RacGAP50C is sufficient to signal cleavage furrow formation during cytokinesis

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Summary

Several studies indicate that spindle microtubules determine the position of the cleavage plane at the end of cell division, but their exact role in triggering the formation and ingression of the cleavage furrow is still unclear. Here we show that in *Drosophila* depletion of either the GAP (GTPase-activating protein) or the kinesin-like subunit of the evolutionary conserved centralspindlin complex prevents furrowing without affecting the association of astral microtubules with the cell cortex. Moreover, time-lapse imaging indicates that astral microtubules serve to deliver the centralspindlin complex to the equatorial cortex just before furrow formation. However, when the GAP-signaling component was mislocalized around the entire cortex using a membrane-tethering motif, this caused ectopic furrowing even in the absence of its motor partner. Thus, the GAP component of centralspindlin is both necessary and sufficient for furrow formation and ingression and astral microtubules provide a route for its delivery to the cleavage site.

Supplementary material available online at
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Key words: Cytokinesis, Centralspindlin, Cleavage formation, *Drosophila*

Introduction

Cytokinesis ensures the proper partitioning of nuclear and cytoplasmic materials between the two daughter cells at the end of cell division. In a typical animal mitosis, a cleavage furrow forms at the equator of a cell and ingresses inwards to bisect the dividing cell. The nature of the signal that determines the position of the cleavage plane is one of the most intriguing questions in cell biology. Although it has been known for more than four decades that the spindle microtubules play a crucial role in establishing the division axis (Rappaport, 1961), their exact role has been long debated (Burgess and Chang, 2005; D'Avino et al., 2005). Recent studies indicate that astral microtubules promote furrowing. For example, we have shown that in *Drosophila* mitotic and male meiotic cells a subpopulation of astral microtubules contact the cortex at the cleavage site just before furrow ingression and then merge with an array of antiparallel and interdigitating microtubules, the central spindle, which forms between the separating anaphase chromosomes (Inoue et al., 2004). In spermatocytes these astral microtubules bundle to form a peripheral central spindle structure that subsequently merges with a distinct, interior population of central spindle microtubules during furrow ingression (Inoue et al., 2004). Similarly, in mammalian Ptk1 cells a particularly stable subpopulation of microtubules interacts with the cell cortex at the cleavage site and appears to promote furrowing (Canman et al., 2003; Shannon et al., 2005). It is, however, still unclear whether spindle microtubules directly trigger furrow formation or instead deliver a signal to the equatorial cortex.

The formation and ingression of the cleavage furrow is driven by the assembly and contraction of actomyosin filaments and several observations indicate that this cytoskeletal rearrangement is orchestrated by the family of small Rho GTPases (D'Avino et al., 2005; Wang, 2005). In particular, RhoA appears to be a major player in regulating this process because its inactivation prevents cytokinesis in most systems (Piekny et al., 2005). Moreover, it has been shown recently that the active form of RhoA accumulates at the future cleavage site in a microtubule-dependent manner, suggesting that spindle microtubules promote furrowing through activation of this GTPase (Bement et al., 2005). Studies in *Drosophila* and mammals also indicate that RhoA activation at the cleavage furrow requires the RhoGEF Pebble-ECT2 (Kimura et al., 2000; Prokopenko et al., 1999; Tatsumoto et al., 1999).

A possible link between the spindle microtubules and the activation of RhoA at the cleavage furrow became apparent after the discovery that the RhoGEF Pebble interacts with the Rho family GAP component of the centralspindlin complex (Somers and Saint, 2003). This evolutionary conserved two-protein complex is required for cytokinesis and central spindle assembly in all metazoans, from nematodes to humans (Mishima et al., 2002; Somers and Saint, 2003). The motor component of the complex, dubbed ZEN-4 in *C. elegans*, Pavarotti (PAV) in *Drosophila* and MKLP-1 in mammals, is a kinesin-like protein that displays plus-end directed motor activity (Adams et al., 1998; Nislow et al., 1992; Powers et al., 1998; Raich et al., 1998). The GAP partner (CYK-4 in nematodes, RacGAP50C or Tum in flies and MgcRacGAP in mammals) has been reported to downregulate the in vitro activity of Rac and Cdc42 GTPases more efficiently than Rho (Hirose et al., 2001; Jantsch-Plunger et al., 2000; Somers and

Saint, 2003). Consistent with this observation, genetic evidence in *Drosophila* suggests that RacGAP50C inhibits the activity of Rac GTPases in vivo (D'Avino et al., 2004). The two complex components are tightly associated throughout the cell cycle (Zhao and Fang, 2005) but their interaction with microtubules is prevented before anaphase onset through phosphorylation of the motor component by the cyclin-dependent kinase-1 (CDK-1) (Mishima et al., 2004). These data led to the proposal that stimulation of cytokinesis could be triggered by the microtubule-mediated delivery of the centralspindlin complex to the cortex where its RacGAP component would activate the RhoGEF Pebble and, in turn, RhoA (Saint and Somers, 2003). The recent finding that MgcRacGAP interacts with ECT2 in mammals indicates that this mechanism has been conserved during evolution (Kamijo et al., 2006; Nishimura and Yonemura, 2006; Yuce et al., 2005; Zhao and Fang, 2005). Here we provide further evidence in support of this model. We used time-lapse microscopy to show that the centralspindlin complex localized to the equatorial cortex just before furrow formation. Moreover, depletion of either member of the complex abolished furrowing without preventing the association of microtubules with the cortex. Strikingly, mislocalization of the RacGAP component around the cell cortex is sufficient to induce the formation of multiple ectopic furrows.

Results

Astral microtubules contact the equatorial cortex in centralspindlin-depleted cells

Several reports indicate that the centralspindlin components are required for furrow formation and ingression in *Drosophila* and mammalian cells (Adams et al., 1998; Kamijo et al., 2006; Nishimura and Yonemura, 2006; Somers and Saint, 2003; Yuce et al., 2005; Zhao and Fang, 2005), but it is unclear whether these furrowing defects result from abnormal astral microtubule behavior. To address this question, we depleted by RNAi either PAV or its partner RacGAP50C in a *Drosophila* cell line stably expressing a Tubulin::GFP transgene (Goshima and Vale, 2003). In our conditions, the vast majority of Tubulin::GFP cells became polyploid after 72 hours of RNAi treatment directed against either member of the centralspindlin complex. Therefore, to avoid aberrations due to supernumerary centrosomes and chromosomes, we filmed cells 48 hours after the addition of dsRNA. As shown in Fig. 1A, the level of PAV or RacGAP50C was drastically reduced after 48 hours of RNAi treatment. We also found that depletion of one component of the complex caused a reduction in the amount of its respective partner (Fig. 1A), confirming and extending previous results indicating that the abundance or stability of PAV is dependent on RacGAP50C (Zavortink et al., 2005).

In all Tubulin::GFP control cells (n=16), astral microtubules probed and contacted the cortex at the equator shortly after anaphase onset, while the central spindle began to assemble (Fig. 1B, 02:30 time point and Movie 1 in supplementary material). Soon thereafter a cleavage furrow formed at the contact site (Fig. 1B, 04:00 time point). This furrow rapidly ingressed to divide the two daughter cells (Fig. 1B, 08:00 time point) and formed a midbody (Fig. 1B, 21:30 time point). In *pav* RNAi cells, spindle assembly and chromosome segregation occurred as normal (Fig. 1B; *pav* RNAi, and Movie 2 in supplementary material) and astral microtubules contacted the cortex at approximately the same time as in control cells (Fig. 1B; *pav* RNAi, 03:00 time point). However, no furrowing activity was observed in 9 out of 10 cells, even though astral microtubules were in contact with the equatorial cortex (Fig. 1B; *pav* RNAi, 06:30 and 08:00 time points). In addition, the central spindle began to form but disassembled and cytokinesis ultimately failed (Fig. 1B; *pav* RNAi, 27:00 time point). A very similar phenotype was observed in all *RacGAP50C* RNAi cells filmed (n=12) (Fig. 1B; *GAP* RNAi and Movie 3 in

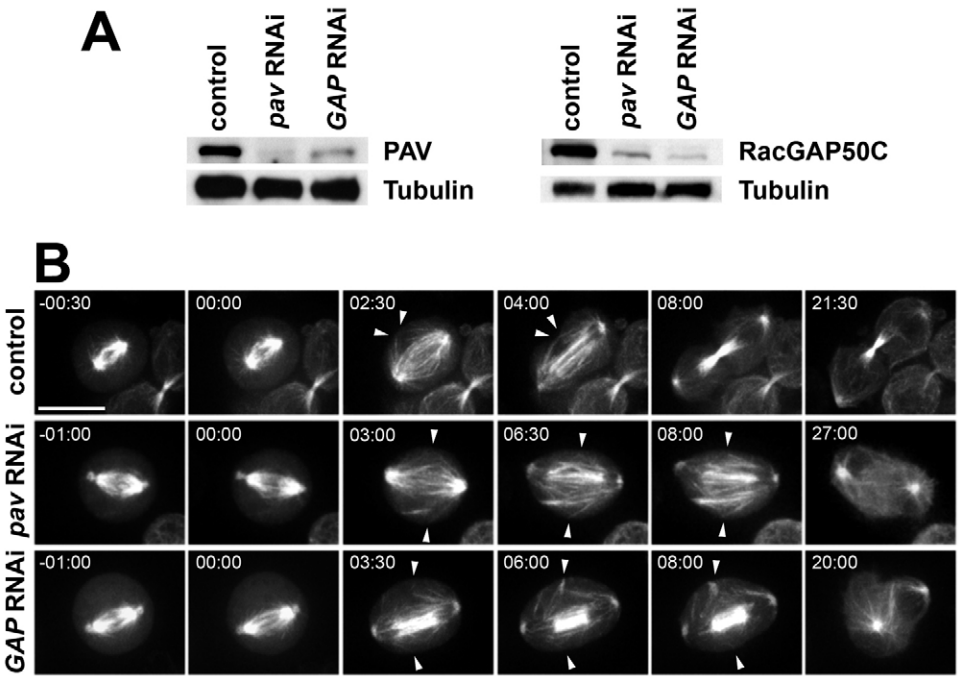

Fig. 1. Inactivation of the centralspindlin complex prevents furrowing without affecting astral microtubule dynamics. (A) Western blot analysis of protein levels after RNAi treatment. Tubulin::GFP cells were incubated with dsRNAs directed against PAV (*pav* RNAi), RacGAP50C (*GAP* RNAi) or no dsRNA as a control. After 48 hours, the proteins were extracted and separated on a 10% SDS gel, transferred onto a PVDF membrane and probed with antibodies against PAV, RacGAP50 and α-tubulin. (B) Selected images from time-lapse recordings of *Drosophila* S2 cells expressing a Tubulin::GFP transgene. Cells were treated for 48 hours with dsRNAs directed against the two members of the centralspindlin complex: PAV (*pav* RNAi) and RacGAP50C (*GAP* RNAi) or no dsRNA as a control. The arrowheads indicate the astral microtubules contacting the equatorial cortex. Time is in minutes:seconds relative to anaphase onset. Bar, 10 μm.

supplementary material). Astral microtubules probed and contacted the cortex shortly after anaphase onset (Fig. 1B; *GAP* RNAi, 03:30 time point) with no indication of furrow formation (Fig. 1B; *GAP* RNAi, 06:00 and 08:00 time points), finally resulting in cytokinesis failure (Fig. 1B; *GAP* RNAi, 20:00 time point). Notably, severe depletion of either member of the complex did not prevent the initial assembly of the central spindle, suggesting that either very low levels of centralspindlin are sufficient for the formation of this structure or redundant mechanisms exist to compensate for its absence. These results demonstrate that inactivation of the centralspindlin complex leads to failure in furrow formation without grossly altering astral microtubule behavior.

Centralspindlin localizes to the plus ends of the astral microtubules that promote furrowing

To study centralspindlin dynamics, we tagged both PAV and RacGAP50C with green fluorescent protein (GFP) and generated independent cell lines stably expressing either of these transgenes under the control of the *actin 5C* promoter (see Materials and Methods). These cell lines were fully viable and did not display any mitotic or cytokinetic defects. Both tagged components exhibited a subcellular distribution identical to that in their endogenous counterparts and always co-localized with their respective partners (Fig. 2A). The tagged motor component, however, appeared brighter than RacGAP50C::GFP and therefore we used the PAV::GFP cell line in our time-lapse experiments. As shown in Fig. 2B and Movie 4 in supplementary material, the tagged motor protein localized along the ends of the microtubules contacting the future cleavage site (Fig. 2B; 03:00 and 04:30 time points) and then merged with the central spindle population during furrow ingression (Fig. 2B; 08.30 time point). To analyze PAV dynamics at higher resolution, we also studied its behavior in dividing primary spermatocytes. These cells are particularly suited for the analysis of astral microtubule dynamics because spindle and astral microtubules are physically separated by a series of membranes and mitochondria. Time-lapse imaging in spermatocytes showed that, shortly after anaphase onset, GFP::PAV translocated to and accumulated near the plus ends of astral microtubules contacting the equatorial cortex as well as on the central spindle (Fig. 3A,B; 04:00 time point and Movies 5 and 6 in supplementary material). Furrows initiated several minutes after GFP::PAV concentrated on the forming microtubule bundles of the peripheral central spindle (Fig. 3A,B; 08:00 time point and Movies 5 and 6 in supplementary material). The peripheral and central spindle signals then compacted into a common equatorial band during furrow ingression (Fig. 3A, 16:00 time point). Thus, in both mitotic and male meiotic cells, the plus-end-directed motor activity of PAV appears to translocate centralspindlin to the ends of the astral microtubules that determine the cleavage plane.

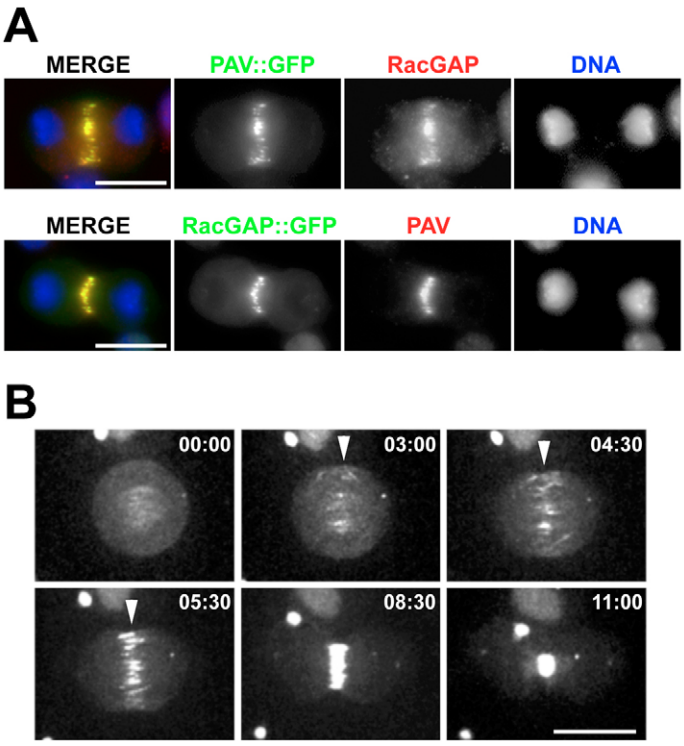

Fig. 2. Centralspindlin localizes to the equatorial cortex just before furrow ingression. (A) Localization of PAV::GFP and RacGAP::GFP fusion proteins in cleaving S2 cells. Cells were fixed and stained to detect GFP (green in the merged panels), DNA (blue in the merged panels) and either RacGAP50C or PAV (red in the top and bottom merged panels, respectively). (B) Selected frames from a time-lapse series showing PAV::GFP dynamics in S2 cells. The white arrowheads indicate PAV::GFP localization at the cell equator. Time is in minutes:seconds relative to anaphase onset. Bars, 10 μm.

A membrane-tethered version of RacGAP50C induces ectopic furrowing

The experiments described so far suggest that the PAV plus-end motor activity delivers the GAP-signalling subunit to the equatorial cortex where it may stimulate furrowing. Since the entire cortex has the ability to generate furrows (Shannon et al., 2005), we reasoned that expression of a membrane-tethered version of RacGAP50C should provoke ectopic furrowing. To test this, we fused an integral plasma membrane component, the human T-cell receptor CD8, with the full coding region of *RacGAP50C* and a GFP tag was inserted between the two genes to follow its distribution. Because of potential toxicity, the *CD8::GFP::RacGAP50C* fusion gene and its control *CD8::GFP* were expressed under the control of an inducible metallothionein (Mt) promoter (Fig. 4A). These constructs were then used to generate stable cell lines. Western blot analysis showed that, after induction, CD8::GFP::RacGAP50C protein expression was comparable with that of the endogenous RacGAP50C (Fig. 4B). Both CD8::GFP and CD8::GFP::RacGAP50C proteins accumulated at the plasma membrane and in cytoplasmic vesicles in interphase cells, consistent with previous studies (Fig. 4C) (Lee and Luo, 1999; Zito et al., 1997). Notably, the membrane protrusions seen in control cells appear reduced in cells expressing the membrane-tethered version of RacGAP50C, possibly because of inhibition of Rac activity (Fig. 4C). A more diffuse cytoplasmic signal was observed in metaphase, probably as a result of the Golgi fragmentation that occurs during cell division (Fig. 4D). Virtually all CD8::GFP cells showed normal cytokinesis figures (97.8%; n=93) whereas the majority (68.2%; n=85) of telophase cells expressing CD8::GFP::RacGAP50C exhibited numerous cortical constrictions in addition to the expected equatorial furrow (Fig.

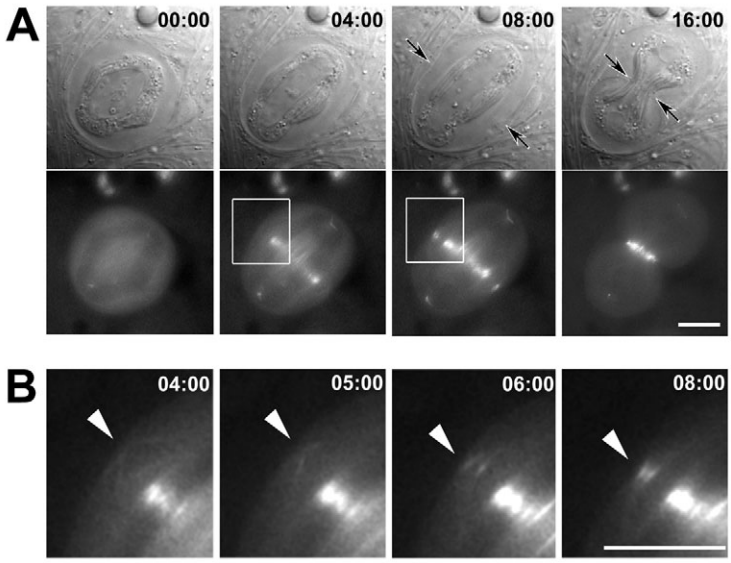

Fig. 3. PAV::GFP dynamics in primary spermatocytes. (A) Selected frames from a time-lapse series showing PAV::GFP dynamics in *Drosophila* primary spermatocytes. Corresponding DIC images are at the top. The black arrows indicate furrow ingression whereas the white arrowheads mark PAV::GFP localization at the cell equator. The boxed regions indicate the area magnified in B. Time is in minutes relative to anaphase onset. (B) Magnified selected frames from the time-lapse recording of the cell shown in A. Bars, 10 μm.

5). Two key observations indicate that these constrictions represent ectopic furrows and not simple membrane blebs. First, they accumulated the contractile ring components Anillin and the septin Peanut (Fig. 5B,C) (Field and Alberts, 1995; Neufeld and Rubin, 1994). Second, these ectopic constrictions were able to generate cytoplasts and constrict or bisect the dividing nuclei as they advanced (Fig. 5A-C). PAV, however, was not detected at these ectopic sites (Fig. 5D), indicating that the membrane-tethered version of RacGAP50C can induce furrowing in the absence of its motor partner. To confirm this latter finding, we depleted PAV by RNAi in cells expressing the membrane-tethered version of RacGAP50C. As expected, no furrows were observed in CD8::GFP control cells after RNAi knockdown of the motor component of the complex (Fig. 6, top panels). By contrast, multiple ectopic furrows still formed in CD8::GFP::RacGAP50C cells depleted of PAV, even in those that, judging by the size of the dividing nuclei, appeared to have already failed cytokinesis once (Fig. 6,

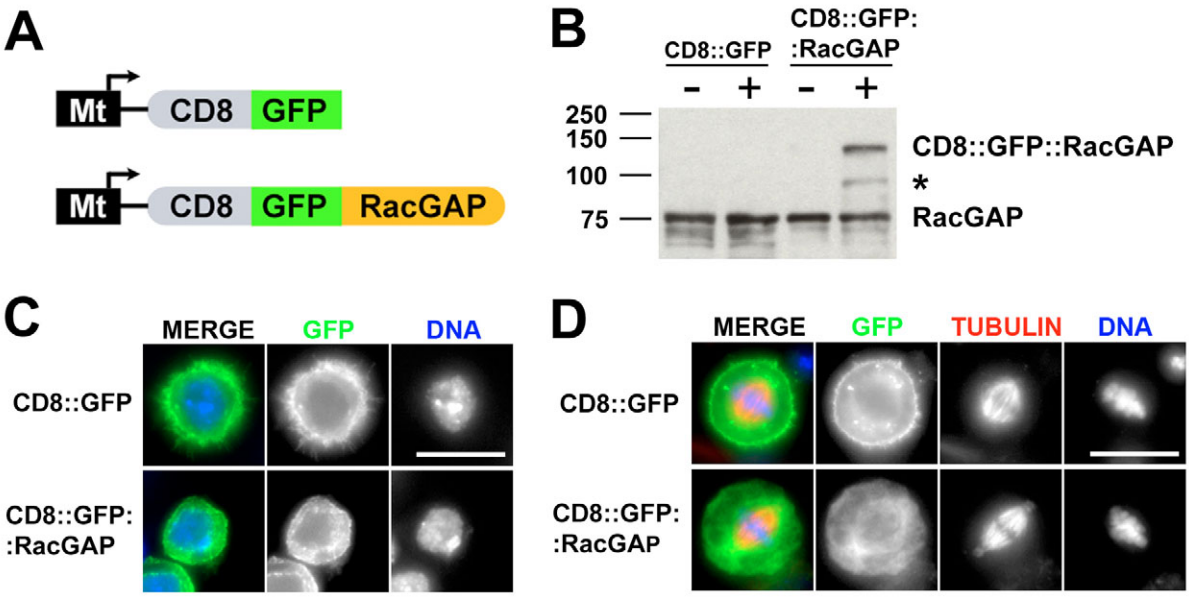

Fig. 4. Expression and localization of the CD8::GFP and CD8::GFP::RacGAP50C fusion proteins. (A) Schematic diagram of the transgenes used to generate the stable cell lines. (B) Western blot analysis of CD8::GFP::RacGAP50C expression. CD8::GFP::RacGAP50C and CD8::GFP cells were cultured in completed medium with (+) or without (–) 0.7 mM $CuSO_4$ for 5 hours and then harvested for protein extraction. Protein extracts were separated on a 10% SDS gel, transferred onto a PVDF membrane and probed with a RacGAP50 antibody. The asterisk indicates a breakdown product that corresponds in size to a GFP::RacGAP50C protein. (C,D) Localization of the CD8::GFP and CD8::GFP::RacGAP50C fusion proteins in S2 cells. Expression of CD8::GFP and CD8::GFP::RacGAP50C was induced by incubating the cultures in complete medium containing 0.7 mM $CuSO_4$ for 5-7 hours. The cells were then fixed and stained to detect GFP (green in the merged panels), DNA (blue in the merged panels) and tubulin (red in the merged panels in C). Interphase and metaphase cells are shown in C and D, respectively. Bars, 10 μm.

bottom panels). Unfortunately, we were unable to document the ectopic furrowing activity by time-lapse microscopy because CD8::GFP::RacGAP50C cells were poorly adherent and rotated extensively during cell division. Interestingly, CD8::GFP::RacGAP50C appeared to accumulate on central spindle microtubules independently of PAV (Fig. 5D and Fig. 6). Since RacGAP50C does not contain any recognizable microtubule-binding domain (Somers and Saint, 2003), we speculate that this localization reflects the interaction of the membrane-tethered variant with an unidentified microtubule-associated protein. This interaction does not appear necessary for the formation of ectopic furrows, because these were clearly observed in regions devoid of microtubules (Fig. 5A and Fig. 6).

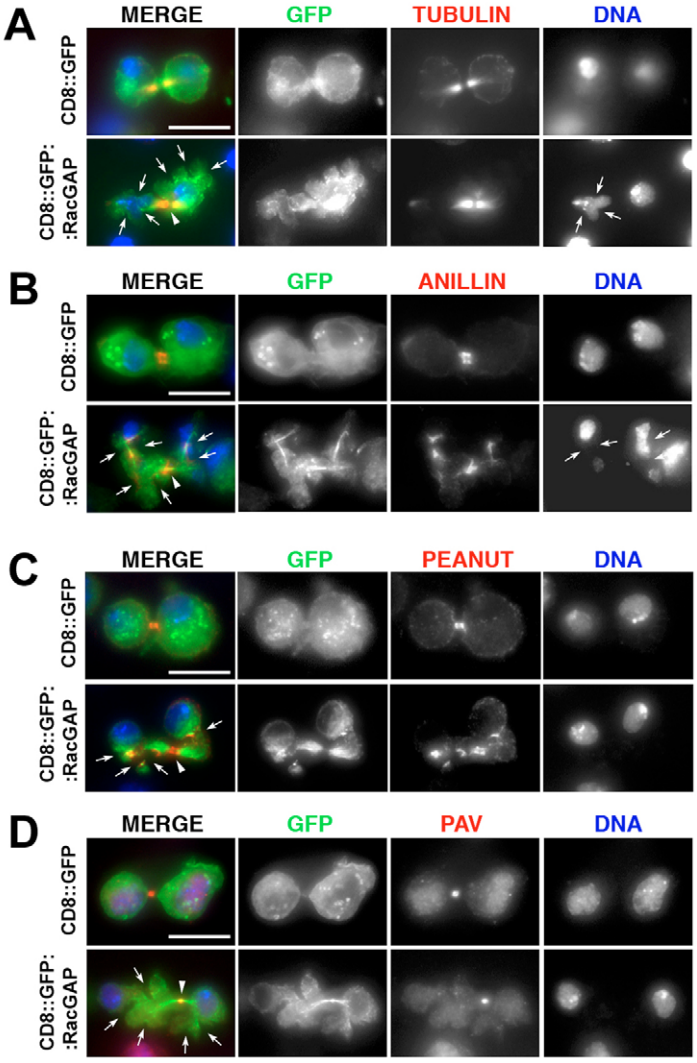

Fig. 5. Mislocalization of RacGAP50C causes ectopic furrowing. Expression of CD8::GFP and CD8::GFP::RacGAP50C was induced by incubating the cultures in complete medium containing 0.7 mM $CuSO_4$ for 5-7 hours. The cells were then fixed and stained to reveal GFP (green in the merged panels), DNA (blue in the merged panels) and either tubulin (A), Anillin (B), Peanut (C) or PAV (D) (red in the merged panels). The arrows indicate the ectopic furrows whereas the arrowhead indicates the equatorial furrow. Note the misshapen and bisected nuclei in A and B and the small cytoplast in C. Bars, 10 μm.

Discussion

Our results demonstrate that centralspindlin localizes to the cleavage site before cytokinesis onset and that its GAP component is both necessary and sufficient for furrowing, strongly indicating that RacGAP50C is a furrow-inducing signal. Interestingly, two lines of evidence indicate that the ability of this GAP to induce furrowing does not require its partner PAV. First, this motor protein does not accumulate at the ectopic furrows induced by the membrane-tethered version of RacGAP50C (Fig. 5D). Second, RNAi silencing of PAV does not prevent ectopic furrowing in CD8::GFP::RacGAP50C cells (Fig. 6). The apparent inability of CD8::GFP::RacGAP50C to recruit PAV at ectopic furrows would also suggest that the affinity of this motor for microtubules is stronger than its affinity for RacGAP50C. We cannot exclude, however, the possibility that low levels of PAV, undetectable by immunostaining, were also present at ectopic furrowing sites.

How does RacGAP50C stimulate furrowing? Recent findings indicate that the GAP subunit of the centralspindlin complex activates RhoA during cytokinesis through its interaction with the RhoGEF Pebble-ECT2 (Kamijo et al., 2006; Nishimura and Yonemura, 2006; Somers and Saint, 2003; Yuce et al., 2005; Zhao and Fang, 2005). Pebble localizes to the cell cortex in *Drosophila* and thus it could mediate the ectopic furrowing activity triggered by the membrane-tethered version of RacGAP50C (Fig. 4) (Prokopenko et al., 1999). Consistent with this hypothesis, no equatorial or ectopic furrowing activity was observed in CD8::GFP::RacGAP50C cells after RNAi depletion of the RhoGEF Pebble (data not shown). We were unable to determine whether Pebble localized to these ectopic furrows because of the lack of an antibody suitable for immunostaining. In addition, we also cannot rule out the possibility that the membrane-tethered version of RacGAP50C is able to recruit other factors that co-operate with it to induce ectopic furrows. Several other molecules required for cytokinesis display a similar localization to the plus-ends of both central spindle and astral microtubules. These include microtubule-associated proteins (MAPs), kinesins and signaling molecules such as the Polo kinase and the Aurora-B passenger protein complex (for a review, see D'Avino et al., 2005). However, in many cases the role of these proteins in cleavage furrow formation is unclear, mostly because their requirement during metaphase prevents an accurate analysis of their functions during cytokinesis. For certain gene products, such as the kinesin KLP67A (KIF18 in vertebrates) and the MAPs FEO (PRC1) and Orbit/MAST (CLASP in mammals), careful phenotypic analysis of cytokinesis in mutant primary spermatocytes and/or RNAi-depleted cells have clearly indicated that these proteins are not essential for the initiation of cleavage furrows (Gatt et al., 2005; Inoue et al., 2004; Verni et al., 2004). Recent data also suggest that the Aurora-B complex may not be necessary for furrow formation as one of its components, INCENP, does not accumulate at the ectopic furrows generated by taxol-stabilized microtubules (Shannon et al., 2005). Moreover, addition of the Aurora-B kinase inhibitor hesperadin to HeLa cells just after anaphase onset does not prevent furrow formation and ingression (Guse et al., 2005).

In mammals, the interaction between ECT2 and MgcRacGAP is inhibited before anaphase onset by CDK-1-

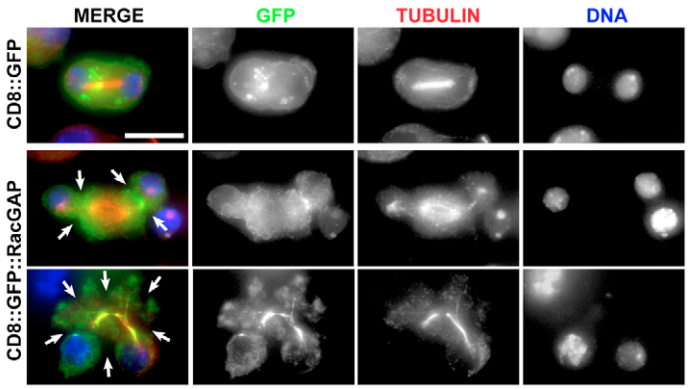

Fig. 6. The membrane-tethered version of RacGAP50C can induce ectopic furrows in the absence of PAV. CD8::GFP and CD8::GFP::RacGAP50C cells were incubated with dsRNA directed against PAV for 48 hours and then the expression of transgenes induced by incubating the cultures in complete medium containing 0.7 mM $CuSO_4$ for 5-7 hours. The cells were then fixed and stained to reveal GFP (green in the merged panels), tubulin (red in the merged panels) and DNA (blue in the merged panels). The arrows mark the ectopic furrows. Note that the size of the nuclei of the CD8::GFP::RacGAP50C cell shown at the bottom indicates that this cell is tetraploid and therefore already failed cytokinesis once. Nonetheless, multiple ectopic furrows still formed and CD8::GFP::RacGAP50C co-localized with the remnant of the central spindle. Bar, 10 μm.

mediated phosphorylation of ECT2 (Yuce et al., 2005). Although it is unclear whether the same occurs in flies, this could explain why expression of the membrane-tethered variant of RacGAP50C induced ectopic furrowing only in post-anaphase cells (Figs 3 and 4). In addition, it is also emerging that this GAP down-regulates the activity of Rac GTPases and that this inhibition is crucial for cytokinesis (D'Avino et al., 2004; Yoshizaki et al., 2004). Consistent with these results, the GAP domain of RacGAP50C is necessary for furrowing (Zavortink et al., 2005).

In conclusion, our results support a model whereby the microtubule-mediated delivery of the GAP subunit of the centralspindlin complex to the cleavage site triggers a local reorganization of the actomyosin cytoskeleton via antagonistic regulation of Rho and Rac signaling pathways.

Materials and Methods

Fly stocks, cell culture and RNAi treatments

The Ubiquitin-GFP::PAV fly strain has been previously described (Minestrini et al., 2003). Flies were grown at 25°C with 80% humidity. The *Drosophila* Tubulin::GFP S2 cell line (Goshima and Vale, 2003) was grown in Schneider's medium supplemented with 10% FCS (Sigma). For all other tissue culture experiments we used the DMEL strain of S2 cells (Invitrogen) that has been adapted to grow in serum-free medium (SFM). Generation of dsRNAs and RNAi treatments were performed as described (D'Avino et al., 2004); oligonucleotide sequences are available upon request.

DNA constructs, generation of stable cell lines and western blot analysis

A Gateway® (Invitrogen) vector to express C-terminal-tagged EGFP proteins in S2 cells under the control of the Actin 5C promoter was constructed by inserting a 'Gateway-EGFP' cassette from pUASp-DEST13 (a gift from F. Wirtz-Peitz, IMBA, Vienna, Austria; see also http://dgrc.cgb.indiana.edu/vectors/store/vectors.html) into a pAc.5/V5-HisA vector (Invitrogen) lacking V5 and His sequences. To express CD8::EGFP fusion variants N-terminally under the metallothionein (Mt) promoter, a Gateway® plasmid was generated by inserting a 'CD8::EGFP-Gateway®' cassette from pUAST-DEST16 (a gift from F. Wirtz-Peitz; see also http://dgrc.cgb.indiana.edu/vectors/store/vectors.html) into a pMt/V5-HisA vector (Invitrogen) lacking V5 and His sequences. The coding regions of *pav* and *RacGAP50C* were PCR amplified using cDNAs as templates and cloned into Gateway® vectors. The PCR products were sequenced on both strands. To generate stable cell lines, 3×10^6 DMEL cells were transfected with 5 μg of the appropriate expression vector along with 0.5 μg pCoBlast, a plasmid carrying a resistance to the antibiotic blasticidin (Invitrogen), and 15 μl Cellfectin (Invitrogen). Resistant cells were selected using SFM media containing either 30 (Actin5C plasmids) or 20 (Mt plasmids) μg/ml blasticidin. For induction of Mt-regulated constructs, cells were incubated in complete medium containing 0.7 mM $CuSO_4$ for 5-7 hours. For western blot analysis, protein extracts were prepared, fractioned on a 10% SDS-PAGE and transferred on a PVDF membrane as previously described (D'Avino et al., 2004). A RacGAP50C antibody was generated in rabbits against the region comprised between amino acids 68-225. The serum was used at a dilution of 1:5000 for western blot and 1:500 for immunofluorescence.

Microscopy

Immunocytochemistry was carried out as previously described (D'Avino et al., 2004) except that images were captured using a Coolsnap HQ camera on a Zeiss Axiovert 200 microscope. Antibodies and working dilutions have been described before (D'Avino et al., 2004).

For time-lapse experiments, S2 cells were plated on No. 1 1/2 thickness acid cleaned coverslips 48 hours after RNAi treatment. Imaging was performed on a Zeiss Axiovert 200 microscope with a 100× (NA 1.4) lens. Specimens were maintained at 25°C by using a dual stage and objective heating unit (Tempcontrol 37-2 digital; Zeiss). At 30-second intervals a *z*-series of ten 1-μm sections was captured using a Perkin Elmer spinning disk confocal head. All images shown are maximum intensity projections. Time-lapse recordings of primary spermatocytes have been described (Inoue et al., 2004).

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