: nucleation and barbed-end association. *Science* **297**, 612-615.

Raich, W. B., Moran, A. N., Rothman, J. H. and Hardin, J. (1998). Cytokinesis and midzone microtubule organization in Caenorhabditis elegans require the kinesin-like protein ZEN-4. *Mol. Biol. Cell* **9**, 2037-2049.

Rappaport, R. (1986). Establishment of the mechanism of cytokinesis in animal cells. *Int. Rev. Cytol.* **105**, 245-281.

Rittinger, K., Walker, P. A., Eccleston, J. F., Nurmahomed, K., Owen, D., Laue, E., Gamblin, S. J. and Smerdon, S. J. (1997). Crystal structure of a small G protein in complex with the GTPase-activating protein rhoGAP. *Nature* **388**, 693-697.

Rothwell, W. F. a. W. S. (2000). Fluorescent analysis of Drosophila embryos. In *Drosophila Protocols* (ed. M. A. W. Sullivan and R.S. Hawley), pp. 141-157. New York: Cold Spring Harbor Laboratory Press.

Saint, R. and Somers, W. G. (2003). Animal cell division: a fellowship of the double ring? *J. Cell Sci.* **116**, 4277-4281.

Severson, A. F., Hamill, D. R., Carter, J. C., Schumacher, J. and Bowerman, B. (2000). The aurora-related kinase AIR-2 recruits ZEN-4/CeMKLP1 to the mitotic spindle at metaphase and is required for cytokinesis. *Curr. Biol.* **10**, 1162-1171.

Shu, H. B., Li, Z., Palacios, M. J., Li, Q. and Joshi, H. C. (1995). A transient association of gamma-tubulin at the midbody is required for the completion of cytokinesis during the mammalian cell division. *J. Cell Sci.* **108**, 2955-2962.

Skop, A. R., Liu, H., Yates, J., 3rd, Meyer, B. J. and Heald, R. (2004). Dissection of the mammalian midbody proteome reveals conserved cytokinesis mechanisms. *Science* **305**, 61-66.

Smallhorn, M., Murray, M. J. and Saint, R. (2004). The epithelial-mesenchymal transition of the Drosophila mesoderm requires the Rho GTP exchange factor Pebble. *Development* **131**, 2641-2651.

Somers, W. G. and Saint, R. (2003). A RhoGEF and Rho family GTPase-activating protein complex links the contractile ring to cortical microtubules at the onset of cytokinesis. *Dev. Cell* **4**, 29-39.

Somma, M. P., Fasulo, B., Cenci, G., Cundari, E. and Gatti, M. (2002). Molecular dissection of cytokinesis by RNA interference in Drosophila cultured cells. *Mol. Biol. Cell* **13**, 2448-2460.

Sotillos, S. and Campuzano, S. (2000). DRacGAP, a novel Drosophila gene, inhibits EGFR/Ras signalling in the developing imaginal wing disc. *Development* **127**, 5427-5438.

Tatsumoto, T., Xie, X., Blumenthal, R., Okamoto, I. and Miki, T. (1999). Human ECT2 is an exchange factor for Rho GTPases, phosphorylated in G2/M phases, and involved in cytokinesis. *J. Cell Biol.* **147**, 921-928.

Theurkauf, W. E. (1992). Behavior of structurally divergent alpha-tubulin isotypes during Drosophila embryogenesis: evidence for post-translational regulation of isotype abundance. *Dev. Biol.* **154**, 205-217.

Tominaga, T., Sahai, E., Chardin, P., McCormick, F., Courtneidge, S. A. and Alberts, A. S. (2000). Diaphanous-related formins bridge Rho GTPase and Src tyrosine kinase signaling. *Mol. Cell* **5**, 13-25.

Wu, J. Q., Kuhn, J. R., Kovar, D. R. and Pollard, T. D. (2003). Spatial and temporal pathway for assembly and constriction of the contractile ring in fission yeast cytokinesis. *Dev. Cell* **5**, 723-734.

Yasuda, S., Oceguera-Yanez, F., Kato, T., Okamoto, M., Yonemura, S., Terada, Y., Ishizaki, T. and Narumiya, S. (2004). Cdc42 and mDia3 regulate microtubule attachment to kinetochores. *Nature* **428**, 767-771.

Yoffe, K. B., Manoukian, A. S., Wilder, E. L., Brand, A. H. and Perrimon, N. (1995). Evidence for engrailed-independent wingless autoregulation in Drosophila. *Dev. Biol.* **170**, 636-650.

Yoshizaki, H., Ohba, Y., Parrini, M. C., Dulyaninova, N. G., Bresnick, A. R., Mochizuki, N. and Matsuda, M. (2004). Cell type-specific regulation of RhoA activity during cytokinesis. *J. Biol. Chem.* **279**, 44756-44762.

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