Introduction

In animal cells, microtubule dynamics are dominated by events at the plus ends of individual microtubules, whose minus ends are anchored within γ-tubulin-rich microtubule-organising centres (Akhmanova and Steinmetz, 2008). Microtubules extend through the addition of tubulin-GTP monomers to their plus ends and, following a switch in state termed ‘catastrophe’ (Desai and Mitchison, 1997), are disassembled from the same ends (Akhmanova and Steinmetz, 2008). Microtubule behaviour is therefore dominated by environmentally induced changes in the rates of these transitions between discrete phases of growth and shrinkage (Desai and Mitchison, 1997), triggered for example by contact with the cell cortex, probably as a result of the loss of a protective plus-end β-tubulin-GTP cap (Akhmanova and Steinmetz, 2008). Significantly, this ability of individual growing microtubule tips to probe their local environment enables the population of microtubules as a whole to efficiently explore cellular space (Mitchison and Kirschner, 1985). Moreover, through their ability to direct polar cell transport and to generate forces through active polymerisation and depolymerisation, these same microtubules help to organise global cell architecture.

Establishing and maintaining a dynamic microtubule cytoskeleton that is sensitive to cell shape, mechanics and local polarity cues rests on the activities of a host of plus-end binding proteins (Akhmanova and Steinmetz, 2008). These form a visible comet at the growing tip of the microtubule, which results from the transient binding of a large number of proteins, probably through recognition of structural features within the growing microtubule GTP-tubulin cap (Akhmanova and Steinmetz, 2008). At the core of this plus-end binding complex is a protein called EB1 (Akhmanova and Steinmetz, 2008; Vaughan, 2005). EB1 is conserved from fungi to humans, binds directly to the plus ends of growing microtubules and is therefore able to track them in vitro (Bieling et al., 2007; Schuyler and Pellman, 2001). When bound to microtubules, EB1 recruits a host of other plus-end tracking proteins (Akhmanova and Steinmetz, 2008) including CLIP170 (Dixit et al., 2009; Goodson et al., 2003), CLASPs (Mimori-Kiyosue et al., 2005), RhoGEF2 (Rogers et al., 2004), ACF7/Kakapo/Shot (Kodama et al., 2003) and APC (Nathke et al., 1996). Together with EB1, these and other associated proteins dictate the behaviour and binding properties of the growing microtubule and regulate microtubule dynamic instability. As a result, in vivo, the loss of the tip-binding complex is correlated with catastrophe and microtubule depolymerisation (Akhmanova and Steinmetz, 2008). Interestingly, mechanical force can mimic the effect of these biochemical changes and induce catastrophe (Foethke et al., 2009; Janson et al., 2003), perhaps because it physically limits plus-end polymerisation, compromising the maintenance of the transient GTP-tubulin plus-end cap (Molodtsov et al., 2005).

Despite the importance of the regulation of microtubule plus ends for understanding microtubule cytoskeletal organisation and dynamics, little is known about the signalling mechanisms that trigger changes in their behaviour in space and time. Here, we report the identification of a conserved, microtubule-associated protein kinase Drosophila Tao-1 (Hutchison et al., 1998; Johne et al., 2008; Mitsopoulos et al., 2003; Zihni et al., 2007) as a protein whose ability to reduce microtubule stability is required for functional interactions between growing microtubule plus ends and the actin-rich cell cortex.
Results
Identification of Tao-1 as a regulator of cell shape
In a series of RNA inhibition (RNAi) screens in Drosophila cells in culture, we have identified a number of genes that share a common RNAi phenotype characterised by the loss of lamellipodia and the formation of multiple microtubule-based protrusions (Kiger et al., 2003; Kunda et al., 2003; Liu et al., 2009). The majority of these genes proved to encode known and novel regulators of the actin cytoskeleton, including Cdc42, Rac and components of the SCAR/WAVE and Arp2/3 complexes (Kunda et al., 2003; Rogers et al., 2003). Interestingly, however, our screens for dsRNAs that generate cells with microtubule-rich protrusions also identified several genes known to be associated with microtubule biology (supplementary material Table S1, left column). These included the kinesin-13 family member Kip1p10a (Mennella et al., 2005; Sharp et al., 2005), the microtubule-binding protein Shot [short stop/kakapo (Gregory and Brown, 1998; Kodama et al., 2003; Prokop et al., 1998; Roper et al., 2002)], dynein heavy chain Dhc64c (Rasmussen et al., 1994), the dynein light-chain binding protein SW (Song et al., 2007) and Tao-1 (Liu et al., 2009), a relatively poorly studied STE20 kinase family member implicated in the regulation of apoptosis, JNK (Zihni et al., 2007) and spindle-checkpoint signalling (Draviam et al., 2007). Because Tao-1 homologues had been described as binders to microtubules (Hutchison et al., 1998; Johne et al., 2008; Mitsopoulos et al., 2003; Zihni et al., 2007) and actin regulators (Johne et al., 2008), we chose to explore its function as a potential regulator of the actin and microtubule cytoskeleton and cell shape in more detail.

The genome of Drosophila melanogaster contains a single member of the conserved Tao protein kinase family, which has been reported to affect cell death in the Drosophila germline (Sato et al., 2007). The corresponding protein Tao-1 consists of an N-terminal kinase domain and two C-terminal coiled-coil domains. It also has a central region, which, according to the NCBI Conserved Domains tool, is predicted to contain a Smc domain (supplementary material Fig. S1A). A phylogenetic analysis of Tao kinase evolution (supplementary material Fig. S1B) suggests that Drosophila Tao-1 is related in a similar way to each of the three human Tao homologues.

RNAi-mediated depletion of Tao-1 induced the formation of microtubule-rich protrusions and bundled microtubule filaments, and exerted a strong effect on S2R+ cell shape (Fig. 1A,B). Using non-overlapping dsRNAs, we confirmed the specificity of this Tao-1 knockdown phenotype, which was associated with the loss of >95% of Tao-1 mRNA [measured relative to a control message (Rp49) by two-step RT-QPCR (Fig. 1C)]. To study the corresponding protein, we raised polyclonal antibodies against the phosphorylated form of the protein (Fig. 2D, top panel). These antibodies were used to identify a single protein band that ran at approximately 120 kDa in western blots of S2 cell lysates, a signal that was depleted 5 days after treatment with Tao-1 dsRNA (Fig. 2C). To further confirm the specificity of both Tao antibodies, we overexpressed a GFP-tagged version of full-length and truncated Tao-1-A423-900 in S2R+ cells. Using lysates from these cells, we were able to detect bands of protein running at approximately 150 kDa for the full-length, and 100 kDa for the truncated version, using our two anti-Tao antibodies and an anti-GFP antibody (Fig. 2D), as expected based upon the size of the GFP (30 kDa). We also used a brief treatment with lambda protein phosphatase to confirm that the antibody against phosphorylated Tao specifically recognised the phosphorylated form of the protein (Fig. 2D, top panel). These data show that Tao-1 is present as a single isoform in Drosophila cells in culture, and that a proportion of dominant-active form (Tao-1-α) dsRNAs. (A) Following Tao-1 silencing, microtubules lose their normal organisation and form very long, microtubule-rich protrusions. Scale bar: 10 μm. (B) This phenotype was quantified in 100 randomly picked cells in triplicate. Spiky microtubule-rich cells were seen in an average of 90% of Tao-1 RNAi cells and in 3% of control lacZ RNAi cells. (C) Q-RT-PCR confirmed the knockdown of Tao-1 mRNA in S2R+ cells after a 5-day RNAi treatment. The ribosomal housekeeping gene Rp49 was used as an internal control. Values represent the means ± s.d. of triplicate Q-PCR values from duplicate samples.

Fig. 1. Tao-1 RNAi phenotype in Drosophila S2R+ cells. Drosophila S2R+ cells were fixed and stained for microtubules using antibodies against α-tubulin 5 days after treatment with Tao-1 or control (lacZ) dsRNAs. (A) Following Tao-1 silencing, microtubules lose their normal organisation and form very long, microtubule-rich protrusions. Scale bar: 10 μm. (B) This phenotype was quantified in 100 randomly picked cells in triplicate. Spiky microtubule-rich cells were seen in an average of 90% of Tao-1 RNAi cells and in 3% of control lacZ RNAi cells. (C) Q-RT-PCR confirmed the knockdown of Tao-1 mRNA in S2R+ cells after a 5-day RNAi treatment. The ribosomal housekeeping gene Rp49 was used as an internal control. Values represent the means ± s.d. of triplicate Q-PCR values from duplicate samples.

that the antibody against phosphorylated Tao specifically recognised the phosphorylated form of the protein (Fig. 2D, top panel). These data show that Tao-1 is present as a single isoform in Drosophila cells in culture, and that a proportion of dominant-active form (Tao-1-Δ423-900, see below) is activated through phosphorylation of the T-loop during exponential growth. When we used the anti-Tao antibody to immunostain fixed Drosophila S2R+ cells, endogenous Tao-1 was colocalised with α-tubulin along a subset of interphase microtubules (Fig. 2A,B). Similarly, during mitosis, this antibody stained the mitotic spindle (supplementary material Fig. S2D). By contrast, the antibody against phosphorylated Tao yielded a punctate staining pattern in interphase Drosophila cells (Fig. 2A-B; punctae were frequently seen closely associated with the tips of microtubule bundles) and was localised to peri-centrosomal material in mitotic cells, where the plus-tip binding protein EB1 has been shown to be concentrated (Rogers et al., 2002) (supplementary material Fig. S2D). As with the western blot analysis, the endogenous pool of Tao-1 was significantly depleted following Tao-1 RNAi (supplementary material Fig. S2A) and increased following overexpression of RFP-Tao-1 (supplementary material Fig. S2B), confirming that the signal corresponded to Tao-1. Inspection of cells by immunofluorescence in which ectopic Tao-1 was expressed at levels of 120 kDa in western blots of S2 cell lysates, a signal that was depleted 5 days after treatment with Tao-1 dsRNA (Fig. 2C). To further confirm the specificity of both Tao antibodies, we overexpressed a GFP-tagged version of full-length and truncated Tao-1-A423-900 in S2R+ cells. Using lysates from these cells, we were able to detect bands of protein running at approximately 150 kDa for the full-length, and 100 kDa for the truncated version, using our two anti-Tao antibodies and an anti-GFP antibody (Fig. 2D), as expected based upon the size of the GFP (30 kDa). We also used a brief treatment with lambda protein phosphatase to confirm that the antibody against phosphorylated Tao specifically recognised the phosphorylated form of the protein (Fig. 2D, top panel). These data show that Tao-1 is present as a single isoform in Drosophila cells in culture, and that a proportion of dominant-active form (Tao-1-Δ423-900, see below) is activated through phosphorylation of the T-loop during exponential growth. When we used the anti-Tao antibody to immunostain fixed Drosophila S2R+ cells, endogenous Tao-1 was colocalised with α-tubulin along a subset of interphase microtubules (Fig. 2A,B). Similarly, during mitosis, this antibody stained the mitotic spindle (supplementary material Fig. S2D). By contrast, the antibody against phosphorylated Tao yielded a punctate staining pattern in interphase Drosophila cells (Fig. 2A-B; punctae were frequently seen closely associated with the tips of microtubule bundles) and was localised to peri-centrosomal material in mitotic cells, where the plus-tip binding protein EB1 has been shown to be concentrated (Rogers et al., 2002) (supplementary material Fig. S2D). As with the western blot analysis, the endogenous pool of Tao-1 was significantly depleted following Tao-1 RNAi (supplementary material Fig. S2A) and increased following overexpression of RFP-Tao-1 (supplementary material Fig. S2B), confirming that the signal corresponded to Tao-1. Inspection of cells by immunofluorescence in which ectopic Tao-1 was expressed at relatively low levels (e.g. GFP-Tao-1) revealed localisation of this fusion protein to a subset of microtubules in both interphase (supplementary material Fig. S2C) and mitotic (supplementary material Fig. S2E) cells. These localisation data are consistent with published data suggesting that Tao-1 homologues in human cells (Tao kinase 1-3) are associated with and act on microtubules (Johne et al., 2008; Mitsopoulos et al., 2003; Zihni et al., 2007).
Deconstructing Tao-1 protein structure and function

To carry out a structure-function analysis of Tao-1, we generated a number of expression constructs encoding mutant forms of the protein tagged with mRFP (Fig. 3A). S2R+ cells were transfected with these constructs and then fixed and stained to visualise both microtubules and the RFP-tagged protein. While the full-length RFP-tagged protein had a somewhat weak and variable effect on microtubule organisation and cell shape in this cell type (Fig. 3B), quantified in supplementary material Fig. S3A), the kinase-dead RFP-Tao-1 K56A protein, lacking a lysine residue at the catalytic core of the kinase domain, reproducibly induced the formation of microtubule-rich protrusions (Fig. 3C) similar to those seen in Tao-1 RNAi cells. A similar phenotype was also seen following the overexpression of RFP-Tao-1 itself is sufficient to dismantle the microtubule network and to inhibit cell spreading (compare control and RFP-Tao-1 cells, supplementary material Fig. S3A,B). In addition, Tao-1 over-expression in these cells delayed the ability of the microtubule network to recover following the removal of a microtubule inhibitor, colcemid, from the medium (compare control and RFP-Tao-1 cells, supplementary material Fig. S3A,B). Taken together, the loss- and gain-of-function phenotypes suggest that Tao-1 functions to destabilise microtubules.

The role of microtubules in the generation of the Tao-1 phenotype

As mentioned above, Tao-1 was identified in screens by an RNAi phenotype similar to that seen when the actin cortex is compromised through loss of Arp2/3-dependent actin filament formation – namely, microtubule-based protrusions and a starfish-like cell shape (Kunda et al., 2003; Rogers et al., 2003) (Fig. 1). Following the loss of lamellipodial actin (as a result of RNAi or the addition of actin inhibitors), these microtubule-based protrusions are generated as growing microtubules push on and deform the weakened cell cortex (Kunda et al., 2003; Rogers et al., 2003). These observations imply that the normally isotropic cell shape is maintained by an active dialogue between actin filaments and microtubules. Thus, a similar
phenotype could be generated by increased microtubular growth acting on a relatively normal cell cortex, as is seen in S2R+ cells treated with dsRNA to knock down Klp10A and Short stop/Kakapo (data not shown), where microtubules and microtubule-cortical interactions are likely to be perturbed (Sanchez-Soriano et al., 2010; Sharp et al., 2005; Applewhite et al., 2010). To determine whether microtubules have a direct role in the generation of the spiky shape that is characteristic of Tao-1 RNAi cells, we began by using a cold-shock regimen (Rogers et al., 2008) to test the effects of microtubule depolymerisation and recovery on cell shape 5 days after treatment with Tao-1 dsRNA. In this experiment, dsRNAs against lacZ and SCAR were used as controls.

The analysis revealed several important aspects of the Tao-1 RNAi phenotype. First, although both SCAR/WAVE and Tao-1 knockdown cells had long microtubule-rich protrusions before cold shock, after 2.5 hours in an ice-cold environment, microtubules were visible in Tao-1 RNAi cells (Fig. 4A shows a representative cell; note the loss of microtubule spikes), even though this treatment eliminated visible microtubules in the vast majority of control cells. This suggests that microtubules are unusually stable in cells lacking Tao-1. Nevertheless, after 4 hours on ice, tubulin polymer was completely disassembled in all control and RNAi cells (data not shown). Interestingly, under these conditions ~80% of Tao-1 RNAi cells resumed the isotropic shape characteristic of untreated S2R+ cells (Fig. 4B), whereas SCAR RNAi cells remained spiky. [A similar difference between SCAR and Tao-1 RNAi cells was also seen when microtubules were depolymerised using the drug nocodazole (data not shown).] However, microtubule-rich processes were rapidly re-established in Tao-1 RNAi cells following the return to room temperature (Fig. 4C). These data suggest that although the loss of lamellipodial actin appears to be the primary cause of the spiky SCAR RNAi phenotype (Kunda et al., 2003), microtubules have a crucial role in the generation of the spiky Tao-1 RNAi phenotype.

To examine the aetiology of Tao-1 versus SCAR RNAi phenotypes in more detail, we next performed a time-course dsRNA experiment to see how changes in microtubules and the actin cortex develop at 2, 3 and 4 days after treatment with dsRNA (supplementary material Fig. S4). Although the majority of SCAR and Tao-1 RNAi cells exhibited the expected microtubule-based protrusions after 4 days of RNAi, at 2 days and 3 days following dsRNA treatment, we observed a significant population of Tao-1 RNAi cells (20% and 30% respectively) with visibly bundled microtubules and a normal actin cytoskeleton (supplementary material Fig. S4A, arrow in Day 3 image). As expected for an intermediate state, the number of cells with this phenotype was reduced to 15% by day 4, when over 80% of Tao-1 RNAi cells had developed microtubule-based protrusions. During the same time-course in the corresponding control experiment, fewer than 5% of SCAR RNAi cells exhibited a normal actin cortex and a visibly disturbed microtubule cytoskeleton (see supplementary material Fig. S4B for the quantification). Again, this experiment supports the idea that although both Tao-1 and SCAR have a similar terminal RNAi phenotype, resulting from defects in the cross-talk between the actin and microtubule cytoskeletons, the two RNAi phenotypes arise in distinct ways: through a primary defect in the ability to nucleate lamellipodial actin in the case of SCAR, and through deregulation of microtubule dynamics in the case of Tao-1.

To further explore this potential role for Tao-1 in the regulation of microtubule dynamics and the dialogue between the actin cortex and the microtubule cytoskeleton, we used Drosophila EB1, a

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Fig. 3. Structure-function analysis of Tao-1. A structure-function analysis of Drosophila Tao-1 was carried out in Drosophila S2R+ cells to test the effects of mutating (K56A) or deleting the S-T protein kinase domain (ΔS1-354), the central region containing homology to Smc (Δ423-900) and the C-terminal coiled-coil domain (Δ900-1039). (A) Schematic of the mutation and deletion constructs used. (B) RFP-Tao-1 has a variable, minor effect on microtubule organisation. Microtubules are shown in green, RFP-Tao-1 in red. Scale bars: 10 μm and 7.5 μm (bottom row). (C) Expression of the kinase point mutant RFP-Tao-1 K56A causes a spiky phenotype similar to that of Tao-1 RNAi, and localises with microtubules at the cell cortex and at the tip of microtubule bundles. (D) Expression of the kinase-domain deletion mutant ΔS1-354 RFP-Tao-1 induces a phenotype similar to that seen following expression of RFP-Tao-1 K56A. (E) The coiled-coil deletion mutant, Δ900-1039 RFP-Tao-1, accumulates in the nucleus and does not induce a visible microtubule phenotype. (F) Expression of the central region deletion construct Δ423-900 RFP-Tao-1 causes drastic microtubule disassembly, which inhibits cell spreading.
Microtubules were depolymerised with a cold-shock regimen in S2R+ cells that had been treated with Tao-1, SCAR or control dsRNA for 5 days, then fixed and stained for microtubules. (A) After 2.5 hours in an ice-cold environment, many long microtubules remain visible in Tao-1 RNAi cells but are completely eliminated in control cells. Cell shape was quantified in control, SCAR and Tao-1 RNAi cells after 4 hours on ice (B) and after a 10 minute recovery at room temperature (C). In each case, results show the average of 30 cells analysed in triplicate. Cells that failed to spread were not included in the analysis. Error bars represent s.d.

When we quantified the effects of overexpressing the Tao-1 dominant-negative K56A protein on microtubule catastrophe rates in cells expressing EB1-GFP (Fig. 5E), K56A was found to induce a threefold increase in the average lifetime of microtubule plus ends remaining paused at the cell cortex before catastrophe (30±5.6 seconds), compared with the control (10±1.6 seconds) and with cells overexpressing RFP-Tao-1 (8±1.4 seconds) (Fig. 5E). These data confirm a role for Tao-1 in the ability of growing microtubules to undergo catastrophe in response to contact with the actin-based cortex. If it is the case that Tao-1 acts primarily to destabilise microtubules, we reasoned that it should be possible to reduce the rate of formation of microtubule-induced protrusions in cells with a weakened actin cortex by expressing dominantly activated Tao-1. To test this, we examined the growth of individual microtubules in EB1-GFP S2 cells expressing control or RFP-D423-900 Tao-1 after the disruption of the actin cortex. As shown in supplementary material Fig. S5A (top row), growing microtubules rapidly induced the formation of protrusions in control cells following the addition of latrunculin B, but this effect was significantly reduced in cells overexpressing RFP-D423-900 Tao-1 protein (supplementary material Fig. S5A, bottom three rows).

To determine how Tao-1 might alter interactions between the plus ends of microtubules and the cell cortex, we tracked microtubule plus ends as they passed between central and peripheral regions of the cell, defined based on automatically thresholded background fluorescence levels (Fig. 6A). We then used EB1-GFP comets to measure the speed of individual growing microtubules within comparable regions of control cells, Tao-1 RNAi cells, and cells expressing RFP-Tao-1 and Tao-1 K56A (Fig. 6B-E). In both control cells and in cells overexpressing full-length Tao-1, microtubules were seen growing with a wide range of speeds (Fig. 6B). In both cases, the rate of microtubule polymerisation was dependent on the location of the microtubule plus end; specifically, EB1-GFP comets moved rapidly as if unimpeded when passing through the centre of these cells, and slowly upon entering the thin lamellipodial region of the cell (Fig. 6F). In striking contrast, in cells with reduced Tao-1 activity, growing microtubules formed a single kinetic population whose speeds were found to be independent of their position within the cell. This is shown most clearly in Fig. 6F, where the ratio of peripheral to internal velocity was found to be close to 1 for both Tao-1 RNAi cells and cells overexpressing the K56A-Tao-1 variant. Thus, plus ends appear blind to their position within the cell when Tao-1 function is compromised. When we examined unimpeded plus-end growth in cells immediately after recovery from drug-induced microtubule depolymerisation, the average growth rates of new plus-ends growing from the cell centre were found to be indistinguishable in control and Tao-1 K56A cells (data not shown). Thus, the slight reduction in the average velocity of EB1-GFP comets in the central part of cells lacking Tao-1 activity probably reflects a decrease in the available pool of tubulin monomer, which limits the speed of unimpeded growth of microtubule plus ends in these cells.

Finally, in an attempt to identify proteins that function together with Tao-1 in the regulation of microtubule dynamics, we performed a small modifier RNAi screen in S2R+ cells (Kiger et al., 2003) for microtubule-associated proteins that genetically interact with Tao-1. We simultaneously silenced Tao-1 and several other microtubule regulators, and looked for dsRNAs that modified the spiky microtubule Tao-1 RNAi phenotype (supplementary material Table S1, right column). Of the genes tested, only EBI RNAi was able to induce a partial but significant rescue of the Tao-1 phenotype (supplementary material Fig. S5B). This supports the idea that Tao-1 acts on the plus-end binding complex to regulate microtubule dynamics and the interactions between microtubule plus ends and the actin cortex.

Discussion

In this paper, we show that a conserved microtubule-associated kinase, Tao-1, regulates microtubule dynamics and is required to limit the growth of microtubule plus ends as they contact the actin-based cell cortex. The idea that Tao-1 acts on microtubules confirms
Tao-1 limits microtubule plus-end growth

the conclusions of previous work on the mammalian homologues of Tao-1. It should be noted however that one of its human homologues, Tao kinase 2, is reported to stabilise microtubules (Mitsopoulos et al., 2003), implying that the different homologues might have taken on divergent functions in human cells (Hutchison et al., 1998; Mitsopoulos et al., 2003).

Interestingly, in Drosophila cells in culture, the phenotypic effect of Tao-1 silencing mirrors the effects of a loss of lamellipodial actin, as seen for example following SCAR/WAVE RNAi. In this case, the actin cortex is weakened, allowing normally growing microtubules to generate microtubule-based protrusions as they polymerise and push on the plasma membrane (Kunda et al., 2003). By contrast, the spiky Tao-1 RNAi phenotype appears to develop in stages. First, cells appear to have a macroscopically normal actin cytoskeleton. The cell edge then becomes gradually distorted over time (supplementary material Figs S5 and S4) as a result of the unchecked growth of microtubules that fail to undergo catastrophe when reaching the cortex (Fig. 5). Although we cannot completely rule out an additional role for Tao-1 in the regulation of actin filaments in this process, as previously proposed (Johne et al., 2000).

Fig. 5. Microtubules push on the cortex of cells depleted for active Tao-1, generating protrusions. (A-D) Stably expressed EB1-GFP visualised in control S2 cells (A), in cells overexpressing (OE) K56A RFP-Tao-1 (B), in Tao-1 RNAi cells (C) and in cells following treatment with latrunculin B (D). Times between frames are indicated in seconds. Scale bars: 2.5 μm. (E) The average (mean) lifetimes of GFP-EB1 comets pausing at the cell cortex before undergoing catastrophe determined by analysing frames taken every 2 seconds in movies of control, RFP-Tao-1- and K56A RFP Tao-1-expressing cells. Error bars represent s.d.

Fig. 6. Tao-1-induced changes in growth rates of microtubule plus-ends vary with context. (A) Microtubule-plus-end growth rates were calculated from stills of S2 cells stably expressing EB1-GFP imaged every 2 seconds. Automatic thresholding identified distinct cell domains based on plus-end growth speeds, the cell edge (red) and cell centre (blue), to determine plus-end growth in different contexts. (B-E) The growth rates of individual microtubule plus ends at the edge (red, upper panels) or within the cell centre (blue, lower panels) determined using semi-automated image analysis. The average velocity of microtubule growth measured using EB1-GFP was quantified for individual microtubules in control and Tao-1 RNAi cells, and in cells expressing full-length RFP-Tao-1 or a K56A RFP-Tao-1 mutant. Data from individual samples was grouped in 20 nm/second bins. N denotes the number of individual microtubules analysed in each case. (F) The ratio of mean peripheral versus central velocity of EB1 comets determined using semi-automated image analysis. The average velocity of microtubule growth measured using EB1-GFP was quantified for individual microtubules in control and Tao-1 RNAi cells, and in cells expressing full-length RFP-Tao-1 or a K56A RFP-Tao-1 mutant. Data from individual samples was grouped in 20 nm/second bins. N denotes the number of individual microtubules analysed in each case. (F) The ratio of mean peripheral versus central velocity of EB1 comets.
al., 2008), our analysis suggests that Tao-1 acts primarily on the microtubule cytoskeleton. The change in microtubule dynamics then alters the balance of forces acting to determine S2R+ cell shape, as the mechanically strong actin-rich cortex resists the protrusive forces generated by microtubule plus-end growth. A similar finely tuned balance has been proposed to underlie interactions between the actin cytoskeleton and microtubules in the regulation of axonal extension (Bradke and Dotti, 1999). Moreover, mechanical force has been shown to contribute directly to microtubule catastrophes and has been proposed to have a significant role in guiding microtubule organisation in yeast (Foethke et al., 2009; Janson et al., 2003). Force could therefore have a significant role in the Tao-1-dependent regulation of microtubule dynamics.

The ability of Tao-1 to regulate interactions between microtubule plus ends and the cell cortex could be mediated by a variety of molecular mechanisms. Because endogenous Tao-1 localises both to microtubules and at the cortex, as suggested by studies of the mammalian Tao-1 homologues (Johne et al., 2008; Mitsopoulos et al., 2003; Zihni et al., 2007), the kinase could act to bridge the interface between microtubules and actin filaments, as previously proposed for other microtubule-binding proteins such as ACF7/Kakapo/Shot (Gregory and Brown, 1998; Kodama et al., 2003; Prokop et al., 1998; Wu et al., 2008), CLIP170 (Akhanova et al., 2001; Goode et al., 2000) and CLASPs (Lansbergen et al., 2006; Mimori-Kiyosue et al., 2005). In this context, we note that some algorithms identify a region within Tao-1 with homology to Smc proteins within its central regulatory domain, which was recently proposed to function as an actin-dependent ATPase in the context of ACF7 (Wu et al., 2008). The role of Tao-1 in the ability of microtubules to sense and respond to these cortical forces is also reminiscent of the recent report that Drosophila Shot (a hit in our screen) acts in concert with cortical F-actin to check the growth of microtubules in neuronal growth cones (Sanchez-Soriano et al., 2010), suggesting that Tao-1 is part of a hitherto unappreciated class of proteins that can execute this important function of linking the two major cytoskeletal systems.

A detailed biochemical analysis will be required to reveal the molecules phosphorylated by Tao-1 to negatively regulate this important function of linking the two major cytoskeletal systems. Tao-1 with homology to Smc proteins within its central regulatory domain is also reminiscent of the recent report that Drosophila Shot (a hit in our screen) acts in concert with cortical F-actin to check the growth of microtubules in neuronal growth cones (Sanchez-Soriano et al., 2010), suggesting that Tao-1 is part of a hitherto unappreciated class of proteins that can execute this important function of linking the two major cytoskeletal systems. Tao-1 with homology to Smc proteins within its central regulatory domain is also reminiscent of the recent report that Drosophila Shot (a hit in our screen) acts in concert with cortical F-actin to check the growth of microtubules in neuronal growth cones (Sanchez-Soriano et al., 2010), suggesting that Tao-1 is part of a hitherto unappreciated class of proteins that can execute this important function of linking the two major cytoskeletal systems.
equivalent amount of buffer was added instead of enzyme. After enzymatic treatment, samples were loaded and run on 12% Laemmli buffer resolubilized gels. After 10 minutes before loading onto 8% SDS-PAGE gels. Protein was then transferred to Immobilon-P (Millipore) by western blotting. For non-phospho-specific antibodies, membranes were blocked with 5% non-fat dry milk in Tris-buffered saline with 0.05% Triton X-100 for 1 hour; for phosphorylated Tao antibody, membranes were instead blocked in 5% BSA in TBS-T (0.01% Triton X-100). All primary antibody incubations were carried out at room temperature for several hours with either 1% milk (non-phospho) or 1% BSA (phospho) [1:1000 for the P-Tao-1 antibody; 1:75 for the Tao-1 antibody; 1:1000 for GFP antibody (Molecular Probes A11212), 1:1500 for the loading control dSra1], and then washed five times with TBS-T (same proportions as above) after each addition as used for blocking. Membranes were then incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (DAKO, 1:1000 in TBS-T for 1 hour, and washed as before. Enhanced chemiluminescent reagent (Amersham/GE Healthcare) was used for detection on Hyperfilm EC (Amersham/GE Healthcare). When re-probing was required, membranes were stripped in 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.8, at 50°C for 30 minutes with agitation, then washed twice for 10 minutes in TBS-T. Membranes were tested with ECL to confirm absence of signal before re-washing and re-blocking as normal.

Drosophila cell culture, RNAi and cell biology methods

The Drosophila cell lines S2R+ and GFP-EBi S2 used in this study were all cultured with Shields and Sang M3 insect medium (Sigma) with 10% heat-inactivated fetal bovine serum (JRH Biosciences) and 1% penicillin-streptomycin (Sigma). Drosophila RNAi experiments were carried out as previously described (Baum B. and Chubb FH, 2003). Cells were seeded in serum-free medium at a concentration of 2×10^5 cells/ml mixed with dsRNA designed and prepared as described (Liu et al., 2009), to give a final concentration of 30 ng/µl centrifuged briefly in their culture vessels, and then incubated at 24°C for 15 minutes before addition of complete medium. Cells were grown for 2-7 days at 24°C, depending on the experiment. For microscopy, cells were rinsed twice with FBS- or ConA-coated Trition X-100 (Sigma) as used for blocking. To analyse the effect of microtubule-destabilising drugs on the behaviour of Tao-1, fixed embryos were first treated with 0.5 µg/ml colcemid, and at 4 and 12 hours after RNAi. The cells were allowed to spread for 30 minutes before they were imaged with confocal microscopy; a 40× oil lens was used for taking 512×512-resolution live images at a frequency of one frame every 2-4 seconds for 60 time-points. Representative movies are available as supplementary material: supplementary material Movie 1 is the control, supplementary material Movie 2 is the K56A null mutant and supplementary material Movie 3 is the Tao-1 RNAi condition. Analysis of GFP-EB1 comets was carried out in ImageJ on processed images as follows. Because the lamellipodia manifested lower GFP background fluorescence when compared with the central portion of the cell, thresholding was used to differentially identify the two regions, and pixels with grey values below and above this identified threshold were reassigned with values of 255 and 0 respectively. 231-313 EB1-GFP comets from 2-5 individual cells (depending on the conditions) were tracked by hand and recorded as ImageJ ROI files. Finally, localisation of the tracked EB1-ROI spots onto either cell edge or central regions were obtained by taking pixel values (0 or 255) from the cell binary movies at the EB1-ROI positions. The velocity of the EB1-GFP spots was calculated by dividing their displacement by the time interval between frames (2-4 seconds).

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References


