

Mob1: defining cell polarity for proper cell division

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Summary

Mob1 is a component of both the mitotic exit network and Hippo pathway, being required for cytokinesis, control of cell proliferation and apoptosis. Cell division accuracy is crucial in maintaining cell ploidy and genomic stability and relies on the correct establishment of the cell division axis, which is under the control of the cell's environment and its intrinsic polarity. The ciliate *Tetrahymena thermophila* possesses a permanent anterior–posterior axis, left–right asymmetry and divides symmetrically. These unique features of *Tetrahymena* prompted us to investigate the role of *Tetrahymena* Mob1. Unexpectedly, we found that Mob1 accumulated in basal bodies at the posterior pole of the cell, and is the first molecular polarity marker so far described in *Tetrahymena*. In addition, Mob1 depletion caused the abnormal establishment of the cell division plane, providing clear evidence that Mob1 is important for its definition. Furthermore, cytokinesis was arrested and ciliogenesis delayed in *Tetrahymena* cells depleted of Mob1. This is the first evidence for an involvement of Mob1 in cilia biology. In conclusion, we show that Mob1 is an important cell polarity marker that is crucial for correct division plane placement, for cytokinesis completion and for normal cilia growth rates.

Key words: Mob1, Cell polarity, *Tetrahymena*, Basal bodies, Cilia, Cytokinesis

Introduction

The accuracy of cell division is crucial in maintaining cell ploidy and genomic stability. In fact, events like DNA replication, chromosome segregation, mitosis completion and cytokinesis must be tightly controlled, and their deregulation is closely associated with cancer development (Kops et al., 2004; Weaver and Cleveland, 2007). The correct positioning of the division plane of the cell, which is dependent on the polarity axis, is crucial in ensuring proper cell division. This polarity axis is established both by internal and external clues, and the mitotic spindle must be oriented along it, allowing chromosomes to be equally segregated into the two daughter cells (Chan and Amon, 2010; Caydasi et al., 2010).

In *Saccharomyces cerevisiae*, which presents an asymmetric cell division, whenever the mitotic spindle is mispositioned, the spindle position checkpoint (SPOC) pathway prevents the activation of the mitotic exit network (MEN) until the correct orientation of the spindle is achieved (Caydasi et al., 2010). Therefore, the success of mitosis completion and cytokinesis is dependent on correct spindle orientation, itself a consequence of correct cell polarization.

MEN is a signaling cascade that controls mitosis to interphase transition. The final outcome of MEN signaling is the activation of the Cdc14 phosphatase, which then promotes the degradation of mitotic cyclins. MEN-regulated Cdc14 activation is dependent on the kinase activity of the complex Mob1–Dbf2 (Bardin and Amon, 2001). In yeast, Mob1 mutant cells arrest in late anaphase and show impaired cytokinesis (Luca et al., 2001).

Most components of the MEN pathway, such as Cdc15, Mob1 and Dbf2, have metazoan homologs. Importantly, the mammalian

nuclear Dbf2-related kinases (NDR) LATS1 and LATS2, and MST1/2 (Cdc15 orthologous), or the *Drosophila melanogaster* Hpo and Warts form, together with Mob1 and Sav1 (the core kinase module of the Hippo pathway), were recently established as a major conserved mechanism governing cell contact inhibition, organ size control and cancer development both in mammals and in *Drosophila* (for a review, see Zeng and Hong, 2008). Furthermore, most MEN proteins are associated with the centrosome, the major microtubule organizing center in animal cells (Glover et al., 1998; Mailand et al., 2002; Wilmeth et al., 2010). Human MOB1 was also described as a tumor suppressor and is required for centrosome duplication (Wilmeth et al., 2010; Hergovich et al., 2009).

A phylogenetic analysis showed that Mob proteins are usually encoded by more than one gene, ranging from two in yeast (*mob1* and *mob2*), to three in *Drosophila* (*Mob1*, *Mob2* and *Mob3*) and at least six human Mob homologous genes (*MOB1A* and *MOB1B* that encode two closely related proteins, *MOB2*, *MOB3A*, *MOB3B* and *MOB3C*) (Chow et al., 2010; Hergovich, 2011). Although a complete picture of the role of the distinct Mob proteins in vivo is still missing, some of these proteins seem to have specialized functions. For example, in budding and fission yeasts Mob2 interacts with the Cbk1 and Orb6 kinases, respectively, and is required for cell cycle progression and regulates polarized growth and morphogenesis (Hou et al., 2003; Weiss et al., 2002; Colman-Lerner et al., 2001). Interestingly, Mob1 and Mob2 proteins are not functionally interchangeable (Hou et al., 2003, 2004). In *Drosophila*, although the role of Mob2 is far from being understood, it is known to interact with Trc kinase, playing a role in wing hair morphogenesis (He et al.,

2005) and photoreceptor cell development (Liu et al., 2009). In humans, MOB2 seems to be involved in the regulation of MOB1 by competing for the NDR1/2 kinases (Kohler et al., 2010). The existing data on Mob3 proteins is sparse and their role elusive (Hergovich, 2011). By contrast, the ciliate *Tetrahymena thermophila* possesses only one gene (*mob1*) encoding a Mob1-like protein. *Tetrahymena* is a highly differentiated organism that in contrast to budding yeast divides symmetrically and possesses a permanent anterior–posterior axis and left–right asymmetry. Moreover, this unicellular organism contains a vegetative macronucleus (MAC) that divides amitotically and a germ line micronucleus (MIC) presenting an acentrosomal spindle (Frankel, 2000). These unique features of *Tetrahymena* prompted us to investigate the functions of *Tetrahymena* Mob1.

Here, we report that *Tetrahymena* Mob1 is a molecular polarity marker of the posterior pole basal bodies, the first so far described in *Tetrahymena*. Furthermore, *Tetrahymena* Mob1 is involved in cilia biogenesis and is a crucial factor for the correct establishment of the cell division plane and cytokinesis.

Results

Tetrahymena Mob1 downregulation leads to incorrect cell division planes and impaired cytokinesis

A bioinformatics analysis on the *T. thermophila* genomic database (TGD Wiki) revealed that, unlike the majority of eukaryotes, *Tetrahymena* only possesses one gene coding for a Mob1-like protein, whose amino acid sequence identity with Mob1 proteins throughout the eukaryote lineage ranges from 49 to 62% (supplementary material Fig. S1A). Interestingly, the lowest value (49%) is found between *Tetrahymena* Mob1 and the budding yeast counterpart, whereas 61% identity is found with human Mob1 (supplementary material Fig. S1A).

To investigate the function of *Tetrahymena* Mob1, we constructed a *Tetrahymena* Mob1 knockdown strain (Mob1-KD). Using this strategy, we disrupted the endogenous *mob1* locus in the polyploid macronucleus MAC (45n) by the introduction in the coding region of a Neo4 cassette that confers resistance to paramomycin (Gaertig et al., 1994) (supplementary material Fig. S1B). Transformants were selected with increasing paramomycin doses until the sub-lethal concentration of 2.8 mg/ml, thus ensuring, by phenotypic assortment (Sonneborn, 1974), the maximum number of *mob1* disrupted alleles (supplementary material Fig. S1B). The correct insertion in the MAC genome was confirmed by PCR analysis, which showed the presence of the *mob1* wild-type allele with the expected size of 3.5 kb and the corresponding knockout allele of 4.6 kb containing the inserted Neo4 cassette (see supplementary material Fig. S1B). The presence of the *mob1* knockout allele was further confirmed by sequence analysis using the primers described in supplementary material Table S1 (see Materials and Methods). In the resulting *Tetrahymena* strain, we achieved an average decrease of 70% of the *mob1* mRNA levels (supplementary material Fig. S1C).

A morphological analysis by light microscopy of the *Tetrahymena* Mob1-KD population revealed the presence of cells with dramatically abnormal shapes. A range of defects were observed in these cells (see later) including ‘heart shape’, ‘boomerang shape’ and big cell masses or ‘monsters’. None of these phenotypes was observed in wild-type cells. These classes of defects might be explained by the phenotypic assortment that originates in cell populations, with individual cells presenting

different ratios of wild-type to disrupted alleles (supplementary material Fig. S1B).

Immunofluorescence confocal microscopy of *Tetrahymena* Mob1-KD cells, using antibodies against α -tubulin and centrin, indicated that the different morphologies are due to the failure of cell division. Wild-type *Tetrahymena* cells divide symmetrically, the division plane being perpendicular to the anterior–posterior axis in the midzone of the cell, just above the newly formed oral apparatus (Fig. 1A,B). We observed that in *Tetrahymena* Mob1-KD cells the division plane is frequently displaced anteriorly along the anterior–posterior axis. Moreover, the angle that the division plane established with the anterior–posterior axis was severely affected, ranging from 90° to 0° (Fig. 1G).

In a dividing wild type cell, the new oral apparatus is the first visible structure assembled prior to cell division and its position is determined by the distance to the old oral apparatus, which is directly proportional to the cell length (Lynn and Tucker, 1976). We observed that in the majority of *Tetrahymena* Mob1-KD cells this distance was reduced (Fig. 1C,D) or abolished (Fig. 1E), suggesting that the new oral apparatus was either assembled at a wrong place or had migrated towards the old oral apparatus during the elongation process (Frankel, 2008). The oral apparatus being an anterior polarity landmark in *Tetrahymena*, the occurrence of these Mob1-KD phenotypes suggested that these cells possess altered polarity, which compromised the correct definition of the division plane. Importantly, Mob1-depleted cells seemed to have started the formation of the cleavage furrow but then aborted division, failing cytokinesis (Fig. 1C–F). In these cases, the normal gap observed between the cilia longitudinal rows was not formed and the rows were continuous across the cleavage furrow (Fig. 1D, right, 1E). Strikingly, these errors did not arrest the cell cycle because the cells continued trying to divide, originating gigantic monsters (Fig. 1F). Also, basal body duplication was not impaired because the distance between them decreased over the longitudinal cilia rows (Fig. 1F). Moreover, the MAC and the MIC divisions seemed to occur normally, although the two MAC could fail to segregate correctly (Fig. 1D, left).

Tetrahymena Mob1 accumulates at basal bodies with a polarized gradient distribution towards the posterior pole

In order to further define the function of *Tetrahymena* Mob1, we analyzed its cellular distribution. For that purpose we constructed a *Tetrahymena* strain expressing Mob1–GFP through the introduction, by DNA homologous recombination, of a Mob1–GFP construct into a β -tubulin locus, under the control of the cadmium (Cd^{2+})-inducible promoter metallothionein 1 (MTT1) (supplementary material Fig. S2A). The levels of Mob1–GFP in response to Cd^{2+} addition for different times were evaluated by western blot using an anti-GFP antibody (supplementary material Fig. S2B). Mob1–GFP was detected at 15 minutes and reached the highest levels after 30 minutes of Cd^{2+} induction. As a control, a *Tetrahymena* strain expressing just GFP was also created.

An immunofluorescence confocal microscopy analysis of control cells expressing GFP alone showed it to be distributed in the cytoplasm (Fig. 2A). Unexpectedly, Mob1–GFP, was clearly accumulated in posterior pole basal bodies (Fig. 2B–E), as confirmed by colocalization with centrin (Fig. 2C,E). Remarkably, Mob1–GFP follows a gradient along the anterior–posterior axis of the cell, with a high signal at the posterior pole

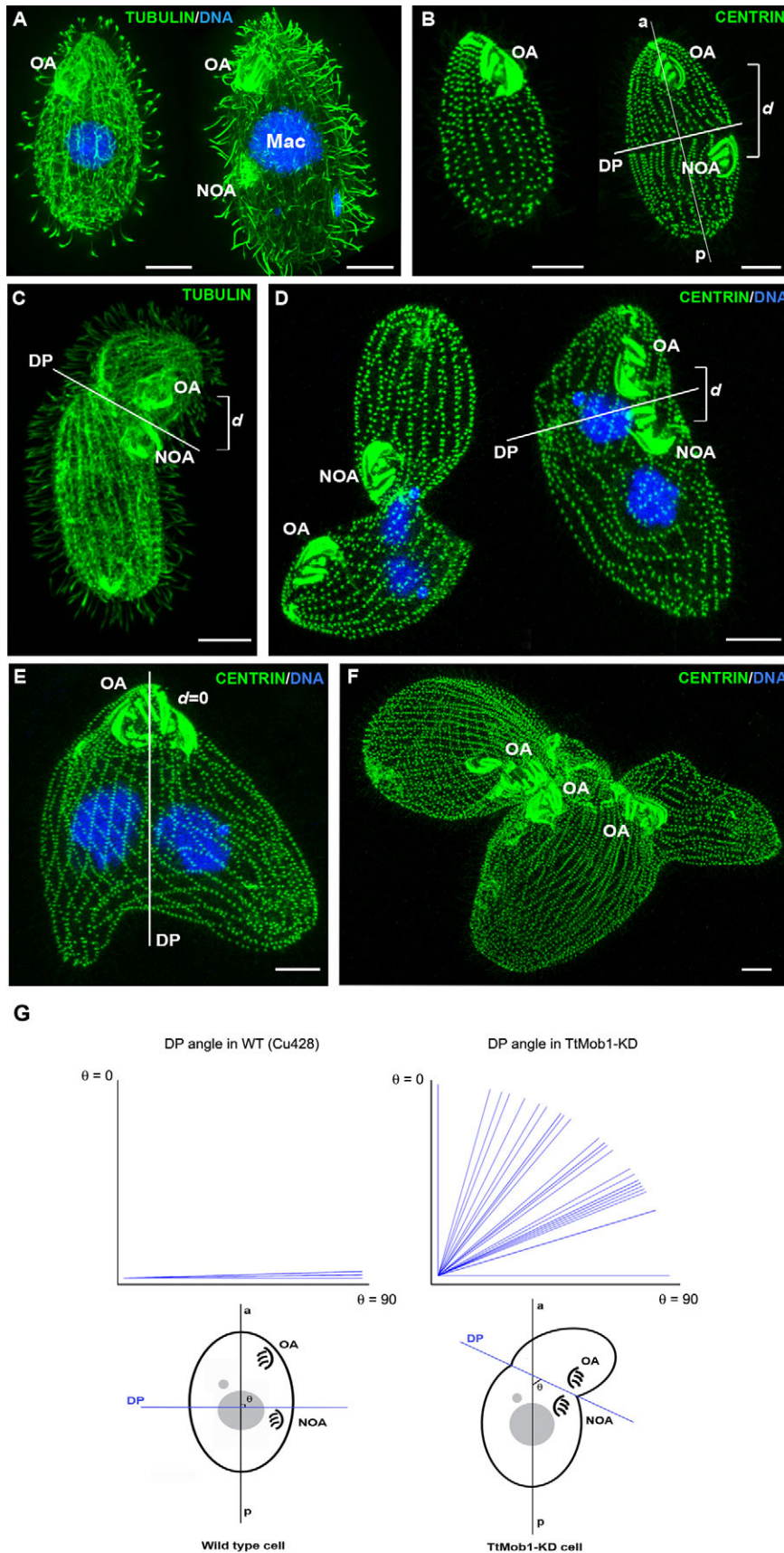


Fig. 1. Mob1 depletion in *Tetrahymena* causes incorrect positioning of the cell division plan and impaired cytokinesis. (A–F) Immunofluorescence of α -tubulin and centrin in *Tetrahymena* wild-type (Cu428 strain) (A,B) and Mob1-KD cells (C–F), co-stained with TOTO-3-iodide to label DNA (A,D,E). In wild-type cells, the division plane (DP) is established perpendicularly to the anterior (a)–posterior (p) axis, in the midzone of the cell. The oral apparatus (OA) and the new oral apparatus (NOA) show a conserved distance (d) that depends on cell length (B). Mob1-KD cells present a DP angle deviation relatively to the anterior–posterior axis and fail cytokinesis (C–F). (G) Graphic representation of the angle (θ) established between the DP and the anterior–posterior axis in wild-type ($n=14$) and Mob1-KD ($n=33$) cells. Measurement of angle (θ) was carried out using ImageJ software. Scale bars: 10 μ m.

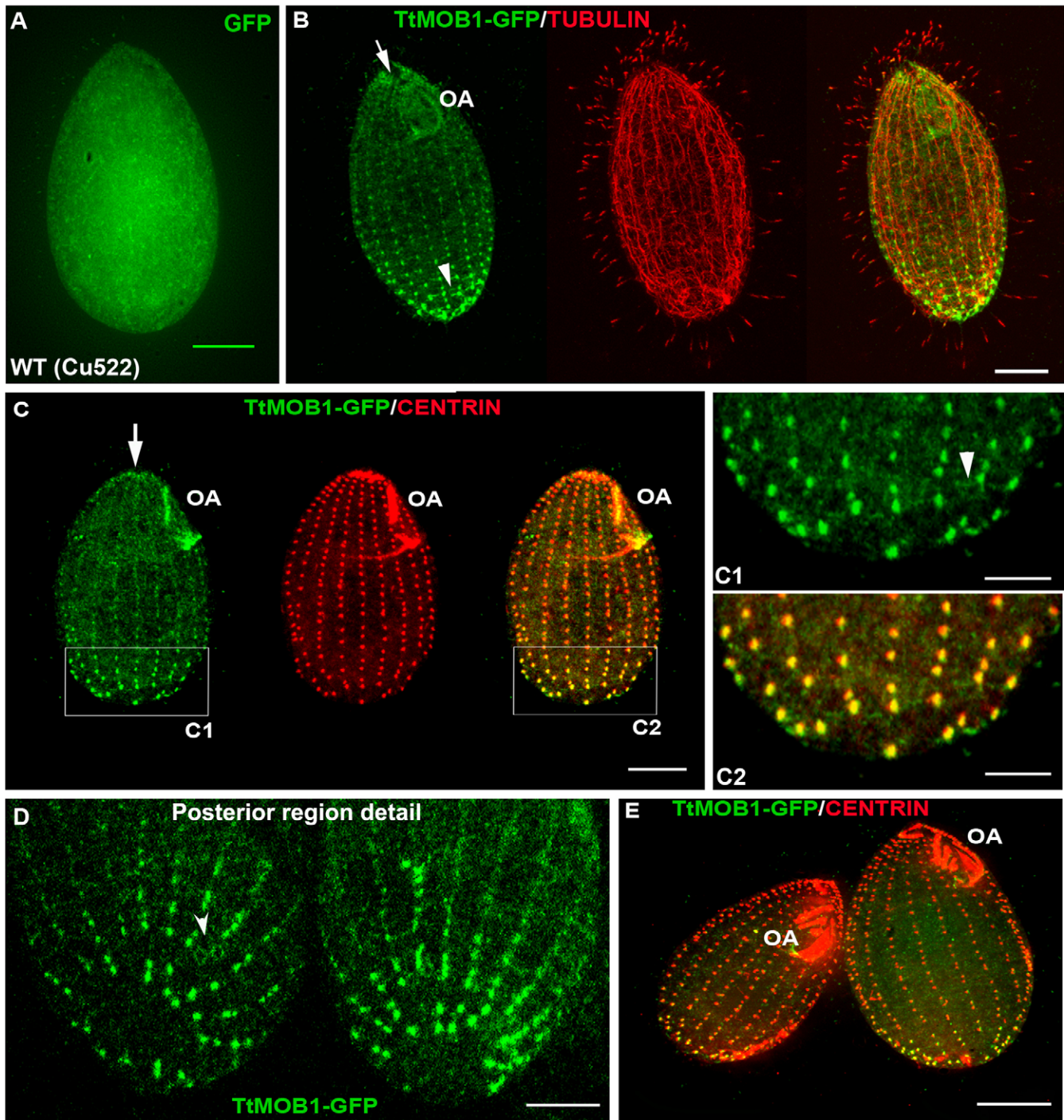


Fig. 2. *Tetrahymena* Mob1 localizes preferentially at the posterior pole basal bodies. (A) *Tetrahymena* cells expressing GFP were stained only with anti-GFP antibody. (B–E) Immunofluorescence microscopy of *Tetrahymena* cells expressing Mob1–GFP using anti-GFP (all images), anti- α -tubulin (B) and anti-centrin (C–E) antibodies. Mob1–GFP accumulates preferentially at the posterior pole basal bodies, co-localizing with centrin (C,E). Panoramic view of the posterior region of Mob1–GFP-expressing cells is shown in D; arrowhead indicates where contractile vacuole pores are visible. Mob1–GFP could also be detected at the oral apparatus (OA), at the posterior pole transversal microtubules (indicated by arrowheads in B and C1) and at the asymmetric anterior crown (arrows in B and C). Scale bars: 10 μ m (A,B,C,E); 2 μ m (C1,C2,D).

that progressively and substantially decreases towards the anterior pole (Fig. 2B–E). Therefore, in *Tetrahymena*, distinct basal bodies clearly present different molecular compositions and abilities to recruit and/or concentrate pools of proteins involved

in establishing cell polarity, which suggests that in addition to cilia assembly they might present specialized functions inside a single cell. Mob1–GFP was also detected in some posterior transversal microtubules, contractile vacuole pores and in the oral

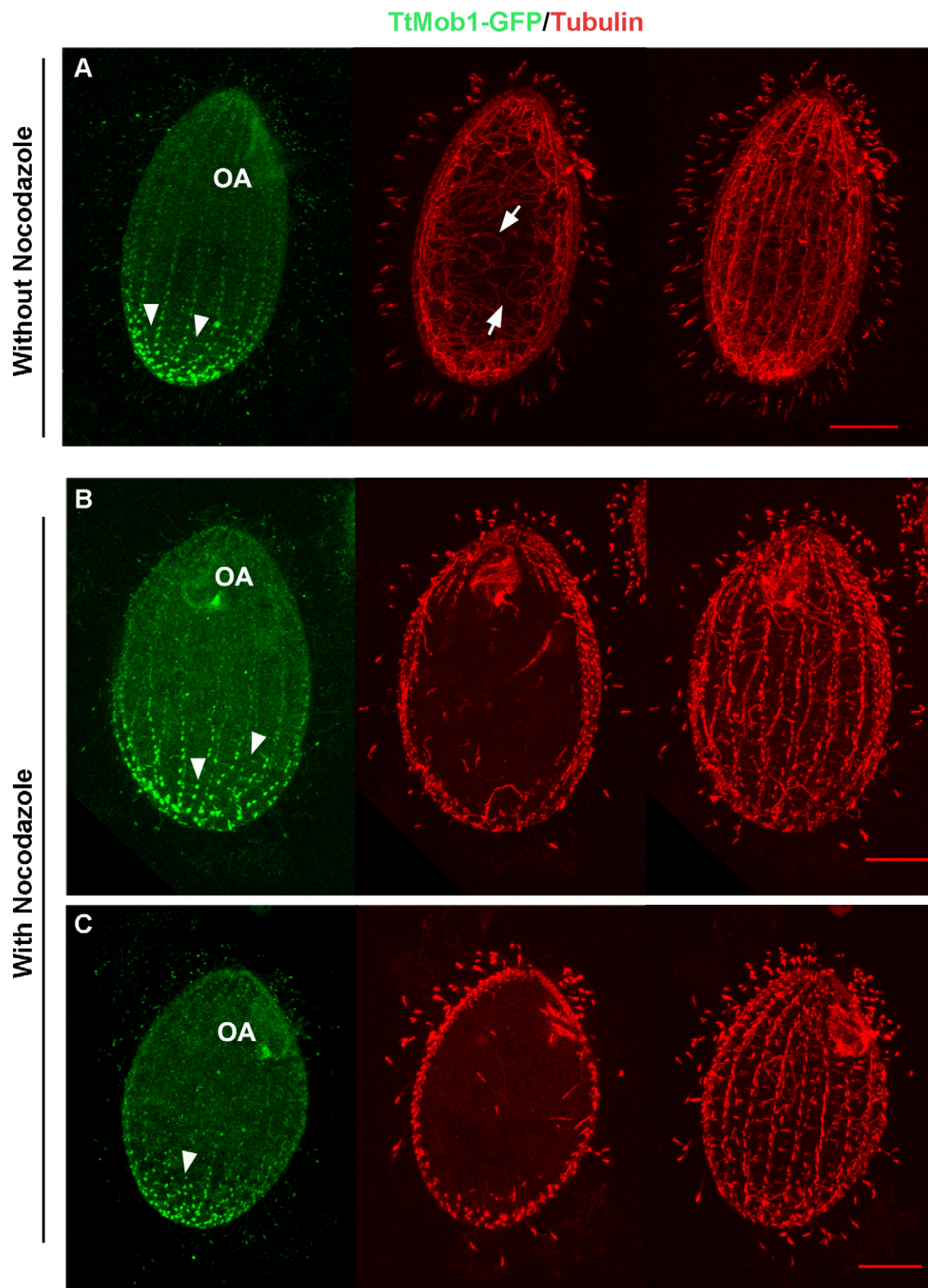


Fig. 3. *Tetrahymena* Mob1 basal body localization does not depend on intracytoplasmic microtubules. (A) *Tetrahymena* cells expressing Mob1-GFP were grown in culture medium supplemented with Cd^{2+} (2.5 $\mu\text{g/ml}$) and show the typical polar localization of Mob1-GFP in the oral apparatus (OA) and at the basal bodies of the posterior pole. (B,C) Mob1-GFP-expressing cells were treated with nocodazole (30 μM) to depolymerize internal cytoplasmic microtubules (arrows). In these cells, internal microtubules are not visible, but Mob1-GFP maintains the same pattern of localization in the OA and in posterior basal bodies (arrowheads). Scale bars: 10 μm .

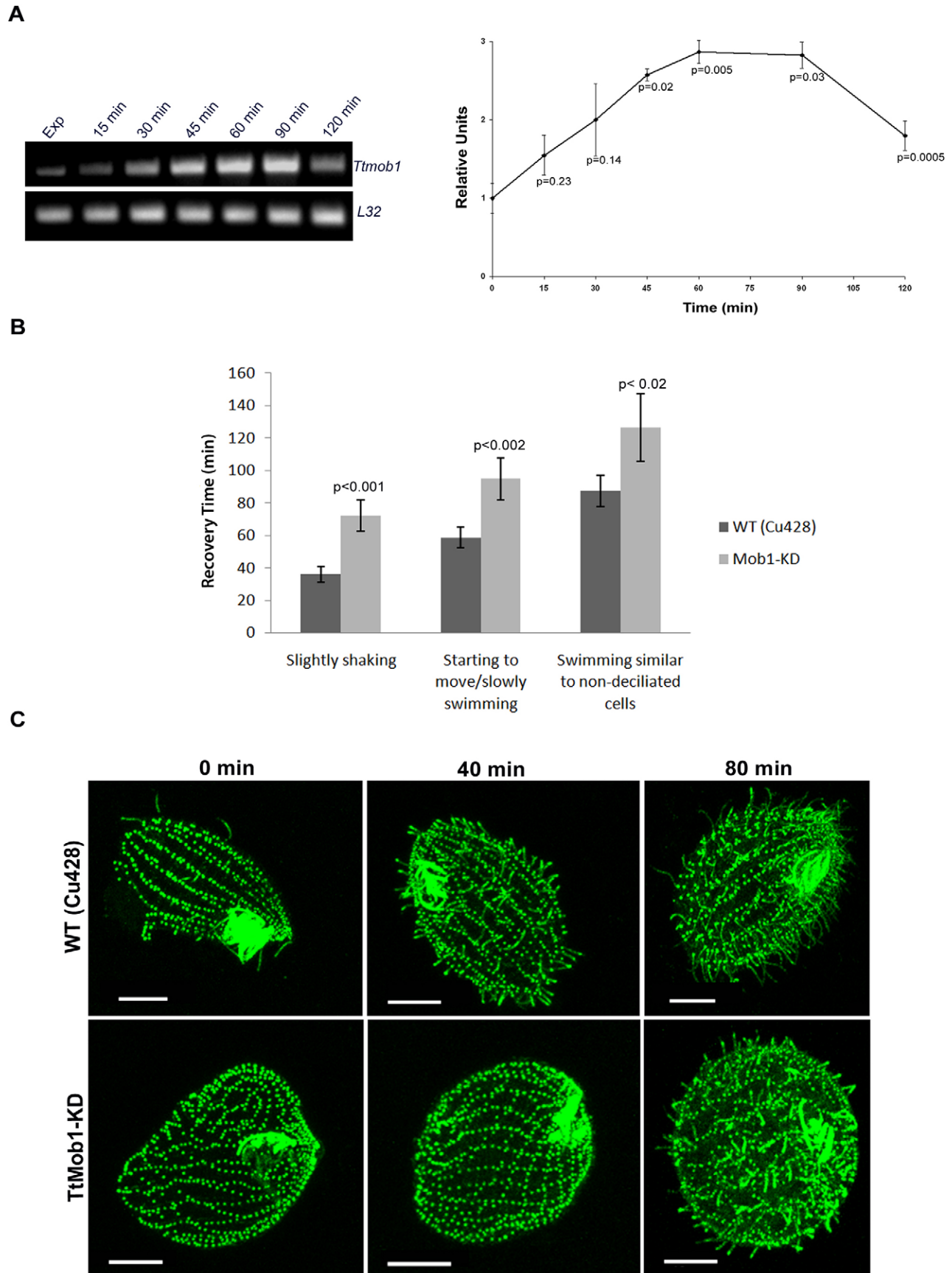


Fig. 4. See next page for legend.

apparatus (Fig. 2B–E). We therefore investigated whether the localization of basal bodies and oral apparatus in *Tetrahymena* expressing Mob1–GFP was dependent on microtubules by treating the cells with the microtubule-depolymerizing agent nocodazole. In nocodazole-treated *Tetrahymena* cells, Mob1–GFP had the same localization pattern as in non-treated cells, indicating that this specific localization of *Tetrahymena* Mob1 is independent of intracytoplasmic and cortical nocodazole-sensitive microtubules (Fig. 3).

***Tetrahymena* Mob1 depletion leads to a delay in cilia recovery**

Given the localization of *Tetrahymena* Mob1 at basal bodies, we wondered whether it had an involvement in cilia biology. *Tetrahymena* has a complex cortical microtubule cytoskeleton where basal bodies and cilia are found in repeated units (see Fig. 1A,B), and is capable of reciliation if cilia are removed (Soares et al., 1994). Therefore, we investigated the behavior of *Tetrahymena* Mob1-KD cells deciliated by osmotic or mechanical stress. Interestingly, we found that *Tetrahymena mob1* expression was upregulated during cilia recovery after ablation (Fig. 4A), responding to cilia biogenesis, with the highest levels detected after 45–90 minutes of reciliation. In agreement, we observed that *Tetrahymena* Mob1-KD cells have delayed cilia recovery compared with wild-type cells (Fig. 4B,C). The swimming behavior of *Tetrahymena* Mob1-KD cells was also investigated using bright field microscopy and, whereas wild-type reciliating cells are able to swim in a similar fashion to non-deciliated cells after 87.5 ± 9.6 minutes, the Mob1-KD cells take longer to reach normal swimming behavior (126.7 ± 20.8 minutes) (Fig. 4B). Noteworthy, analysis of these cells by immunofluorescence microscopy, using an antibody against glutamylated tubulin, showed no difference in the recovery rates for cilia of different cell regions (Fig. 4C), suggesting that high Mob1 levels at the posterior basal bodies are not important for cilia biogenesis. Our results do not rule out the hypothesis that the delayed cilia biogenesis is related to an abnormal spatial organization and/or altered polarity of the cell. Nonetheless, this is the first time that Mob1 has been implicated in cilia biology. Additional investigation would allow a better understanding of this new function.

Fig. 4. *Tetrahymena* Mob1 depletion leads to a delay in cilia recovery.

(A) RT-PCR analysis shows the expression pattern of *mob1* gene in *Tetrahymena* wild-type (Cu428 strain) exponentially growing cells (Exp) and cells recovering their cilia for different times (15–120 minutes; left panel). The gene encoding ribosomal protein L32 was used as internal control. Time-course analysis of changes in *mob1* mRNA levels during reciliation (right panel). The data represent average values from four independent experiments and are expressed relative to the amount of mRNA in Exp cells. Error bars indicate s.d. Statistical significance was calculated using the Student's *t*-test. (B) Comparison of the time required by *Tetrahymena* wild-type (Cu428 strain) and Mob1-KD cells to acquire different swimming behaviors during cilia recovery. The deciliated populations were continuously followed over time until swimming behavior was similar to that of non-deciliated cells. Values show the specific time points at which the majority of the population of cells presented the swimming behaviors indicated. The data are from four independent experiments. (C) Immunofluorescence microscopy of wild-type (Cu428 strain) and Mob1-KD cells with anti-glutamylated-tubulin to evaluate cilia recovery. At 40 minutes, many Mob1-KD cells have no visible cilia. Scale bars: 10 μ m.

***Tetrahymena* Mob1 recruitment to the cell midzone defines the division plane**

Normally in eukaryotic cells the cell division plane, where the cleavage furrow forms and ultimately cytokinesis occurs, bisects the preformed mitotic spindle (Oliferenko et al., 2009). In *Tetrahymena*, macronuclear chromosomes lack centromeres and the MAC divides amitotically without a typical spindle being assembled. However, intranuclear microtubules that organize perpendicularly to the cleavage region have been implicated in MAC division (Smith et al., 2004; Fujiu and Numata, 2000; Wunderlich and Speth, 1970; Tamura et al., 1969). These microtubules seem to be nucleated at a large number of sites inside of the nucleus (Fujiu and Numata, 2000). By contrast, the MIC divides mitotically and the spindle also assembles perpendicularly to the furrow but does not present organized structures at its poles (Frankel, 2000). Also, *Tetrahymena* cell division involves several morphological events at the cell cortex, beginning with the formation of an oral primordium, which starts with an anarchic field of basal bodies that will develop into the new oral apparatus (Fig. 5A–C) (Frankel, 1967). In dividing cells, we detected *Tetrahymena* Mob1–GFP in the oral apparatus immediately at the beginning of its assembly (Fig. 5B1), where it remained (Fig. 5B–F). As the cell cycle progressed, Mob1–GFP started to accumulate in the midzone basal bodies, just above the region where the cleavage furrow would form (between the old oral apparatus and the new one) (Fig. 5B–F). Therefore, Mob1–GFP accumulates where the new posterior pole of one of the siblings is to be formed (Fig. 5G). These observations, together with the knockdown results, strongly suggest that *Tetrahymena* Mob1 is involved in the establishment and maintenance of the anterior–posterior polarity of the cell. Next, we created a *Tetrahymena* Mob1–GFP ShuttON/OFF strain (supplementary material Fig. S2C) for two different purposes: firstly, to confirm that the *Tetrahymena* Mob1-KD phenotypes were specific and due to the downregulation of Mob1; and secondly, to investigate whether Mob1–GFP recruitment to the cleavage furrow is crucial for defining the cell division plane. For this, the *Tetrahymena* Mob1–GFP-expressing strain previously described was transformed with the *Tetrahymena* Mob1-KD DNA construct (supplementary material Fig. S1B). With this strategy, we were able to induce or repress Mob1–GFP expression in an endogenous Mob1 depletion background by addition or removal of Cd^{2+} (Fig. 6A). We observed that in the presence of Cd^{2+} (ShuttON), the cells were indistinguishable from wild-type cells (Fig. 6B,C). On the other hand, cells growing in Cd^{2+} -depleted medium (ShuttOFF) showed the same phenotypes as *Tetrahymena* Mob1-KD cells (Fig. 6B,D–G), clearly demonstrating that the phenotypes result from low levels or absence of Mob1. In abnormally dividing cells, low levels of Mob1–GFP could be seen in old posterior poles. In addition, the abnormal establishment of the division plane and failure of cytokinesis clearly correlated with the absence or trace levels of Mob1–GFP at the oral apparatus and cell midzone where the cleavage furrow should be formed (Fig. 6D–G). Whenever ShuttOFF cells showed some Mob1–GFP at the equatorial region, cell division seemed to occur symmetrically (Fig. 6D). These results strongly reinforce the idea that the polarized distribution of *Tetrahymena* Mob1 plays a crucial role in the definition of the division plane and, consequently, in cytokinesis.

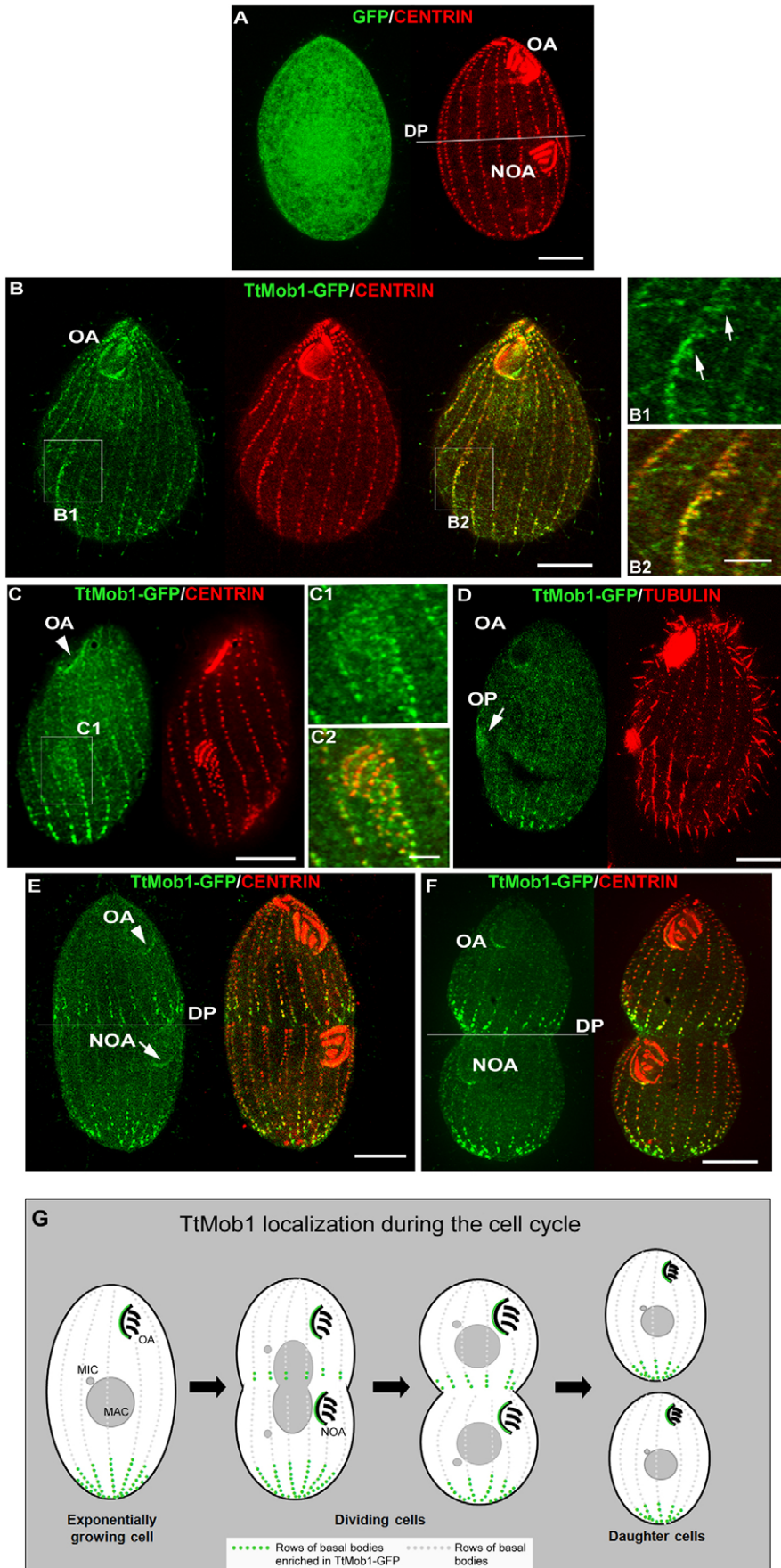


Fig. 5. *Tetrahymena* Mob1 localizes at the division plane basal bodies and new oral apparatus in dividing cells. (A–F) Immunofluorescence microscopy of *Tetrahymena* cells labeled with anti-GFP antibody (A–F), anti-centrin (A–C,E,F) and anti-glutamylated-tubulin (D). (A) In a dividing cell expressing GFP, no specific GFP localization is observed. (B–F) Mob1-GFP-expressing cells through different stages of cell division. In dividing cells, Mob1-GFP is visible in different stages of development of the oral primordium (OP; arrow in D) (B–D) and in the equatorial region where the division plane (DP) will be established (E,F). Magnifications of B and C (B1,B2 and C1,C2) show detail of the first steps of the new oral apparatus (NOA; arrow in E) formation. Note that in B1, Mob1 is already recruited to the first basal bodies of NOA (arrows). Arrowheads point to oral apparatus (OA). (G) Schematic representation of Mob1-GFP localization during the *Tetrahymena* cell cycle. Scale bars: 10 μ m; 2 μ m (in magnifications).

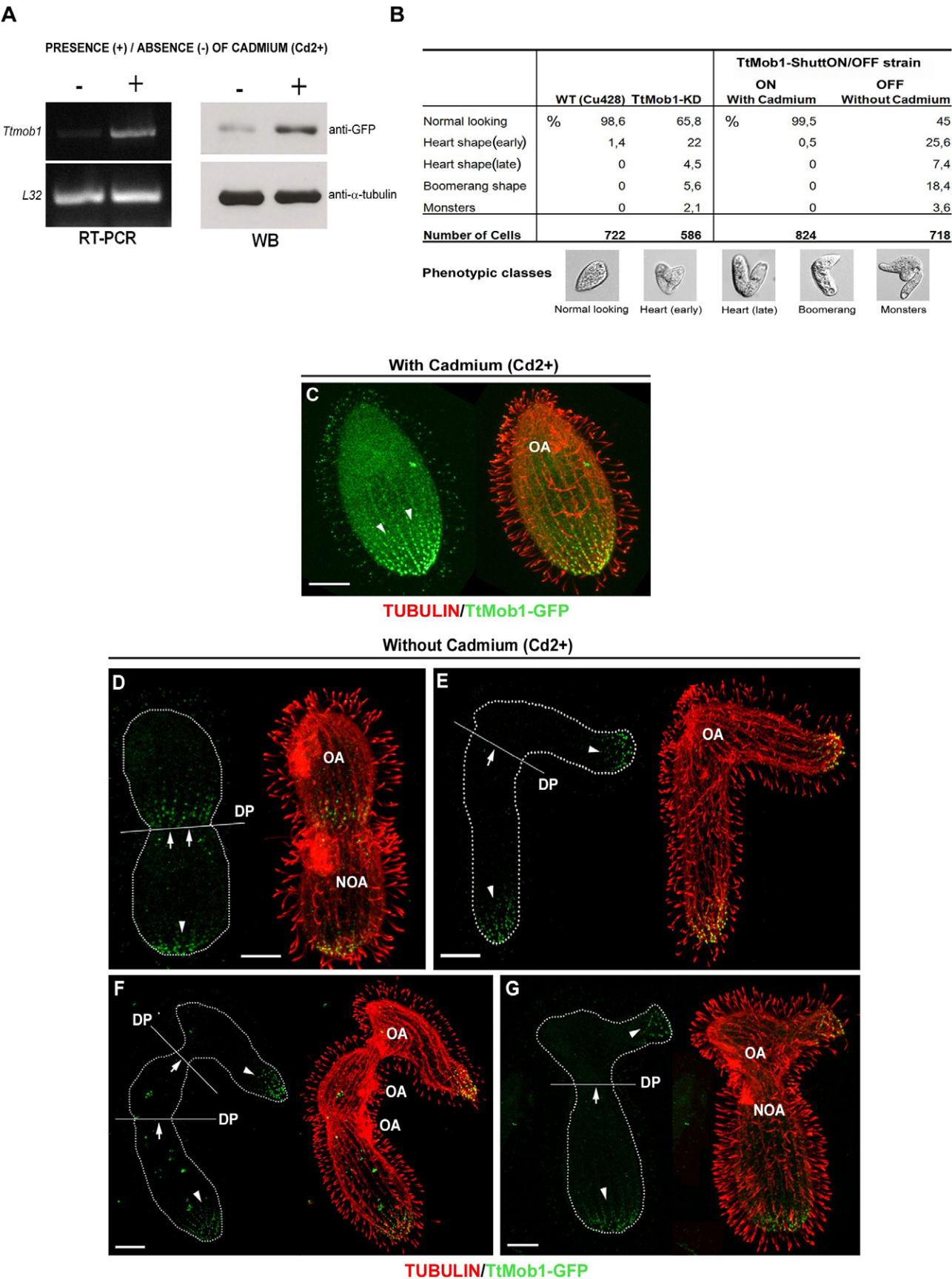


Fig. 6. See next page for legend.

Discussion

Because *Tetrahymena* is a permanently polarized unicellular protozoa that divides symmetrically, it is an attractive model for investigating how intrinsic cell polarity is related to symmetric cell division. Here we present the first studies concerning the function of the unique *Tetrahymena* Mob1-encoding gene (*mob1*) that presents 61% identity with the human counterpart, *MOB1*, which is a member of the MEN cascade.

Strikingly, *Tetrahymena* Mob1 accumulates in the posterior pole basal bodies, creating a gradient through the anterior–posterior axis. Moreover, during cell division *Tetrahymena* Mob1 is also recruited to the basal bodies at the cell equatorial zone where the cleavage furrow will be formed. To our knowledge, this is the first molecular polarity marker of the posterior pole at the posterior pole basal bodies so far described in *Tetrahymena*. This specific polarized localization of *Tetrahymena* Mob1 points to the importance of basal bodies possessing distinct compositions, creating specialized landmarks inside a single-celled organism. In fact, the presence of cortical gradients in *Tetrahymena* was previously postulated based on the spatial patterns of basal body proliferation that precede cleavage furrow constriction (Kaczanowski, 1978; Kaczanowska et al., 1999). In addition, studies of structural pattern mutants (Frankel, 2008) and on fenestrin localization below the fission line and accumulating at the anterior region in dividing cells (Nelsen et al., 1994; Cole et al., 2008) indicate the existence of an asymmetry in the cleavage furrow region.

The observed phenotypes in the *Tetrahymena* Mob1-KD cells and Mob1–GFP ShuttON/OFF strain clearly show that the Mob1 gradient throughout the anterior–posterior axis is essential for maintaining cell polarity, which is crucial for the establishment of the cell division plane (see Fig. 5). As described, *Tetrahymena* cells lacking Mob1 at the constriction region present dramatically altered orientations of the division planes. In these cells, cytokinesis aborts, although cells continue trying to divide and generate giant monsters with incorrect polarity axes. In yeast, Mob1 mutants also arrest in late anaphase but continue proliferation after cytokinesis failure and undergo several mitotic cycles, resulting in cell chains (Luca et al., 2001). Impaired cytokinesis was likewise described in both *Trypanosoma* and human *MOB1*-depleted cells (Hammarton et al., 2005) (C. Florindo, Study of human genes *MOB*, *hsMob4A*

and *hsMob4b*, PhD thesis, New University of Lisbon, Portugal), which suggests a conserved role for Mob1 throughout the eukaryotic lineage.

Although the basal body localization of *Tetrahymena* Mob1 and its recruitment to the constriction zone in dividing cells parallels the yeast spindle pole body localization and recruitment to the neck bud (Luca et al., 2001; Visintin and Amon, 2001), mutations in yeast Mob1 do not affect the spindle orientation, showing that Mob1 is only required for cytokinesis and mitotic exit (Luca et al., 2001). Interestingly, in yeast, MEN is only activated when a set of proteins, like Tem1 and Bub2/Bfa1 (SPOC pathway), are asymmetrically distributed in the two spindle pole bodies such that they are more abundant at the one that will migrate to the bud (Caydasi et al., 2010). This suggests that MEN is under the control of polarity factors. Our results show that the *Tetrahymena* Mob1 is itself a polarity factor required for the establishment of the polarity axis and consequently for the correct orientation of the division plane and successful cytokinesis. The differences between *Tetrahymena* and *S. cerevisiae* could be related to the fact that yeast possesses two distinct Mob genes (*mob1* and *mob2*). *Tetrahymena* Mob1 presents 49% and 42% amino acid sequence identity with yeast Mob1p and Mob2p proteins, respectively. Interestingly, in yeast, Mob2p localizes at the growing bud tip and is required for polarized cell growth and activation of daughter-specific genes necessary for cell separation after cytokinesis (Colman-Lerner et al., 2001; Weiss et al., 2002). Furthermore, in fission yeast, Mob2p is required for coordinated polarized growth with the onset of mitosis (Hou et al., 2003), and for the maintenance of polarisome components at hyphal tips in *Candida albicans* (Gutiérrez-Escribano et al., 2011). In neuronal mouse cells, Mob2 localizes at the base of protrusive neurite, at the branching point and at the tip of the neuritis, promoting neuritis differentiation (Lin et al., 2011). These data clearly show that Mob2 is a key factor for cell polarity establishment. At a first glance it seems that the *Tetrahymena* Mob1 fulfills all the Mob protein functions. If this is true, is tempting to speculate that the duplications of Mob-encoding genes throughout eukaryote lineage probably lead to the distribution of specific functions of the ancestral gene throughout the distinct members of the gene family. However, this analysis might be simplistic. In humans, *MOB1* localizes at the centrosome, at the spindle midzone and midbody (Wilmeth et al., 2010) (C.F., personal communication). It is worth noting that at the end of mitosis Mob1 is detected only in the centriole that moves closer to the midbody (C.F., personal communication), which is probably the mother centriole. The asymmetry of mother and daughter centrioles in a centrosome, and consequently between duplicated centrosomes, is also a crucial feature of asymmetric cell divisions (Yamashita et al., 2007). Interestingly, it was recently demonstrated that proteins related to cell and tissue polarity associate asymmetrically to the mother centriole (Jakobsen et al., 2011). Therefore, it is tempting to suggest that the role of Mob1 in *Tetrahymena* could also be replicated in metazoan cells.

Collectively, the data clearly supports the view that *Tetrahymena* Mob1 is a key factor for the establishment of intrinsic cell polarity and this is probably its most ancient role. Moreover, our results also show that Mob1 is involved in cilia biogenesis because its depletion delays cilia recovery, which is well correlated with the fact that *Tetrahymena mob1* is upregulated in response to cilia regeneration. Finally, we

Fig. 6. *Tetrahymena* Mob1-KD phenotype recovery in a Mob1–GFP ShuttON/OFF strain. (A) Left: RT-PCR analysis of *mob1-GFP* expression in *Tetrahymena* Mob1–GFP ShuttON/OFF cells grown in the absence (–) or presence (+) of Cd^{2+} . Right: Western blot (WB) of total proteins obtained from Mob1–GFP ShuttON/OFF cells under the same conditions. Both mRNA and protein levels of Mob1 are decreased in the absence of Cd^{2+} , which supports the efficiency of the ShuttON/OFF strategy. (B) Frequency of Mob1 depletion phenotypes in *Tetrahymena* wild-type (Cu428 strain), Mob1-KD, Mob1–GFP ShuttON and Mob1–GFP ShuttOFF cells. (C–G) Immunofluorescence microscopy of *Tetrahymena* Mob1–GFP ShuttON/OFF cells with anti- α -tubulin and anti-GFP antibodies. (C) In the presence of Cd^{2+} , Mob1–GFP is expressed, suppresses the Mob1-KD phenotypes, resulting in cells that are phenotypically indistinguishable from Mob1–GFP-expressing cells. (D–G) *Tetrahymena* cells grown for 18 hours in Cd^{2+} -depleted medium show Mob1-KD phenotypes, including normal-looking cells (D). Mob1 levels are almost inexistent at the division plane (DP) (arrows), indicating a requirement of the protein for correct establishment of the cleavage furrow. At the posterior pole, Mob1–GFP levels are also decreased (arrowheads). Scale bars: 10 μm . OA, oral apparatus; NOA, new oral apparatus.

wonder whether tumor formation in Mob1 mutants (Lai et al., 2005) is related to possible alterations in cell polarity. In fact, several studies implicate cell polarity pathways in tumor formation and progression, as in the case of the human polarity protein LKB1 (PAR-4 ortholog) whose mutations cause the Peutz–Jeghers Syndrome, which is characterized by benign hematomas and a high frequency of carcinomas (Baas et al., 2004). Our work, therefore, brings new clues about how cell polarity is linked for accurate cell division and cytokinesis and how it can be related to cancer.

Materials and Methods

Plasmid construction

To construct the *Tetrahymena* Mob1-KD plasmid, we amplified the 5' and 3' untranslated regions (UTRs) of the *Tetrahymena mob1* gene (5'-*mob1*UTR and *mob1*UTR-3'), using specific primers with attached restriction sites. The two homology arms were cloned in the pNeo4 vector (a kind gift from Jacek Gaertig, University of Georgia, Athens, GA) that has an inducible promoter responsive to Cd²⁺ (MTT1) and a neomycin resistance marker (Neo4). The resulting plasmid was as follows: 5'-*mob1*UTR–MTT1–Neo4–*mob1*UTR-3'. For the *Tetrahymena* Mob1–GFP construct, we amplified the *Tetrahymena mob1* gene open reading frame (ORF) (THERM_00716080; XP_001031965) with specific primers (supplementary material Table S1) containing the restriction sites to clone the *mob1* coding sequence in the pMTT1–GFP vector (kind gift from Jacek Gaertig), in frame with GFP in the C-terminal region. This plasmid contains the 5' and 3' UTRs of one of the two β -tubulin genes of *Tetrahymena* (*BTU1*) and also the inducible promoter MTT1. The Mob1–GFP construct used was as follows: 5'-*BTU1*UTR–MTT1–*mob1*–GFP–*BTU1*UTR-3'.

Tetrahymena strains

All cell strains used were grown in super proteose peptone (SPP) medium (1% proteose peptone, 0.1% yeast extract, 2.2% glucose, and 0.003% Fe-EDTA) at 30°C, with gentle shaking.

The constructs described were used to biolistically transform the MAC of exponentially growing *Tetrahymena* cells (Cassidy-Hanley et al., 1997). For the *Tetrahymena* Mob1-KD strain, Cu428 cells were transformed with the *Tetrahymena* Mob1-KD plasmid, which was inserted in the endogenous locus of *mob1*. Transformed cells were selected with rising doses of paromomycin, until the sub-lethal concentration of 2.8 mg/ml, to force an increase in the number of disrupted copies by phenotypic assortment. Cu522 cells were transformed with the Mob1–GFP construct, which was inserted in the ectopic locus of *BTU1*. Transformants were selected with 20 μ M paclitaxel. To obtain the *Tetrahymena* Mob1–GFP ShuttON/OFF strain, the Mob1–GFP-expressing strain was transformed with the Mob1-KD construct and transformants selected with paromomycin until the sub-lethal dose of 9mg/ml. The expression and repression of Mob1–GFP in *Tetrahymena* Mob1–GFP ShuttON/OFF cells was controlled by the addition of 2.5 μ g/ml of CdCl₂ or depletion of Cd²⁺. Cd²⁺ was eliminated from SPP by depleting the medium with 5% Chelex-100 beads (Bio-Rad) followed by complementation with trace metals (Seixas et al., 2010).

Nocodazole treatment

Tetrahymena exponentially growing cells expressing Mob1–GFP were treated with 30 μ M of nocodazole for 30 minutes. After the nocodazole treatment, 2.5 μ g/ml of CdCl₂ was added to the medium. After 30 minutes, cells were washed in 10 mM Tris-HCl (pH 7.5) and processed for immunofluorescence. Nocodazole-untreated Mob1–GFP-expressing cells with 2.5 μ g/ml of CdCl₂ in the medium were used as control.

Immunofluorescence assays

For staining *Tetrahymena* Mob1-KD cells, 40–60 cells were isolated into 20 μ l of 10 mM Tris-HCl (pH 7.5) on a coverslip previously coated with poly-L-lysine (Sigma). For staining *Tetrahymena* Mob1–GFP-expressing and Mob1–GFP ShuttON/OFF cells, exponentially growing cultures were washed and diluted in 10 mM Tris-HCl (pH 7.5). Then, 20 μ l of cells at 20,000 cells/ml were placed on a poly-L-lysine-coated coverslip. The following primary antibodies were used: mouse 20H5 anti-centrin (1:100; kind gift from Jeffrey Salisbury, Mayo Clinic, Rochester, MN), mouse 12G10 anti- α -tubulin (1:10; University of Iowa, Developmental Studies Hybridoma Bank), anti-glutamylated-tubulin (GT335) (kind gift from Carsten Janke, Institut Curie, Orsay, France) and rabbit anti-GFP (1:200; A11122, Invitrogen). Secondary antibodies used were Alexa-Fluor-488-conjugated goat anti-mouse, Alexa-Fluor-594-conjugated goat anti-mouse and Alexa-Fluor-488-conjugated goat anti-rabbit (1:500; Molecular Probes). Coverslips were processed for immunofluorescence labeling as described by Thazhath and co-workers (Thazhath et al., 2002). Cells were viewed using a Leica

SP5 Spectral Confocal with resonant scanner (using a 63 \times oil immersion lens). Images were assembled using NIH ImageJ 1.42q and Adobe Photoshop CS4 extended version 11.0.

Western blot and electrophoresis analysis

To analyze the expression of the tagged Mob1–GFP protein in *Tetrahymena* cells expressing Mob1–GFP and in *Tetrahymena* Mob1–GFP ShuttON/OFF cells (with/without Cd²⁺), total protein extracts from 120,000 cells of both strains and from wild-type cells were prepared. Cells were pelleted at 1600 *g* for 3 minutes, suspended in 1 ml of 10 mM Tris-HCl (pH 7.5) and concentrated in a final volume of 50 μ l. Cell pellets were lysed with 50 μ l of lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β -mercaptoethanol and Bromophenol Blue). Protease inhibitors were added at a final concentration of 0.5 mg/ml leupeptin, 10 mg/ml chymostatin and 15 mg/ml antipain. The mixture was boiled for 5 minutes at 95°C and 50 μ l of each sample was used for SDS-PAGE. Electrophoresis and western blot analysis of SDS-PAGE (12% w/v) gels were carried out as described by Soares and colleagues (Soares et al., 1997). Primary antibody used was rabbit anti-GFP (1:2000; A11122, Invitrogen). The secondary antibody used was goat anti-rabbit IgG (H+L) (1:1000; Zymed). The LMW-SDS Marker Kit (GE Healthcare) was used to mark molecular mass.

Deciliation assay

Tetrahymena Mob1-KD and wild-type cells exponentially growing were washed in 10 mM Tris-HCl (pH 7.5). Cells were concentrated in one twentieth of the initial volume and deciliated in drops. Briefly, cells from both strains were transferred from 25 μ l of Tris-HCl to 50 μ l of 50 mM sodium acetate, 10 mM EDTA-Na₂ pH 6; then after 30 seconds 25 μ l of water was added. After a further 30 seconds, 2.5 μ l of 0.4 M CaCl₂ was added to the drop and after 15 seconds the cells were transferred to SPP drops and allowed to recover cilia. Cells were observed over time using bright field microscopy to evaluate movement. Immunofluorescence was performed as described above at different time points of cilia recovery.

Gene expression analysis by RT-PCR

Total RNA samples from *Tetrahymena* wild-type, Mob1-KD, reciliating and Mob1–GFP ShuttON/OFF cells (with/without Cd²⁺) were prepared using the RNeasy Mini Kit (Qiagen, Germantown, MD) followed by cDNA synthesis using Superscript II RT (Invitrogen). Primers were designed to amplify *Tetrahymena* Mob1 and L32, which was used as an internal control. PCR was performed for 30 cycles.

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