Mini spindles, the XMAP215 homologue, suppresses pausing of interphase microtubules in *Drosophila*

Amy L Brittle and Hiroyuki Ohkura*

Wellcome Trust Centre for Cell Biology, Institute of Cell Biology, School of Biological Sciences, The University of Edinburgh, Edinburgh, UK

***Drosophila* Mini spindles (Msps) protein belongs to a conserved family of microtubule-associated proteins (MAPs). Intriguingly, this family of MAPs, including *Xenopus* XMAP215, was reported to have both microtubule stabilising and destabilising activities. While they are shown to regulate various aspects of microtubules, the role in regulating interphase microtubules in animal cells has yet to be established. Here, we show that the depletion or mutation of Msps prevents interphase microtubules from extending to the cell periphery and leads to the formation of stable microtubule bundles. The effect is independent of known Msps regulator or effector proteins, kinesin-13/KinI homologues or D-TACC. Real-time analysis revealed that the depletion of Msps results in a dramatic increase of microtubule pausing with little or no growth. Our study provides the first direct evidence to support a hypothesis that this family of MAPs acts as an antipausing factor to exhibit both microtubule stabilising and destabilising activities.**

The EMBO Journal (2005) 24, 1387–1396. doi:10.1038/sj.emboj.7600629; Published online 17 March 2005

Subject Categories: cell & tissue architecture

Keywords: bundle; interphase; microtubule; pause; RNAi

Introduction

The microtubule network of eukaryotic cells plays important roles in a variety of cellular processes, including cell division, morphogenesis, polarity, migration and intracellular transport. Microtubules are polymers consisting of subunits of α - and β -tubulin, and display behaviour, described as dynamic instability, both *in vivo* and *in vitro* (Mitchison and Kirschner, 1984; Cassimeris *et al.*, 1988; Shelden and Wadsworth, 1993). Individual microtubules independently undergo polymerisation or depolymerisation with infrequent changes between the two states. In addition, the third state, pausing, in which microtubules undergo no significant growth or shrinkage is often observed in cells (Shelden and Wadsworth, 1993; Tirnauer *et al.*, 1999; Rusan *et al.*, 2001), but its functional significance is yet to be understood.

*Corresponding author. Wellcome Trust Centre for Cell Biology, Institute of Cell Biology, School of Biological Sciences, The University of Edinburgh, Edinburgh EH9 3JR, UK. Tel.: +44 131 650 7094; Fax: +44 131 650 8650; E-mail: h.ohkura@ed.ac.uk

Received: 15 December 2004; accepted: 23 February 2005; published online: 17 March 2005

In order for microtubules to fulfil their various functions within the cell, microtubules need to be organised into an array of different structures. While microtubules form only one basic structure, the bipolar spindle, in dividing cells, microtubules form a vast array of different arrangements in interphase cells depending on the cell type. Compared to spindle formation, little information is available on how interphase microtubules are organised.

A number of microtubule-associated proteins (MAPs), both motor or nonmotor proteins, collectively regulate microtubule dynamics and organisation in the cell. The Dis1/TOG family is a family of nonmotor MAPs highly conserved from yeast to plants and humans (Ohkura *et al.*, 2001; Kinoshita *et al.*, 2002). The *Xenopus* homologue, XMAP215, has been characterised *in vitro* as a major regulator of microtubule plus end growth (Gard and Kirschner, 1987; Vasquez *et al.*, 1994). In addition, in *Xenopus* egg extracts, the protein acts as a suppressor of microtubule catastrophes by antagonising the activity of XKCM1, a member of the microtubule destabilising kinesin-13/KinI family (Tournéize *et al.*, 2000). These two proteins are proposed to be the principal regulators of microtubule dynamics, as addition of the two proteins to pure tubulin results in microtubule dynamics similar to those seen *in vivo* (Kinoshita *et al.*, 2001). Dis1/TOG proteins have long been regarded as microtubule stabilising proteins; however, recent reports indicate that, at least under some conditions, XMAP215 and the budding yeast homologue Stu2 can act as microtubule destabilising proteins (van Breugel *et al.*, 2003; Shirasu-Hiza *et al.*, 2003). While it is clear that the Dis1/TOG family are important regulators of microtubule dynamics, detailed understanding of the activities of the protein remains to be elucidated.

Functional studies of Dis1/TOG proteins *in vivo* have revealed a role for the proteins in the organisation and function of the spindle. Most of the Dis1/TOG proteins localise to the spindle poles as well as the microtubules (Wang and Huffaker, 1997; Charrasse *et al.*, 1998; Matthews *et al.*, 1998; Cullen *et al.*, 1999; Graf *et al.*, 2000; Tournéize *et al.*, 2000; Garcia *et al.*, 2001; Nakaseko *et al.*, 2001). Dramatic spindle defects are seen in mutants of the *Drosophila* homologue, *mini spindles* (*msps*) (Cullen *et al.*, 1999; Cullen and Ohkura, 2001). Very similar defects were subsequently described in human cells following depletion of the human homologue, TOGp (Gergely *et al.*, 2003; Holmfeldt *et al.*, 2004). Additionally, defects in astral microtubules are also seen in mutants of budding yeast *stu2* and the worm homologue *zyg-9* (Matthews *et al.*, 1998; Usui *et al.*, 2003). Kinetochore function has been described for the fission yeast, Dis1 and Alp14, and in budding yeast Stu2 (Garcia *et al.*, 2001; Kosco *et al.*, 2001; Nakaseko *et al.*, 2001; Severin *et al.*, 2001).

There are some indications that the Dis1/TOG family also plays a role in interphase microtubule regulation. Two

temperature-sensitive alleles of the plant homologue, *mor-1*, have revealed a function for the protein in cortical microtubule organisation (Whittington *et al*, 2001). Defects in interphase microtubules have also been described in fission yeast and budding yeast (Radcliffe *et al*, 1998; Kosco *et al*, 2001; van Breugel *et al*, 2003). In contrast to clear evidence in yeasts and plants, the roles in regulating interphase microtubules are not well established in animal cells. Reports describing depletion of the human TOG protein state that no interphase microtubule defects were observed in cells, although microtubule dynamics were not examined (Gergely *et al*, 2003; Holmfeldt *et al*, 2004). Very recently, defects in cytoplasmic microtubules and the localisation of *bicoid* mRNA in *Drosophila* oocytes were described in *msps* mutants (Moon and Hazelrigg, 2004).

To understand the role of Msp protein in interphase microtubule regulation, we decided to use *Drosophila* culture cells, which allow the visualisation of individual microtubules and are also amenable to RNA interference (RNAi). Here we demonstrate that Msp is a major regulator of interphase microtubule organisation, and that this activity is independent of known regulators and effectors, the kinesin-13/KinI homologues and D-TACC. Further analysis of microtubule dynamics indicates that Msp acts as a microtubule antipause factor in interphase cells.

Results

*Msp*s is associated with interphase microtubules

As the first step to understand the cellular roles of Msp during interphase, we examined Msp protein localisation in *Drosophila* S2 culture cells. When these cells were grown on a concanavalin A (con A)-coated surface, they spread and the interphase microtubules extend outwards to the cell periphery, enabling visualisation of individual microtubules in the

flattened region of the cell (Rogers *et al*, 2002). S2 cells were subjected to immunostaining using antibodies against Msp (Cullen *et al*, 1999) and α -tubulin. During mitosis, Msp concentrated on the centrosomes and the spindle microtubules, as seen in *Drosophila* embryos. During interphase, a high level of Msp staining is seen in the central region of the cell where microtubule density is high (Figure 1A and Supplementary Figure 1). Punctate staining was also seen along individual microtubules that extended out toward the cell cortex. Additionally, Msp staining was seen strongly on the plus ends of microtubules at the edge of the cell (Figure 1A, arrowheads). These stainings were greatly reduced after RNAi of Msp (Supplementary Figure 2), indicating that the stainings represent Msp protein. The association of Msp protein with microtubules in interphase prompted us to study the roles of Msp in the regulation of interphase microtubules.

Depletion of Msp by RNAi disrupts interphase microtubule organisation

To study the role of Msp protein in interphase, we depleted Msp from S2 cells by RNAi. Incubations with double-stranded RNAs (dsRNAs) corresponding to different non-overlapping parts of *msps* gave the same results, while incubation with unrelated control dsRNA (bacteria β -lactamase) showed no effects (Figures 1C and 3D). A reduction in the level of Msp was seen at 24 h and more than 70% of the protein was depleted after 48 h (Figure 1B), while at 120 h greater than 95% was depleted (Supplementary Figure 2). No change in Msp protein level was detected in the cells treated with the control dsRNA (Figure 1B).

Msp protein forms a complex with D-TACC protein (Cullen and Ohkura, 2001; Lee *et al*, 2001), and this interaction is conserved in other organisms (Bellanger and Gönczy, 2003; Gergely *et al*, 2003; Srayko *et al*, 2003; Sato *et al*, 2004).

Figure 1 Msp depletion disrupts the organisation of interphase microtubules. (A) Localisation of Msp along interphase microtubules with concentration in plus ends in S2 cells. *Drosophila* S2 cells were plated out on the con A-coated surface and stained with antibodies against α -tubulin and Msp. The yellow box indicates the area magnified in the right bottom panel. The arrowheads indicate Msp staining, which coincides with microtubule plus ends. (B) Depletion of Msp by RNAi. S2 cells were incubated with dsRNA corresponding to a part of the *msps* gene (m), or bacteria β -lactamase as control (c). Immunoblots indicated that Msp protein was well depleted by *msps* RNAi. In addition, the amount of D-TACC, the binding partner of Msp, was decreased to a lesser extent. (C) Interphase microtubule organisation of cells treated with Msp dsRNA or control dsRNA. Most of cells treated with control dsRNA showed normal 'extended' microtubule organisation in which interphase microtubules extend towards the periphery of the cell from a microtubule-dense region in the centre of the cells. The depletion of Msp induced abnormal microtubule organisation in which microtubules are not extended from the central region ('compact') or form thick bundles in the central region ('bundled'). Standard deviations are represented as lines at the top of corresponding bars. (D) Typical microtubule organisation in control and Msp-depleted cells. The yellow dotted lines indicate the outlines of the cell. Bar = 10 μ m.

In the case of *Caenorhabditis elegans*, depletion of the Mps homologue Zyg-9 reduces the amount of D-TACC homologue TAC-1 (Bellanger and Gönczy, 2003). Immunoblotting indicated that the amount of D-TACC protein was indeed reduced in Mps-depleted cells, but at earlier time points, the level of D-TACC depletion was significantly lower than that of Mps depletion (Figure 1B). Nevertheless, as the depletion of D-TACC does not result in the defects seen in Mps RNAi (see below), it alone cannot account for the defects caused by Mps depletion.

Microtubule organisation in Mps-depleted cells was examined by α -tubulin immunostaining. Abnormal spindles were observed in mitotic cells, accompanied by an elevated mitotic index. Abnormalities included broad or split spindle poles and multiple bipolar spindles with some shared poles (Supplementary Figure 3), similar to those seen in cells of the larval central nervous systems in *mps* mutants (Cullen *et al*, 1999) and also to the phenotype seen by RNAi in human cultured cells (Gergely *et al*, 2003; Holmfeldt *et al*, 2004).

In interphase cells, Mps depletion dramatically altered microtubule organisation. In the control cells, microtubules are typically extended towards the cell periphery from a mass of microtubules in the centre of the cell (Figure 1D). Following Mps depletion, in many cells, the microtubules were concentrated only in the central region of the cell and did not extend out to the periphery (Figure 1D). At 72 h, normal extended microtubules were observed in only 28% of cells. In addition, thick bundles of microtubules formed around the nucleus situated in the cell centre in some cells. At 120 h, extensive microtubule bundling was found in 34% of cells.

In our typical protocol, cells were fixed after culturing them for 2 h on a con A-coated surface (Materials and methods). It was therefore possible that Mps depletion was affecting the speed of microtubule rearrangement and extension towards the cell periphery after cell spreading. To exclude this possibility, S2 cells were continuously cultured on a con A-coated surface from the start of Mps depletion. The same microtubule defects were observed as those seen with 2 h spreading (Supplementary Figure 4), indicating that Mps is important for normal microtubule organisation.

The dramatic affect of microtubule organisation could have secondary effects on cell architecture. Staining with rhodamine-labelled phalloidin indicated that Mps depletion did not affect spreading on the con A-coated surface or actin distribution (Supplementary Figure 5).

Effects of depletion is independent of kinesin-13/KinI proteins or D-TACC

Vertebrate homologues of Mps are known to antagonise the activity of a catastrophe factor, MCAK/XKCM1, which belongs to the kinesin-13/KinI family of kinesin-like proteins (Tournebise *et al*, 2000). The *Drosophila* genome has three members of the kinesin-13/KinI family, KLP10A, KLP59C and KLP59D (Rogers *et al*, 2004). To test if the kinesin-13 proteins were involved in generation of the interphase microtubule phenotype seen upon Mps depletion, RNAi was carried out on the three kinesin-13 proteins in combination with Mps.

Single RNAi of the three kinesin-13 proteins produced no obvious interphase microtubule phenotype (data not shown). Depletion of KLP10A and KLP59C was confirmed by the appearance of mitotic phenotypes as previously re-

ported (Rogers *et al*, 2004). Consistent with the previous report, RNAi of KLP59D produced no mitotic phenotype. Considering the robustness of RNAi in S2 cells (Goshima and Vale, 2003), successful depletion of KLP59D is also likely.

Individual depletion of each of the three kinesin-13 proteins in combination with Mps did not rescue the interphase microtubule defects caused by Mps depletion (Figure 2A). Interestingly, codepletion of KLP10A with Mps resulted in more severe microtubule defects. The number of cells with extensive microtubule bundling rose from 24% (in cells singly depleted of Mps) to 58% (in cells doubly depleted of Mps and KLP10A; Figure 2A). In contrast, depletion of KLP10A partially rescued mitotic spindle defects caused by Mps depletion (Supplementary Figure 6).

To exclude the possibility that effects of kinesin-13 depletion were masked by functional redundancy between the kinesin-13 proteins, all three proteins were simultaneously depleted with Mps. The triple depletion did not rescue the

Figure 2 Interphase function of Mps is independent of known effectors or regulators, kinesin-13/KinI and D-TACC. (A) Interphase microtubule organisation of cells that were depleted of the kinesin-13/KinI homologues and Mps (120 h after dsRNA addition). Mps was depleted by RNAi alone (*mps* RNAi), together with single kinesin-13/KinI homologues (*mps* klp), and all three kinesin-13 (*mps* All KinI). KLP10A depletion enhanced the effect of Mps depletion. Simultaneous depletions of kinesin-13/KinI homologues did not rescue interphase microtubule defects of Mps depletion. (B) Depletion of the Mps binding partner D-TACC by RNAi. Immunoblots using Mps and D-TACC antibodies show that D-TACC was effectively depleted, while Mps was not affected. An α -tubulin antibody was used as a loading and blotting control. (C, D) Mps localisation to microtubules in D-TACC-depleted cells. D corresponds to the area marked by the yellow box in panel C. The preferential localisation to microtubule plus ends was observed as in untreated cells. Bar = 10 μ m.

interphase microtubule defects resulting from Msp1 depletion (Figure 2A), indicating that the defect is caused by a mechanism independent of kinesin-13/KinI proteins. Therefore, Msp1 function in regulating interphase microtubule organisation is not mediated by an antagonising kinesin-13 activity.

Msp1 protein forms a complex with D-TACC protein and the localisation and function of Msp1 to spindle poles depend on D-TACC in female meiosis and early embryos (Cullen and Ohkura, 2001; Lee *et al.*, 2001). Because Msp1 depletion resulted in the reduction of D-TACC in S2 cells (Figure 1B), we examined whether D-TACC may be involved in interphase microtubule organisation. In D-TACC RNAi experiments in S2 cells, we found that D-TACC protein was well depleted by 120 h, but the amount of Msp1 protein was unaffected in these cells (Figure 2B). Immunostaining indicated that depletion of D-TACC had no significant effects on interphase microtubule organisation (Figure 2C). Additionally, Msp1 protein was able to localise to interphase microtubules in D-TACC-depleted cells (Figure 2D). These results indicate that Msp1 protein function in interphase microtubule organisation is independent of D-TACC.

The N-terminal half of the Msp1 protein partially rescues Msp1 depletion

To gain a further insight into interphase Msp1 function, we located an active region within Msp1. The N-terminal region of Msp1 is made of five TOG domains, each consisting of five HEAT repeats (Figure 3A). We expressed the N-terminal 1133

amino acids of Msp1 (Msp1-N; Figure 3A) containing four repeats of the TOG domains in S2 cells to test whether the fragment can rescue the microtubule defects caused by Msp1 depletion. The equivalent region in XMAP215 has only a weak affinity to microtubules, but has been shown to have both microtubule stabilising and destabilising activities (Popov *et al.*, 2001; Shirasu-Hiza *et al.*, 2003). As a control, we created a mutant that contains an alteration of a highly conserved amino acid (E190K) in the first TOG domain (Figure 3A and B). This mutation is equivalent to a temperature-sensitive mutation in the *Arabidopsis* homologue (*mor1-2*), which disrupts cortical microtubules (Whittington *et al.*, 2001).

To examine if Msp1-N can carry out interphase function, the endogenous full-length Msp1 protein was depleted by RNAi and then Msp1-N or Msp1-N[E190K] was ectopically expressed by transfection of a corresponding plasmid. To avoid dsRNA destroying the RNA encoding Msp1-N, dsRNA corresponding to the C-terminal region was used for depletion of endogenous Msp1 protein. Immunoblotting using an antibody against the N-terminal region of Msp1 confirmed that full-length Msp1 was successfully depleted and the Msp1-N protein expressed (Figure 3C).

The Msp1-N and Msp1-N[E190K] proteins were found to localise throughout the cytoplasm with some granular concentrations (Supplementary Figure 7). Consistent with results from the *Xenopus* homologue (Popov *et al.*, 2001), this fragment does not localise to the centrosomes in mitosis (data not

Figure 3 N-terminal region of Msp1 partially rescues depletion of the endogenous Msp1. (A) Domain structures of Msp1 protein and mutants used in this study. A full-length Msp1 protein consists of five TOG domains and the C-terminal conserved region. Each TOG domain is made of up to five HEAT repeats. Constructs were made to express the N-terminal 1133 amino acids (Msp1-N) and the same region with one mutation (Msp1-N[E190K]) that results in a change (glutamine to lysine) of the conserved 190th amino acid. (B) Sequence comparison of the last HEAT repeats of the first TOG domain among Msp1 homologues. E190 in Msp1 is well conserved. (C) Expression of Msp1-N and depletion of endogenous full-length Msp1. Endogenous full-length Msp1 was depleted (Msp1 RNAi) using dsRNA that corresponds to the C-terminal region. dsRNA corresponding to the β -lactamase gene was used as a control. Msp1-N and Msp1-N[E190K] were expressed under the native *msp1* promoter by transfections of plasmids. Msp1 proteins were detected by an antibody that recognises the N-terminal half of Msp1. An α -tubulin antibody was used as a loading and blotting control. (D) Partial rescue of Msp1 depletion by the N-terminal region of Msp1. Interphase microtubule phenotypes were examined 4 days after the addition of dsRNA. Defects caused by Msp1 depletion are partially rescued by expression of Msp1-N but not Msp1-N[E190K].

shown). Expression of Msp^s-N protein in cells treated with control dsRNA did not affect microtubule organisation (Figure 3D). When Msp^s-N was expressed in Msp^s-depleted cells, the proportion of cells with abnormal microtubule organisation was reduced. In particular, the proportion of cells with extensive microtubule bundling dropped from 43 to 13% in Msp^s-depleted cells. The mutation, Msp^s-N[E190K], abolished the ability of the protein to rescue the defects of Msp^s depletion (Figure 3D), although it may be partly due to slightly lower protein level.

These results indicate that the Msp^s-N can, at least partially, rescue the defects caused by Msp^s depletion. This N-terminal region is likely to contain an activity that is important for Msp^s regulation of interphase microtubules and the conserved residue in the first TOG domain is crucial for the activity.

Extensive microtubule pausing in Msp^s-depleted cells

In order to achieve a better understanding of interphase microtubule defects, we examined dynamics of individual microtubules in the Msp^s-depleted cells. For real-time analysis of microtubule dynamics, we used an S2 cell that stably expresses α -tubulin fused with GFP (Goshima and Vale, 2003). The depletion of Msp^s by RNAi in this cell line showed the same microtubule organisation defects as those observed in S2 cells (data not shown). Since individual microtubules cannot be resolved in most cells after 72 h, we observed cells incubated with dsRNA for 48 h at which time microtubule organisation is mildly affected (Figure 1C).

We followed the behaviour of individual microtubules both in control cells and Msp^s partially depleted cells by live-cell fluorescent microscopy. Microtubules in control cells exhibited dynamic instability (Figure 4A and C), alternating between growth phase and shrinkage phase. In addition, microtubule ends spent time in a paused state with little growth or shrinkage. We noticed that microtubule behaviour is different at the cortex than when microtubule ends are internal. Internal microtubules tend to grow fairly continuously with few catastrophes (0.22 events per minute). When microtubules reach the cortex, they tend to pause (time spent in a paused state rises from 29.7 to 48.4%; Figure 4E) and catastrophe frequency more than doubles (0.52 events per minute).

Microtubule behaviour was very different in Msp^s-depleted cells. The most significant change was the increase in the amount of time spent pausing (Figure 4E). For internal microtubules in control cells, on average, 29.7% of their time is spent pausing, while internal microtubules in Msp^s-depleted cells are paused for 71.4% of their lives. Microtubules that reach the cortex also spend more time in paused state than control cells (76.1 versus 48.4%).

Life history plots of microtubules from Msp^s-depleted cells show that long periods of pause are interspersed with short grow periods or shrinkage events, but unlike control cells, it was rare to see consistent periods of growth followed by shrinkage (Figure 4D). The rate of growth in Msp^s-depleted cells (2.1 μ m/min) is not significantly different from control cells (2.2 μ m/min) while the shrinkage rate is reduced in Msp^s-depleted cells from 9.5 to 4.8 μ m/min (Figure 4E).

For further understanding of the increased paused state, transition frequencies between the three states of growth, pause and shrinkage were determined. For internal micro-

tubule ends, a significant increase in the transition from growth to pause, and a significant decrease in transition from pause to growth were observed (Figure 4F). Overall, this will result in a shift towards the paused state from a growth state.

It is possible that Msp^s depletion eliminated a population of dynamic microtubules rather than changed dynamics of individual microtubules. We do not think this is the case for the following reasons. Firstly, in control RNAi cells, we did not find microtubules behaving like those in Msp^s RNAi cells. Secondly, we did not find any significant differences in the amount of tubulin in polymers between control and Msp^s RNAi cells (Supplementary Figure 8).

In summary, partial depletion experiments indicate that Msp^s protein suppresses pause state by promoting the transition from pause to growth and reducing the transition from growth to pause.

Microtubule dynamics are greatly reduced in Msp^s-depleted cells

Individual microtubule dynamics could not be observed in cells well depleted of Msp^s. Therefore, two alternative strategies—use of a depolymerising drug and fluorescent recovery after photo bleaching (FRAP) analysis—were employed to gain insights into microtubule dynamics in cells with over 90% Msp^s depletion.

Firstly, we compared the stability of microtubules between control and Msp^s-depleted cells by monitoring resistance to the microtubule depolymerising drug colchicine. In control cells, nearly all microtubules were depolymerised when exposed to 200 μ M colchicine for 16 h (Figure 5A and B). In contrast, more than half of Msp^s-depleted cells still contained bundles of microtubules after the same treatment (Figure 5A and B). This result indicates that microtubules are stable in Msp^s-depleted cells in terms of resistance to a microtubule destabilising drug.

A second approach for assaying microtubule dynamics was to examine turnover of tubulin dimers in microtubule bundles by FRAP. The GFP-tubulin-expressing line was again used in these experiments. An area of microtubules was bleached in the cell and the recovery followed by time-lapse photography (Figure 5C). The percentage of recovery after 5 min was measured for control and Msp^s dsRNA-treated cells. After 5 min, control cells had recovered, on average, to 57% of the prebleach level, while after the same time period Msp^s-depleted cells had recovered on average to 23% of the prebleach level (Figure 5D). Msp^s microtubule bundles have a lower turnover of tubulin dimers, indicating an attenuation of microtubule dynamics.

These results from drug and FRAP experiments are consistent with real-time observations of individual microtubule dynamics, which indicate that microtubules are stabilised by depletion of Msp^s.

Defective organisation of interphase microtubules in haemocytes from msp^s mutant larvae

Our results demonstrated that Msp^s protein is a major regulator of interphase microtubule organisation in S2 cultured cells. To confirm the role of Msp^s in interphase microtubule regulation, we looked for evidence of interphase microtubule defects in *msp^s* mutant flies.

Figure 4 Extensive microtubule pausing is induced by Msp depletion. **(A)** Time-lapse images of microtubule plus ends in control GFP-tubulin cells. Growing, shrinking and pausing ends were marked with green, red and yellow dots. Numbers at the bottom left represent time in seconds. Microtubules show dynamic instability with occasional pauses. Bar = 10 μm. **(B)** The dynamics of microtubule plus ends in cells partially depleted of Msp. Cells were observed 48 h after the addition of dsRNA. Microtubule plus ends exhibit a prolonged pausing. **(C)** Life history plots of microtubules in control cells. Microtubule plus ends show persistent growth and occasional shrinkages with some pausing. **(D)** Life history plots of microtubules in cells partially depleted of Msp. Microtubules spent most of their time pausing with occasional growth and shrinkage. **(E)** Parameters of microtubule dynamics. Times spent in pause are shown in percentages. In control cells, microtubule plus ends near the cell periphery (within 0.2 μm) spent more time in pause than those inside of the cell (internal). In both cases, Msp partial depletion increased time spent in pause. Average growth and shrinkage rates were shown with standard deviations (lines). **(F)** Transition frequencies between three microtubule states: growth, shrinkage and pause. Only the transition frequencies marked with red arrows and numbers were significantly different in Msp-depleted cells from those of control cells ($P < 0.05$). The transition from pause to growth significantly decreased and the transition from growth to pause increased. Transition frequencies from shrinkage to growth or pause may be increased, but they were not proved to be statistically significant due to small sample size of time microtubules spent in shrinkage.

We isolated haemocytes from wild-type and *msps* mutant third-instar larvae, and incubated them on the con A-coated surface. Microtubule organisation of these cells was examined by immunostaining. In wild-type cells, the microtubule organisation was similar to that of S2 cells, with an extended microtubule network reaching the cell periphery (Figure 6A). Haemocytes were obtained from a number of *msps* mutants. The null alleles produced very few haemocytes, probably due to defects in mitosis and were therefore not analysed (unpublished results). However, two hypomorphic alleles, *msps*^P

and *msps*¹⁴⁶ (Materials and methods), were found to produce enough haemocytes to investigate the microtubule phenotype. Both mutant alleles showed a reduction in the proportion of cells with an extended microtubule array and a rise in the proportion of cells with a compact circle of microtubules in the centre of the cell (Figure 6A). The more severe phenotype was demonstrated by the *msps*^P mutant, in which 60% of haemocytes had compact microtubule organisation, compared to less than 15% in wild-type flies (Figure 6B). Additionally, 15% of *msps*^P cells showed bund-

Figure 5 Msp1 depletion results in stable interphase microtubules. (A) Microtubule organisation after addition of colchicine, a microtubule destabilising drug. Control and Msp1-depleted cells (5 days after dsRNA addition) were observed after 16 h incubation with colchicine. Some interphase microtubules remained in Msp1-depleted cells, while nearly all microtubules were depolymerised in control cells. (B) Frequencies of cells that have residual microtubules after colchicine treatment. Lines indicate standard deviations from three independent experiments. (C) FRAP of GFP-tubulin. Small areas (arrowheads) of cells expressing GFP-tubulin were bleached and the recovery of fluorescence monitored for 5 min. Cells were treated with dsRNA for 5 days before the photobleach. (D) Percentages of the fluorescent recovery 5 min after photobleaching. Lines indicate standard deviations. Decreased turnover of tubulin dimers into microtubules was observed in Msp1-depleted cells, indicating decreased microtubule dynamics. The estimated maximum recovery and $t_{1/2}$ are $74 \pm 5\%$ and 54 ± 6 s in control, and $39 \pm 9\%$ and 63 ± 18 s in Msp1 RNAi.

ling of microtubules, while none were seen in wild-type cells. These defects are similar to those in Msp1-depleted S2 cells.

These results from *msps* mutants indicate that Msp1 is a major regulator of interphase microtubule organisation in flies as well as in cultured cells.

Discussion

The Dis1/TOG proteins are one of the most conserved families of MAPs among eukaryotes. Studies in various systems revealed that this family of MAPs regulates a variety of microtubule functions in the cell (Ohkura *et al*, 2001). However, the focus of most studies has been on functions in mitosis, while interphase microtubule regulation has featured less prominently. Crucially, *in vivo* roles of this family of MAPs in interphase animal cells need to be established. Here, we have reported the first detailed functional study of the role of a member of the Dis1/TOG family in interphase microtubule dynamics in animal cells.

Our study revealed that the depletion of Msp1 results in a dramatic increase in the amount of time spent in a paused state. Strikingly, even the partial depletion of Msp1 has stronger effects than nearly complete depletion of EB1, the only protein previously shown to have antipause activity (Rogers *et al*, 2002). Previous *in vitro* experiments revealed activities of the Msp1 homologues to increase growth and shrinkage rates, and to suppress catastrophe frequency (Gard

Figure 6 Abnormal interphase microtubule organisation in haemocytes from *msps* mutants. (A) Interphase microtubule organisation of haemocytes from wild-type larvae and *msps* mutants (allele 146 and P) after being plated out on a con A-coated surface. Most haemocytes from wild type have 'extended' microtubule organisation (left), while those from *msps* mutants contain 'compact' (middle) or 'bundled' microtubule organisation as seen in *msps*-depleted S2 cells. (B) Quantitative analysis of interphase microtubule organisation in *msps* mutants.

and Kirschner, 1987; Vasquez *et al*, 1994; Tournebize *et al*, 2000; Kinoshita *et al*, 2001). However, antipause activity has not been reported in other Msp homologues *in vivo* or *in vitro* except the budding yeast homologue *stu2*, the deletion of which modestly increases pausing of cytoplasmic microtubules (Kosco *et al*, 2001). It would be interesting to see whether microtubule dynamics are altered by the depletion of TOG in mammalian cells in the light of our finding.

Dynamic instability is an intrinsic property of microtubules. Microtubules assembled *in vitro* from purified tubulin either grow or shrink, with infrequent alternation between the two states (Mitchison and Kirschner, 1984). In cells, however, microtubule dynamics are strongly influenced by additional proteins, and microtubule plus ends often pause without significant growth or shrinkage. This pausing is more frequent at the cortex (this study and others; Komarova *et al*, 2002), indicating that it is regulated by cellular factors. This regulation is potentially critical for linking microtubule ends to the cell cortex. Despite being a common feature of microtubules in cells, relatively little attention has been paid to the pause state, and consequently only limited information is available on the nature and regulation of this state.

The most enigmatic aspect of this family of MAPs is to have apparently opposing activities, both stabilising and destabilising microtubules (Tournebize *et al*, 2000; van Breugel *et al*, 2003; Shirasu-Hiza *et al*, 2003). *In vitro* functional analysis of XMAP215 indicates that the protein has the intrinsic ability to promote both the polymerisation and depolymerisation of microtubules (Tournebize *et al*, 2000; Shirasu-Hiza *et al*, 2003). Shirasu-Hiza *et al* (2003) proposed that the two opposing activities of XMAP215 could be reconciled if it functions as an antipause factor. This model is based on the hypothesis (Tran *et al*, 1997) that the pause state may be the obligate intermediate between growth and shrinkage. It is also proposed (Arnal *et al*, 2000) that a pause corresponds to blunt-ended ultrastructures distinct from those of growth (sheet-like) and shrinkage (curled). Destabilising the pause state will promote the transitions from pause to both growth and shrinkage, therefore it can act as a microtubule stabiliser as well as a destabiliser.

Our results in *Drosophila* cells provide the first direct experimental evidence to support the hypothesis proposed by Shirasu-Hiza *et al* (2003) that Msp acts as an antipause factor. Consistent with the hypothesis, our further analysis shows that Msp promotes transitions from pause to growth and inhibits transitions from growth to pause. On the other hand, we did not see that Msp significantly promoted the transition from pause to shrinkage. However, this may be due to partial depletion of Msp, which allows direct observation of microtubule dynamics. In cells that are well depleted of Msp, microtubules are more resistant to the depolymerising drug colchicine, suggesting that catastrophe or depolymerisation is inhibited.

We showed that Msp associates with microtubule plus ends in the cell. Therefore, it could directly destabilise the paused state by altering polymer conformation or it could act indirectly by preventing a pause factor from accessing the polymer. In *Xenopus* egg extracts both from mitosis and interphase, XMAP215 is shown to stabilise microtubules mainly by antagonising the microtubule depolymerising activity of the kinesin-13/KinI family kinesin, XKCM1 (Tournebize *et al*, 2000). In contrast, our study shows that

Msp regulation of interphase microtubules in S2 cells is unlikely to be through the kinesin-13 proteins. We also found that Msp activity and localisation to interphase microtubules are independent of its binding partner D-TACC, suggesting this interphase function is independent of known regulators or effectors. As our knowledge on microtubule pausing is very limited, we cannot speculate on the precise mechanism of the antipause activity. However, our finding represents a significant advance towards the molecular understanding of this third state of microtubule dynamics.

Msp depletion dramatically altered interphase microtubule organisation in *Drosophila* S2 cells. Microtubules are unable to extend to the periphery of the cell and occasionally form extensive bundles. The importance of Msp for microtubule organisation in developing flies was confirmed by the presence of similar defects in haemocytes from *msps* mutant larvae. In addition, a very recent report (Moon and Hazelrigg, 2004) has described defects in microtubule organisation in *msps* mutant oocytes. Therefore, Msp is likely to be a general regulator of interphase microtubules in *Drosophila*. Mutations in *msps* homologues have a dramatic impact on the organisation of interphase microtubules in yeasts, *Dictyostelium* amoebae and plants (Garcia *et al*, 2001; Whittington *et al*, 2001; Graf *et al*, 2003). In contrast, the depletion of the mammalian homologue, TOG, by RNAi does not alter interphase microtubule organisation (Gergely *et al*, 2003; Holmfeldt *et al*, 2004), although none of these studies have examined microtubule dynamics.

Extensive pausing can explain the failure of interphase microtubules to extend out to the cell cortex seen in Msp-depleted cells. It is not clear whether the microtubule bundling phenotype is a secondary consequence of extensive pausing or whether Msp somehow acts directly as an anti-bundling factor. It is also unclear whether microtubule stabilisation caused bundling or *vice versa*. Interestingly, although microtubule bundling is commonly observed during overexpression of MAPs (Bu and Su, 2001; Haren and Merdes, 2002; Raemaekers *et al*, 2003), Msp is the only protein that, as far as we are aware, induces microtubule bundling when it is depleted.

In conclusion, our functional study of Msp demonstrated an essential role in the organisation and dynamics of interphase microtubules *in vivo*, and uncovered the antipause activity, which provides a crucial insight into this family of MAPs.

Materials and methods

Cell culture and RNAi

Drosophila S2 cells were cultured and RNAi was performed as described (Clemens *et al*, 2000; Rogers *et al*, 2002). dsRNAs were produced by *in vitro* transcription using T7 polymerase (Ambion) and templates made as follows. About 0.6 kb within an exon of the target gene was first amplified from genomic DNA or cDNA using gene-specific primers containing half of the T7 promoter sequence, and further amplified using primers with a full T7 sequence. dsRNAs corresponding to the bacterial β -lactamase gene were used for control RNAi throughout.

For expression of the N-terminal 1133 amino acids (Msp-N), the 6.2 kb *SpeI*-*KpnI* genomic DNA containing the promoter and 5' half of *msps* gene were cloned into pBluescript (Stratagene). A point mutation, E190K, was made using the site-directed mutagenesis kit (Stratagene). These constructs were transfected into S2 cells that had been treated with control or Msp dsRNA for 2 days. Cells were transfected using Effectene Transfection Reagent (Qiagen). After

transfection, 15 µg of dsRNA was added to the cells to ensure depletion of the protein.

Molecular and immunological techniques

Standard molecular techniques were followed throughout (Harlow and Lane, 1988; Sambrook *et al.*, 1989). For immunoblotting, 5×10^4 S2 cells were loaded per lane. An α -tubulin antibody (DM1A; Sigma) was used throughout this study to examine tubulin distribution and as a loading control. In addition to an antibody against the C-terminal of Msp^s (264; Cullen *et al.*, 1999), rabbit antibodies were made against the following bacterially produced proteins: middle region (746–1225 amino acids) of Msp^s fused with GST (268), MBP-D-TACC containing the N-terminal 2–433 amino acids or the C-terminal 250 amino acids (Gergely *et al.*, 2000; gifts from J Raff). Antibodies were affinity purified as described (Smith and Fisher, 1984). Peroxidase-conjugated secondary antibodies (Jackson Lab) and the ECL kit (Amersham) were used for detection.

Immunofluorescence microscopy

For immunostaining, S2 cells were cultured on a con A-coated coverslip for 2 h and fixed with 90% methanol, 3% formaldehyde and 5 mM sodium carbonate at -80°C as described (Rogers *et al.*, 2002). Haemocytes were collected from third-instar larvae by tearing the cuticle in 100 µl Schneiders media (Tirouvanziam *et al.*, 2004), and were then left to attach onto a con A-coated coverslip for 2 h before fixation as above. Secondary antibodies conjugated with Alexa488 or Cy3 (Jackson Lab or Molecular Probes) and DAPI (Sigma) were used for visualisation. For quantification, a total of at least 300 cells were routinely counted in three separate experiments. Images were captured using an Axioplan2 microscope (Zeiss) attached with a CCD camera (Hamamatsu) controlled by OpenLab (Improvision) or an Axiovert microscope attached with LSM510Meta confocal scan head (Zeiss), and processed using Photoshop (Adobe).

Microtubule dynamics in live S2 cells

For observation of individual microtubule dynamics, cells expressing GFP-tubulin (a gift from R Vale; Goshima and Vale, 2003) were treated with 15 µg of control or msp^s dsRNA for 2 days. The cells were then grown on con A-coated coverslips for 1–2 h before being observed on the confocal microscope described above. Images were acquired every 5 s for 5 min. Microtubule length was measured from an arbitrary chosen point on the microtubule over time using ImageJ (<http://rsb.info.nih.gov/ij/>). Only microtubules that could be followed for at least 3 min were included in the analysis. Pauses

were defined in this study as events without significant change in the microtubule length ($<0.5 \mu\text{m}$) for 30 s or more. The transition frequencies between growth, pause and shrinkage were calculated as the total number of transitions (from A to B) divided by the total time spent in the pretransition state (A). Microtubule ends at the cell periphery (within $0.2 \mu\text{m}$ from the cell edge) and in the internal region were analysed separately.

FRAP

S2 cells expressing GFP-tubulin treated with either control or Msp^s dsRNA for 5–6 days were plated on con A-coated coverslips. The FRAP analysis was carried out using the confocal microscope described above. An area $2.5 \mu\text{m} \times 6 \mu\text{m}$ was bleached on 60% laser power for 50 iterations. Images were recorded prebleach, immediately after bleaching and subsequently images were captured every 20 s for at least 5 min. Signals in a nonbleached area were also measured to compensate a reduction of fluorescence caused by image captures. For a comparison, per cent fluorescence recovery was calculated as the amount of recovery (the difference between images immediately after photobleach and 5 min later) relative to photobleach (the difference before and immediately after photobleach).

Fly stocks

Standard techniques for fly manipulation were followed (Ashburner, 1989). All stocks were grown at 25°C in standard cornmeal media. *w¹¹¹⁸* flies were used as wild type. msp^s alleles were kept over TM6C (Lindsley and Zimm, 1992). msp^s^P is a P-element insertion allele, which greatly reduced Msp^s protein level (Cullen *et al.*, 1999), and msp^s¹⁴⁶ contains a mutation in a splicing junction (unpublished). msp^s^P dies as early pupae and msp^s¹⁴⁶ dies as pharate, while null alleles die as early third-instar larvae. Their lethality is rescued by the wild-type msp^s transgene.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We are grateful to S Rogers and R Vale (UCSF) for making GFP-tubulin cell line available, to J Raff for D-TACC constructs and antibody and to Marissa Brown and Duncan Sproul for helping with antibody production and EMS screening. The work is supported by The Wellcome Trust and BBSRC.

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