Spatiotemporal regulation of microtubule function by human End Binding proteins

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Spatiotemporal regulation of microtubule function by human End Binding proteins

Regulação espacial e temporal da função dos microtúbulos pelas proteínas humanas "End Binding"

Supervisor: Prof. Doutor Helder Maiato

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A presente dissertação foi escrita em Inglês na sua quase totalidade devido ao facto de alguns dos trabalhos terem sido realizados com colaboração internacional.

"The gift of the great microscopist is the ability

to think with the eyes and see with the brain.

Deep revelations into the nature of living

things continue to travel on beams of light"

Daniel Mazia, circa 1996

Para a Célia

... por tudo

Para os meus Pais, Irmão e Avós

por acreditarem sempre

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ABSTRACT

Regulation of microtubule dynamics is crucial in many cellular processes, which include cell division, migration and morphogenesis. These processes can be regulated both temporally and spatially by the binding of different microtubule associated proteins (MAPs). Notably, amongst the larger group of MAPs, there is a diverse array of proteins that specifically recognizes and binds to the distal part of the microtubule and is known as plus-end tracking proteins (+TIPs). In this thesis we focused on the multiple cellular functions of human End-Binding (EB), highlighting their crucial role in the regulation of microtubule dynamics and defining how they help control mitotic progression and exit.

In this thesis we show that EB proteins differentially regulate mitotic progression and exit. EB1 controls spindle positioning during early mitosis by regulating astral microtubule nucleation, which is required for alignment of the spindle in relation to the substrate (z-axis). EB3 is necessary for limiting lateral spindle movements (in the xy-axis) by controlling the dynamic behaviour of a mitotic actin wave. Moreover, EB3 is also required for coordinated daughter cell spreading and cytokinesis. These late mitotic functions require distinct EB3 functional states which are dependent on an Aurora B kinase mediated phosphorylation gradient. Phosphorylation of EB3 at serine 176 by Aurora kinase in the spindle midzone/midbody is required for successful completion of cytokinesis. On the other hand, dephosphorylation of EB3 near the substrate ensures coordinated attachment of the daughter cells to the substrate.

In addition, we also demonstrate that these post-mitotic cell adhesion effects are relevant during interphase. Accordingly, EB1 and EB3 have differential roles in cell motility and adhesion, which depend on their ability to regulate microtubule dynamics and stabilize focal adhesions (FAs). EB1 is necessary for the normal turnover of FAs and its depletion leads to loss of normal cell motility. EB3 is required for the stabilization of FAs and in its absence, cells exhibit higher motility.

Finally, we demonstrate that association of EB proteins to the microtubule plus-ends is regulated in a cell cycle dependent manner and relies on a phospho-regulatory mechanism controlled by Aurora kinase B. Moreover, we show that phosphorylation of EB3 at serine 176 increases the affinity of EB proteins towards the plus-ends and changes microtubule dynamic behaviour. In addition, this association specifically depends on the EB1/EB3 heterodimer and provides the first clue for the physiological relevance heterodimer formation.

Taken together, our results help clarify the spatial and temporal regulation of EB proteins at the microtubule plus-ends and provide new insight on their mitotic and post-mitotic functions.

RESUMO

A regulação da dinâmica dos microtúbulos é essencial em numerosos processos, incluindo a divisão celular, a migração celular e a morfogénese. Estes processos podem ser regulados tanto espacialmente como temporalmente através da ligação de diversas proteínas associadas aos microtúbulos ("Microtubule-Associated Proteins"; MAPs). Entre as diferentes MAPs, podemos encontrar algumas proteínas que reconhecem especificamente a parte distal do microtúbulo e, como tal, são conhecidas como proteínas associadas à extremidade positiva ("Plus-end tracking proteins"; +TIPs). No decurso desta tese, tentamos esclarecer as diferentes funções celulares das proteínas humanas "End-Binding" (EB), com especial ênfase no seu papel de regulação da dinâmica dos microtúbulos, bem como no controlo da progressão e saída de mitose.

Assim, demonstramos que as proteínas EB regulam diferencialmente a progressão e saída de mitose. A proteína EB1 controla a posição do fuso mitótico durante as fases iniciais de mitose, através da sua capacidade de regular a nucleação de microtúbulos astrais, o que é necessário para a manutenção da posição do fuso paralela em relação ao substrato (eixo zz). Por outro lado, a proteína EB3 é necessária para controlar os movimentos laterais do fuso (no eixo xy), através da sua capacidade de influenciar a dinâmica de polimerização da actina. Além disso, a proteína EB3 é necessária para a adesão simultânea das células filhas ao substrato e para completar citocinese. Estas últimas funções dependem de diferentes estados funcionais da proteína EB3, que estão sob o controlo de um gradiente de fosforilação mediado pela cínase Aurora B. Fosforilação da EB3 na serina 176 pela cínase Aurora B na zona média e corpo médio das células mitóticas é necessária para que a citocinese possa ocorrer. Por outro lado, a EB3 deve ser desfosforilada junto ao substrato para que as células filhas possam aderir coordenadamente à matriz extracelular.

Os processos regulados pelas proteínas EB durante a saída de mitose são igualmente relevantes durante interfase. Como tal, verificamos que a EB1 e EB3 têm efeitos díspares na regulação da mobilidade e adesão celulares, que dependem essencialmente da sua capacidade de regular a dinâmica dos microtúbulos e estabilizar os pontos de adesão focal ("Focal Adhesions"; FAs). A EB1 é necessária para a rotatividade normal das FAs e a sua deplecção leva a uma diminuição da mobilidade celular. Por outro lado, a EB3 é necessária para a estabilização das FAs e a sua eliminação leva a um aumento da mobilidade celular.

Por último, demonstramos que a associação das proteínas EB aos microtúbulos durante o ciclo celular é regulada por fosforilação, mediada pela cínase Aurora B. Neste contexto, a fosforilação da EB3 na serina 176 aumenta a afinidade das proteínas EB para os microtúbulos, o que interfere com sua dinâmica. Além disso, esta associação depende especificamente da formação do heterodímero EB1/EB3 o que demonstra, pela primeira vez, a relevância funcional desta estrutura.

Em conclusão, os nossos resultados permitem esclarecer quais os mecanismos de regulação espacial e temporal da função das proteínas EB nos microtúbulos, enquanto demonstram uma diversidade funcional da EB1 e EB3 na progressão e saída de mitose.

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I. GENERAL INTRODUCTION

(invited review for International Review of Cell and Molecular Biology)

1 – General overview

Division of one cell into two genetically identical daughter cells occurs in a process known as mitosis. In order to do so, cells have to assemble a microtubule-based structure known as the mitotic spindle. During prophase, when chromosomes are still condensing, the centrosomes, which are the main Microtubule Organizing Centres (MTOCs) in animal cells, start to move away from each other. Upon Nuclear Envelope Breakdown (NEB), microtubules invade what was originally the nuclear space and come into contact with a specialized structure on the chromosomes known as kinetochores. Initially, kinetochores will mostly capture microtubules from one spindle pole. Only when the sister kinetochore captures microtubules emanating from the opposite spindle pole, will the chromosome become bioriented, move towards the equator of the cell and align in the metaphase plate. Upon anaphase onset, sister chromatids will start migrating towards the poles. In a first instance, chromatids separate and move towards opposite spindle poles (which is termed anaphase A), whereas in a later stage, the spindle itself will elongate, which is known as anaphase B. During telophase, each daughter cell will reform the nuclear envelope and decondense the sister chromatids. This is accompanied by the formation of a cleavage furrow, which will give rise later on, to the midbody. This last step is crucial for the final separation of the two daughter cells.

Microtubules are crucial in many steps of mitosis. They are inherently unstable polymers that switch between phases of growth and shrinkage in a mechanism defined as dynamic instability (Mitchison and Kirschner, 1984). It is now widely known that their behaviour is modulated by a number of microtubule associated proteins (MAPs), which can influence dynamic instability parameters, but also directly impact on mitotic progression. Many of these MAPs share the interesting ability to recognize only the distal part of a polymerizing microtubule. For this reason, these are known as microtubule plus-end tracking proteins (+TIPs) (Akhmanova and Steinmetz, 2008; Schuyler and Pellman, 2001). In this chapter we will cover a range of topics which include: 1) how tubulin is assembled and dynamically regulated; 2) how +TIPs can specifically recognize and bind to the plus-ends of microtubules; 3) how they are able to modify microtubule behaviour by inducing growth or shrinkage; and finally 4) how different +TIPs interact with each other to coordinate progression and exit from mitosis.

2- Microtubule structure and dynamics

2.1 - Tubulin

Microtubules are cylindrical filamentous structures composed of tubulin subunits that are involved in a number of cellular processes, including cell division, motility, differentiation and sub-cellular transport. They are structurally composed of two major subunits (Figure 1): α– and β–tubulin (Krauhs et al., 1981; Ponstingl et al., 1981), which are organized in heterodimers (Lowe et al., 2001; Nogales et al., 1998).

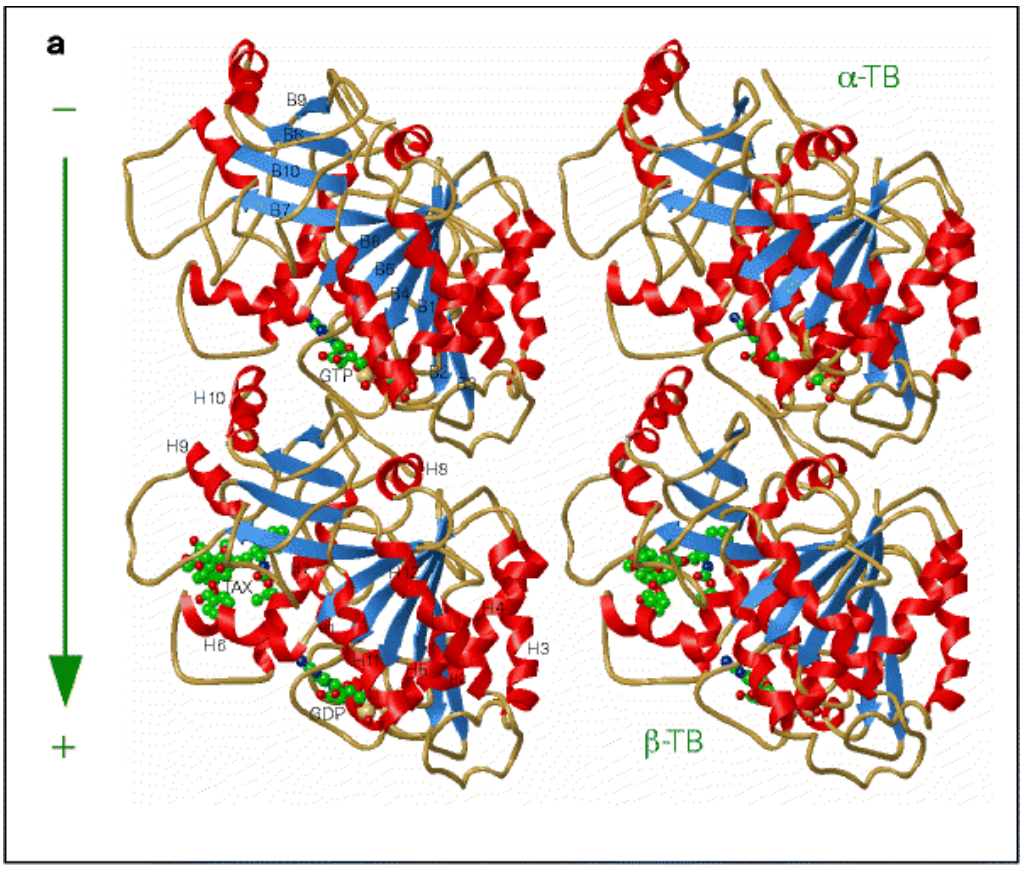

Figure 1 – Structure of the αβ tubulin heterodimer. The structure shows labels for strands (in the α-subunit) in blue and helices (in the β-subunit) in red. The arrow indicates the direction of the protofilament and microtubule axis. Adapted from (Nogales et al., 1998).

Tubulin was first isolated because of its ability to associate with colchicines. This drug can prevent microtubule polymerization and arrests cells in mitosis (Borisy and Taylor, 1967a; Borisy and Taylor, 1967b). The individual tubulin subunits have an approximate molecular

weight of 55 kDa and interact with each other in a non-covalent manner to create the heterodimeric, functional form of the protein (Nogales et al., 1998). The monomers contain three functional regions, which include a GTP-binding N-terminal domain, a C-terminal domain with two α-helices (and presumably serves as a binding site for motor proteins), as well as an intermediate domain which includes the taxol binding site in β tubulin (McKean et al., 2001; Nogales et al., 1998). The monomers of α and β tubulin share about 40% aminoacid identity and are comprised of a core of two β-sheets surrounded by α-helices (Nogales et al., 1998). The heterodimer has a size of approximately 8 nm in length and interacts both longitudinally and laterally to form structures known as protofilaments (Downing and Nogales, 1998).

Although the tubulin subunits are very similar, they also have distinctive features that allow for their functional separation. In fact, whereas GTP is found associated with α–tubulin, GDP can be found associated with β–tubulin (Howard and Hyman, 2003). Further studies revealed that GTP hydrolysis specifically occurs at the E-site of β–tubulin during microtubule polymerization (Manser and Bayley, 1987; Weisenberg et al., 1976). This hydrolysis locks GDP in the protofilament. Although both subunits can bind to GTP, the nucleotide associated with the α subunit cannot be hydrolyzed during polymerization (Spiegelman et al., 1977). The GTP-binding site in the α subunit is known as the N-site and it has been suggested to have no role in microtubule assembly.

There are multiple isoforms of tubulin in eukaryotic cells. Although the most abundant are α– and β– isoforms, other tubulin isotypes have been described which include gamma (γ), delta (δ), epsilon (ε), zeta (ζ), eta (η) iota (ι) and kappa (κ) tubulins (Dutcher, 2003; McKean et al., 2001). Interestingly, these isoforms are distinct in their aminoacid composition but also have differential sub-cellular localization and functions (Table I). The first tubulin isoform to be identified after the canonical αβ isoforms was γ–tubulin. (Oakley and Oakley, 1989). It was first discovered in *Aspergillus* as an interactor of β–tubulin and shares about 30% sequence identity with the αβ isoforms. γ–tubulin is associated with Microtubule Organizing Centres (MTOCs) such as the spindle pole body of fungi, centrosomes and basal body of animal cells and is also associated with the mitotic spindle. At these structures, it plays an important role in microtubule nucleation.

Tubulin	Localization/Function
αβ	Heterodimeric. Basic building block of microtubules
γ	Essential role in initiating microtubule assembly at MTOC, such as spindle pole body, centrosome and basal body.
δ	Localized to the centrosome or basal body. δ-tubulin mutants exhibit basal body defects (doublet rather than triplet arrangement) due to loss of C-tubule
ε	Localized to the centrosome in a cell cycle-specific manner
ζ	Localized to the basal body in *Trypanosoma brucei*
η	No localization data available. *Paramecium* η–tubulin mutants exhibit rare defects in basal body (lacking microtubules from triplets).
ι	Present in *Tetrahymena* and *Paramecium* only.
κ	No available data. Predicted by genome browsing

Table I – Localization and function of different tubulin isoforms in cells. αβ (alpha/beta), γ (gamma), δ (delta), ε (epsilon), ζ (zeta), η (eta), ι (iota) and κ (kappa). Adapted from (Dutcher, 2003; McKean et al., 2001).

Although MTOCs are the major sites of γ–tubulin localization, in a large number of cells there is a substantial soluble pool (Moudjou et al., 1996; Wiese and Zheng, 1999). δ-tubulin was identified as the product of the UNI3 gene in *Chlamydomonas* and its deletion leads to the appearance of doublet instead of triplet microtubules at the basal body (Dutcher and Trabuco, 1998). Careful analysis showed that the C-tubule is missing along most of the length of the basal body but is still present at the distal ends (Dutcher, 2003). Therefore, δ-tubulin was deemed to be necessary for the stability of the C-tubule but not for its formation. In eukaryotic cells, data is more contradictory. One report describes centrosomal localization (Chang and Stearns, 2000), whereas other study reports both cytoplasmic and nuclear staining with no colocalization with microtubules (Smrzka et al., 2000). ε–tubulin was shown to localize to centrosomes in a cell–cycle dependent manner, first associating only with the older centrosome and later associating with both centrosomes (Chang and Stearns, 2000). In addition, ε–tubulin seems to be necessary for centrosome duplication and microtubule

organization (Chang et al., 2003), but not microtubule nucleation. In fact, centrosomes can still nucleate microtubules even in the absence of ε–tubulin (Chang and Stearns, 2000). Other tubulin isoforms are less well known. ζ–tubulin was first identified in *T. brucei* and it seems to localize to the basal body (Vaughan et al., 2000). η–tubulin was first described in *Paramecium* and is important for basal body duplication (Ruiz et al., 2000). However, no data is available on the role of these tubulin isoforms in vertebrate cells.

In addition to multiple isoforms, cells can also change tubulin behaviour by introducing multiple post-translational modifications. These modifications are evolutionarily conserved and include acetylation, detyrosination, polyglutamylation, and polyglycylation [reviewed in (Hammond et al., 2008)]. Tubulin phosphorylation was the first described modification (Larsson et al., 1977). However, it is still not clear to what extent phosphorylation impacts on microtubule function. One possibility has to do with the regulation of microtubule dynamics at specific cell-cycle stages. In fact, CDK1 was proposed to phosphorylate β-tubulin at serine 172 leading to changes in microtubule dynamics during mitosis (Fourest-Lieuvin et al., 2006). Tubulin acetylation mainly occurs on lysine 40 of α-tubulin which is embedded in the tubulin subunit structure and is normally associated with stable microtubules (Hammond et al., 2008; L'Hernault and Rosenbaum, 1983). This modification seems to occur through the action of alpha-tubulin acetyltransferases (TATs) (Maruta et al., 1986; Shida et al., 2010). Inversely, tubulin deacetylation seems to occur through the action of two main enzymes: HDAC6 and Sirt2 (Hubbert et al., 2002; North et al., 2003). The effect of tubulin acetylation on microtubule function is still unclear. However, data suggests that acetylated microtubules are more resistant to depolymerization (Piperno et al., 1987) and affect motor-based activities (Janke and Bulinski, 2011). In addition, HDAC6 has also been implicated in the regulation of microtubule dynamics (Zilberman et al., 2009).

Tubulin tyrosination/detyrosination cycles occur on the C-terminal tyrosine of α-tubulin. Detyrosination involves the removal of the C-terminal tyrosine of α-tubulin in microtubules by a C-terminal carboxypeptidase (Argarana et al., 1978). This generates detyrosinated microtubules (which are also called Glu-MTs) and are normally considered to be stable microtubules. On the other hand, addition of a tyrosine residue to the new C-terminal glutamate α-tubulin occurs on soluble tubulin heterodimers and is catalyzed by the tubulin tyrosine ligase (TTL) (Westermann and Weber, 2003). The tyrosination/detyrosination cycle of tubulin can affect the recruitment of MAPs such as +TIPs and molecular motors. In fact, the recruitment of CAP-Gly proteins (a subset of +TIPs) to the microtubule plus-ends is highly

dependent on the tyrosination status of tubulin (Peris et al., 2006). In addition, the motor protein kinesin-1 has been shown to preferentially bind detyrosinated microtubules (Konishi and Setou, 2009) and induce microtubule disassembly driven by tyrosination (Peris et al., 2009).

A less common modification is polyglutamilation, in which glutamate side chains of variable lengths are introduced on a glutamate residue on the C-terminal tails of α- and β-tubulin (Janke et al., 2008). This modification was shown to impact on axonemal function by modulating dynein-microtubule interactions (Kubo et al., 2010). Polyglycylation involves the adition of glycine residues of variable length on the on the C-terminal tails of α- and β-tubulin (Redeker et al., 1994). Glycylation is mainly limited to axonemal tubulin present in motile cilia and flagella (Thazhath et al., 2002).

2.2 – Microtubule structure

The first reports on microtubule structure describe "slender tubules, 230 to 270 A in diameter and indeterminate length" (Ledbetter and Porter, 1963). These observations were later confirmed by finer EM analyses that showed microtubules as hollow cylinders of 25 nm in diameter and variable length (Figure 2). Further X-ray crystallography data revealed that these structures are composed of heterodimeric $\alpha\beta$ tubulin subunits (Nogales et al., 1998). These subunits are organized in a head-to-tail configuration to form a linear protofilament. Lateral interactions between 13 of these protofilaments generate the common microtubule structure as it is normally seen *in vivo* (Tilney et al., 1973). However, *in vitro* studies also show that microtubules can vary from 8 to 17 protofilaments (Chretien et al., 1992) and in certain organisms it is common to find variable protofilament numbers (Davis and Gull, 1983). Remarkably, the presence of centrosomes constrains the microtubule lattice to assemble in the 13 protofilament structure usually found *in vivo* (Evans et al., 1985).

Lateral interactions between protofilaments can give rise to A-type lattices when α subunits from one filament interact with β subunits from the adjacent filament. In a B-type lattice α and β subunits interact with α and β subunits from the adjacent filament, respectively (Desai and Mitchison, 1997). Experimental evidence showed that the B-type lattice is the preferential configuration adopted by microtubules *in vivo* although the A-type lattice can still be found under certain conditions (McIntosh et al., 2009).

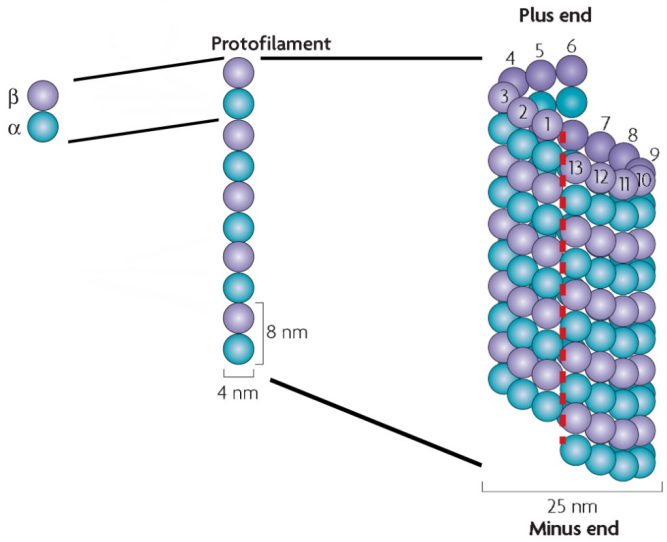

Figure 2 – Microtubule structure. Microtubules are composed of 13 protofilaments that form a hollow tube, whose main axis is parallel to the protofilament alignment axis. The surface lattice is described by a left-handed 3-start helix, with a seam that marks the tube closure (red line) along which monomers of one type can interact laterally with monomers of the other type. In this configuration, alignment of the protofilaments maintains dimer asymmetry, so that the microtubule ends are different. The minus-end exposes α-tubulin to solution whereas the plus-end exposes β-tubulin. Adapted from (Akhmanova and Steinmetz, 2008).

The B-type lattice gives rise to a structure called 3-start helix due to lateral interactions between tubulin subunits. Each turn of the helix results in a shift 1.5 times the dimer size. Because of the nature of the lateral interactions in the B-type lattice, there is also a region of discontinuity called the seam (Desai and Mitchison, 1997). This seam creates a region of weaker binding, which gave rise to the hypothesis that microtubules first grow as a sheet and only after create a closed structure (Chretien et al., 1995).

2.3 – Dynamic behaviour of microtubules

Microtubules are polarized structures that exhibit an inherent difference between the more dynamic, fast growing plus-ends and the relatively stable minus-ends (Summers and Kirschner, 1979). In fact, Summers and Kirschner, describe in their paper “one end growing at three times the rate of the other”. Later studies revealed that these differences could be partially explained by the fact that minus-ends are usually embedded on the centrosome or

other stabilizing sites (Mogensen et al., 2000; Wiese and Zheng, 2000), which makes it more difficult for microtubules to grow and shrink from this end. Inversely, microtubule plus-ends emanate outwards from MTOCs to probe the entire cell and are free to grow and shrink (Rodionov and Borisy, 1997; Sammak and Borisy, 1988). Differences in microtubule dynamic behaviour are also observed *in vivo* and can occur via two distinct mechanisms. One involves the addition and loss of tubulin subunits at the same end of microtubules – a mechanism known as **dynamic instability** (Mitchison and Kirschner, 1984; Sammak and Borisy, 1988; Schulze and Kirschner, 1988). The second mechanism occurs through gain of tubulin at the plus-ends of microtubules and loss of tubulin at the minus-end of the microtubule – a mechanism known as **treadmilling** (Margolis and Wilson, 1978; Rodionov and Borisy, 1997).

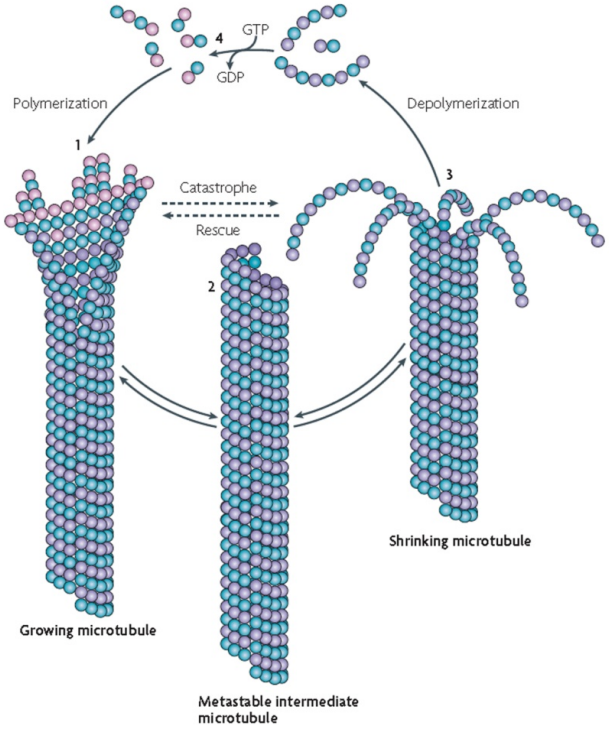

Figure 3 – Microtubule dynamic instability. (1) GTP-bound tubulin assembles at the microtubule plus-end creating a stable GTP cap that prevents microtubules from depolymerising. (2) Closure of the tubulin sheet creates a metastable intermediate. (3) When GTP hydrolysis occurs, the microtubule becomes unstable and depolymerises by the outward curving of individual protofilaments, which leads to destabilization of the microtubule structure. (4) When GDP is substituted for GTP in the disassembled tubulin subunits, the cycle begins again. Adapted from (Akhmanova and Steinmetz, 2008).

Dynamic instability was first described by Mitchison and Kirschner when they observed that centrosomal microtubules constantly interconverted between stages of growth and shrinking (Mitchison and Kirschner, 1984). This observation was later confirmed *in vivo* and was shown to have a major role in the reorganization of the microtubule network (Sammak and Borisy, 1988). Interestingly, at the time, additional cytoplasmic factors other than tubulin itself were also proposed to play a role in the regulation of dynamic instability (Schulze and Kirschner, 1988). Episodes of transition between microtubule growth and shortening are both abrupt and stochastic. Switches from growth to shortening are defined as catastrophes and transitions from shortening to growth are defined as rescues. Overall, dynamic instability became the major mechanism to explain microtubule dynamics both *in vitro* and *in vivo*.

Dynamic instability is driven mainly by GTP hydrolysis (Hyman et al., 1992). Tubulin subunits are incorporated into a protofilament when bound to GTP (Figure 3). After incorporation, GTP hydrolysis occurs very rapidly (Desai and Mitchison, 1997). This means that the microtubule lattice is enriched in GDP-tubulin. As a consequence, microtubules are less stable and tend to adopt a curved conformation, favouring depolymerization (Desai and Mitchison, 1997; Melki et al., 1989). Given this, how is it then possible for microtubules to stabilize and polymerize? Hydrolysis of GTP does not occur in the last subunit added to the protofilament but in the one before last. For this reason, it was proposed that microtubules have a GTP β-tubulin cap that would be sufficient to stabilize them (Mitchison and Kirschner, 1984). The exact size of the GTP cap is still unclear and many studies have reached different conclusions, with values ranging from 40 GTP subunits (Voter et al., 1991) to a single GTP subunit on each protofilament (Caplow and Shanks, 1996; Drechsel and Kirschner, 1994). Interestingly, recent *in vivo* data demonstrated that GTP-bound tubulin can also be found on the microtubule lattice and the authors proposed that these GTP-tubulin sites would work as a "memory" for where rescues occurred (Dimitrov et al., 2008).

Four parameters are currently used to define dynamic instability: growth velocity, shrinking velocity, rescue frequency and catastrophe frequency (Walker et al., 1988). In the same study, the authors determined that growth velocity depends on soluble tubulin concentration and the rate of association of GTP-tubulin to the microtubule. On the other hand, shrinking velocity is independent of tubulin concentration but depends on the dissociation rate of GDP-tubulin. Therefore, increasing tubulin concentration can increase growth rate which, in turn, leads to a decrease in microtubule shortening rate. Catastrophe/Rescue frequency is defined as the number of catastrophes/rescues a microtubule undergoes during the total growth time of a microtubule, respectively. These

dynamic parameters can easily be visualized using kymographic tools (Pereira and Maiato, 2010), which provide a visual representation of the microtubule plus-end over time (Figure 4).

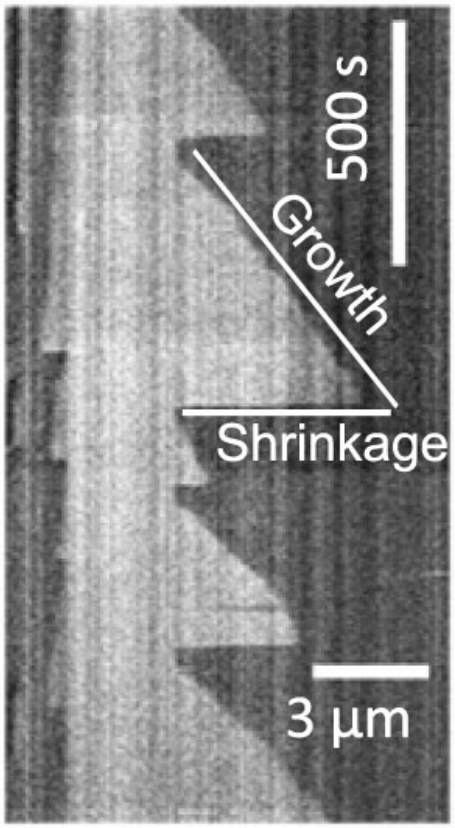

Figure 4 – Typical kymograph (plot of distance versus time) with changes in microtubule length and transitions over time. Microtubules will normally switch stochastically between growth and shrinkage. Highlighted are one growth phase and a subsequent shrinkage phase (white lines). A rescue event corresponds to a transition from shrinkage to growth and a catastrophe corresponds to a transition from growth to shrinkage. Adapted from (Gardner et al., 2012).

Although there are significant differences between dynamic parameters *in vitro* and *in vivo*, it is generally accepted that *in vitro* measurements share similarities with values obtained for living cells and thus, can give good estimates on how microtubules behave. As an example, microtubules isolated from HeLa cells are very static, spending most of their time in the "paused" state when compared to the same type of microtubules in living HeLa cells (Newton et al., 2002). This implies that additional factors have to be taken into account when analyzing dynamic instability other than tubulin itself (Schulze and Kirschner, 1988). In fact, with minimal components such as microtubules, a microtubule stabilizer and a microtubule destabilizer, it was possible to create a near-physiological system (Kinoshita et al., 2001). Typical growth velocities of microtubules *in vitro* are around 1-5 μm/min and depend on temperature, tubulin concentration and ions (Herzog and Weber, 1977) but these values can be much higher in living cells. Microtubule shortening velocities are in the order of 10-50 μm/min and are normally 10 times higher than growing velocities. Exactly what controls the rates of

polymerization/depolymerization is still unclear, but several hypotheses have been put forward. These include structural features of the microtubules themselves, which were shown to have an impact on assembly rates (Gildersleeve et al., 1992) or defects in microtubule closure, which would induce mechanical stress and lead to catastrophe events (Hunyadi et al., 2005). Interestingly, EB1 (a +TIP that controls persistent microtubule growth) was described to be involved in microtubule sheet closure and favour polymerization events (Vitre et al., 2008). Hydrolysis of GTP, which occurs at the microtubule plus-ends also plays a crucial role in this transition (Hyman et al., 1992). In fact, hydrolysis causes tubulin to adopt a curved conformation, ultimately leading to destabilization of the lattice (Melki et al., 1989). Because these GDP-tubulin subunits are not allowed to completely curve while in the lattice, energy released from hydrolysis is stored as mechanical strain within the microtubule (Caplow et al., 1994). This means that when catastrophe events occur, protofilaments adopt an outwards curvature, leading to rapid depolymerization of the microtubule. Curiously, when GMPCPP (a slowly hydrolysable analogue of GTP) is used, curvature of the protofilaments is reduced by two-fold without any significant changes on lattice structure (Hyman et al., 1995). It would be interesting to define exactly how biochemical and structural changes on the microtubule integrate to regulate polymerization/depolymerization rates.

Microtubule treadmilling was first proposed when it was observed that isolated bovine brain tubulin continuously incorporated into microtubules at a constant rate, while the microtubule length remained constant (Margolis and Wilson, 1981). This mechanism implies that (1) there has to be a unidirectional flow of tubulin subunits with incorporation at the plus-end and dissociation at the minus-end and (2) the rate of tubulin association has to be similar to the rate of tubulin dissociation (Margolis and Wilson, 1981). Similarly to dynamic instability models, microtubule treadmilling also requires GTP hydrolysis (Margolis, 1981). Further work has also revealed that in MAP-rich and MAP-depleted conditions, microtubule length can change even in the presence of treadmilling (Farrell et al., 1987). This means that dynamic instability and treadmilling can co-exist. However, it was shown that treadmilling can exist independently of MAPs in conditions where dynamic instability is inhibited (Panda et al., 1999). Moreover, the same work suggested that to obtain treadmilling all that is required is a difference in the critical concentration of tubulin subunits at the opposite MT ends. The treadmilling model implies that this mechanism could be bi-directional, depending on the available tubulin concentration at each given moment. In fact, fluorescence speckle microscopy techniques demonstrated a lack of polarity in treadmilling (Grego et al., 2001), suggesting that stochastic differences in dynamics between plus- and minus-ends (and not

unidirectional treadmilling) are responsible microtubule turnover. Evidence for treadmilling *in vivo* has been more difficult to collect mainly due to the fact that dynamic instability is more prevalent. However, it seems that treadmilling can be observed in some specific conditions. Namely, when microtubules detach from the centrosome, leaving their minus-ends free (Rodionov and Borisy, 1997). In any case, these findings reveal that minus-end dynamics and, more specifically, treadmilling can be as important as plus-end dynamic instability.

The role of microtubule dynamics is crucial for many cellular functions. Therefore, it is not surprising that spatial and temporal regulation of microtubule dynamic instability is coupled to processes as diverse as mitotic chromosome capture (Holy and Leibler, 1994; Kirschner and Mitchison, 1986; Mimori-Kiyosue and Tsukita, 2003), cell migration (Watanabe et al., 2005) or neuronal growth cone motility (Tanaka et al., 1995). Some of these characteristics and features will be addressed in the following section.

2.4 – Regulation of microtubule dynamics during the cell cycle

Microtubule dynamics during mitotic entry

Cell cycle progression is accompanied by changes in microtubule dynamics at very specific stages. This was first shown when cultured cells were injected with labelled tubulin. Under these conditions, mitotic cells incorporated tubulin within seconds, whereas interphase microtubules required minutes (Saxton et al., 1984; Wadsworth and Sloboda, 1983). In addition, later experiments using *Xenopus* egg extracts and live cells that were arrested either in interphase or mitosis, demonstrated that differences in microtubule dynamics were mainly due to an increase in catastrophe frequency of mitotic microtubules (Belmont et al., 1990; Rusan et al., 2001). More detailed reports described that this increase in microtubule dynamics specifically occurred with NEB and could be important for spindle morphogenesis (Piehl and Cassimeris, 2003; Zhai et al., 1996). Interestingly, these changes are accompanied by a decrease in tubulin polymer (Zhai and Borisy, 1994; Zhai et al., 1996) and a reorganization of the remaining microtubules towards the nuclear envelope, where they interact with dynein/dynactin and components of the nuclear pore complex (Bolhy et al., 2011; Piehl and Cassimeris, 2003). These abrupt differences in microtubule polymer led to the hypothesis that microtubule stabilizers would have to be inactivated upon mitotic entry. This hypothesis was supported by the fact that addition of cyclins or activated p34/cdc2 (Cyclin Dependent Kinase 1; CDK1) protein kinase to *Xenopus* extracts was sufficient to induce a mitotic-like catastrophe

rate of microtubules (Belmont et al., 1990; Murray and Kirschner, 1989). Furthermore, phosphorylation of MAPs (such as MAP4) by CDK1 did not prevent MAP binding to microtubules, but abolished its microtubule stabilizing activity (Ookata et al., 1995), confirming that phosphorylation of these proteins (such as MAP4, XMAP215 and CLASPs) upon mitotic entry is necessary to alter their function, thus leading to changes in microtubule stability. In this context, it is possible that MAP4 binding to microtubules during interphase (when Cyclin B/CDK1 complexes are inactive), is sufficient to counteract promotion of microtubule catastrophes (Holmfeldt et al., 2002). Immediately before NEB, active Cyclin B/CDK1 complexes accumulate in the nucleus (Gavet and Pines, 2010). Upon NEB, active Cyclin B/CDK1 complexes are released and free to associate with spindle microtubules due to its interaction with MAP4, leading to MAP phosphorylation and consequent microtubule destabilization (Ookata et al., 1995). Curiously, inactivation of CDK1 upon anaphase onset was shown to require intact microtubules (Andreassen and Margolis, 1994) and inhibition of CDK1 promotes microtubule growth (Moutinho-Pereira et al., 2009; Skoufias et al., 2007), which provides an interesting crosstalk between all components.

Why is it necessary to dramatically alter microtubule dynamics upon mitotic entry? The first mechanism that accounted for this was the “search-and-capture” model in which dynamic mitotic microtubules would search the cell space to capture chromosomes (Kirschner and Mitchison, 1986). Although many mathematical models provided backup to this “search-and-capture” mechanism (Hill, 1985; Holy and Leibler, 1994), it became evident that changes in microtubule dynamics alone cannot account for efficient capture of chromosomes within the normal time frame of mitosis (Magidson et al., 2011; Wollman et al., 2005). One other aspect that requires a tight regulation of microtubule dynamics is correct interaction of astral microtubules with the cell cortex, which has implications in spindle positioning and can affect mitotic outcome (Shaw et al., 1997; Toyoshima and Nishida, 2007). In fact, a number of microtubule regulators such as the +TIPs EB1 and APC (Green et al., 2005; Rogers et al., 2002), but also MAP4 and CLASP1 (Samora et al., 2011) were shown to be necessary for this process. These regulators allow astral microtubules to reach the cell cortex where they are caught by dynein/dynactin complexes (O'Connell and Wang, 2000). These complexes then can exert a pulling force on astral microtubules and fine tune spindle position (Kiyomitsu and Cheeseman, 2012).

Assembly or disassembly of microtubules can also generate force without direct contribution of motor proteins (Dogterom and Yurke, 1997; Koshland et al., 1988) and these are sufficient to move sub-cellular structures such as chromosomes, organelles or assist in

mitotic spindle positioning (Dogterom et al., 2005; Inoue and Salmon, 1995; Mogilner and Oster, 2003; Tolic-Norrelykke, 2008). Pushing forces are generated by means of microtubule polymerization. Addition of tubulin subunits to the microtubule plus-end will induce a compression when the microtubule hits an object and this leads to a movement of the microtubule in the opposite direction, unless the microtubule is attached to a structure (Dogterom and Yurke, 1997; Holy et al., 1997). Due to their nature, these forces can only be exerted over very short distances, because microtubules have a tendency to buckle when they grow too long (Dogterom et al., 2005; Dogterom and Yurke, 1997). For that reason, *in vivo* evidence regarding microtubule-generated pushing forces has been lacking. So far, microtubule pushing forces were shown to be required for nuclear positioning in fission yeast (Tran et al., 2001) and, more recently, it was reported that growing microtubules can exert pushing forces to position the oocyte nucleus (Zhao et al., 2012). However, there is no direct proof that microtubules can push chromosomes *in vivo*. Pulling forces, on the other hand, are exerted by means of microtubule depolymerization. This was first demonstrated *in vitro*, when it was shown that depolymerising microtubules could generate sufficient pulling force to move chromosomes (Koshland et al., 1988). Subsequent reports demonstrated that the objects being pulled moved at about 30 μm/min in an ATP-independent manner and thus, were dependent only on microtubule depolymerization (Coue et al., 1991). This pulling force exerted purely by microtubule depolymerization should be distinguished from pulling forces generated by motor proteins at the cell cortex (O'Connell and Wang, 2000) although they can act in concert.

Microtubule dynamics in the metaphase spindle

Upon establishment of the metaphase spindle, its length and shape appear relatively stable. However, the spindle itself is quite heterogeneous and dynamic, mainly due to constant microtubule turnover and flux (Kwok and Kapoor, 2007). As was said earlier, metaphase cells have a microtubule half-life in the order of seconds, whereas interphase microtubules have a half-life of approximately 3 minutes (Saxton et al., 1984). Later experiments confirmed these observations in many different systems and demonstrated that spindle microtubule turnover was likely derived from the high dynamic instability of non-kinetochore microtubules (Buster et al., 2007; Gorbsky et al., 1990; Salmon et al., 1984; Zhai et al., 1995). Similar measurements made in kinetochore microtubules showed that, although still capable of turnover, they do so at much lower rates (approximately 10x) (Zhai et al., 1995). Interestingly, this report also

indicated a striking reduction of microtubule turnover rates and microtubule transport at anaphase onset, suggesting that kinetochore-microtubule attachment is stabilized upon anaphase (Gorbsky and Borisy, 1989; Zhai et al., 1995). This further demonstrates that microtubules can also change their dynamic parameters during mitosis. In addition to dynamic instability, a second mechanism also ensures proper spindle dynamics, which is microtubule poleward flux (Mitchison, 1989). This is a highly conserved feature of the mitotic spindle in eukaryotes and involves the incorporation of microtubule subunits at the microtubule plus-end and disassembly of subunits at the microtubule minus-ends (Mitchison et al., 1986). This mechanism is different from microtubule treadmilling that was originally proposed (Margolis and Wilson, 1978), because the later does not take into account contributions from microtubule motors.

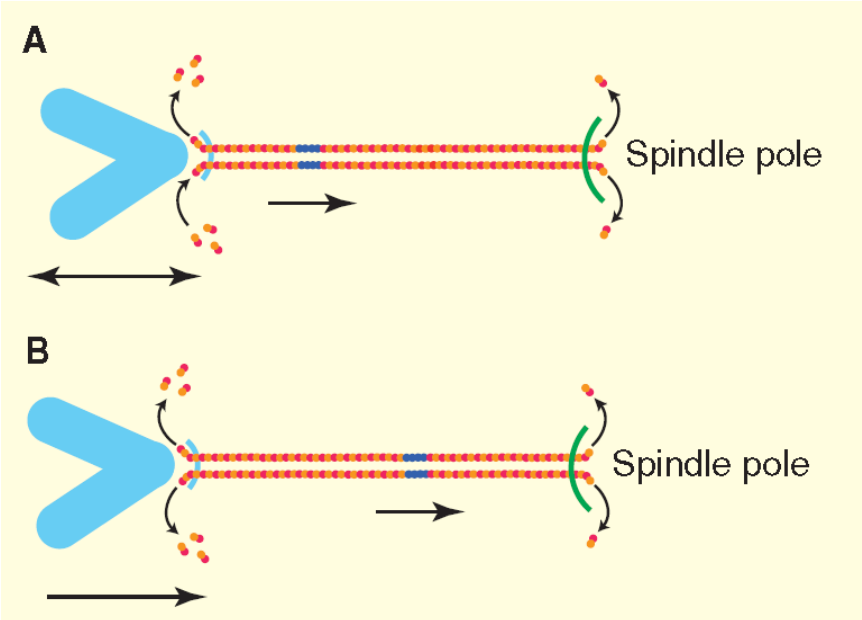

Figure 5 – Microtubule poleward flux during mitosis. (A) During metaphase, tubulin subunits are continuously removed at the minus-ends near the spindle poles. At the same time, the plus-ends near the kinetochore continuously switch between addition and removal of tubulin. As a result, chromosomes remain stationary while tubulin subunits move towards the spindle poles. (B) Upon anaphase onset, kinetochores will switch to a depolymerization mode only, resulting in poleward movement of chromosomes on microtubules ("Pac-man" model). At the same time, microtubules continue to depolymerise at their minus-ends, reeling in the chromosomes ("Traction fibre" model). Adapted from (Khodjakov and Kapoor, 2005).

Curiously, poleward flux was initially proposed to occur independently of chromosomes, centrosomes and anti-parallel microtubules (Sawin and Mitchison, 1994). This was later shown no to be totally accurate, as anti-parallel microtubules can also contribute to poleward flux (Mitchison et al., 2004). Current models proposed to explain microtubule flux take into account the following premises: active incorporation of tubulin subunits at the kinetochore and disassembly of tubulin subunits at the centrosome (Buster et al., 2007; Kwok and Kapoor, 2007; Mitchison, 1989), sliding of microtubules through the action of plus-end directed motors (Brust-Mascher and Scholey, 2002) and possible interaction of microtubules with a "matrix" or actin-related proteins so that microtubules can be displaced poleward (Forer et al., 2007; Silverman-Gavrila and Forer, 2000). The net result is the stabilization of the spindle size, while maintaining the structure highly dynamic.

The metaphase-anaphase transition

As was mentioned earlier, different microtubule populations have distinct dynamic properties in the metaphase spindle. Non-kinetochore microtubules have a higher turnover when compared to kinetochore microtubules (Cassimeris et al., 1990; Mitchison et al., 1986; Saxton et al., 1984). These differences will be reflected on microtubules as cells enter anaphase. In fact, turnover of kinetochore microtubules was shown to decrease as cells enter anaphase by as much as fivefold when compared to the same population of microtubules in metaphase cells (Gorbsky and Borisy, 1989; Zhai et al., 1995). Likewise, poleward flux of kinetochore fibres is also reduced upon anaphase entry but turnover of non-kinetochore fibres is not affected during the transition from metaphase to anaphase (Zhai et al., 1995). Additional evidence has also indicated that the total amount of tubulin polymer remains unchanged during the metaphase-anaphase transition (Zhai and Borisy, 1994), although anaphase chromosome movement requires a shortening of kinetochore fibres, be it from the plus-end (Gorbsky et al., 1987) or minus-end of microtubules (Waters et al., 1996). Shortening of kinetochore fibres during anaphase should occur either by active depolymerization of microtubules at the pole region (known as the "Traction Fiber" model; Figure 5) or disassembly of microtubules at the kinetochore (known as the "Pacman" model; Figure 5) (Maiato, 2010). The "Traction Fiber" model does not take into account the fact that structures other than microtubules can contribute to chromosome movement. For this reason, it was proposed that kinetochores themselves could also contribute actively to the generation of movement, and not be only passive anchorage points, which gave rise to the "Pacman" model (Cassimeris and

Salmon, 1991; Gorbsky et al., 1988; Nicklas, 1989). This implies that tubulin subunits are actively removed from the kinetochore interface. Initial approaches suggested that microtubule depolymerization *per se* was sufficient to drive chromosome movement *in vitro* (Coue et al., 1991; Koshland et al., 1988) and this was also confirmed when it was shown that microtubule depolymerization alone could generate force (Grishchuk et al., 2005). However, there is evidence to suggest that microtubule depolymerization appears to be insufficient to move chromosomes without the assistance of motor proteins (Desai and Mitchison, 1995; Pfarr et al., 1990). Interestingly, a recent report also demonstrated that the bidirectional microtubule depolymerizer kinesin-13 MCAK was able to generate force by acting on both microtubule ends (Oguchi et al., 2011), which provides support for this theory. In addition, it seems that transition from metaphase to anaphase also requires that spindle microtubules become less dynamic and this transition is regulated by the phosphatase Cdc14 (Higuchi and Uhlmann, 2005; Mallavarapu et al., 1999). If phosphatase action is inhibited, microtubules are maintained in a highly dynamic state, which leads to problems in chromosome movement (Higuchi and Uhlmann, 2005).

Mitotic exit and remodelling of microtubule cytoskeleton

In addition to its role in chromosome movement, microtubules are also necessary for changes in cell shape and size during anaphase and telophase. As was mentioned earlier, tubulin levels remain constant during the metaphase-anaphase transition (Zhai and Borisy, 1994). This means that depolymerization of spindle microtubules has to be compensated by an increase in astral microtubule polymerization or elongation during anaphase (Morrison and Askham, 2001; Strickland et al., 2005b). Elongation of astral microtubules is necessary for their interaction with the cell cortex and definition of the cytokinetic furrow, but apparently is not essential for anaphase progression itself, as the cytokinetic furrow can still be formed even in the absence of astral microtubules (Rankin and Wordeman, 2010; Strickland et al., 2005a; Strickland et al., 2005b; Sullivan and Huffaker, 1992). This reorganization of microtubules has to be controlled both temporally as well as spatially, in order to coordinate chromosome segregation with post-mitotic events. In fact, it was shown that microtubule reorganization involves the inactivation of the mitotic kinase CDK1 and this triggers the formation of anaphase microtubules and the midbody (Wheatley et al., 1997). Strikingly, a similar phenomenon was also observed in *Drosophila* S2 cells which had acentriolar microtubule-organizing centres (aMTOCs) but lacked centrosomes. These aMTOCs were still able to induce

formation of cytoplasmic microtubules upon CDK1 inhibition at anaphase onset (Moutinho-Pereira et al., 2009) and this was dependent on the activity of Msps/XMAP215 and KLP10A/kinesin-13. In addition to biochemically triggered changes, there are also structural changes occurring at anaphase onset. It was reported that during anaphase there is a fragmentation of the centrosome with a consequent release of microtubule fragments and this was postulated as a mechanism of microtubule remodelling in post-mitotic cells (Rusan and Wadsworth, 2005). Furthermore, remodelling of the microtubule cytoskeleton correlates with migration of the centrosome towards the midbody (Piel et al., 2001). Cytokinesis is dependent on microtubules in several ways. Firstly, definition of the cleavage plane is specifically determined by astral microtubules (and not spindle microtubules) as furrowing still occurs in the presence of asters without any intervening spindle (Rieder et al., 1997). In addition, if anaphase astral microtubule formation is suppressed by interfering with the +TIP EB1 or with dynactin, cytokinesis is delayed (Strickland et al., 2005b), which supports the necessity of microtubule interaction with the cortex to define cleavage plane localization (Bement et al., 2005; Strickland et al., 2005a). At this stage, regulation of microtubule dynamics seems to be dispensable, as contact of microtubules with the cortex is sufficient to trigger the process (Strickland et al., 2005a). In contrast with earlier stages of cytokinesis, microtubules are essential for completion of the process (Savoian et al., 1999). The microtubules that establish the midbody are highly stable (Margolis et al., 1990), were shown to be acetylated (a marker of stable microtubules) and are resistant to nocodazole treatment (Foe and von Dassow, 2008; Piperno et al., 1987). Nevertheless, some midbody microtubules are still able to exhibit a highly dynamic behaviour as can be seen by live imaging of microtubule plus-ends with EB proteins, with plus-ends moving in and out of the midbody (Rosa et al., 2006). Thus, it is not surprising that γ-tubulin was found in the midbody during late cytokinesis (Julian et al., 1993). Final disassembly of the midbody requires that microtubules are severed. This is accomplished by a mechanism that involves the microtubule-severing enzyme spastin (Guizetti et al., 2011).

3 – Microtubule plus-end tracking proteins (+TIPs)

3.1 - Regulation of microtubule dynamics by +TIPs

Microtubule plus-ends are essential structures that play a crucial role in the regulation of microtubule dynamic instability. Curiously, many of the functions played by microtubules such as chromosome movement or organelle transport require interaction of these structures with the microtubule plus-ends (Howard and Hyman, 2003). A large number of MAPs specifically recognize this terminal portion of the microtubule (Table II). These are known as microtubule plus-end tracking proteins or +TIPs (for review see (Akhmanova and Steinmetz, 2008). These are different from other MAPs that normally associate with the entire microtubule lattice. When these +TIPs are labelled with a fluorescent tag, they appear as "comets" in the microtubule tip, moving throughout the cell as the microtubule grows and disappearing when the microtubule shrinks (Howard and Hyman, 2003). Despite their common localization, these different +TIPs have very distinct effects on microtubule dynamics (Table II).

The first description of tip-tracking behaviour came from work of Kreis and collaborators, when they demonstrated that Cytoplasmic LInker Protein (CLIP) 170 was able to associate with the plus-end of polymerizing microtubules (Diamantopoulos et al., 1999; Perez et al., 1999). Later, it was demonstrated that Tip1p, the fission yeast homologue of CLIP170 was capable of spatially organizing microtubule dynamics by confining catastrophes to certain cortical areas (Brunner and Nurse, 2000). The CLIP170 budding yeast homologue Bik1 also interferes with microtubule function as depletion of the protein leads to very short microtubules (Berlin et al., 1990). Association between budding yeast Bim1 (EB1) and Bik1 (CLIP170) is essential in the regulation of microtubule dynamics and, under these conditions, CLIP170 acts as a catastrophe-promoting agent (Blake-Hodek et al., 2010). Furthermore, this interaction is essential for loading CLIP170 into the plus-ends (Bieling et al., 2008; Dixit et al., 2009). Overall, both CLIP170 and CLIP115 regulate microtubule dynamics in a redundant manner through their ability to promote microtubule rescues (Komarova et al., 2002) and this relies exclusively on the N-terminal region of the proteins (Arnal et al., 2004). The exact mechanism of CLIP170-mediated microtubule rescue is still unclear but could involve stabilization of the curved protofilaments by the N-terminus of CLIP170 (Arnal et al., 2004) or binding of CLIP170 to GTP islands in the microtubule lattice, which would provide a structural basis for rescue events when catastrophes reach this point (Dimitrov et al., 2008). Interestingly, although CLIPs track only growing microtubule plus-ends, they also influence the behaviour of depolymerising microtubules. This is a puzzling observation and indicates that

CLIPs function is not totally understood. Indeed, studies of Bik1 and Kip2 reported that these proteins were observed to follow depolymerising microtubules (Carvalho et al., 2004) but, so far, there is no evidence of the same happening with human CLIP170.

End-binding (EB) proteins are currently viewed as major microtubule dynamics regulators. This happens because EB proteins interact with and are responsible for loading many of the other +TIPs to the microtubule plus-ends such as CLIP170 (Dixit et al., 2009), APC (Askham et al., 2000), ACF7/MACF1 (Subramanian et al., 2003) and CLASPs (Mimori-Kiyosue et al., 2005). Many reports have addressed the roles of EB proteins in microtubule dynamics regulation but results are still not definitive. The budding yeast homologue of EB1, Bim1, is involved in the regulation of microtubule dynamics by promoting microtubule assembly (Blake-Hodek et al., 2010) and Bim1 deletion leads to shorter and more static cytoplasmic microtubules (Tirnauer et al., 1999). Similarly, *Drosophila* EB1 also makes microtubules more dynamic by stimulating catastrophes and growth and reducing microtubule pause time (Rogers et al., 2002). In addition, in *Xenopus* egg extracts, EB1 increased the frequency of microtubule rescues and decreased catastrophes, which resulted in augmented microtubule polymerization (Tirnauer et al., 2002b). Finally, in mammalian cells, EB proteins were reported to suppress microtubule catastrophes in a process that seems to depend on dimerization, without any significant effect on microtubule growth rate or rescue frequency (Komarova et al., 2009). Inversely, studies *in vitro* demonstrated that recombinant EB1 seems to induce microtubule elongation without increasing microtubule polymerization (Ligon et al., 2003) and another report showed that EB proteins promote rather than inhibit catastrophes (Komarova et al., 2009). The discrepancy between *in vitro* and *in vivo* results could be explained by the fact that in cells, EB proteins could counteract the function of microtubule destabilizing factors, either directly, by physically shielding binding sites on the microtubules, or indirectly, by altering the microtubule tip structure (Komarova et al., 2009). The effect of EB1 on microtubule dynamics also appears to be spatially regulated. In fact, in mouse fibroblasts a complex of EB1-APC-mDia stabilizes microtubules near the cell membrane (Wen et al., 2004). Under these conditions, expression of full length EB1 was sufficient to induce microtubule stabilization. Proposed models for EB1 action on microtubules were based on *in vitro* observations that EB1 would bind and stabilize growing microtubule sheets and/or seam (Sandblad et al., 2006; Vitre et al., 2008). However, recent reports contradict this model and propose that Mal3, the fission yeast EB1, directly binds to the protofilaments (and not the seam) through the Calponin Homology (CH) domain and protects the microtubule from depolymerization (Maurer et al., 2012). Nevertheless, it seems that EB1 binding to the microtubule might promote binding of other

MAPs by stabilizing microtubule structure which favours growth over shrinkage. The roles of EB2 and EB3 in microtubule dynamics regulation are less clear. EB2 is also able to tip-track but shows a more diffuse pattern on the microtubule lattice and does not have the catastrophe-suppression activity of other EBs (Komarova et al., 2009). In addition, EB2 does not show the same preference for heterodimerization as EB1 and EB3 (De Groot et al., 2009). EB3, on the other hand, readily heterodimerizes with EB1 (De Groot et al., 2009) and shows the same ability to regulate microtubule dynamics as the later (Komarova et al., 2009). Interestingly, EB3 seems to regulate microtubule dynamics in more specialized or differentiated cell types. It was shown that it can locally regulate microtubule dynamics in the cell cortex and is essential for myoblast elongation and fusion (Straube and Merdes, 2007). In this situation, EB3 is responsible for maintaining microtubules in a more "paused" state by reducing their growth time and allowing capture and stabilization of microtubules at cortical areas, possibly through CLASPs (Lansbergen and Akhmanova, 2006; Mimori-Kiyosue et al., 2005). EB3 is also crucial in microtubule regulation of neuronal cells. In this context, EB3-mediated microtubule dynamics regulation is crucial for synaptic plasticity and dendritic spine morphology by interacting with p140/Cap and cortactin and establishes a link between the microtubule and actin cytoskeletons (Jaworski et al., 2009).

Some +TIPs do not promote microtubule growth but instead stabilize it. CLIP-associating proteins (CLASPs) and also APC seem to function this way. CLASPs are loaded onto the plus-end by EB1 (Mimori-Kiyosue et al., 2005). In the case of CLASP2, this interaction is mediated by the central region of the protein, which contains a Ser-Arg-Pro rich region with two tandemly repeated SxIP motifs. These SxIP motifs were shown to be the EB1-binding motif required for many +TIPs to tip-track on microtubules (Honnappa et al., 2009). When associated to the distal ends of microtubules, CLASPs prevent catastrophes and promote pausing and rescue events (Al-Bassam et al., 2010; Mimori-Kiyosue et al., 2005; Sousa et al., 2007). These rescue events mediated by CLASPs involve the direct binding of the protein to tubulin dimers and their incorporation on the microtubule lattice (Al-Bassam et al., 2010). The CLASP-mediated incorporation of tubulin dimers at the microtubule plus-ends is also observed in mitotic cells. In fact, MAST/Orbit, the Drosophila homologue of CLASP was shown to be required for the incorporation of tubulin subunits into K-fibres and this is necessary for microtubule poleward flux during mitosis and for maintaining a steady-state spindle length (Maiato et al., 2005). The direct binding of +TIPs to the tubulin dimer does not seem to be a conserved feature. Although CLIP170 can also directly bind to non-polymerized tubulin dimers (Diamantopoulos et al., 1999), results for EB1 are not so clear.

+TIP	Homologues	Interaction partners	Protein domain	Main functions
EB1 family (EB1, EB2, EB3)	Mal3 (Sp) Bim1 (Sc)	Most +TIPs (with SxIP and CAP-Gly domains)	Calponin homology	Regulation of microtubule growth and dynamics; loading of other +TIPs to plus-ends
CLIP family (CLIP170, CLIP115)	Tip1 (Sp) Bim1 (Sc) CLIP190 (Dm)	EB family CLASP family p150glued Cytoplasmic dynein	CAP-Gly	Microtubule rescue and stabilization; Targeting of dynein to plus-ends; microtubule interaction with cell cortex, kinetochores and vesicles
APC family (APC, APC2/APC-L)	Kar9 (Sc) APC1/2 (Dm) APR-1 (Ce)	EB family XKCM1		Microtubule stabilization; anti-catastrophe activity; interaction with cell cortex and kinetochores
CLASP family (CLASP1, CLASP2)	Peg1 (Sp) Stu1 (Sc) MAST/Orbit (Dm) Cls-2 (Ce)	EB family CLIP170 CLIP115	HEAT repeat	Microtubule rescue and stabilization; microtubule interaction with cell cortex, kinetochores and Golgi apparatus
Motor proteins (kinesin-7, kinesin-14, dynein)	**Kinesin-7** CENP-E (Hs) Tea2 (Sp) Klp2 (Sc); **Kinesin-14** Ncd (Dm) KLP2 (Sp) Kar3 (Sc); **Cytoplasmic Dynein**	EB family Bik1, Tip1 Kar9 Dynein; EB family; Dynactin (p150glued) LIS1	Plus-end directed motor Minus-end directed motor Dynein minus-end directed motor	Microtubule stabilization; promoter of microtubule growth Minus-end directed transport; antiparallel microtubule sliding; microtubule depolymerization Minus-end directed transport; pulling of microtubules at cell cortex; transport of microtubules
Kinesin-13 family (Kif2A, Kif2B, Kif2C/MCAK	XKCM1 (Xl) KLP10A (Dm)	EB family XMAP215 CLIP170 APC	Kinesin-13 motor	Microtubule depolymerization; catastrophe-inducer
TOG family (ch-TOG)	XMAP215 (Xl) Dis1 Alp14(Sp) Stu2 (Sc) Msps (Dm) ZYG-9 (Ce)	EB family Bik1 Tip1 Kar9 Dynein	HEAT repeats	Microtubule stabilization; promoter of microtubule growth; interaction with the cell cortex
Lis1	Pac1 (Sc) NudF (An)	CLIP170 (Bik1) Dynein Dynactin	WD40 repeats	Dynein activation; interaction with the cell cortex

Table II – Main +TIP families with respective homologues, essential functional domains and main cellular functions. Sc - *Saccharomyces cerevisiae*; Sp - *Schizosaccharomyces pombe*; Dm – *Drosophila melanogaster*; An – *Aspergillus nidulans*; Ce - *Caenorhabditis elegans*; Hs – *Homo sapiens*. Adapted from (Akhmanova and Hoogenraad, 2005; Lansbergen and Akhmanova, 2006).

One study describes direct association between EB1 and tubulin dimers (Juwana et al., 1999), but subsequent studies failed to find the same association (Gache et al., 2005). As with other +TIPs, CLASPs are also involved in regional regulation of microtubule dynamics through the interaction with other factors, which include PI3-kinase and GSK3β (Akhmanova et al., 2001). Strikingly, CLASPs also show a spatial asymmetry in migrating cells leading them to decorate the entire lattice and not only the plus-ends of lamella microtubules, in a process regulated by GSK3β (Kumar et al., 2009; Wittmann and Waterman-Storer, 2005).

Not all +TIPs have a positive impact on microtubule growth, as many can act as microtubule destabilizers. Members of the kinesin-13 family (such as Kif2C/MCAK) are known to be potent microtubule depolymerizers (Mennella et al., 2005; Walczak, 2003). This family of kinesins is very peculiar because, unlike other families, it possesses ATPase activity which they use to depolymerise microtubules from both ends (Desai et al., 1999; Helenius et al., 2006; Hunter et al., 2003). In fact, these kinesins are able to induce a conformational change in microtubule plus-ends which leads to their destabilization (Desai et al., 1999). Recently, MCAK was described as a major microtubule remodeler by preventing microtubule aging and inducing random catastrophes (Gardner et al., 2012). This ability of MCAK makes it important during mitosis in order to correct erroneous kinetochore-microtubule attachments but also assist in spindle assembly and chromosome congression (Kline-Smith et al., 2004; Sampath et al., 2004; Wordeman et al., 2007). In fact, a recent report clarified the role of MCAK activity at microtubule tips. This specific pool of MCAK was proposed to be essential in the restriction of spindle length and in the promotion of stable kinetochore microtubule attachments (Domnitz et al., 2012).

3.2 – Microtubule recognition by +TIPs

Accumulation at the microtubule plus-ends is what defines a +TIP (Figure 6). Although their localization can be confined to a small terminal region of the microtubule, they employ different mechanisms to recognize and move along microtubules. This section will focus on how +TIPs recognize the plus-end and how they are able to move along the microtubule lattice.

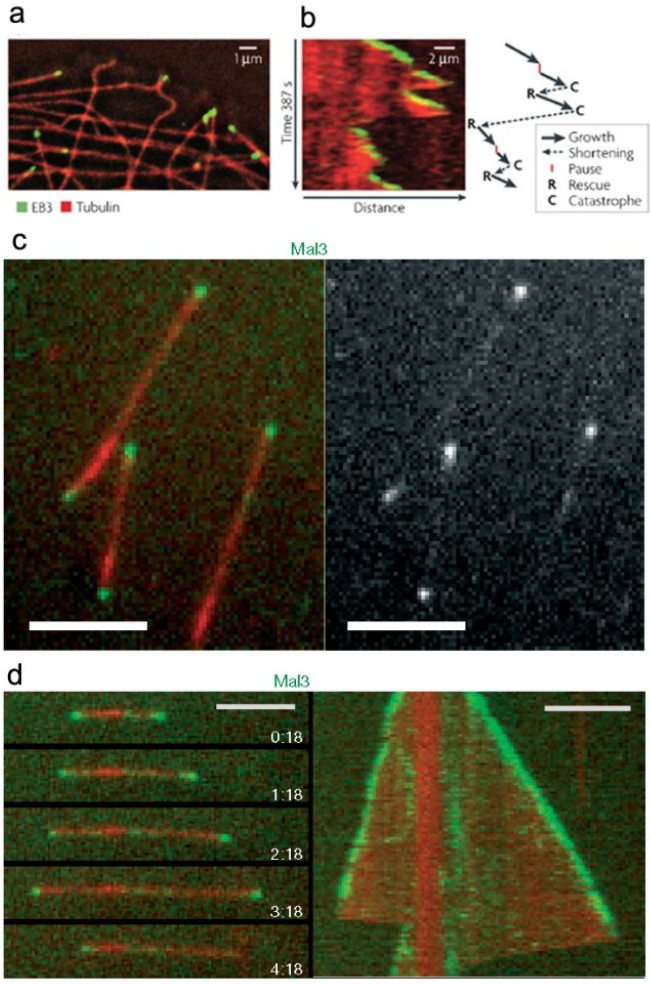

Figure 6 – Association of EB proteins with microtubule plus-ends. (a) EB3 tagged with GFP associates with the plus-ends of microtubules in cells. (b) A representative kymograph of a microtubule labelled with EB3-GFP showing dynamic instability. (c) Overlaid TIRF images of Mal3-488 and dynamic Alexa-568 labelled microtubules (left) and Mal3-488 only (right). (d) EB3 associates only with the growing ends of microtubules and dissociates during catastrophes. In an *in vitro* system, Mal3 autonomously associates with both plus- and minus-ends of growing microtubule in the absence of any other factors, but is much more effective in plus-end association. Red, tubulin; green, +TIP. Scale bars, 5 µm. Adapted from (Akhmanova and Steinmetz, 2008; Bieling et al., 2007).

Tip tracking behaviour implies that +TIPs must either have the ability to directly bind tubulin or microtubules or, in alternative, be recruited indirectly through binding to other factors. The fact that many different classes of proteins can exhibit tip-tracking (Table 1), led to the proposal of four models to account for this behaviour: end binding, copolymerization, directed transport and hitchhiking. Curiously, it seems that the same +TIP can exhibit different behaviours depending on the conditions or on the organism. For example, in mammalian cells,

APC can be loaded to the plus-ends in an EB1-dependent manner (Slep et al., 2005) but it can also tip-track autonomously (Kita et al., 2006). On the other hand, loading of CLIP170 to plus-ends can be mediated by motors in yeast (Busch et al., 2004; Carvalho et al., 2004; Maekawa and Schiebel, 2004), whereas in mammalian cells it involves direct binding and treadmilling on plus-ends (Perez et al., 1999).

Recognizing the microtubule end

How is it that some +TIPs are able to directly associate to the growing end of microtubules? This question is of great importance because EB proteins are responsible for loading the majority of other +TIPs, including CLIPs, CLASP and APC (Lansbergen and Akhmanova, 2006) and can influence drastic changes in microtubule dynamics. This means that they must recognize specific structural features on plus-ends that are different from the lattice (Figure 7). The first obvious hypothesis is the GTP cap itself. Recently, it was reported that introducing GTPγS (a slowly hydrolysable form of GTP) on plus-ends, mimicked the EB-binding site (Maurer et al., 2011). This is in line with a report stating that EB1 can recognize the nucleotide state of tubulin independently of its location. Under these conditions, EB1 recognizes the GMPCPP microtubule lattice as opposed to the GDP lattice (Zanic et al., 2009). This was extended further, when a different study revealed that EBs can recognize the nucleotide state of the plus-end and this is crucial both for EB binding and for stabilizing a structural cap that protects the microtubule from depolymerization (Maurer et al., 2012). Although it is tempting to assume that the nucleotide state of tubulin alone is sufficient to determine plus-end binding, there is evidence that argues against such a simple model. In fact, the GTP cap size is thought to be very small when compared to the region decorated by the EB comet. Typical comets can vary between 0.5-3 μm in length, depending on the growth rate but not +TIP concentration (Bieling et al., 2007). This means that comets have to encompass several hundreds or thousands of tubulin subunits, which is much bigger than the assumed GTP cap size. It should be noted, however, that recent studies propose the existence of longer GTP caps that exhibit dynamic behaviour and could partly account for this discrepancy (Schek et al., 2007).

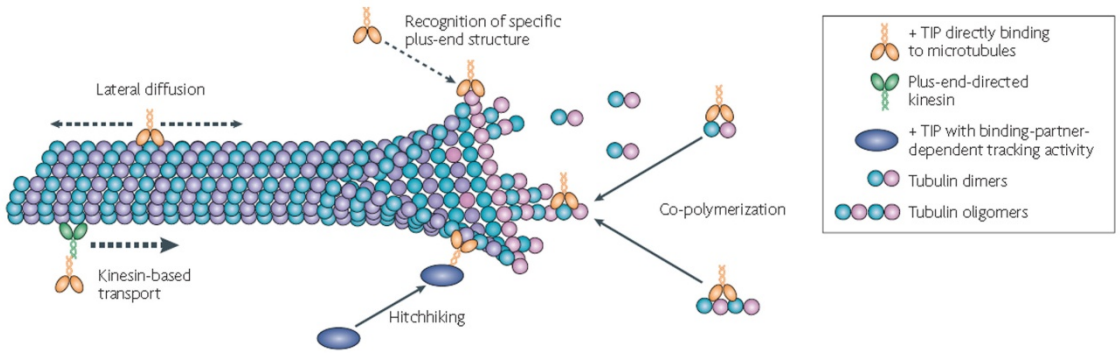

Figure 7 – Mechanisms of plus-end recognition by +TIPs. +TIPs can arrive at the plus-end by diffusion along the microtubule lattice or diffusion in the cytoplasm. In alternative, they can be transported by kinesins or associate with the growing end of microtubules by attaching to another +TIP (hitchhiking). Some +TIPs can recognize special structural features of the plus-ends of microtubules or they may co-polymerize with tubulin dimers or oligomers.

Furthermore it was suggested that EB1, instead of binding the protofilaments themselves (Maurer et al., 2012), could bind to tubulin while still in the sheet conformation. This means that EB1 would promote sheet closure and bind to the microtubule seam instead of the protofilaments (Vitre et al., 2008). In fact Mal3, the EB1 homologue, was reported to act as a molecular zipper by binding to the seam and leading to changes in microtubule structure (des Georges et al., 2008; Sandblad et al., 2006). Overall these results indicate that the nucleotide state of tubulin plays an important role in plus-end binding but there may be additional mechanisms that contribute to +TIP-microtubule association. An alternative explanation for specific EB association to the plus-end could depend on the electrostatic interactions between the C-terminal portion of EB1 and the microtubule lattice (Buey et al., 2011). The authors propose that long-range electrostatic repulsive interactions between the C-terminus of EB1 and the microtubule lattice are able to drive accumulation of EBs on growing microtubule ends. In fact, replacing the negatively charged C-terminal portion for a neutral coiled-coil increased dwell time of EB1 on the microtubule without affecting interaction with the plus-end. Other possible mechanisms may involve the post-translational modification of EB proteins themselves. In fact, recent reports demonstrated that phosphorylation of EB proteins could have an important role in their association to the plus-end. One study described a mutation on the linker region of Mal3 that is sufficient to reduce the affinity of the protein for microtubules (Iimori et al., 2012) while another demonstrated that phosphorylation of Bim1p

by Aurora/Ipl1p was sufficient to remove the +TIP from static and dynamic microtubules (Zimniak et al., 2009). Curiously, the same authors demonstrate that both dimerization of Bim1p and the presence of the linker domain are both required for efficient tip tracking (Zimniak et al., 2009).

Copolymerization

In addition to recognizing microtubule plus-ends, some +TIPs such as CLIP170 also have the ability to directly bind tubulin subunits (Arnal et al., 2004; Folker et al., 2005). This means that, in order to tip-track these proteins must copolymerize with tubulin into the microtubule and then quickly dissociate from the older part of the microtubule as it grows (Akhmanova and Hoogenraad, 2005). In addition, these +TIPs must have a higher affinity for free GTP tubulin subunits than the GTP or GDP polymer itself. Association of CLIP170 to free GTP tubulin subunits is thought to occur through its CAP-Gly domain which is able to bind directly the EEY/F motif on the C-terminal α-tubulin tail (Mishima et al., 2007). Interestingly, the CAP-Gly domain of CLIP170 also interacts with EB1 and explains how it recognizes a composite binding site on microtubules plus-ends composed of EB1 (including its C-terminal tyrosine) and tyrosinated α-tubulin (Bieling et al., 2008; Mishima et al., 2007). Taken together, these results provide a model for copolymerization of CLIP170 with tubulin, but they do not explain how it dissociates from the growing microtubule.

While copolymerization seems to explain CLIP170 behaviour, it falls short of explaining general +TIP behaviour. Firstly, other +TIPs such as EB1 do not use the copolymerization mechanism. In fact, EB1 seems to bind the tubulin polymer but not the individual subunits (Gache et al., 2005). Furthermore, *in vitro* tracking systems were able to recreate plus-end tracking without the presence of enzymes, which means that you can have tip-tracking behaviour independently of GTP or GDP and argues against its role in microtubule recognition (Bieling et al., 2008; Bieling et al., 2007; Maurer et al., 2011). Recent experiments using Fluorescence Recovery After Photobleaching (FRAP) demonstrated that +TIPs associate very transiently with the plus-end of microtubules (Dragestein et al., 2008; Wittmann and Waterman-Storer, 2005). Furthermore, turnover measurements of CLIP170 and EB3 demonstrated that they show diffusion both at the plus- and the minus-ends of microtubules, which is inconsistent with the copolymerization model (Dragestein et al., 2008). Using reconstituted *in vitro* systems it was possible to demonstrate that Mal3 did not bind tubulin subunits and that accumulation of Mal3 on plus-ends was independent of tubulin

concentration (Bieling et al., 2007). Taken together, these results argue against the role of copolymerization as the major contributor to plus-end tracking.

Dissociation from the plus-end

The balance between association-dissociation must be tightly regulated so that +TIPs remain confined to the plus-end of microtubules. This is clearly seen when +TIPs are over-expressed and label the entire microtubule lattice (Schwartz et al., 1997; Tirnauer and Bierer, 2000). Dissociation of +TIPs from the microtubule may involve changes in the microtubule lattice (such as GTP hydrolysis) or a structural change in the +TIP itself (Akhmanova and Hoogenraad, 2005). One very convenient mechanism would be +TIP phosphorylation. In fact, CLIP170 association to the microtubule is negatively regulated by phosphorylation (Rickard and Kreis, 1991). So far, several kinases have been described to affect CLIP170 phosphorylation, including mTOR (Choi et al., 2002), Plk1 and CK2 (Li et al., 2010) and AMPK (Nakano et al., 2010). Surprisingly, inhibition of mTOR decreases the binding of CLIP170 to microtubules, whereas inhibition of AMPK increases the binding of CLIP170 to microtubules. This data emphasizes that multiple layers of regulation must exist that control association of CLIP170 to the microtubule. Experiments with the EB1 homologue in yeasts have also suggested phosphorylation as a possible mechanism for EB binding to microtubules in a cell cycle dependent manner (Iimori et al., 2012; Zimniak et al., 2009). However, there is no evidence so far for a phospho-regulatory mechanism that specifically controls association/dissociation of individual +TIPs to microtubules other than affecting the total +TIP pool.

While the mechanism that regulates +TIP association to the microtubule is still intriguing, considerable progress has been made in how these proteins turnover at the plus-end. The first model proposed that +TIPs bind only once to the plus-end then dissociate when the microtubule lattice becomes “mature” (Carvalho et al., 2003; Galjart, 2005). This is called **treadmilling** because of the similarities with the flux of tubulin subunits within the microtubule (Figure 8). While it seems that +TIPs are moving along as the microtubule grows, they are in fact stationary and it is the addition of new +TIPs and tubulin at the plus-end that creates this optical illusion (Carvalho et al., 2003). The treadmilling model also implies that fluorescence decay observed in the comet`s tail is due to the dissociation of +TIPs as the microtubule matures. Initial approaches using Fluorescent Speckle Microscopy (FSM) techniques proposed

that EB1 and CLIP170 probably used treadmilling on microtubule plus-ends (Perez et al., 1999; Tirnauer et al., 2002b; Waterman-Storer et al., 1998).

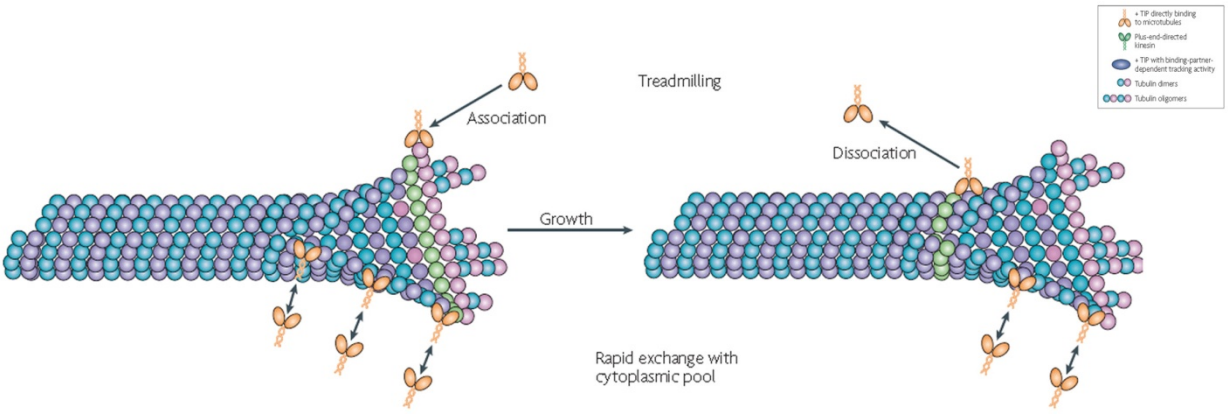

Figure 8 – Mechanisms of +TIP dissociation from the microtubule. +TIPs that associate with the microtubule may remain attached to the structure until the plus-end is converted into a regular lattice (highlighted in green) and then dissociates, which is known as treadmilling. On the other hand, +TIPs may exchange rapidly with the cytoplasmic pool at their binding sites in the plus-end, while these sites decay exponentially over time. Adapted from (Akhmanova and Steinmetz, 2008).

However, recent studies demonstrated that this is not the case. Based on single molecule studies and FRAP experiments of microtubule plus-ends (Bieling et al., 2007; Dragestein et al., 2008; Kumar et al., 2009), a model of **fast exchange** was proposed (Figure 8). This is supported by the observation that the microtubule +TIP decoration time is much longer than the dwell time of single molecules of Mal3 (Bieling et al., 2007). This means that Mal3 molecules must continuously turnover on the plus-end until it changes into a "mature" microtubule structure. In addition, FRAP experiments demonstrated that individual +TIPs are very dynamic and can repeatedly bind to the same plus-end with low affinity. Both CLIP170 and EB3 molecules exhibit a rapid turnover behaviour on plus-ends as shown by FRAP experiments (Dragestein et al., 2008). This rapid turnover means that several molecules can bind to the same binding site on the microtubule and continuously exchange with the cytoplasmic pool, as was shown for EB3 (Dragestein et al., 2008). Further studies confirmed that EB1 also exhibited the same behaviour (Dixit et al., 2009). Taken together this means that +TIP turnover is much higher than binding site turnover, which is in disagreement with the treadmilling model. As a consequence, these experiments show that accumulation of +TIPs in a comet-like structure depends on the exponential decay of EB binding sites in the microtubule. EBs will bind and dissociate very rapidly which creates a large number of binding sites for other

+TIPs. This is in agreement with studies that demonstrate a necessity of CLIP170 to bind simultaneously to EB1 and tubulin composite sites (Bieling et al., 2008). In addition, other +TIPs also show a slower dissociation rate from the microtubule, when compared to EB proteins (Bieling et al., 2007; Dragestein et al., 2008), which further supports the role of EBs in facilitating the binding of +TIPs to microtubules.

Diffusion vs motor-based transport

Accumulation of +TIPs does not necessarily involve direct binding to the plus-end in all situations. Sometimes +TIPs will bind to the lattice and move towards the plus-end of the microtubule where it accumulates. To do so, these proteins use two different mechanisms: **simple diffusion** and **motor-based transport**.

Diffusional motility is defined as a one-dimensional walk along the microtubule lattice driven solely by thermal energy (Cooper and Wordeman, 2009). Simple diffusion of molecules along a microtubule is a simple, low-energy mechanism that also has the advantage of allowing bi-directional movement (Figure 9). This mechanism is represented by the same mathematical equations that define Brownian motion, although diffusion coefficients tend to be smaller (Ali et al., 2007; Gestaut et al., 2008; Helenius et al., 2006). The first observations of single molecule diffusional motility on microtubules was performed using non-processive kinesin motors (Inoue et al., 2001; Okada and Hirokawa, 1999). While kinesin motor proteins usually "walk" along microtubules using ATP hydrolysis, they can sometimes show a "biased diffusion". This has already been demonstrated for a number of kinesins which include KIF1A, CENP-E, Ncd and Eg5 (Furuta and Toyoshima, 2008; Kim et al., 2008; Kwok et al., 2006; Okada and Hirokawa, 1999). It has been proposed that this type of motor protein motility occurs when the motor domain is not so tightly bound to the microtubule. In accordance, in experiments where ADP is added instead of ATP, these proteins exhibit a pure diffusional movement presumably because of weaker binding to the microtubule (Kwok et al., 2006; Okada and Hirokawa, 1999). In addition to kinesins, dynein and other MAPs have also been shown to exhibit this type of motility. Dynein molecules exhibit a combination of processive movement and dimensional diffusion which is blocked when the ATPase domain of dynein is inhibited, suggesting a bidirectional movement (Reck-Peterson et al., 2006; Vale et al., 1989). In addition, certain microtubule associated proteins show diffusional motility on the

microtubule lattice such as XMAP215 (Brouhard et al., 2008), Ndc80 (Powers et al., 2009), Tau (Konzack et al., 2007), the DAM1 complex (Ramey et al., 2010) and MCAK (Helenius et al., 2006). Interestingly, MCAK not only diffuses along the microtubule but also recognizes the curvature of the microtubule tip and interacts with EB proteins, which suggests that it could also bind directly to the plus-end through these proteins (Lee et al., 2008; Moore et al., 2005).

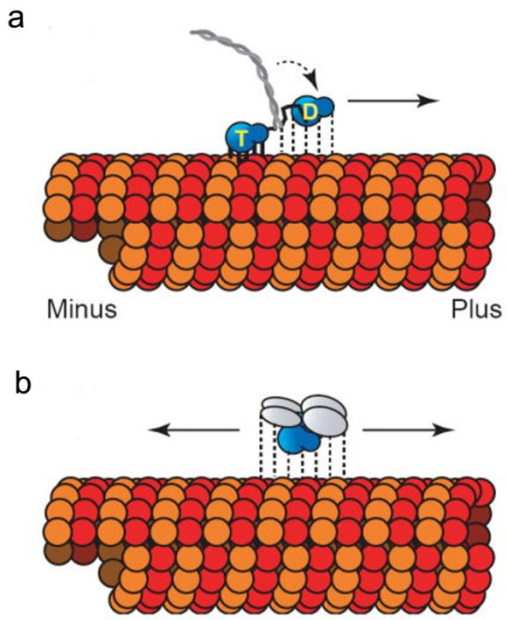

Figure 9 – Motor-based and diffusive modes of microtubule directed motility. Proposed **m**odel for motor-based directional motility on microtubules (a). The two motor domain heads can be strongly bound (solid lines) or weakly bound (dashed lines) to the microtubule. The strongly bound head has ATP (labelled "T") in its nucleotide-binding pocket, while the weakly bound head has ADP (labelled "D"). Proposed model of unbiased diffusional motility of molecules on microtubules (b). Depicted in the model is MCAK with its motor domain in blue and N-terminal and C-terminal dimerization domains in gray. This model allows random movement of the molecule in either direction along the microtubule lattice without losing attachment. Adapted from (Cooper and Wordeman, 2009).

This type of diffusion appears to occur ubiquitously and provides some advantages over motor-based movement. Firstly, it makes the system more flexible by allowing unbiased binding of proteins at both microtubule ends. Interestingly, many microtubule associated

proteins (such as MCAK) that require localization at both the plus- and minus-end of microtubules use this mechanism (Oguchi et al., 2011). Secondly, by making weaker attachments to the lattice, in theory it could allow these proteins to overcome obstacles that may exist along the microtubule by jumping between protofilaments in a side-step manner (Wang et al., 1995). Thirdly, diffusion does not require ATP consumption to move proteins. Finally, over short distances (<1 μm) diffusional motility is faster than directed motility which may allow quicker delivery of molecules to the plus-ends (Cooper and Wordeman, 2009). However, because it is a random process, it is very ineffective over longer distances.

Contrary to unbiased diffusional motility, molecules can exhibit a directional movement on microtubules that is dependent on motor proteins. This mechanism requires the action of kinesin motor proteins which can contribute to plus-end accumulation. However, kinesin action alone is not sufficient to induce the formation of a comet, as there must be some retention mechanism that allows the +TIP to remain associated with the plus-end (Galjart and Perez, 2003). CLIP170 homologues Tip1 and Bik1 are transported to the plus-end by the action of kinesins Tea2 and Kip2, respectively (Busch et al., 2004; Carvalho et al., 2004). Interestingly, Tea2 action requires interaction with the EB homologue Mal3 both for loading and tip-tracking behaviour (Bieling et al., 2007). Although not so common in mammals, APC also requires kinesin-2 for plus-end accumulation (Jimbo et al., 2002) and myosin Va (an actin-based motor) also interacts with plus-ends through EB1 (Wu et al., 2005). In addition, +TIPs CLIP170 and dynactin can also recruit motor proteins such as dynein to the plus-end which will allow minus-end transport of vesicular cargo (Lansbergen et al., 2004; Vaughan et al., 1999; Vaughan et al., 2002). Interestingly, +TIPs that are transported by motors can also track depolymerising microtubules in a mechanism known as backtracking (Carvalho et al., 2004). This accumulation occurs if the motor transport velocity is higher than microtubule polymerization/depolymerization velocity (Busch et al., 2004; Carvalho et al., 2004).

Hitchhiking

Loading +TIPs via a motor-based transport requires that they hitchhike on motor proteins. However, most +TIPs accumulate at plus-ends indirectly by hitchhiking on other +TIPs (Lansbergen and Akhmanova, 2006). This implies that a core component of +TIPs must exist that is able to associate to microtubules independently of any other factor (Figure 7). EB proteins can perform this function because it has been shown that they can autonomously

track microtubule plus-ends (Bieling et al., 2008; Bieling et al., 2007), and are known to interact with most other known plus-end associated proteins. The interaction of EBs with other +TIPs occurs through the EB C-terminal domain (also known as End Binding Homology – EBH – domain) (Bu and Su, 2003). The list of interactors includes APC, CLIP170, MCAK, CLASPs, ACF7/MACF1, Stim1, p150glued. Although all these proteins share the ability to tip-track, there is no apparent functional similarity between them. Thus, one may ask: is there any common feature between them that accounts for their behaviour? In fact, it is now established that these interactions occur through the SxIP and CAP-Gly domains.

Proteins that have a CAP-Gly domain were the first to be identified that exhibit tip-tracking behaviour (Perez et al., 1999) and these include CLIP170, CLIP115 and p150glued, among others (Steinmetz and Akhmanova, 2008). The CAP-Gly domain is highly conserved in eukaryotes, can exist in either single or multiple copies and is involved in the regulation of protein interactions and formation of protein networks (Akhmanova and Steinmetz, 2008; Galjart, 2005). Structural data derived from X-ray crystallographic analyses of these domains demonstrated that the conserved motif GKNDG is essential for interaction with the EEY/F motif of α-tubulin, EB1 and CLIP170 (Steinmetz and Akhmanova, 2008; Weisbrich et al., 2007). CAP-Gly proteins are unable to bind tubulin dimers that lack the C‑terminal tyrosine of EE Y/F (Peris et al., 2006) and moreover, mutations in the Lys-Asn of the GKNDG or the EEY/F motives are sufficient to abolish this interaction (Steinmetz and Akhmanova, 2008; Weisbrich et al., 2007). These interactions with CAP-Gly proteins have dissociation constants in the micromolar range, which is similar to what is observed for the interaction of the C-terminal of EB1 (EB1c) with an APC C-terminal peptide (APCp1) and indicates they are very dynamic (Honnappa et al., 2005; Honnappa et al., 2006; Mishima et al., 2007; Weisbrich et al., 2007).

In addition to CAP-Gly interactions, proteins can also use the SxIP motif to bind to EB proteins and hitchhike on microtubules. The first report on the role of a Ile-Pro peptide in +TIP interaction came from structural work on the EB1c-APC interaction (Honnappa et al., 2005). The authors demonstrated that this Ile-Pro peptide was part of the APC region that bound EB1 and that mutating it was sufficient to impair the interaction. Further studies revealed that many +TIPs such as MCAK, CLASPs, APC and ACF7/MACF1 have a similar motif embedded within basic and proline/serine rich sequence regions: the Ser-x-Ile-Pro (SxIP) motif (Honnappa et al., 2009). In addition, it was also demonstrated that this SxIP motif is sufficient to load these +TIPs to the microtubule plus-ends through interaction with EB1. Two interesting observations were derived from this study: it is possible to abolish the interaction between these +TIPs and EB1 and, as a consequence, inhibit tip-tracking by simply mutating the SxIP

motif (for instance by substituting the Ile-Pro with Asparagines); it also became possible to "transform" a protein into a +TIP by introducing the SxIP motif in its aminoacid sequence. Taken together, these findings allow the establishment of a general microtubule tip localization signal (MtLS) and create a unifying mechanism for plus-end targeting.

Conceptually, the hitchhiking mechanism implies that +TIPs that use this mechanism are not able to interact efficiently with tubulin or microtubules, but this is not always the case. Notably, proteins that contain CAP-Gly domains can efficiently associate with tubulin (Dixit et al., 2009; Folker et al., 2005; Mishima et al., 2007). In addition, although some proteins with SxIP domains such as RhoGEF2 and melanophilin do not bind tubulin directly, many others are able to do so (Al-Bassam et al., 2010; Helenius et al., 2006; Rogers et al., 2004; Wu et al., 2005). Although direct binding to tubulin or microtubules circumvents the necessity for hitchhiking, it seems that plus-end accumulation of this class of +TIPs depends on the hitchhiking mechanism. In fact, CLIP170 requires EB1 to tip-track (Dixit et al., 2009) and both EB1 and EB3 enhance the binding of CLIPs to the microtubule plus-ends (Komarova et al., 2005). Interestingly, hitchhiking seems to be necessary for the loading but not dissociation of +TIPs. These results were based on observations that CLIP170 (which hitchhikes on EB1) *in vitro* remains associated with the microtubule longer than EB1 itself (Dixit et al., 2009). This probably happens because CLIP170, besides binding to EB1, is able to bind directly to the C-terminal tails of tubulin (Mishima et al., 2007).

3.3 – CLIP family

The CLIP family of proteins is comprised of two members in mammalians: CLIP170 and CLIP115. Notably, CLIP170 was first identified as a linker between microtubules and endocytic carrier vesicles (Pierre et al., 1992) and later was the first protein shown to exhibit a tip-tracking behaviour (Perez et al., 1999). CLIP115 is a brain-specific CLIP that shares functional similarities with CLIP170 (De Zeeuw et al., 1997). These proteins have a characteristic CAP-Gly domain (Figure 10) which is necessary for interaction with tubulin and EB1 (Weisbrich et al., 2007). These CAP-Gly domains are surrounded by basic, serine-rich residues that assist in the binding to microtubules (Hoogenraad et al., 2000). In order to perform its function, CLIP170 was reported to exist as a parallel homodimer. Each monomer is composed of an N-terminal domain required for microtubule binding (with two CAP-Gly domains per monomer), a central coiled-coil domain required for dimerization and a C-terminal metal-binding domain (with two

zinc fingers per monomer; Figure 10) (Gupta et al., 2009b; Pierre et al., 1994; Scheel et al., 1999). Both the CAP-Gly domains at the N-terminus and the zinc fingers at the C-terminus are thought to play an important role in the auto-regulation of CLIP170 (Hayashi et al., 2007; Lansbergen et al., 2004). In accordance, it was shown that they can interact with each other, creating a doughnut shaped molecule that no longer interacts with microtubules. In addition, these auto-inhibitory interactions use the same binding determinants as CLIP170's intermolecular interactions with p150glued, suggesting that regulation of microtubule binding by +TIPs occurs through direct competition between homologous binding interfaces (Hayashi et al., 2007). Although CLIP115 lacks the C-terminal domain of CLIP170, they share a similar N-terminal domain, which means they could regulate microtubule dynamics in a similar fashion (Hoogenraad et al., 2000; Komarova et al., 2002). In fact, both *in vivo* and *in vitro* studies using the N-terminus of CLIP170 demonstrated that these proteins act by preventing catastrophes or promoting rescue events (Arnal et al., 2004; Komarova et al., 2002). This is in agreement with earlier studies which showed that fission yeast Tip1 prevented microtubule catastrophes (Brunner and Nurse, 2000) and that loss of Bik1 in budding yeast lead to very short microtubules (Berlin et al., 1990). Taken together, these studies demonstrate that CLIPs can stabilize interphase microtubules, promoting rescues or inhibiting catastrophes (Steinmetz and Akhmanova, 2008). Furthermore, CLIP170 interacts with the minus-end directed motor dynein and is also required for its recruitment to the plus ends (Lomakin et al., 2009; Vaughan et al., 1999). This interaction is most likely indirect and occurs through p150glued/dynactin. It is known that CLIP170 associates with microtubules and EB1 through the CAP-Gly domains and dynactin interacts with CLIP170 through the zinc fingers in the C-terminus (Gupta et al., 2009a; Lansbergen et al., 2004). Curiously, this mechanism also provides an explanation for the original function of endocytic vesicle transport that was described for CLIP170 (Pierre et al., 1992).

Figure 10 – **Typical structure of a CLIP-like protein**. CLIPs contain Basic/Ser stretches (blue). In addition, there are CAP-Gly domains (pink) which interact with EB proteins. Yellow represents a coiled coil region. Orange boxes represent the Zinc Fingers. Red highlights the EEY/F sequence.

Besides their role in interphase, CLIPs also play an important role in mitosis. CLIP170 was shown to localize to kinetochores upon mitotic entry (Dujardin et al., 1998). Subsequent works demonstrated that CLIP170 is necessary for mitotic progression (Wieland et al., 2004). CLIP170 localizes specifically to unattached kinetochores and facilitates the formation of kinetochore-microtubule attachments (Tanenbaum et al., 2006). However, the mitotic defects that were attributed to loss of CLIP170 in cultured cells were not confirmed in mouse models of CLIP170 deficiency, which raises the possibility that loss of CLIP170 *per se* is not essential for establishing kinetochore-microtubule attachments (Akhmanova et al., 2005). Interestingly CLIP190, the *Drosophila* homologue of CLIP170, was also reported to localize to unattached kinetochores and this was shown to be dynein/dynactin-dependent (Dzhindzhev et al., 2005). In addition to its more general roles in interphase microtubule regulation and mitotic kinetochore-microtubule attachments, CLIP170 was also reported to have a role in spermatid manchette development (Akhmanova et al., 2005) and to be overexpressed in Reed-Sternberg cells of Hodgkin's disease (Bilbe et al., 1992). The other family member CLIP115 also plays an important role in the nervous system, consistent with its brain-specific localization. In fact, mutations in the *CYLN2* gene (which encodes CLIP115) in mice were associated with brain abnormalities which are reminiscent of Williams Syndrome (Akhmanova et al., 2005).

3.4 – EB family

EB proteins are part of a highly conserved family that, in mammalians, comprises three members encoded from three separate MAPRE genes: EB1, EB2 (RP1) and EB3 (EB3F) (Su and Qi, 2001). EB1 was the first member identified in a yeast two hybrid screen as an interactor of the C-terminus of the Adenomatous Polyposis Coli (APC) tumour suppressor protein, thus receiving the name End Binding protein (Su et al., 1995). While there is only one form of EB1, EB2 can be found in two alternative forms derived from alternative initiation codons. EB3, on the other hand, has two forms expressed from alternatively spliced mRNAs (Su and Qi, 2001). Both EB1 and EB3 seem to be ubiquitously expressed, whereas EB2 expression is restricted to only certain cell types (Su and Qi, 2001). Normally, EB1 is expressed in higher levels when compared to other EBs. However, EB3 is also highly expressed in specific cell types. It was originally reported in neurons, where it was shown to interact with a brain specific form of APC (APC2), but it is also highly expressed in muscle cells (Nakagawa et al., 2000; Straube and Merdes, 2007). EB proteins are also widely expressed in other organisms such as *S. pombe* (Mal3) (Beinhauer et al., 1997), *S. cerevisiae* (Bim1)(Schwartz et al., 1997), *Xenopus* (Mimori-

Kiyosue et al., 2000b), *Drosophila* (Rogers et al., 2002) and plants (AtEB1a/AtEB1-Homolog2, AtEB1b/AtEB1, and AtEB1c/AtEB1-Homolog1)(Chan et al., 2005; Chan et al., 2003; Mathur et al., 2003).

EBs are relatively small elongated proteins (around 32 kDa) with conserved structural features (Figure 11). All EB members have an N-terminal, microtubule-binding portion containing a calponin homology (CH) domain with a highly conserved fold (Akhmanova and Steinmetz, 2008). The structural basis for EB1 binding to microtubules have been described recently (Hayashi and Ikura, 2003; Slep and Vale, 2007). It was shown that this CH domain is both required and sufficient for binding to microtubule plus-ends (Hayashi and Ikura, 2003; Komarova et al., 2009). The C-terminal portion of EB1, on the other hand, contains a coiled-coil region which is necessary for EB dimerization (Su and Qi, 2001).

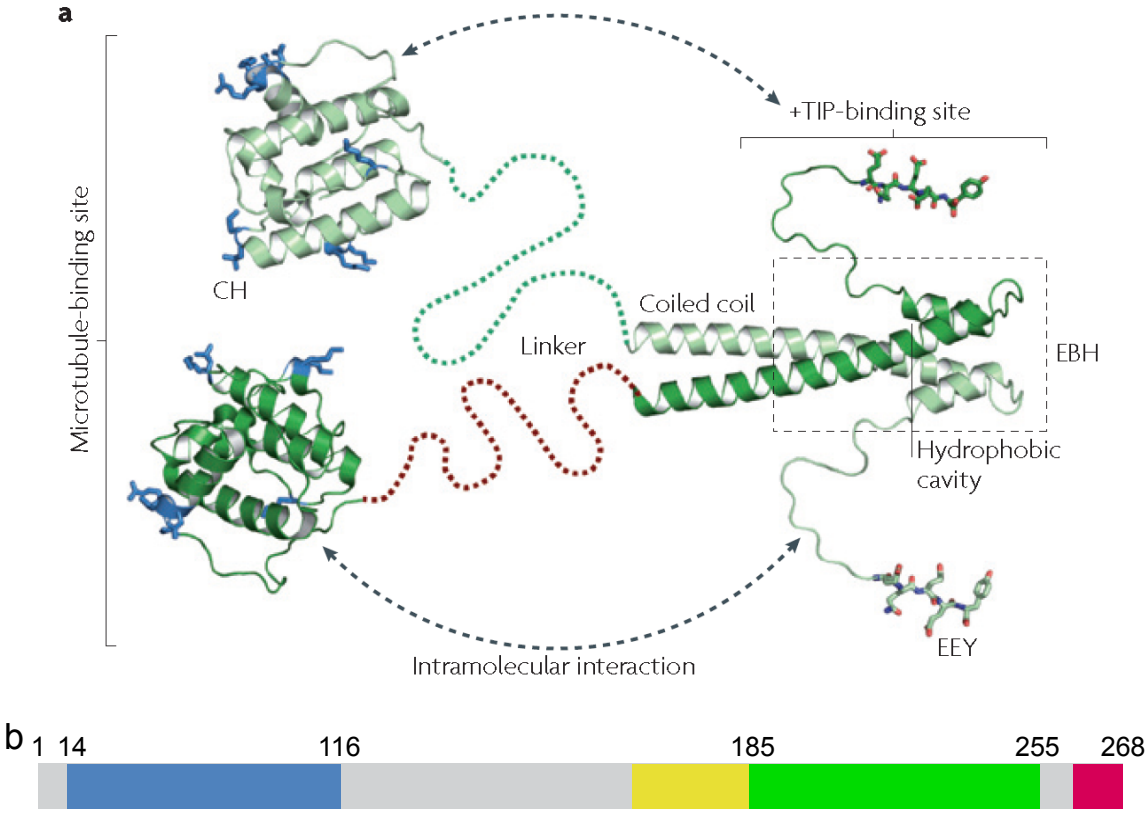

Figure 11 – (a) Molecular model of homodimeric EB protein structure (adapted from Akhmanova and Steinmetz, 2008) and (b) diagram highlighting the main structural domains of EB proteins. The structure of the Calponin Homology (CH) domain and the Coiled Coil are depicted as ribbons. The CH domain is responsible for microtubule binding, while the ENH domain is required for interaction with partner proteins. Dashed curved lines indicate the flexible linker region. Blue represents the CH domain. Yellow represents the coiled coil region. Green highlights the EBH domain and red represents the EEY/F sequence.

This is essential not only because they need two CH domains to interact with microtubules but also to form the functional C-terminal domain (Buey et al., 2011; Honnappa et al., 2005). Recently it was shown that EB1 and EB3 prefer heterodimerization to EB1/EB1 or EB3/EB3 homodimers (De Groot et al., 2009). This chain exchange between EBs can be suppressed by specific EB interaction partners, which indicates an extra layer of regulation of EB function (De Groot et al., 2009). Furthermore, the authors also demonstrate that EB2 does not show preferential association with any other EB member. The coiled-coil region partially overlaps the End Binding Homology (EBH) domain, which was shown to be required for efficient interaction with EB binding partners (Akhmanova and Steinmetz, 2008; Bjelic et al., 2011). Solving the C-terminal structure of EB1 (EB1c) by X-ray crystallography demonstrated that the coiled-coil terminates in a 4-helix bundle with a hydrophobic cavity (Honnappa et al., 2005; Slep et al., 2005). In addition, EB1c has an EEY/F motif that is very similar to the one found in α-tubulin and CLIP170 (Komarova et al., 2005; Mishima et al., 2007; Weisbrich et al., 2007). As was described above, this motif might be important to help in the regulation of EB1/CLIP170/tubulin association (Bieling et al., 2008; Mishima et al., 2007). Both EB1 and EB3 have very similar structures, which is highlighted by the fact that they share a functional similarity (Komarova et al., 2005). On the other hand, EB2 appears to have fewer similarities with the other two family members. Not only are the interaction partners substantially different between this and other EBs, but EB2 does not readily dimerize with EB1 or EB3 nor does it promote persistent microtubule growth or restore CLIP association to the microtubule plus-ends (De Groot et al., 2009; Komarova et al., 2009; Komarova et al., 2005). In fact, EB2 does not interact to the same extent with MCAK, APC or CLIP170 (Bu and Su, 2003; Komarova et al., 2005; Lee et al., 2008). This can be explained by the fact that the C-terminal domain of EB2 is significantly different from EB1 and EB3, with fewer acidic residues. Furthermore, EB2 has a longer N-terminal region, containing approximately 40 aminoacids in excess when compared to EB1 and EB3 (Komarova et al., 2009). Interestingly, this difference in the N-terminal domain is clustered around the sequence SRHD in the CH domain, which is essential for microtubule binding and can explain the differences observed between EB2 and the other family members in this aspect (Komarova et al., 2009).

EB proteins are associated with microtubule plus-ends both in interphase and mitotic cells [Figure 12; (Berrueta et al., 1998; Mimori-Kiyosue et al., 2000b; Morrison et al., 1998)]. The first report regarding the possible role of EB proteins in microtubule dynamics came from the observation that, when over-expressed, these proteins induced the formation of microtubule bundles that were resistant to nocodazole treatment and were acetylated (Bu and

Su, 2001). In addition, their ability to tip-track microtubules led to the possibility that they might be involved in microtubule dynamics regulation, particularly in promoting microtubule growth (Nakamura et al., 2001; Tirnauer and Bierer, 2000). This was confirmed in many independent studies using different model organisms such as budding and fission yeast, Drosophila and human cells but also *in vitro* systems (Beinhauer et al., 1997; Coquelle et al., 2009; Komarova et al., 2009; Nakamura et al., 2001; Rogers et al., 2002; Tirnauer et al., 1999).

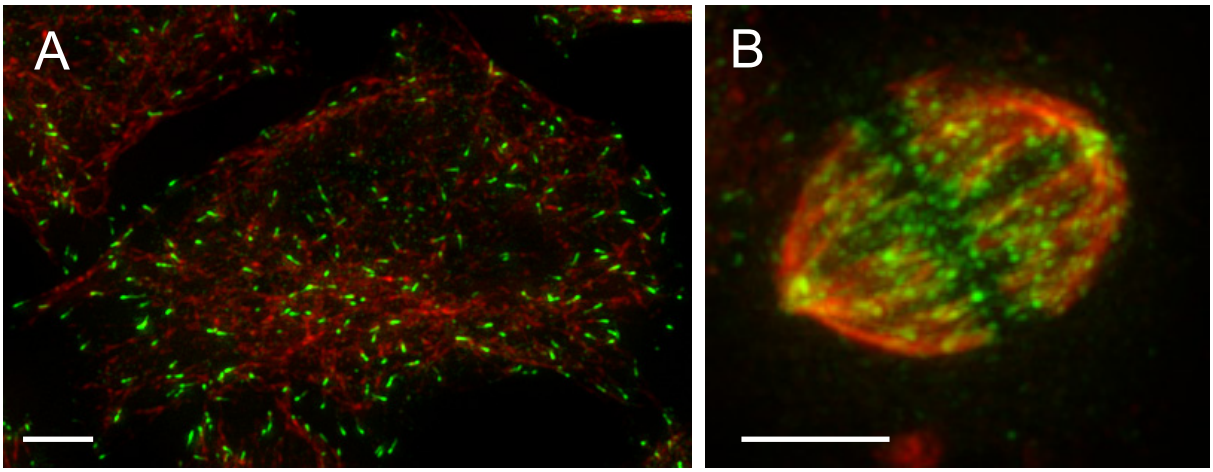

Figure 12 – Immunolocalization of EB1 during (A) interphase and (B) mitosis. EB proteins associate with the growing ends of microtubules throughout the cell cycle. In addition, EB1 also associates with the centrosome. Scale bars, 5 μm.

The overall picture that has emerged confirms the role of EB proteins in the regulation of microtubule dynamics but their precise effect is still not fully characterized. In mouse fibroblasts, EB1 depletion leads to an increase in microtubule pausing and a decrease in microtubule growth time (Kita et al., 2006). In addition, EB1 was also shown to induce microtubule stabilization by interacting with mDia and APC (Wen et al., 2004). In this study, the authors show that EB1 localized to stable Glu-microtubules and knockdown of EB1 leads to the appearance of more dynamic microtubules, as demonstrated by the concomitant decrease in Glu-microtubules. EB3 also interferes with microtubule dynamics. In fact, it was shown in myoblasts that EB3 depletion induced an over-growing of microtubules near the cell cortex and a significant decrease in the shrinkage rate of cortical microtubules (Straube and Merdes, 2007). This was further emphasized when it was shown that EB1 and EB3 in mammalian cells

promote persistent microtubule growth by suppressing microtubule catastrophes (Komarova et al., 2009). In HeLa cells EB1 and EB3 regulate both the growth and shrinkage rates of cortical microtubules, but while EB3 depletion leads to more dynamic microtubules with increased rescue frequencies, EB1 depletion leads to reduced rescue frequencies (our unpublished observations). The impact on interphase microtubule dynamics can also involve the interaction of EBs with other +TIPs. It has been suggested that differences in the expression and regulation of several +TIPs in different cell types may be responsible for the differences observed in microtubule dynamics (Ligon et al., 2003). Data derived from *in vitro* assays demonstrated that EB1 can act cooperatively with other +TIPs such as CLIP170 in the regulation of microtubule dynamics (Lopus et al., 2012). Furthermore, interaction of yeast EB1 and APC is critical for the regulation of microtubule polymerization (Nakamura et al., 2001). These reports indicate that additional studies are required to help clarify the role of mammalian EBs either in the direct regulation of microtubule dynamics or via the recruitment of other +TIPs to the growing ends of microtubules.

Besides its plus-end localization, EB proteins were also shown to bind other sub-cellular structures either directly (centrosome) or indirectly (F-actin and membranes). In fact, EB1 is a functional component of centrosomes and binds to this structure independently of microtubules through its C-terminal domain (Louie et al., 2004). Curiously, the C-terminal domain of EB1 is also required for the recruitment of γ-tubulin to centrosomes and anchoring of microtubules to this structure (Askham et al., 2002). Moreover, EB1 was shown to interact with the centrosomal protein FOP (Yan et al., 2006). This interaction is essential for recruitment of EB1 to the centrosome and its later association with CAP350, forming a microtubule anchoring complex. EB3 also localizes to the centrosome (Ban et al., 2009) and this localization persists even after microtubule depolymerization with nocodazole (our unpublished observations). EB proteins are also important for cilia biogenesis. EB1 is required for primary cilia formation in mouse fibroblasts (Schroder et al., 2007) and recently it was shown that both EB1 and EB3 (but not EB2) are essential in cilia biogenesis by interacting with proteins involved in minus-end anchoring such as PCM1 and Cep290 (Schroder et al., 2011). EBs can also interact indirectly with actin filaments or membrane structures. EB1 was shown to interact with the spectraplakin ACF7/MACF1, providing a link between the microtubule and actin cytoskeletons (Kodama et al., 2003). Moreover, melanophilin and myosin Va can interact with EB1 allowing the transfer of melanosomes from microtubules to actin filaments (Wu et al., 2005). EB3 also interacts with the actin-binding protein drebrin and this is important to coordinate interactions between microtubules and actin filaments (Bazellieres et al., 2012;

Geraldo et al., 2008). Microtubule plus-ends are also linked to ER tubules through the interaction of EB1 with STIM1, an ER transmembrane protein (Grigoriev et al., 2008).

In addition to their interphase role, EB proteins have also been implicated in mitosis. This was first postulated when it was observed that EB1 localized to spindle microtubules (Berrueta et al., 1998). Both immunofluorescence analyses and live imaging using EB1 tagged with GFP showed that it is able to localize to the growing ends of microtubules throughout mitosis (Berrueta et al., 1998; Morrison et al., 1998; Piehl and Cassimeris, 2003). More in-depth observations demonstrated that EB1 can target to kinetochores with attached growing microtubules (Tirnauer et al., 2002a). In *Drosophila*, EB1 was shown to be required for bipolar spindle assembly (Rogers et al., 2002) but in mammalian cells, lack of EB1 does not seem to interfere with spindle assembly. However, EB1 interaction with APC was proposed to play a role in chromosome alignment without inducing mitotic arrest (Green et al., 2005). One other way in which EBs can influence mitotic progression is by affecting the localization or activity of essential mitotic players. In fact, EB1 binds Aurora B *in vitro* and *in vivo* (Sun et al., 2008). Furthermore, the authors show that EB1 protects Aurora B from inactivation by Protein Phosphatase 2A (PP2A) but EB1 itself is not phosphorylated by Aurora B (Sun et al., 2008). Inversely, EB3 is a substrate of both Aurora A and Aurora B kinases (Ban et al., 2009). The phosphorylation of EB3 stabilizes the protein during mitosis but it is not clear what the functional relevance of this modification is. These interactions between EB proteins and Aurora kinases are highly conserved and can also be observed in yeast (Zimniak et al., 2009). The Aurora homologue Ipl1 phosphorylates the single EB1-like protein Bim1 and this is required for spindle elongation and proper disassembly of the spindle midzone. In HeLa cells, EB1 is also essential for correct positioning of the metaphase spindle by promoting the nucleation/stabilization of astral microtubules (Toyoshima and Nishida, 2007). This could be accomplished by the interaction of EB1 with the motor protein Kif18B, a plus-end directed motor that can modulate microtubule dynamics and has been shown to regulate astral microtubule length in early mitosis (Stout et al., 2011). So far, EB3 has not been described to play any role in metaphase spindle positioning. Overall, EB proteins act, either directly or through interaction with a partner, as regulators of cellular functions by mediating microtubule dynamics. More detailed data on the physiological relevance of EB proteins still awaits the development of mammalian knockout models.

3.5 – CLASP family

CLASPs are CLIP-associating proteins (hence, the name CLASP), which are involved in the regulation of microtubule dynamics and their stabilization (Akhmanova et al., 2001). This family of proteins is highly conserved and in humans is composed of two members: CLASP1 and CLASP2 (Akhmanova et al., 2001; Galjart, 2005). Homologues include *Drosophila* Orbit/Mast (Inoue et al., 2000; Lemos et al., 2000), *Xenopus* Xorbit (Hannak and Heald, 2006), S. *cerevisiae* Stu1 (Pasqualone and Huffaker, 1994), S. pombe Peg1/CLASP (Grallert et al., 2006) and *A. Thaliana* CLASP (Ambrose et al., 2007). CLASP1 is widely expressed, whereas CLASP2 is more abundant in the brain. These proteins have several isoforms with a molecular weight of 140-170 kDa, that differ between themselves in the N-terminus sequence (Galjart, 2005). Interestingly, one of the CLASP2 isoforms has an N-terminal palmitoylation motif which is crucial for its anchoring to membranes (Akhmanova et al., 2001). Structurally, CLASPs have an N-terminal domain very similar to the sequences present in the Dis1/TOG family, which are known microtubule stabilizers (Figure 13). In the middle region of the protein there is a Serine- and Arginine-rich region, a series of HEAT repeats and two tandem SxIP motifs. This region is essential for binding both to microtubules and EB1 (Honnappa et al., 2009; Mimori-Kiyosue et al., 2005). In addition, the C-terminal region contains a coiled-coil domain that is essential for CLASPs dimerization as well as for interacting with partners such as CLIPs, CENP-E and the chromokinesin Kid (Akhmanova et al., 2001; Al-Bassam et al., 2010; Maffini et al., 2009; Patel et al., 2012). This C-terminal region is also essential for interaction with the Golgi apparatus and the cell cortex (Efimov et al., 2007; Lansbergen et al., 2006). CLASPs localize to the plus-ends of microtubules. They can bind directly to tubulin and microtubules but are normally loaded on to the microtubules by EB1 (Akhmanova et al., 2001; Al-Bassam et al., 2010; Mimori-Kiyosue et al., 2005). The association of CLASP to microtubules is phospho-regulated by GSK3β. In fact, GSK3β targets the serine residues around the SxIP motif leading to an impairment of the plus-end tracking of CLASP (Kumar et al., 2009) and inhibition of GSK3β increases the affinity of CLASP2 to microtubules (Akhmanova et al., 2001).

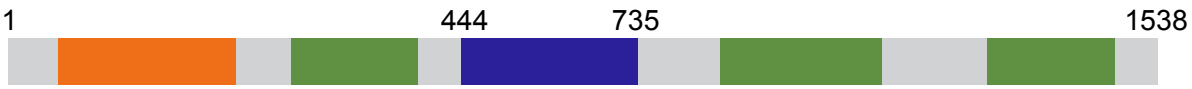

Figure 13 – Diagram of CLASPs structure. Residue numbers are based on CLASP1 sequence. CLASPs have a TOG-like domain in the N-terminal region (shown in orange). In addition, there are three helical regions (in green) and one Basic/Ser rich region (in blue), which contains the SxIP motives required for interaction with EB proteins.

Interestingly, phosphorylation of CLASP2 by CDK1 and GSK3β also disrupts the association of EB1 with CLASP2 and completely abolishes the plus-end tracking of the proteins (Kumar et al., 2012). Taken together, these observations confirm that phosphorylation in the vicinity of SxIP motifs negatively regulates the localization of +TIPs to microtubule plus-ends by interfering with their ability to interact with EB1 (Honnappa et al., 2009). CLASPs are microtubule stabilizers because they promote microtubule "pausing" (Mimori-Kiyosue et al., 2005; Sousa et al., 2007) and this is important for the role they play at the cell cortex, where they bind and stabilize microtubules (Lansbergen et al., 2006). Furthermore, this requires interaction of CLASPs with LL5β, ELKS and the microtubule-actin crosslinking factor ACF7/MACF1 (Drabek et al., 2006; Lansbergen et al., 2006; Wu et al., 2008). When CLASPs are either depleted by RNAi or blocked by antibody injection, microtubules fail to stabilize and attach to the cortex (Mimori-Kiyosue et al., 2005). This lack of microtubule stabilization coincides with a decrease in acetylated tubulin and microtubule density. However, it should be noted that individual CLASP1 or CLASP2 depletion does not induce the same effect (Mimori-Kiyosue et al., 2005), suggesting a partial functional redundancy. Inversely, over-expression of CLASPs leads to accumulation of these proteins along the whole microtubule length, which induces their stabilization and bundling (Akhmanova et al., 2001; Maiato et al., 2003a). In addition to its plus-end localization, CLASP2 also localizes to the Golgi apparatus, centrosome, cell cortex and leading edge of motile fibroblasts (Efimov et al., 2007; Lansbergen et al., 2006; Wittmann and Waterman-Storer, 2005).

The first description of CLASP in *Drosophila* revealed that during early mitosis the protein localized to the spindle microtubules, centrosomes, kinetochores and spindle midbody (Lemos et al., 2000). Later work on human cells in culture described a similar localization pattern for CLASP1 (Maiato et al., 2003a). CLASPs remain associated with the kinetochores even after chromosome congression. After anaphase, CLASPs are transferred to the spindle midzone and, later on, associate with the midbody (Lemos et al., 2000; Maiato et al., 2003a; Pereira et al., 2006). The first functional studies done in *Drosophila* S2 cells and *mast/orbit* mutants described defects in chromosome congression and formation of monopolar spindles, mainly due to a shortening of kinetochore microtubules which lead to spindle colapse (Lemos et al., 2000; Maiato et al., 2003a; Maiato et al., 2003b; Maiato et al., 2002). Subsequent experiments revealed that CLASP is required for microtubule poleward flux. If mature kinetochore fibres are laser ablated after CLASP depletion, the microtubule stub attached to the kinetochore is no longer capable of regrowing (Maiato et al., 2005). Fluorescence Recovery After Photobleaching (FRAP) experiments on these mature k-fibres further confirmed that

CLASPs are essential for microtubule flux (Maiato et al., 2005). Overall, these results suggest that CLASPs are essential to regulate kinetochore-microtubule attachments.

In mammalian cells, CLASPs seem to play similar and redundant roles in mitotic spindle organization, which is reinforced by the fact that they co-localize throughout mitosis (Mimori-Kiyosue et al., 2006; Pereira et al., 2006). FRAP studies also demonstrated that CLASPs are highly dynamic both at kinetochores and centrosomes during mitosis (Pereira et al., 2006), suggesting that interfering with CLASPs function during mitosis could impact on mitotic fidelity. Indeed, simultaneous CLASPs depletion leads to an increase in the mitotic index. Furthermore, loss of CLASPs gives rise to a large number of mitotic abnormalities which include monopolar, disorganized, and multipolar spindles as well as several aneuploidy and chromosome missegregation events (Mimori-Kiyosue et al., 2006; Pereira et al., 2006). These phenotypes can be partially explained by the fact that loss of CLASPs produces an increase in chromosome oscillations, which indicates a defect in kinetochore-microtubule attachments (Mimori-Kiyosue et al., 2006), although this is not observed when CLASP1 is depleted by antibody injection (Maiato et al., 2003a). In fact, CLASPs were defined as outer-kinetochore components, where they are necessary to regulate kinetochore-microtubule attachments (Maiato et al., 2003a). Considering that, during the metaphase-anaphase transition, kinetochore-microtubule interactions are less dynamic (Gorbsky and Borisy, 1989), one can attribute this (at least partially) to the fact that CLASPs transfer from kinetochores to the midzone at this exact point. Interestingly, the regulation of this attachment may involve the differential interaction of CLASPs with the Astrin/Kif2b complex (Manning et al., 2010). In fact, the authors show that CLASP1 interacts with Kif2b in prometaphase and this is essential to maintain more dynamic interactions, which are easier to correct. On the other hand, in metaphase CLASP1 interacts with Astrin and stabilizes kinetochore-microtubule attachments. This provides an interesting mechanism that helps explain how CLASPs differentially regulate the stability of kinetochore microtubule attachments. Recently, it was also shown that CLASP1 together with MAP4 are important for positioning the mitotic spindle (Samora et al., 2011). While the above reports explain the mitotic defects associated with kinetochore-microtubule dynamics such as monopolar spindles and missegregation defects, they provide no explanation for the multipolar phenotype observed upon CLASPs depletion. This was addressed by a recent report that proposed a role for CLASPs (together with Ninein) in ensuring spindle-pole integrity after bipolarization in response to CENP-E- and Kid-mediated forces derived from misaligned chromosomes (Logarinho et al., 2012). In addition to their roles in early mitosis, CLASPs are also required for late mitotic events. During anaphase CLASP1 helps to stabilize interpolar

microtubules by interacting with the microtubule stabilizer PRC1 and this allows normal chromosome congression and cytokinesis (Liu et al., 2009). Overall, these results support a role for CLASPs in the formation and maintenance of a stable bipolar spindle and for mitotic progression.

3.6 – APC family

The Adenomatous Polyposis Coli (APC) protein is a product of the *Apc* gene which encodes a large protein of approximately 300 kDa (Smith et al., 1993). In humans, an additional form of APC can be found which is a product of the *APCL/APC2* gene (Nakagawa et al., 1998). Interestingly, this APC2 can complement some of the functions of the original APC, although it lacks some of its structural features, such as the β-catenin binding sites (van Es et al., 1999). This gene was first identified in 1991 as the site for mutations in families with Familial Adenomatous Polyposis (Kinzler et al., 1991; Nakamura et al., 1992). Additional work uncovered a functional relationship between APC mutations and the appearance of colorectal cancer in a mouse model (Su et al., 1992). In structural terms, APC is composed of several domains (Figure 14). Close to the N-terminus there is an Armadillo Repeat Domain (ARD) necessary for Asef binding.

Figure 14 – Diagram of APC structure. APC contains coiled coil regions (in yellow). In addition, there is a stretch of Armadillo repeats necessary for Asef binding (in red). In the middle of the protein there is a helical region (in green). In the C-terminal domain of APC there are Basic/Ser rich sequences in blue) which contain the SxIP motif necessary for EB1 binding.

Asef is a small guanine nucleotide binding (G)-protein exchange factor (GEF) which interacts with APC to promote membrane ruffling and the formation of lamellipodia in mammalian tissue-culture cells (Kawasaki et al., 2000).

In the middle of APC there are β-catenin-binding motifs, Axin-binding motifs and also a mutation cluster region (MCR). In addition, there is a KKKK stretch, which is postulated as a

putative Nuclear Localization Signal (NLS). In the N-terminus, there is a microtubule binding domain and an EB1-binding domain (Bienz, 2002). The interaction of EB1 with APC was first mapped to a small region in the C-terminus of APC which comprises the aminoacids 2559-2843 (Su et al., 1995) and is independent of the microtubule binding domain. Subsequent work narrowed this to the last 170 aminoacids of APC (Askham et al., 2000) and finally, this interaction was attributed to a basic, serine-rich sequence in the C-terminus of APC named APCp1 (Honnappa et al., 2005). More specifically, interaction of APC with EB1 depends on the SxIP motif (Ile2805 and Pro2806) of APC (Honnappa et al., 2009; Honnappa et al., 2005). Interestingly, mutations within this region are sufficient to abolish EB1 interaction and also the ability of APC to tip-track (Honnappa et al., 2009; Honnappa et al., 2005).

The first function assigned to APC was in the regulation of the β-catenin, a key effector of the Wnt signalling pathway (Rubinfeld et al., 1993; Su et al., 1993). In the cell, APC shuttles between the nucleus and the cytoplasm and this is thought to be relevant for its role in the regulation of β-catenin transcriptional activity (Bienz, 2002). In addition to this, APC is also involved in the regulation of microtubule function. In fact, APC directly associates with microtubules and promotes their polymerization and stabilization *in vitro* (Munemitsu et al., 1994; Nakamura et al., 2001; Zumbrunn et al., 2001). As was mentioned above, interaction of APC with EB1 seems to be important for its ability to track microtubule plus-ends (Mimori-Kiyosue et al., 2000a). However, this might not be the only mechanism that APC uses to localize to growing microtubule ends, as APC association to the microtubule can occur even in the absence of EB1 (Kita et al., 2006). Nevertheless, it seems that APC is mainly loaded onto plus-ends by hitchhiking on EB1 (Honnappa et al., 2009). This interaction seems to be important because it can help regulate microtubule stability and promote cell migration (Wen et al., 2004), although another study with MEFs derived from mice carrying a truncated *Apc* allele demonstrated that this APC-EB1 interaction is not essential for microtubule stabilization (Drabek et al., 2006). APC is also involved in the interaction between the microtubule and actin cytoskeletons (Moseley et al., 2007) and in the regulation of cell polarity (Etienne-Manneville and Hall, 2003).

In addition to its localization in the plus-ends of microtubules, APC also localizes to the kinetochores and centrosomes during mitosis (Kaplan et al., 2001; Louie et al., 2004). This mitotic distribution led to the assumption that APC might be involved in the regulation of microtubule – kinetochore attachments and chromosome segregation (Fodde et al., 2001; Green et al., 2005; Kaplan et al., 2001). In addition, APC was also involved in regulating

metaphase spindle position (Green et al., 2005; McCartney et al., 2001). All of the mitotic functions described for APC involve interaction with EB1, probably due to requirement of EBs to load APC to plus-ends. However, it should be noted that EB1 is not stably associated with kinetochores unless the k-fibre is growing (Tirnauer et al., 2002a), which suggests that APC has to remain bound to kinetochores directly independently of EB1. This function was recently attributed to the fact that APC can interact with the kinetochore-associated BubR1 (Zhang et al., 2007a). BubR1 directly binds and phosphorylates APC *in vitro* and this is essential for the recruitment of APC to kinetochores and stabilization of kinetochore-microtubule attachments.

3.7 – Motor proteins

Mammalian cells have a set of proteins that can travel along microtubules towards the plus-end or the minus-end. Recently, many of these motor proteins have also been identified as +TIPs (Wu et al., 2006). These include the plus-end directed, kinesin-7 family member, CENP-E (Sardar et al., 2010) and minus-end directed dynein (Kobayashi and Murayama, 2009). In this section we will focus on the functional relevance and mechanisms involved in motor protein accumulation at microtubule plus-ends.

Kinesins

Kinesins are plus-end directed motors. The first kinesin was identified in neuronal cells, where it transports cargo towards the plus-end of microtubules (Vale et al., 1985). Kinesins have ATPase activity and generate movement through the motor domain (Vale and Fletterick, 1997). Kinesins are classified according to the position of their motor domain (Miki et al., 2005). This structural analysis led to the separation of kinesins into 15 different families (Hirokawa et al., 2009). In addition to the motor domain, all kinesins have one or more coiled-coil domains. Depending on the kinesin family, they can also have a CAP-Gly domain, a Pleckstrin Homology (PH) domain, a Phox Homology (PX) domain and WD40 repeats (Hirokawa et al., 2009). Any kinesin that does not have a distinguishing feature falls into the orphan kinesin group (Miki et al., 2005). So far, kinesins have been involved in many cellular functions such as organization of the interphase microtubule cytoskeleton, axonal transport, organelle movement and mitotic progression.

Some kinesins have already been described to tip-track microtubules. These include yeast kinesin-7 family members Tea2 and Kip2, microtubule depolymerizer kinesin-13 MCAK and *Drosophila* KLP10A and kinesin-14 Kar3 (Akhmanova and Steinmetz, 2008). In theory, all plus-end directed motors could concentrate on microtubule plus-ends due to their function, but most of them do not. This probably happens because they have to interact with other +TIPs or, in alternative, must show some specificity for the microtubule plus-end to do so (Bieling et al., 2007; Busch et al., 2004). In fact, the kinesin Tea2 needs to interact with Mal3 (the EB1 homologue) to track microtubule plus-ends and to stimulate its ATPase activity (Bieling et al., 2007; Browning and Hackney, 2005; Busch and Brunner, 2004).

In mammalian cells, kinesins play very diverse roles. In neurons, kinesins are involved in bidirectional transport of organelles and vesicles. Anterograde transport inside the axon is driven mainly by N-kinesins, which have plus-end directed motility (Hirokawa et al., 2009). In non-neuronal cells, kinesins are involved in endosome transport (Bananis et al., 2004), vesicles (Peretti et al., 2000) and intraflagellar transport (Ou et al., 2005). During mitosis, kinesins are involved in spindle formation (Haraguchi et al., 2006), chromosome congression (Wood et al., 1997) and interpolar microtubule sliding (Kapitein et al., 2005). Interpolar microtubule sliding is achieved by kinesin-5/Eg5, which is a plus-end directed motor that can also tether microtubule plus-ends (Kapitein et al., 2005). Kinesin-7/CENP-E is a plus-end directed motor required for metaphase chromosome alignment (Wood et al., 1997). In addition, it has been shown *in vitro* that CENP-E accumulates at the plus-ends of microtubules and has critical roles during mitosis including kinetochore-microtubule attachment and movement of monooriented chromosomes to the metaphase plate (Sardar et al., 2010). Although CENP-E is not a conventional +TIP, due to its plus-end directed movement it appears to accumulate in the distal end of microtubules (Sardar et al., 2010). Kinesin-8 family members have the ability to bind microtubule plus-ends. Recently, it was shown that accumulation of kinesin-8 homologue Kip3 at the plus-ends depends on the tail of Kip3 and this tail also inhibits microtubule shrinkage and is required for promoting Kip3-mediated microtubule rescue (Su et al., 2011). On the other hand, microtubule stabilization itself can also trigger the accumulation of kinesin-8/Kif18A (Masuda et al., 2011). During mitosis, kinesin-8/Kif18B binds to EB1 to regulate astral microtubule length (Stout et al., 2011). Furthermore, during metaphase, enrichment of Kif18A at kinetochore-microtubule plus-ends depends on its C-terminal tail domain, while the ability of Kif18A to suppress microtubule growth is conferred by the N-terminal motor domain (Stumpff et al., 2011). In addition, the Kif18A tail contains a second microtubule-binding domain that diffuses along the lattice, suggesting that it tethers the motor to the microtubule

track. One other example of kinesin plus-end tracking comes from kinesin-2/Kif17. Targeting of Kif17 to plus-ends of growing microtubules requires kinesin motor activity and interaction with EB1. In turn, Kif17 targets APC to the plus-ends of a subset of microtubules (Jaulin and Kreitzer, 2010). In addition, kinesin-3/KIF1C also has tip tracking behaviour, localizes to plus-ends together with EB1 and is involved in podosome regulation (Kopp et al., 2006). Interestingly, minus-end directed motors can also bind to microtubule ends such as the *Drosophila* kinesin-14 Ncd (Goshima et al., 2005). This accumulation occurs through interaction with EB1 and is thought to play a role in the capture and transport of kinetochore fibres along centrosomal microtubules and help to form a tightly focused bipolar spindle (Goshima et al., 2005). Finally, in mammalian cells, myosin V also attaches to microtubule plus-ends via the adaptor melanophilin and allows transfer of melanosomes between the microtubule and actin cytoskeletons (Wu et al., 2005).

Dynein

Dynein is a large macromolecular complex with a molecular weight of approximately 1.2 MDa. It is composed of heavy, intermediate, light intermediate and light chains. The heavy chains contain the motor domains with six AAA ATPase domains and a microtubule binding stalk (Oiwa and Sakakibara, 2005). Dynein is a minus-end directed motor that uses ATP hydrolysis to power its movement and requires interaction with the dynactin complex. One of the subunits of the dynactin complex is p150glued. This protein is a +TIP that has a CAP-Gly domain and two coiled-coil regions which are required for dimerization and interaction with the Dynein intermediate chain [Figure 15; (King et al., 2003)]. Early reports of dynein accumulation on microtubule plus-ends came from work with the filamentous fungus *Aspergillus*. In this organism, dynein exhibited plus-end directed movement at velocities similar to microtubule polymerization rates, which suggested that dynein is associated to and moving with the polymerizing ends of microtubules (Xiang et al., 2000). In addition, dynein movements were blocked when microtubules were depolymerised, further suggesting that dynein movement could be microtubule dependent. Subsequent reports described the accumulation of both dynein and NUDF (the homologue of Lis1) at microtubule plus-ends in a come-like structure (Zhang et al., 2003). In the same system, dynein and dynactin required each other for plus-end accumulation but NUDF specifically required dynein to tip-track.

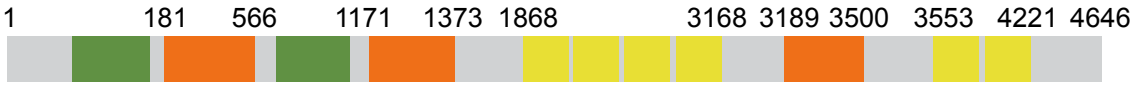

Figure 15 – Diagram of Dynein Heavy Chain structure. Sequence number is based on cytoplasmic Dynein1 heavy chain 1. Dynein contains two helical regions (in green) near its C-terminal domain. There are also three coiled coil regions (in orange) and six AAA ATPase domains (in yellow).

Interestingly, accumulation of dynein and dynactin depends on transport by the kinesin KINA (Zhang et al., 2003). After arriving at the plus-ends, dynein also exhibited some retrograde movement and this movement is also microtubule dependent (Xiang et al., 2000). The interaction of dynein with LIS1 is also important for dynein-mediated retrograde transport, because it allows the release of the dynein-dynactin complex from CLIP-170-decorated microtubule plus-ends (Lansbergen et al., 2004). *In vitro* work estimated that the dynein comet consist of approximately 55 dynein motors. About half of the motors show a slow turnover and are actively kept at the plus-ends by a retention mechanism that requires interaction with dynactin and EB1 (Schuster et al., 2011). Therefore, dynein retention at the plus-ends involves a combination of both stochastic accumulation and active retention to allow formation of the dynein comet and ensure capturing of organelles by minus-end motors (Schuster et al., 2011).

During mitosis, dynein is also essential for force generation and spindle positioning. This happens because cortical dynein can capture astral microtubules and exert a pulling force (Kiyomitsu and Cheeseman, 2012; O'Connell and Wang, 2000). The question arises then of how dynein arrives at its cortical location? It was proposed that in yeast, dynein offloads directly from the microtubule plus-ends to the cell cortex by an active microtubule-mediated delivery (Markus and Lee, 2011). In addition, the neck region of dynein is essential for this process. Longer neck regions allow enhanced off-loading without affecting motor activity, while shorter necks block delivery to the cortex. This led the authors to propose that a conformational change in dynein could be regulated by a masking/unmasking event that controls dynein off-loading from microtubules. In addition, the N-terminal tail domain is essential for targeting dynein to cortical receptor sites, whereas the C-terminal domain is required for plus-end targeting in a Bik1/CLIP170- and Pac1/LIS1-dependent manner (Markus et al., 2009). Curiously,

expression of the motor domain alone blocks the plus-end accumulation of dynein and this can be rescued by over-expression of LIS1.

Additional dynein functions include centrosome and nuclear translocation (Tsai et al., 2007). In neurons, dynein is proposed to work together with Lis1 by pulling microtubule plus-ends to translocate both the centrosome and the microtubule network. Dynein and Lis1 appear to generate tension between the nucleus and the centrosome (Tanaka et al., 2004) and also at the interface between microtubule tips and the cell cortex (Dujardin et al., 2003). Interestingly the role of dynein on nuclear movement appears to be conserved in different cell types. Both dynein and kinesin seem to be required for the bi-directional movement of the nucleus by interacting with the nuclear pore complex (NPC). In fact, interaction of dynein or kinesin-1 with Bicaudal D2 is essential for nuclear and centrosomal position during mitotic entry (Splinter et al., 2010). This may also involve the interaction of dynein/dynactin with a CENP-F- NudE/EL-Nup133 complex (Bolhy et al., 2011).

3.8 – Kinesin 13 family

Members of the kinesin-13 family were named so because of the position of the motor domain in the middle of the protein. The first 12 families (from kinesin-1 to -12) have the motor domain close to the N-terminal domain and kinesin-14 has the motor domain in the C-terminal region (Lawrence et al., 2004; Miki et al., 2005). These kinesins were also initially named M kinesin family (for “Middle Type Motor”) or KinI family (for “Internal Type Motor”). It should be noted that KinI is not related with Kinesin I, which is a member of the kinesin-1 family. Within the kinesin-13 family there are two subfamilies: the ubiquitous KIF24 subfamily and the mammalian specific KIF2 subfamily. This last subfamily is comprised of three members: Kif2A, Kif2B and Kif2C/MCAK. All members of the family have an N-terminal globular domain, followed by a positively charged neck upstream of the centrally located catalytic core, and a C-terminal dimerization domain [Figure 16; (Ogawa et al., 2004; Wordeman, 2005)]. The KIF24 subfamily has the catalytic core close to the N-terminal region, whereas the KIF2 subfamily has the catalytic core closer to the centre of the molecule (Miki et al., 2005). Interestingly, it was demonstrated that MCAK requires dimerization through the coiled-coil domain in the C-terminal region and this has a role in regulating the ATPase activity of the protein [Figure 16; (Ems-McClung et al., 2007)].

Figure 16 – Diagram of MCAK, a kinesin-13 family member. MCAK has a helical region near its N-terminal domain (in dark green). In addition, there is a Basic/Ser rich region, necessary for interaction with EB family members (in blue). In the middle of the protein there is a kinesin domain, which forms the catalytic core (in light green). Near the C-terminal region there is a coiled coil (in orange) that is required for protein dimerization.

Members of the kinesin-13 family have been implicated in vesicle transport (Noda et al., 1995) and, more importantly, in microtubule depolymerization (Desai et al., 1999; Manning et al., 2007). Upon binding to the microtubule end, they induce a conformational change in the microtubule structure that leads to a catastrophe event (Desai et al., 1999). The microtubule-destabilizing properties of kinesin-13 members are unique because they use ATP hydrolysis to induce depolymerization of microtubules from both ends, instead of using it to walk along microtubules (Desai et al., 1999; Helenius et al., 2006; Hunter et al., 2003). The best studied member of the family is MCAK. This kinesin was shown to target the plus-ends of microtubules and once there, 14 MCAK dimers form an ATP-hydrolyzing complex that processively depolymerises the microtubule (Hunter et al., 2003). Normally, MCAK can rapidly depolymerise microtubules in the presence of ATP (Hunter et al., 2003). However, it can still depolymerise microtubules even in the absence of ATPase activity (Desai et al., 1999; Moores et al., 2002) but only when it is in a 1:1 stoichiometry of motor to polymerized tubulin (Moore and Wordeman, 2004b).

One puzzling observation comes from the fact that these proteins, while having potent microtubule depolymerization activity, are able to accumulate in the plus-ends of microtubules (Moore et al., 2005). This suggests that the microtubule depolymerising activity must be inhibited or controlled at this location and raises the question of how does MCAK reach the microtubule tip? Microscopy studies using single molecules demonstrated that MCAK rapidly moves along the microtubule lattice in a random walk (Helenius et al., 2006). Unlike what is necessary for its microtubule depolymerising effect, this diffusion does not require ATP

hydrolysis and is more rapid than direct binding to the plus-end from solution (Helenius et al., 2006). In addition to this, MCAK also associates with EB proteins. In fact, MCAK associates with the C-terminal region of both EB1 and EB3 co-localizes with EB1 at microtubule plus-ends (Lee et al., 2008). This raises the possibility that MCAK can also use an EB-hitchhiking mechanism to bind microtubule plus-ends, in addition to lattice diffusion. These were proposed as complementary mechanisms that would allow MCAK to remain associated with the microtubule even after EB displacement from the plus-end. Recent work demonstrated that MCAK contains an SxIP motif near its C-terminal domain that is crucial associating with EB1 (Honnappa et al., 2009). As expected, mutating the Ile-Pro in this sequence to Asn-Asn completely abrogated MCAK binding to EB1 and its tip-tracking behaviour, but still allowed lattice binding (Honnappa et al., 2009). This property of MCAK seems to be conserved, since KLP10A (the *Drosophila* homologue of MCAK) also associates with EB1 and this is necessary for KLP10A targeting to plus-ends (Mennella et al., 2005). The association of MCAK with the microtubule can also be regulated in a post-translational manner. Indeed, Aurora B was shown to phosphorylate MCAK and this is crucial for its function (Andrews et al., 2004; Lan et al., 2004). In addition, most of these phosphorylation sites seem to cluster in a region close to the SxIP motif, which alters the ability of MCAK to interact with EB1 and tip-track (Honnappa et al., 2009; Moore et al., 2005). Curiously, the other family members Kif2A and Kif2B do not accumulate at microtubule plus-ends and this is explained by the fact that they do not have an SxIP motif (Honnappa et al., 2009).

During mitosis, kinesin-13 members have differential localizations and roles. Kif2A was reported to localize mainly to spindle poles but also to kinetochores (Cameron et al., 2006; Gaetz and Kapoor, 2004; Ganem and Compton, 2004). Localization of Kif2B is not so clear, due to its very low expression levels. However, when over-expressed, Kif2B also localizes to spindle poles and kinetochores (Manning et al., 2007). MCAK, on the other hand, localizes to spindle poles, centromeres, kinetochores, plus-ends of microtubules and also the cytoplasm during mitosis (Ems-McClung and Walczak, 2010; Moore et al., 2005; Wordeman and Mitchison, 1995). During early mitosis, kinesin-13 members are required for bipolar spindle assembly. In fact, knockdown of Kif2A by RNAi leads to a dramatic increase in the number of monopolar spindles (Ganem and Compton, 2004). Perturbing MCAK function leads to the same phenotype, albeit with less pronounced effects (Holmfeldt et al., 2004; Holmfeldt et al., 2005). How do kinesin-13 members regulate spindle bipolarity? When cells depleted of Kif2A are co-depleted for MCAK or treated with low doses of nocodazole, spindle bipolarity is restored (Ganem and Compton, 2004). This means that Kif2A and MCAK must be acting on spindle

bipolarity through their ability to regulate microtubule dynamics. On the other hand, Kif2B was shown to influence the kinetics of centrosome separation (Manning et al., 2007), which indicates there must be different pathways regulated by the different kinesin-13 members. In addition to their role in maintaining spindle bipolarity, kinesin-13s also help regulate spindle microtubule dynamics. For instance, Kif2B is important for k-fibre turnover during prometaphase (Bakhoum et al., 2009). On the other hand, MCAK is required for proper k-fibre turnover during metaphase but has no discernible effect on other spindle microtubules (Rizk et al., 2009; Wordeman et al., 2007). This suggests that there must be a spatial and temporal regulation of kinesin-13 members during mitosis. Given their role in controlling k-fibre turnover, it is not surprising that these proteins can influence chromosome segregation. In fact, MCAK was first shown to be important for chromosome segregation, as knockdown of the protein leads to lagging chromosomes (Maney et al., 1998). Simultaneous knockdown of MCAK and Kif2A induces an even higher number of lagging chromosomes (Ganem et al., 2005). Kif2B knockdown, on the other hand, causes an increase in the number of improperly attached chromosomes, which leads to defective chromosome segregation (Bakhoum et al., 2009).

3.9 – TOG family

The TOG proteins belong to a highly conserved family involved in microtubule dynamics regulation (Al-Bassam and Chang, 2011; Slep, 2009). The initial member of this family XMAP215, was identified in *Xenopus* as a protein that promoted rapid microtubule growth (Gard and Kirschner, 1987). Many homologues have been described since, which include Stu2 in budding yeast (Wang and Huffaker, 1997), Dis1 in fission yeast (Ohkura et al., 1988), *Drosophila* Msps (Cullen et al., 1999) and ch-TOG in humans (Charrasse et al., 1998). In structural terms, these proteins are characterized by the presence of a variable number of Tumour Overexpressed Gene (TOG) domains near the N-terminal region (Figure 17). These domains have approximately 200 aminoacids and comprise between 2-5 units, depending on the organism (Ohkura et al., 2001). Interestingly, each of these contains several HEAT repeats, which are thought to be protein interaction domains (Cassimeris et al., 2001). The human ch-TOG contains five TOG domains near the N-terminal region, regions with sequences rich in serine, glycine and lysine (SK-rich domains) and a conserved C-terminal non-repeat domain (Al-Bassam and Chang, 2011). Interestingly, CLASPs also have TOG-like domains and SR-rich regions, which provide a structural link between the function of both classes of proteins (Lemos et al., 2000; Slep, 2010). Detailed studies revealed that the N-terminal domain

contains a microtubule-stabilizing region, whereas the C-terminal domain is necessary for centrosome and microtubule targeting (Popov et al., 2001).

Figure 17 – Diagram of chTOG/XMAP215. The TOG family of proteins contains a variable number of TOG domains (in red) near the N-terminal region. There is also a Basic/Ser rich region (in blue), as well as helical region (in green) near the C-terminal domain.

TOG proteins localize to microtubule plus-ends but can also bind the microtubule lattice and soluble tubulin. They have an intrinsic ability to promote microtubule elongation from both ends, although they do so more efficiently on the plus-ends (Gard and Kirschner, 1987; Vasquez et al., 1994). *In vitro* studies with recombinant XMAP215 confirmed that these molecules can associate to microtubule plus-ends, stimulating their growth (Brouhard et al., 2008; Kinoshita et al., 2001). These studies further demonstrated that XMAP215 transiently binds the microtubule plus-end and adds 25 tubulin dimers to the microtubule before dissociating (Brouhard et al., 2008). The initial hypothesis for XMAP215 action involved the binding and recruitment of tubulin oligomers to microtubule ends (Cassimeris et al., 2001). However, later it became clear that TOG proteins can only bind one tubulin dimer at a time (Al-Bassam et al., 2006; Brouhard et al., 2008). Curiously, in *Xenopus* egg extracts the N-terminal region is able to stimulate microtubule growth at the plus-ends by inhibiting catastrophes, while the C-terminal region suppresses microtubule growth by promoting catastrophes (Popov et al., 2001). Additional studies in different systems further confirmed the role of TOG proteins in microtubule stabilization and growth (Charrasse et al., 1998; Dionne et al., 2000; Tournebize et al., 2000). ch-TOG has been shown to promote microtubule assembly both in solution and from nucleation centres (Charrasse et al., 1998), and to be essential for the formation of taxol-induced asters in human mitotic extracts (Dionne et al., 2000). In vivo, these proteins increase microtubule growth. Knockdown of the proteins is associated with short interphase microtubules, reduced growth rates and increased catastrophes and pauses (Brittle and Ohkura, 2005; Cullen et al., 1999; Tournebize et al., 2000; Wang and Huffaker, 1997). It was

proposed that the stabilizing effect of these proteins might be due to their interaction with microtubule destabilizing proteins. In fact, XMAP215 seems to stabilize microtubules by opposing the action of destabilizers such as XKCM1 (the *Xenopus* homologue of MCAK).

TOG proteins also bind to the spindle, spindle poles and, at least in yeast, kinetochores which indicates that they also play a role during mitosis. In accordance, in cells lacking Stu2p, mitosis was severely affected, because chromosomes were not able to align in the metaphase plate (Kosco et al., 2001). Furthermore, the fission yeast Dis1 is required for sister chromatid separation but not spindle elongation (Nabeshima et al., 1995; Ohkura et al., 1988). In Drosophila, Msps was shown to localize to kinetochores (Buster et al., 2007) however, it should be noted that in human cells there has been no report of ch-TOG localization to kinetochores. In addition, TOG proteins also localize to spindle poles, where microtubule minus-ends concentrate. In *Drosophila* embryos, the centrosomal protein D-TACC is required to efficiently recruit ch-TOG/Msps to centrosomes (Lee et al., 2001). The role of ch-TOG in spindle organization was proposed to occur in multiple ways. In human somatic cells, ch-TOG is thought to play a major role in organizing spindle poles, and a more minor role in stabilizing spindle microtubules via an interaction with TACC3 (Gergely et al., 2003). Its effect on the spindle involves an interaction with Aurora-A. In fact, depletion of Aurora-A leads to an accumulation of ch-TOG at spindle poles with a concomitant delocalization of MCAK (De Luca et al., 2008). Furthermore, it was suggested that ch-TOG acts on microtubules by protecting k-fibres from depolymerization by MCAK, forming a complex with TACC3 and clathrin, which physically cross-links these fibres and reduces microtubule catastrophes (Barr and Gergely, 2008; Booth et al., 2011). In addition, ch-TOG also has an essential role in centrosomal microtubule assembly independently of MCAK (Barr and Gergely, 2008). Interestingly, these TACC3/ch-TOG/clathrin k-fibre bridges are also regulated by Aurora-A (Cheeseman et al., 2011).

3.10 – Lis1

Lissencephaly 1 (Lis1) proteins were first described as the result of a mutation that leads to severe defect in brain development (Dobyns et al., 1993; Vallee et al., 2001). So far, many orthologs have been identified from yeast (Geiser et al., 1997) to *C. elegans* (!!! INVALID CITATION !!!) and *Drosophila* (Sheffield et al., 2000). Sequences from all orthologs are highly conserved, which suggests a conserved functional role. In structural terms, Lis1 proteins have

three distinct regions (Figure 18). The N-terminal region is called LIS1-homology motif (LisH), which ranges residues 1–39 and has been recently recognized as a ubiquitous motif, found in another 114 eukaryotic proteins (Emes and Ponting, 2001; Kim et al., 2004). The region between aminoacids 40-85 is predicted to be a coiled-coil region which, together with the LisH domain, is involved in dimerization (Tai et al., 2002). Near the C-terminal region, there are seven WD40 repeats which range from aminoacids 96-410 containing a β-propeller domain, which are important for lateral interactions with other proteins (Tarricone et al., 2004).

Figure 18 – Diagram of Lis1 structure. The protein contains a LisH domain near the N-terminal region (in brown) and a coiled coil region (in orange). In the C-terminal region there is a WD40 repeat structure (in blue).

In fact, although it acts as a +TIP, Lis1 seems to target microtubules by WD40-mediated binding to CLIP-170, dynein and dynactin, rather than binding the plus-ends directly (Coquelle et al., 2002; Tai et al., 2002). The interaction of Lis1 with CLIP170 is positively regulated by phosphorylation (Coquelle et al., 2002). During mitosis Lis1 is recruited to the cell cortex and kinetochores in a dynein/dynactin-dependent manner (Coquelle et al., 2002; Faulkner et al., 2000). The C-terminal WD40 repeat region of Lis1 seems to be sufficient for kinetochore targeting (Tai et al., 2002). When over-expressed, Lis1 induces a displacement of CLIP170 from the kinetochores but also interferes with spindle orientation and mitotic progression (Faulkner et al., 2000; Tai et al., 2002; Vallee et al., 2001).

4 - +TIPs in mitosis

4.1 – +TIPs in mitotic spindle organization

The transition from interphase to mitosis involves a dramatic reorganization of the microtubule cytoskeleton. This is accompanied by an increase in microtubule dynamics and an abrupt decrease in microtubule polymer level which tightly correlates with NEB (Zhai et al., 1996). Moreover, mitotic microtubules show increased catastrophe frequencies and spend less time in the "paused" state (Belmont et al., 1990; Rusan et al., 2001). Many of these changes appear to be controlled by phosphorylation-dephosphorylation regulatory mechanisms. Increasing phosphorylation of MAPs during mitosis leads to a decrease in microtubule stability (Andersen, 1998). This also happens with a number of +TIPs and may impact on their ability to regulate microtubule dynamics. Moreover, protein phosphatases PP1 and PP2A have been shown to differentially regulate microtubule dynamics (Tournebize et al., 1997). While PP1 is required for transitions into and out of mitosis, PP2A is required to maintain a steady state spindle length by controlling the level of catastrophes. In addition, CDK1 also plays a role in remodelling the microtubule cytoskeleton, as adding active CDK1 to *Xenopus* extracts increases microtubule dynamics to mitosis-like levels (Verde et al., 1990). Furthermore, CDK1 induces the depolymerization of interphase microtubules when injected into mammalian cells and also leads to the destabilization of microtubules when added to mammalian cell lysates (Lamb et al., 1990; Lieuvin et al., 1994).

Many different classes of +TIPs have been involved in mitotic spindle organization, some of which are regulated by phosphorylation. Notably, CLIP170 is necessary for establishment of spindle bipolarity by interacting with dynein (Tanenbaum et al., 2008). Interestingly, CLIP170 association to the microtubules is regulated by phosphorylation (Choi et al., 2002; Rickard and Kreis, 1991), although it is not known whether this has an impact on spindle organization. Mammalian CLASP1 and CLASP2 have also been shown to play redundant roles in the organization of the mitotic spindle (Mimori-Kiyosue et al., 2006). In fact, simultaneous depletion of CLASPs leads to mitotic spindle defects and abnormal mitotic exit. These results were further confirmed in fibroblasts derived from CLASP2 knockout mice (Pereira et al., 2006). These cells exhibit numerous spindle defects that can be rescued by ectopic expression of either CLASP1 or CLASP2, which further strengthens the claim to their redundancy during mitosis. Recent data further revealed that CLASPs ensure spindle integrity by conferring spindle pole resistance to CENP-E and Kid traction forces exerted during chromosome congression (Logarinho et al., 2012). EB proteins have also been implicated in

spindle organization. The first reports indicated that depletion of EB1 in *Drosophila* by RNAi leads to the formation short spindles and short astral microtubules (Rogers et al., 2002). In *Xenopus* egg extracts, EB1 was involved in spindle organization and chromosome segregation by interacting with XMAP215 (Kronja et al., 2009). In addition, EB1 is also involved in astral microtubule nucleation possibly by interacting with Kif18B (Stout et al., 2011; Toyoshima and Nishida, 2007). In mammalian cells, EB1 (together with APC) is necessary to maintain spindle integrity and ensure proper chromosome alignment (Green et al., 2005; Su et al., 1995). Interestingly,the EB1-interactor APC is hyper-phosphorylated during mitosis, which suggests that its binding to microtubules is regulated by phosphorylation (Bhattacharjee et al., 1996). Moreover, depletion of APC has been shown to compromise the formation of spindles in *Xenopus* extracts (Dikovskaya et al., 2004), although more direct evidence for the role of APC in spindle assembly in animal somatic cells is still lacking. The TOG family of proteins also plays an important role in spindle assembly. In fact, XMAP215 is required for this process in mitotic *Xenopus* extracts and its immunodepletion results in either absence of spindle formation or very short spindles (Tournebize et al., 2000). In mammalian cells, ch-TOG seems to be required for organization of spindle poles but has only a minor role in the stabilization of spindle microtubules (Gergely et al., 2003). The mechanism of ch-TOG-mediated microtubule stabilization is partly mediated by its interaction with TACC3 (Gergely et al., 2003) but it can also directly protect kinetochore microtubules from depolymerization by MCAK (Barr and Gergely, 2008). Overall, ch-TOG contributes to spindle bipolarity by increasing microtubule length and density, focusing microtubule minus-ends at the spindle poles and maintaining centrosome integrity (Cassimeris and Morabito, 2004).

Many motor proteins that act as +TIPs also have an essential role in spindle organization. Dynein is a minus-end directed motor that shows tip-tracking behaviour (Vaughan et al., 1999). Dynein can bind to microtubules and induce their stabilization by tethering the plus-ends (Hendricks et al., 2012; Steuer et al., 1990; Yoshida et al., 1985). Furthermore, cytoplasmic dynein localizes to the cell cortex and serves as an anchor for astral microtubules (Busson et al., 1998). By using their minus-end directed motion, these motors have a two-fold effect: they exert pulling forces that maintain spindle pole separation during mitosis (Laan et al., 2012; Vaisberg et al., 1993) and transport different cargo to the centrosome where they help maintain spindle pole integrity (Purohit et al., 1999; Young et al., 2000). In addition to dynein, there are other motors such as kinesin-13 MCAK that also plays a role in spindle assembly. MCAK localizes to spindle poles and kinetochores/centromeres and also behaves as a +TIP (Ems-McClung and Walczak, 2010). Activity of MCAK at the kinetochores

is required for spindle assembly because it balances the Kif2A-dependent microtubule polymerization (Ganem and Compton, 2004). In fact, in mitotic extracts depleted of XKCM1 (the *Xenopus* homologue of MCAK), there is a 4-fold decrease in catastrophe frequencies, which leads to the formation of very long microtubules and assembly of a monopolar spindle (Walczak et al., 1996).

4.2 - +TIPs at the centrosome

In addition to their tip-tracking ability, many +TIPs have the ability to bind to or interfere with centrosome function. EB1 was first reported to localize to centrosomes in *Dictyostelium*. In this system, EB1 was localized to microtubule-free, isolated centrosomes. Moreover, EB1 is said to be required for initiation of spindle microtubule growth (Rehberg and Graf, 2002). In mammalian cells, EB1 also interacts with centrosomes independently of microtubules, through its C-terminal domain (Louie et al., 2004). In fact, depletion of EB1 leads to a reduction in minus-end anchoring and delays microtubule regrowth from centrosomes. More recently, it was shown that FOP (FGFR1 Oncogene Partner) interacts with the C-terminal region of CAP350 and forms a centrosomal complex necessary for microtubule anchoring. Interestingly, FOP is required for EB1 centrosomal localization (Yan et al., 2006). This localization could also be mediated by an interaction with CDK5RAP2. In fact, in addition to its centrosomal localization, CDK5RAP2 exhibits a tip-tracking behaviour that depends on EB1 binding, through a basic and Ser-rich motif (Fong et al., 2009). Moreover, CDK5RAP2 contains a centrosome-targeting domain that has a high homology to the Motif 2 of centrosomin (CM2) and mediates the association with pericentrin and AKAP450 (Fong et al., 2009; Wang et al., 2010). CLASP1 and CLASP2 also localize to centrosomes during mitosis (Pereira et al., 2006). In fact, loss of CLASPs has been implicated with an increase in the number of multipolar spindles, which suggested they might play a role in centrosome integrity. Indeed, it has been recently demonstrated that human CLASPs ensure spindle-pole integrity after bipolarization in response to traction forces exerted by CENP-E and Kid during chromosome alignment (Logarinho et al., 2012).

The TOG family of proteins also plays a relevant role at the centrosome. As was already mentioned above, depletion of ch-TOG in human somatic cells leads to the appearance of robust but highly disorganized spindles (Gergely et al., 2003). However, ch-TOG also seems to be required for centrosomal microtubule nucleation or stabilization, as absence of the protein

leads to both diminished assembly and less dynamic microtubules (Barr and Gergely, 2008). In addition, ch-TOG also acts by focusing minus-ends at the spindle poles, ensuring centrosome integrity (Cassimeris and Morabito, 2004), but also protects spindle microtubules from MCAK activity at the centrosome, which can lead to multipolar spindles (Holmfeldt et al., 2004). Curiously, the localization of ch-TOG and MCAK at the centrosome and subsequent centrosome stabilization is regulated by Aurora-A (De Luca et al., 2008).

4.3 - +TIPs at the kinetochore

In addition to their role in spindle assembly, many +TIPs are also involved in the regulation of the microtubule-kinetochore attachment. Initial experiments with CLIP170 described its transient association with prometaphase kinetochores, even before CLIP170 was shown to tip-track (Dujardin et al., 1998; Perez et al., 1999). CLIP170 distributes with dynein and dynactin at kinetochores and is required for the formation of kinetochore fibres (Dujardin et al., 1998). Another study concluded that interfering with CLIP170 expression leads to defects in chromosome congression and a decrease in the number of kinetochore-microtubule attachments (Tanenbaum et al., 2006). However, this does not affect microtubule dynamics or the stability of kinetochore-microtubule attachments. These observations indicate that CLIP170 may help in the formation of kinetochore-microtubule attachments by mediating the direct capture of microtubules at the kinetochore (Tanenbaum et al., 2006). Interestingly, this kinetochore-microtubule attachment mechanism may involve phosphoregulation of CLIP170. Indeed, it was proposed that CK2-mediated phosphorylation of CLIP170 is involved in CLIP170 kinetochore localization (Li et al., 2010). Moreover, Plk1 is necessary to enhance this association with CK2. Overall, Plk1- and CK2-associated phosphorylations of CLIP170 are necessary for the timely formation of kinetochore-microtubule attachments during mitosis.

CLASPs also play an important role at the kinetochore. Orbit/MAST, the *Drosophila* homologue of CLASP, was originally reported to be necessary for bipolar spindle assembly (Lemos et al., 2000). Subsequent work in human cells demonstrated that kinetochore localization of CLASPs depends on CENP-E, independently of its motor activity (Logarinho et al., 2012; Maffini et al., 2009). In addition, it was shown that kinetochore targeting requires the C-terminal domain of CLASP1 and CLASP2 but is independent of microtubules or CLIP170 association (Maiato et al., 2003a; Mimori-Kiyosue et al., 2006). At the kinetochores, *Drosophila* CLASP is essential for tubulin subunit incorporation into fluxing k-fibres (Maiato et al., 2005) and maintaining proper microtubule dynamics. This explains why depletion of CLASP leads to

abnormal chromosome congression, collapse of the spindle and attachment of kinetochores to very short microtubules (Maiato et al., 2003a; Maiato et al., 2003b). In mammalian cells, CLASPs depletion leads to a metaphase delay and rapid microtubule growth (Mimori-Kiyosue et al., 2006). For this reason, CLASPs were proposed to provide local stabilization of microtubules by reducing the amplitude of growth/shortening episodes at the plus-end. In fact, while it is true that CLASPs induce stabilization of microtubules in metaphase, this does not represent the full complement of CLASPs roles during mitotic progression. Accordingly, CLASPs were proposed to act as a switch during mitotic progression by interacting with different partners (Manning et al., 2010). In early mitosis, CLASP1 forms a complex with Kif2b at the kinetochores that promotes chromosome movement, kinetochore-microtubule turnover and correction of attachment errors. As cells progress into metaphase, this complex is then replaced by a CLASP1-Astrin complex which promotes kinetochore-microtubule stability and chromosome alignment. Interestingly, these different complexes appear to be mutually exclusive, suggesting that recruitment of these different complexes to kinetochores is enough to change microtubule dynamics. At the transition from metaphase to anaphase, CLASPs are removed from kinetochores and this depends mostly on dynein-dependent minus-end directed movement (Reis et al., 2009).

EB1 localizes to plus-ends of polymerizing microtubules which indicates it may regulate microtubule dynamics during mitosis (Tirnauer et al., 2002a). Although EB1 was originally identified as an APC-interacting protein, it localizes to the plus-ends independently of APC (Berrueta et al., 1998; Morrison et al., 1998). Curiously, the inverse is not true, as APC localization to the plus-ends requires an interaction with EB1 (Askham et al., 2000; Mimori-Kiyosue et al., 2000b). Moreover, the interaction between APC and EB1 seems not to be relevant for EB1 mitotic localization, as immunoprecipitation studies demonstrated that the EB1-APC interaction does not occur during mitosis, possibly because of APC hyperphosphorylation (Askham et al., 2000; Bhattacharjee et al., 1996; Nakamura et al., 2001). In *Xenopus* mitotic extracts, both EB1 and APC interact with kinetochore-associated BubR1 (Zhang et al., 2007a). In this system, BubR1 directly interacts with APC and this is essential for positioning of chromosomes in the metaphase plate. Earlier reports had already identified interaction of APC with checkpoint proteins Bub1 and Bub3 at the kinetochore (Kaplan et al., 2001). This may explain why in cells carrying a truncated APC gene (Min), there are severe defects in chromosome segregation. Furthermore, loss of APC leads to changes in mitotic progression associated with a decrease in metaphase interkinetochore tension (Dikovskaya et al., 2007; Draviam et al., 2006). Similarly, this was accompanied by a decrease in the

association of Bub1 and BubR1 with kinetochores, which suggests that APC may be involved in the loading of these proteins.

The minus-end directed motor dynein also localizes to kinetochores. This localization is regulated by microtubule attachment to the kinetochores but does not depend on tension (King et al., 2000). In fact, dynein binding to the kinetochore is very sensitive, as "fewer than half the normal number of kinetochore microtubules leads to the loss of most kinetochore dynein" (King et al., 2000). A significant pool of kinetochore dynein is regulated by Plk1-mediated phosphorylation, as inhibiting Plk1 severely affects dynein localization to the kinetochore without affecting dynactin or Zw10 (Bader et al., 2011). Under these conditions, inhibition of Plk1 induces defects in microtubule capture and persistent microtubule attachments during prometaphase. What could be the role of dynein at the kinetochore? Dynein associates with kinetochores during prometaphase and, as a minus-end motor, generates a pulling force on microtubules. By interfering with dynein localization at the kinetochore cells fail to achieve efficient chromosome alignment and exhibit problems in microtubule capture (Li et al., 2007). For this reason, kinetochore dynein was proposed to produce a poleward pulling force on monooriented kinetochores that facilitates microtubule capture by the kinetochore and promotes chromosome congression. Depleting or inhibiting kinetochore dynein prevents the rapid poleward motion of attaching kinetochores but does not interfere with kinetochore fibre formation (Yang et al., 2007). In addition, dynein also plays a role in stable microtubule attachment and kinetochore orientation during metaphase, although its kinetochore levels are reduced (Varma et al., 2008; Yang et al., 2007). This effect may be related to the ability of dynein to remove some kinetochore components during mitosis to ensure microtubule stability as was shown for CLASPs (Reis et al., 2009). Interestingly, kinetochore dynein is also required for anaphase chromosome movement independently of poleward microtubule flux (Yang et al., 2007).

In prophase, MCAK localizes to the inner kinetochore. During chromosome congression, MCAK specifically associates with the leading kinetochore (Kline-Smith et al., 2004). Depletion or disruption of MCAK leads to defects in congression, alignment and segregation of chromosomes (Kline-Smith et al., 2004; Zhu et al., 2005). These defects may be the consequence of improper kinetochore-microtubule attachments that arise when MCAK is disrupted and include merotelic or syntelic attachments. For this reason, MCAK was proposed to play a role in the prevention and/or correction of kinetochore-microtubule attachments (Kline-Smith et al., 2004). In fact, additional work demonstrated that MCAK is required for the turnover of kinetochore microtubules and this helps in the directional switching between sister

centromeres (Wordeman et al., 2007). In this context, MCAK`s role would be to contribute to error correction by allowing either the release of microtubules from the kinetochore or by inducing microtubule turnover. Interestingly, this may be mediated by Aurora B phosphorylation. In fact, phosphorylation of MCAK at serine 196 or expression of a phospho-mimic mutant of the protein inhibits MCAK microtubule depolymerizing activity (Andrews et al., 2004; Lan et al., 2004). In addition to this, phosphorylation also seems to play a role in MCAK localization. Expression of a phospho-mimetic mutant of MCAK displays mainly centromeric localization, while the phospho-null form mainly localizes to kinetochores (Andrews et al., 2004).

4.4 – +TIPs regulation during mitosis

Many +TIPs exhibit different localization or behaviour during mitosis. This raises the questions of how are the different +TIPs regulated and could different modifications allow for spatiotemporal regulation of their function? CLIP170 was one of the first +TIPs described to be phosphorylated *in vivo* at multiple sites (Choi et al., 2002). In this report, the authors identify an interaction of CLIP170 with FRAP kinase and treatment with rapamycin interferes with the ability of CLIP170 to associate with microtubules. However, the same report describes several rapamycin-sensitive and –insensitive phosphorylation sites, which indicates there must be several kinases regulating CLIP170 function. Accordingly, both Plk1 and CK2 have been identified as CLIP170 kinases (Li et al., 2010). The CK2-mediated phosphorylation is essential for kinetochore targeting of CLIP170 in a dynactin-dependent manner. In this context, Plk1 seems to act as a priming kinase, which enhances the ability of CLIP170 to bind to CK2. Expression of the phospho-null mutants of CLIP170 is sufficient to displace the protein from the kinetochore and induce defects in the formation of kinetochore fibres, which further highlights the importance of CLIP170 phosphoregulation (Li et al., 2010). This phosphoregulatory mechanism may also be relevant to control CLIP170 association to the plus-ends by inducing conformational changes in the protein. CLIP170 switches between two conformation states that alter its affinity for microtubules (Lansbergen et al., 2004). While in its phosphorylated state, CLIP170 shows enhanced binding between the N- and C-terminal domains and remains in a “closed conformation (Lee et al., 2010). This phosphorylated form of CLIP170 has lower affinity for microtubules and does not interact with p150glued. The phospho-null mutant of CLIP170 is in an “open” conformation and has higher affinity for the

plus-ends of microtubules and p150glued (Lee et al., 2010). This leads to an auto-inhibitory mechanism that confers tighter control of CLIP170 association to the microtubule.

In a similar fashion, CLASPs localization during mitosis is also regulated by phosphorylation both in a direct and in an indirect manner. Astrin and SKAP both bind microtubules directly and are required for CLASP kinetochore localization (Schmidt et al., 2010). However, this complex targets only to bioriented kinetochores due to an Aurora B-antagonizing regulatory mechanism. This provides a spatiotemporal control of kinetochore composition by an Aurora B phosphoregulatory mechanism. In addition to this indirect regulation, CLASP2 can also be directly phosphorylated during mitosis. Although CLASP2 shows tip-tracking behaviour in interphase, it is not easily detected in the plus-ends during metaphase. This raised the possibility that plus-end localization could be under the regulation of specific mitotic kinases. Indeed, Kumar *et al.* showed that priming phosphorylations of CLASP2 by CDK1 and subsequent GSK3β phosphorylation were required to abolish CLASP2 plus-end tracking (Kumar et al., 2012). Interestingly, this tracking behaviour depended on the interaction of CLASP2 with EB1, as imposing the phosphorylations induced a disruption between the two proteins, which led to the displacement of CLASP2 from the plus-ends. Conversely, introducing phospho-null mutations on these specific sites was sufficient to restore binding to the plus-end.

Given their crucial role in the regulation of microtubule dynamics, surprisingly little is known on how EB function is regulated. Much of the recent work has focused on the budding or fission yeast homologues of EB1. The budding yeast EB1 protein Bim1p is regulated by multisite phosphorylation by the Aurora B homologue Ipl1p (Zimniak et al., 2009). EB1 forms a stable complex with Aurora B, which then phosphorylates a serine cluster in the linker region of EB1 and this phosphorylation is sufficient reduce the affinity of EB1 for microtubules. Inversely, a mutation of the fission yeast EB1 protein Mal3 was reported to increase the affinity of the protein to microtubules (Iimori et al., 2012). When the glutamine on position 89 in the CH domain was replaced with an arginine, EB1 no longer behaved as a +TIP but associated with the entire microtubule lattice. This also prevented EB1 from dissociating from the microtubule even when it was not growing, leading to a reduction in the shrinkage rate. Although the association of EB1 to the microtubule is negatively regulated by phosphorylation in the linker region, this mutation is sufficient to abolish this effect, leading to stabilization of the microtubule (Iimori et al., 2012). How do these phosphorylations regulate the affinity of EB1 for microtubules? One may consider that they affect the interaction of EB1 with partner proteins such as CLASP2 and this could impact on the overall affinity of EB1 for the plus-end.

On the other hand, phosphorylation of EB1 could introduce negative charges in the protein which would disrupt the association with the microtubule through electrostatic repulsive interactions as was recently proposed (Buey et al., 2011). Nevertheless it seems plausible that phosphorylation of EB1 leads to an overall decrease of interaction with the microtubule. Curiously, EB3 does not seem to behave in the same manner. During mitosis, EB3 was reported to be phosphorylated by Aurora kinases (Ban et al., 2009). This phosphorylation induces a stabilization of the protein because it prevents its polyubiquitination and proteasome-mediated degradation. At this point, it is not clear whether this phosphorylation has any impact on microtubule dynamics during mitosis.

Motor proteins are also subject to regulation during mitosis. Dynein was shown to be phosphorylated during mitosis when added to *Xenopus* egg extracts. This was dependent on CDK1 and happened specifically after incubation with metaphase but nor interphase extracts (Dell et al., 2000). At least partially, dynein phosphorylation could direct its binding to specific partners or structures. Phosphorylation of dynein intermediate chain favours its association to Zw10 instead of dynactin and this triggers dynein accumulation at the kinetochore (Whyte et al., 2008). Interestingly, this association persists until chromosomes become bioriented, which triggers dynein dephosphorylation. Dephosphorylated dynein then associates preferentially with dynactin and exhibits poleward streaming, which removes it from the kinetochore (Whyte et al., 2008). In addition to CDK1, kinetochore dynein is also phosphorylated by Plk1 (Bader et al., 2011). Interfering with Plk1 induces mislocalization of dynein without significantly affecting dynactin or Zw10 and this leads to defects in microtubule capture at the kinetochore.

MCAK is regulated by phosphorylation through several kinases, of which Aurora B seems to be the most prevalent (Tanenbaum et al., 2011). Addition of phosphates in the N-terminal region of MCAK near the SxIP motif seems to affect its ability to bind EB1 and exhibit tip-tracking behaviour (Honnappa et al., 2009; Moore et al., 2005). Furthermore, Aurora B mediated phosphorylation of MCAK impairs its interaction with TIP150 (Jiang et al., 2009). Overall, these phosphorylations may contribute to a decrease in the recruitment of MCAK to the plus-ends of microtubules, favouring microtubule growth. In agreement, *in vivo* phosphorylation of the neck region of MCAK varies according to the mitotic stage: it is high in early mitosis, but decreases when chromosomes become aligned and kinetochore-microtubule attachments have to be stabilized (Lan et al., 2004). Many phosphorylation sites regulate MCAK binding to spindle poles, kinetochores, centromeres and chromosome arms (Andrews et al., 2004; Lan et al., 2004; Zhang et al., 2008; Zhang et al., 2007b). Moreover, these

phosphorylation events seem to affect specific pools of MCAK. Accordingly, MCAK neck phosphorylation can be found mainly at the centromere, whereas Aurora B phosphorylation at serine 95 inhibits this localization. Strikingly phosphorylation at serine 110 by Aurora B increases centromere binding (Zhang et al., 2007b), which suggests multiple layers of regulation depending on spatiotemporal constraints. A mitotic-specific phosphorylation of MCAK by CDK1 has also been reported (Sanhaji et al., 2010). This modification inhibits MCAK`s microtubule depolymerising activity and can be mimicked by expressing a phospho-mimetic mutant (Sanhaji et al., 2010). However, it is not yet clear what the functional relevance of this modification is. In fact, if microtubules should be more dynamic during mitosis, why should MCAK activity be impaired at this stage? It may be that this phosphorylation (such as happens with Aurora B-mediated phosphorylations), affects only a small percentage of MCAK, which means there must be a local regulation of microtubule dynamics during mitosis. Finally, the C-terminal domain of MCAK which affects its own microtubule depolymerase activity is phosphorylated by Plk1 (Moore and Wordeman, 2004a; Zhang et al., 2010). Unlike CDK1- and Aurora B-mediated phosphorylations, Plk1 phosphorylation promotes the microtubule destabilizing activity of MCAK.

Thus, many layers of regulation ensure that localization and activity of +TIPs during mitosis are tightly regulated and allow successful completion of the process. While phosphorylation emerges as the major regulatory mechanism to control +TIPs interactions, it remains unclear how all these can be integrated to form a coherent picture.

4.5 – Role of +TIPs in positioning the mitotic spindle

Positioning of the mitotic spindle is essential in two ways: first it contributes to the determination of the cleavage plane, which ensures that the two sets of chromatids are equally divided among the daughter cells and second, it helps determine cell fate and tissue organization by responding to intracellular and extracellular cues. In this context, spindle positioning can give rise to two identical daughter cells (symmetric division) or cells with an unequal size or content (asymmetric division). In budding yeast, the site where the bud appears defines where cytokinesis will take place (Chant and Pringle, 1995). Therefore, the spindle will position itself perpendicularly to the predefined cleavage plane (Yeh et al., 1995; Yeh et al., 2000). Positioning of the spindle requires interactions between astral microtubules, the actin cytoskeleton, cortical determinants and many +TIPs (Carminati and Stearns, 1997;

Heil-Chapdelaine et al., 2000; Palmer et al., 1992). Interestingly many of these requirements are conserved in mammalian cells.

The first evidence for +TIP involvement in mammalian spindle positioning came from studies with dynein. In yeast, mutations in the dynein gene affect movement of the spindle into the budding daughter cell without affecting spindle assembly or chromosome segregation (Li et al., 1993). Later work done in mammalian cells showed that when the shape of rat epithelial cells is manipulated during mitosis, the mitotic spindle will always align with the longer cell axis. The spindle alignment can be blocked by inhibiting dynein. This led to the hypothesis that longer astral microtubules, by having a higher number of dynein motors, would be able to generate higher forces on the spindle and align it with the long cell axis (O'Connell and Wang, 2000). This model implies that either astral microtubules are in contact with the actin cortex along their entire length, or that dynein motors can anchor to cytoplasmic complexes and exert a pulling force on microtubules, as was later also suggested for interphase cells (Brodsky et al., 2007). Interestingly, dynein localization seems to depend on both spindle-pole and chromosome-derived signals which affects cortical force generation. Proximity of spindle poles with the cortex displaces dynein to the opposite pole, which results in spindle centering. Activity of Plk1 at the spindle poles is necessary because it regulates the interaction between dynein-dynactin and the cortical factors NuMA and LGN (Kiyomitsu and Cheeseman, 2012). Furthermore, a chromosome-derived RanGTP gradient restricts the localization of NuMA-LGN to the lateral cortex which enforces the spindle orientation axis.

Similarly to dynein, the tumour suppressor and plus-end tracking protein APC is also involved in correct spindle positioning. When a dominant mutated form of APC is expressed in cells or when APC is disrupted, the spindle is displaced from the cell centre (Draviam et al., 2006; Green and Kaplan, 2003). This was also observed when EB1, a known APC interactor, is depleted from cells and correlates with a marked loss of astral microtubules (Draviam et al., 2006; Green et al., 2005). This is all the more relevant because EB1 and Myosin10 were shown to be required for integrin-mediated spindle orientation mechanism (Toyoshima and Nishida, 2007). By stabilizing astral microtubules, EB1 helps orient the spindle parallel to the cell-substrate and provides an additional link between the spindle and the cell cortex. Recently, EB1 was also shown to be required for spindle symmetry. Upon injection of specific antibodies or a dominant-negative form of EB1 in mitotic cells, the resulting daughter cells displayed an unequal content of microtubules and this correlated with an asymmetric spindle pole

movement (Bruning-Richardson et al., 2012). However, in this study there was no significant displacement of the spindle from the cell centre.

Recently, association between CLASP1 and the tau-related protein MAP4 were described as important for maintaining spindle position and defining the division axis in human cells (Samora et al., 2011). This association serves two purposes: whereas CLASP1 is required for astral microtubule capture at the cortex, MAP4 is necessary to prevent engagement of excess dynein motors creating an equilibrium situation. Under these conditions, depletion of MAP4 specifically induces spindle misorientation relative to the substrate. Interestingly, MAP4 depletion does not affect astral microtubule nucleation, which suggests that it is not only the presence of astral microtubules that is required for accurate spindle positioning. Curiously, in *C. Elegans* CLASPs have a partial redundant role in spindle positioning and astral microtubule regulation during asymmetric cell division (Espiritu et al., 2012). In this system, simultaneous depletion of the CLASP homologue CLS-1 together with CLS-2 or CLS3 induces displacement of the spindle, together with changes in spindle length. These cells also have a reduced complement of astral microtubules, which accounts for the positioning phenotype.

It is already well established that astral microtubule nucleation is essential for spindle positioning and, when compromised, spindles will displace inside the cell. However, recently it has been shown that excessive nucleation can also produce the same effect. In fact, when the microtubule depolymerizer MCAK is depleted from HeLa cells, very long astral microtubules are produced (Rankin and Wordeman, 2010). This same effect can be accomplished by treating cells with the microtubule stabilizing drug Taxol. As a consequence, the spindle shows dramatic rocking inside the cell, that is dependent on Myosin II (Rankin and Wordeman, 2010). Overall, these results suggest that regulation of astral microtubule dynamics, together with cortical factors cooperate to ensure proper spindle positioning and successful completion of mitosis.

4.6 - +TIPs in mitotic exit and cytokinesis

The completion of mitosis involves the final separation of sister chromatids into two daughter cells and partitioning of the cytoplasm. This last step requires the formation of an actomyosin ring that will constrict the microtubules in the midzone region (Fujiwara and Pollard, 1976; Schroeder, 1972; Schroeder, 1973). While myosin function in the cytokinetic ring requires astral microtubule interaction with the cortex (Foe and von Dassow, 2008), this seems

to be independent of precise regulation of microtubule dynamics because stabilization of microtubules or increases in microtubule dynamics both induce furrow formation (Strickland et al., 2005a). Changing of the midzone to midbody correlates with furrow ingression and when this is prevented, cells accumulate midzone-like microtubule structures (Straight et al., 2003). Most microtubules that compose the spindle midbody are anti-parallel microtubules that derive from the spindle midzone (Elad et al., 2010; Mullins and Biesele, 1977). As opposed to earlier stages of cytokinesis, microtubules are essential for completion of the process (Savoian et al., 1999). These midbody microtubules are highly stable (Margolis et al., 1990), were shown to be acetylated (a marker of stable microtubules) and are resistant to nocodazole treatment (Foe and von Dassow, 2008; Piperno et al., 1987). However, microtubules in the midbody region can also be nucleated *de novo*, and this may involve γ-tubulin, which is also required for successful completion of cytokinesis (Julian et al., 1993; Shu et al., 1995). Moreover, some midbody microtubules are still able to exhibit a highly dynamic behaviour as can be seen by live imaging of microtubule plus-ends with EB proteins, with plus-ends moving in and out of the structure (Rosa et al., 2006).

Exit from mitosis requires the inactivation of CDK1. This inactivation induces a reorganization of the microtubule cytoskeleton that includes astral microtubule nucleation and midbody formation (Wheatley et al., 1997). In addition to this more general role on microtubule organization, in yeast CDK1 was show to control Aurora kinase by phosphorylating its N-terminal domain (Zimniak et al., 2012). Interestingly, this phosphorylation blocks association of Aurora with the yeast EB1 until anaphase onset. Association between Aurora and EB1 is required for EB1 phosphorylation on its linker region (Zimniak et al., 2009). This phosphorylation is necessary for efficient EB1 tip-tracking of microtubules plus-ends and occurs specifically during anaphase as a means to ensure normal spindle elongation and disassembly of the spindle midzone. Therefore, if CDK1 fails to phosphorylate Aurora kinase, this leads to its premature targeting to the spindle and constitutive EB1 phosphorylation, resulting in problems during mitotic exit (Zimniak et al., 2012). In human cells EB proteins are tightly associated with midzone and midbody microtubules (Berrueta et al., 1998; Morrison et al., 1998). Moreover, EB1 and Aurora B were shown to co-localize in these same structures (Sun et al., 2008). However, unlike in yeast cells, human EB1 is not a substrate of Aurora B, but is required to enhance the kinase activity of Aurora. It does so by preventing association of PP2A with Aurora B and protecting it from dephosphorylation (Sun et al., 2008). This is in apparent contradiction with the studies performed in yeast but one must bear in mind that human cells have more than one EB protein. In fact, both Aurora A and Aurora B were shown

to phosphorylate EB3 during mitosis (Ban et al., 2009). Taken together, the interactions between EB proteins and other mitotic exit-related proteins highlight the importance of +TIPs in this context. Accordingly, if formation of astral microtubules is suppressed during anaphase by interfering with either EB1 or with dynactin, there is a significant delay in cytokinesis (Strickland et al., 2005b). During anaphase phosphorylated MCAK localizes to the spindle midzone and this is important because it allows regulation of MCAK microtubule depolymerization activity (Lan et al., 2004). This phosphorylation is carried out by Aurora B (Fuller et al., 2008; Lan et al., 2004). Recently, a new +TIP termed TIP150 was shown to localize to the plus-ends until anaphase B, in an EB1-dependent manner. TIP150 also interacts with the microtubule depolymerase MCAK and appears to assist in the EB1-mediated recruitment of MCAK to the plus-ends (Jiang et al., 2009). Interestingly, MCAK shares common cellular localizations with EB1 and Aurora B. Taken together this means that, either directly or indirectly, Aurora B and CDK1 seem to regulate the localization or activity of many EB1-associated proteins after anaphase onset and until cytokinesis.

Other EB-associated proteins such as APC or CLASPs have also been shown to independently regulate mitotic exit. During anaphase and telophase both CLASP1 and CLASP2 accumulate in the spindle midzone and midbody (Maiato et al., 2003a; Mimori-Kiyosue et al., 2006) and CLASPs were shown to be required for cytokinesis completion (Pereira et al., 2006). At least CLASP1 recruitment to the central spindle depends on interaction with PRC1 early at anaphase (Liu et al., 2009). Due to the partial redundancy of CLASPs during mitosis (Pereira et al., 2006), it is possible that CLASP2 also shares this late mitotic function, although this remains unknown at the moment. Depletion of CLASP1 or interference with the PRC1-CLASP1 interaction is sufficient to depolymerize the spindle midzone and prevent faithful chromosome segregation (Liu et al., 2009). However, given that both PRC1 and CLASP1 are required for microtubule bundling and PRC1 also allows stabilization of the spindle midzone microtubules (Maiato et al., 2003a; Mollinari et al., 2002), it is possible that PRC1 acts by allowing anti-parallel microtubule elongation and providing stabilization for CLASP1 association with the central spindle microtubules. Interestingly, PRC1 itself is directly regulated by phosphorylation by CDK1 and Plk1, which puts it in the right context for regulating mitotic exit (Hu et al., 2012; Jiang et al., 1998). APC, one of the principal interactors of EB1, has been extensively implicated in cytokinesis completion. Reports on APC mutants demonstrated that these cells become polyploidy over time (Fodde et al., 2001; Kaplan et al., 2001; Tighe et al., 2004), which indicates that APC plays a role in cytokinesis. Although the different APC alleles behave in distinct manner, it is believed that they may interfere with anchoring of the mitotic spindle

(Caldwell et al., 2007). In fact, in a C-terminal truncated mutant of APC, microtubules make fewer contacts with the cell cortex. For this reason, spindles rotate excessively and this leads to cytokinetic failures (Caldwell et al., 2007). Interestingly, both in *Min* mice and APC knockout mice there is an increase in the number of tetraploid cells, which is a hallmark of cytokinesis failure (Caldwell et al., 2007; Dikovskaya et al., 2007).

Other non-EB1-associated proteins play important roles in post-anaphase cells. The kinesin CENP-E, which was shown to accumulate at the plus-ends of microtubules (Sardar et al., 2010), localizes to the midbody where it uses its coiled-coil domain to interact with Skp1 (Liu et al., 2006). These proteins show an inverse correlation at the midbody, with CENP-E levels decreasing as Skp1 associates with this structure. In fact, there is a tight spatiotemporal regulation of CENP-E at the midbody that is essential for completion of cytokinesis and the Skp1 interaction may be essential for CENP-E proteolysis. Dynein may also play a role in cytokinesis, although the mechanism is not completely clear. Dynein light intermediate chain 1 (LIC1) is concentrated at the midbody during abscission (Horgan et al., 2010). Moreover it was recently shown that dynein is necessary for transport of Rab8-positive vesicles to the midbody and this is required for completion of cytokinesis (Kaplan and Reiner, 2011).

In conclusion, +TIPs can impact mitotic exit at many different levels. They interact with the major kinases regulating transition from mitosis to interphase, such as CDK1, Plk1 and Aurora B. Moreover, they are prominently localized to the spindle midzone and midbody which are crucial in the outcome of mitosis. Although they have a significant role in the direct regulation of microtubule function, there is a network of reciprocal interactions at the midzone and midbody which is regulated by phosphorylation by Aurora B or CDK1 and involves many different families of +TIPs.

II. OBJECTIVES

Mammalian EB proteins have emerged as major regulators of microtubule function. Surprisingly, little is known about their roles during mitosis. The main goal of this thesis was to understand how EB proteins regulate mitotic progression and whether they have redundant or distinct roles during mitosis. For this purpose, we set out to characterize the localization and function of EB1 and EB3 in human mitotic cells using an RNAi approach. We analysed the effect of EBs in mitotic spindle positioning through their direct role in the regulation of microtubule dynamics and also in the interaction with a dynamic actin cluster that exists during mitosis and was recently characterized (Fink et al., 2011; Mitsushima et al., 2010). The respective results and discussion are presented in Chapters III and IV and highlight the non-redundant roles of EB1 and EB3 during mitosis.

We then determined whether post-mitotic cells that exhibited defects during mitotic progression were also affected by EB protein depletion. For this purpose, in chapter V we studied the role of EB proteins in the regulation of cell adhesion and motility following an abnormal exit from mitosis. We correlate this with the ability of EB proteins to regulate microtubule dynamics and, consequently, the stability of cell-matrix adhesions.

One of the major unanswered questions in the field of +TIPs is to determine what regulates the association of EB proteins to the microtubule plus-ends. Therefore, in chapter VI, by using live cell imaging, RNAi approaches and small molecule inhibitors we address whether there is a cell-cycle regulated mechanism that ensures EB association to the plus-ends and whether this mechanism is relevant in the context of microtubule dynamics regulation and mitotic progression.

III. DISTINCT EB3 PHOSPHORYLATION STATES REGULATE POST-MITOTIC CELL-SUBSTRATE ADHESION AND CYTOKINESIS

(In revision at the Journal of Cell Biology)

Abstract

During mitosis, human cells round up decreasing their adhesion to extracellular substrates. This must be quickly re-established by yet unclear cytoskeleton remodelling mechanisms that prevent detachment from epithelia, while ensuring the successful completion of cytokinesis. Here we show that the microtubule end-binding proteins EB1 and EB3 play temporally distinct roles during and at the exit from mitosis. While EB1 is required for spindle orientation prior to anaphase, EB3 is required for stabilization of focal adhesions necessary for coordinated daughter cell spreading during mitotic exit. EB3 is also required for midbody stabilization and integrin accumulation at the cleavage furrow necessary for cytokinesis. Importantly, daughter cell spreading and cytokinesis completion rely on distinct phosphorylation states of EB3 on serine 176, an Aurora B-dependent phosphorylation site that regulates microtubule growth capacity. These findings uncover differential roles of EB proteins and are consistent with the existence of an Aurora B phosphorylation gradient important for the spatiotemporal regulation of microtubule function during late mitosis and cytokinesis.

1- Introduction

Human cells round up during mitosis as a result of increased hydrostatic pressure and actomyosin cortex contraction, which counteracts adhesion to extracellular substrates (Stewart et al., 2011). Thus, mitosis represents a short period in the lifetime of the cell where loss of substrate adhesion is maximal. If cell-substrate adhesion is not rapidly re-established upon completion of mitosis, cells may detach from epithelia, which has been proposed as a mechanism for cancer cell dissemination and metastasis (Vasiliev et al., 2004).

Upon mitotic entry, cell-substrate adhesion complexes are disassembled in a process that involves the phosphorylation of Focal Adhesion Kinase (FAK) and its release from other adhesion components such as paxillin and p130/Cas (Yamakita et al., 1999). Stable interaction of mitotic cells with the extracellular matrix is normally achieved through a set of actin-rich structures called retraction fibres (Mitchison, 1992). These not only provide physical attachment of the cell to the substrate but also play an active role during mitosis by providing spatial cues for spindle positioning (Thery et al., 2005). Additional work further highlighted the role of integrin β1-mediated adhesion in spindle alignment with the substrate (Toyoshima and Nishida, 2007). Integrins have also been implicated in the regulation of microtubule growth, spindle assembly (Reverte et al., 2006) and cytokinesis (Aszodi et al., 2003; Pellinen et al., 2008). Thus, a scenario where adhesion complexes act as mediators of the mitotic and cytokinetic machinery is emerging, but how the molecular adhesion machinery cross-talks with cytoskeleton components, microtubules in particular, remains poorly understood.

Microtubule End-Binding (EB) proteins are a conserved family of microtubule plus-end tracking proteins (+TIPs) (reviewed by (Akhmanova and Steinmetz, 2008)). In humans, they consist of three closely related members, EB1, EB2 and EB3. EB1 has been the most widely studied because of its interaction with the C-terminus of Adenomatosis Polyposis Coli (APC), which is often disrupted in colon cancers due to truncating mutations (Askham et al., 2000; Nakamura et al., 2001; Su et al., 1995). During early mitosis, EB1 is involved in spindle orientation in yeast, *Drosophila* and human cells (Green et al., 2005; Lee et al., 2000; Rogers et al., 2002; Toyoshima and Nishida, 2007). This role of EB1 involves stabilization of astral microtubules and possible interaction with APC (Green et al., 2005; Toyoshima and Nishida, 2007). Interaction between EB1 and APC was also shown to promote microtubule stabilization in interphase cells (Mimori-Kiyosue et al., 2000b; Nakamura et al., 2001; Wen et al., 2004). Loss-of-function studies of EB1 have reported somewhat contradictory requirements for mitosis. In fixed cells, it has been proposed that EB1 plays a role in chromosome congression

and segregation (Green et al., 2005; Toyoshima and Nishida, 2007). However, live cell studies revealed that cells depleted of EB1 by RNAi or upon antibody microinjection progress normally throughout mitosis (Bruning-Richardson et al., 2011; Draviam et al., 2006), supporting timely satisfaction of the spindle-assembly checkpoint (SAC).

The roles of EB2 and EB3, on the other hand, are less understood. Similar to EB1, EB3 is ubiquitously expressed in several proliferating cells in culture and localizes to centrosomes and the mitotic spindle throughout mitosis (Bu and Su, 2001; Su and Qi, 2001). EB3 depletion was reported to cause a prometaphase accumulation, resulting in a slight but significant increase in the mitotic index in fixed material (Ban et al., 2009). Curiously, this was not observed upon EB1/EB3 co-depletion. EB3 is also highly expressed in differentiated cells from central nervous system and skeletal muscle, and has been shown to specifically regulate microtubule dynamics at the cell cortex, while being essential for proper myoblast differentiation (Straube and Merdes, 2007). EB2, on the other hand, appears to be the most divergent member of the family with variable expression levels in different cell types (Nakagawa et al., 2000; Su and Qi, 2001).

EB proteins are similar in structure and can adopt a homo- or heterodimeric conformation. EB1 and EB3 readily form heterodimers *in vitro* and in cells, whereas EB2 does not appear to participate in the formation of heterotypic complexes (De Groot et al., 2010). The microtubule-binding domain of EBs that is necessary and sufficient to recognize microtubule plus ends is defined by a calponin homology domain in the N-terminus. In contrast, the dimerization domain of EBs is in the C-terminal and has been implicated in the interaction with numerous EB binding partners, including APC, CLIPs, CLASPs, $p150^{Glued}$/dynactin, spectraplakins, STIM1 and MCAK (Akhmanova and Steinmetz, 2008). Interestingly, the extent of EB1 and EB3 heterodimerization can be controlled by EB binding partners (De Groot et al., 2010), and both EB3 and EB1 have been shown to interact with Aurora kinases, but only EB3 was found to be phosphorylated by Aurora B on serine 176 (S176) (Ban et al., 2009; Sun et al., 2008). These data suggest differential regulation of EB protein function and raise important questions regarding the individual roles of each EB protein in the cell.

In the present study we focus on understanding the respective roles and regulatory mechanism of human EB1 and EB3 in microtubule function throughout mitosis and cytokinesis. We uncovered spatially and temporally distinct roles of EB1 and EB3 in spindle orientation, substrate adhesion during daughter cell spreading and cytokinesis. We provide functional

evidence that the late mitotic and cytokinesis roles of EB3 rely on distinct Aurora B-mediated phosphorylation states of S176 and discuss the implications for the spatiotemporal regulation of microtubule function in light of the recently reported Aurora B gradient at the spindle midzone.

2 - Materials and Methods

Cell culture

All cell lines used were cultured in DMEM with 10% fetal bovine serum (FBS) and grown in a 5% CO2 atmosphere at 37ºC. HeLa H2B-GFP/α-tubulin-mRFP cell line was a gift from Patrick Meraldi (ETH, Zurich, Switzerland). Mouse GE11 integrin β1-null epithelial cell line was a gift from Reinhard Fassler (Max Planck Institute of Biochemistry, Martinsreid, Germany). HeLa Utrophin-GFP/H2B-mCherry, HeLa Myrpalm-GFP/H2B-mCherry and HeLa EB3-GFP/Lifeact-mCherry cell lines were a gift from Matthieu Piel (Institut Curie, Paris, France). For adhesion experiments, cells were seeded on Fibronectin (FBN, 50 μg/ml from Sigma), treated coverslips were either fixed for immunofluorescence or used for live cell imaging on Mattek dishes (Mattek Corporation). For the micropatterning experiments, cells were seeded as described previously (Fink et al., 2011).

Drug treatments

Aurora A inhibitor (MLN8054) was used at a concentration of 250 nM for 1h. Aurora B inhibitor (ZM447439) was used at a concentration of 2-4 μM for 1h. Appropriate controls were performed by incubating the cells with an equivalent amount of solvent.

shRNA and transfection experiments

Cells were transfected using FUGENE HD or X-tremeGENE XP (Roche) 24h after seeding. pSUPER-based vectors were used for RNAi experiments with EB1, EB3 or EB1/EB3 in tandem [sequences were previously described in (Komarova et al., 2005)]. All shRNA-based depletion experiments were performed after 120h. For the rescue experiments, cells were transfected simultaneously with pSUPER-EB1/EB3 and the full length EB3-GFP rescue construct (EB3-FL) or the EB3-GFP MT binding domain (EB3-MT). The EB3-MT construct is comprised of the microtubule binding domain of EB3 (aa 1-122) followed by the linker region and artificially dimerized by the addition of the leucine zipper (LZ) domain of GCN4. pEGFP-FAK was a gift from Peter Wang (UCSD, La Jolla, USA). pFB-Neo-Integrin β1-GFP was a gift from Dr. Martin Humphries (University of Manchester, UK). pEGFP-FAK was a gift from Peter Wang (UCSD, La Jolla, USA). mRFP-FAK was a gift from Gregg Gundersen (Columbia University, USA). To

generate the EB3-S176A-GFP and EB3-S176D-GFP constructs, a synthesized sequence with the respective mutation was introduced in pEGFP-N1 using SalI and BamHI. Rescue experiments with the EB3 mutants were performed by transfecting cells simultaneously with pSUPER-EB3 and either EB3-S176A-GFP or EB3-S176D-GFP constructs.

Antibodies and Immunofluorescence

Cells were grown on FBN-coated coverslips and processed for immunofluorescence as described previously (Maiato et al., 2006). The following primary antibodies were used: mouse anti– α–tubulin clone B512 (1:2000; Sigma-Aldrich), rat anti–α-tubulin YL1/2 1:10 (AbD Serotec), rat anti-EB1 and anti-EB3 1:20 and 1:10, respectively, rabbit anti-EB3 1:300, mouse anti-EB1 1:200 (Abcam), mouse anti-integrin β1 1:400 (DSHB), mouse anti-phosphorylated EB3-S176 1:1 (gift from Takeshi Urano, Japan), rabbit anti-pFAK(Y397) 1:300 (Santa Cruz Biotechnology). Secondary antibodies used were Alexa Fluor 488, 568, and 647 (1:2000; Invitrogen) and 1 μg/ml DAPI.

Time lapse microscopy

Cell lines were grown on FBN-treated coverslips with L-15 medium with 10% FBS. Four-dimensional datasets were acquired with a spinning disc confocal system (Revolution; Andor) equipped with an electron multiplying charge-coupled device camera (iXonEM+; Andor) and a CSU-22 unit (Yokogawa) based on an inverted microscope (IX81; Olympus). Two laser lines (488 and 561 nm) were used for near-simultaneous excitation of GFP and RFP, and the system was driven by iQ software (Andor). Time-lapse imaging of z stacks with 0.7 μm steps covering the entire volume of the mitotic apparatus were collected every 3 min. HeLa S3 cells were grown on FBN-treated coverslips with L-15 medium with 10% FBS. Four-dimensional datasets were collected on a Nikon Eclipse TE2000-U microscope driven by NIS Elements 3.0 software. Time-lapse imaging of z stacks with 10 μm steps covering the entire volume of the cells were collected every 10 min.

Quantitative analysis of spindle angle and length

Quantification of spindle angles and length was performed using Volocity LE. Images were collected by taking z-stacks with a step of 0.3 µm covering the entire volume of the mitotic apparatus. Distances tangent (ΔR) and perpendicular (ΔZ) to the substrate were measured after defining the position of the 2 poles and correcting for projection errors. The angle between the spindle axis and the substrate was defined by the inverse trigonometric function α^{o} = arctg (ΔZ/ΔR). Spindle length is defined as $L=\sqrt{[(\Delta R)^2+(\Delta Z)]^2}$.

Quantification of astral MT area and fluorescence intensity

To determine astral MT area, maximal projections of z stacks covering the entire mitotic apparatus were generated and a freehand selection tool in ImageJ software was used to outline the spindle pole and astral MTs. Astral microtubule fluorescence intensity was performed by selecting the pole regions using the freehand tool of ImageJ and measuring the maximum fluorescence intensity in the entire z stacks. The resulting value was normalized to the corresponding area.

Quantitative analysis of adhesion of the daughter cells to the substrate after mitosis

Individual cells were manually tracked until they exited mitosis and re-spread out in the substrate and the lag time between adhesion of the first and last daughter was quantified.

Quantification of cytokinesis failure

To quantify cytokinesis failure events, we counted the number of mitoses that failed to segregate properly leading to bi-nucleated cells. These were then divided by the total number of mitoses observed, thus defining the % of cytokinesis failures per total mitoses.

Quantification of EB comet distribution and length throughout the cell volume

To determine the comet density in different z slices, we counted the number of comets per slice and normalized for the area of the respective slice, obtaining a comet density (number of

comets per μm^2). Fluorescence intensity of EB3-GFP in different slices of time-lapse movies was measured using the Intensity-vs-Time plugin of ImageJ. Individual EB1 or EB3 comets were quantified using the line measurement tool in Autoquant X2.1.3. In brief, immunofluorescence images of EB1 and EB3 were acquired for interphase or mitotic cells and the individual comet length was measured by applying an exponential curve fit on the measured comet. When either EB1 or EB3 were depleted by RNAi, the comet length for the remaining EB was determined in the same manner.

Fluorescence intensity quantifications

Fluorescence signal quantification in the furrow region was done using a Matlab (The MathWorks, Inc.) script. A sum-projection image of the z-stack is generated upon which the user chooses manually a number of points along the furrow path. A closed cubic spline curve is generated to smoothly connect the points. The spline curve is then convolved with a disc-shaped kernel of variable diameter, which allows control of the Region-of-Interest (ROI) curve thickness. Fluorescence intensity is then calculated as the integrated signal inside the ROI. Results are presented as mean fluorescence intensity (m.f.i.).

Chromo-kymographic analysis of Focal Adhesions

Analysis of FA formation and persistency were done using chromo-kymographic methods that were described previously elsewhere (Pereira and Maiato, 2010). To prepare a kymograph for analysis of focal adhesion assembly, we used a custom routine written in Matlab (The MathWorks, Inc.). A ROI was selected that was parallel to the anaphase axis. Chromo-kymographs are generalized kymographs that retain information contained along the small axis (y) of the ROI by attributing a particular RGB combination to thin ROI sub-slices. RGB components are attributed so that a smoothly varying colour is assigned to objects at different y positions. This allows objects which co-localize in the x-axis to be differentiated by colour. A conventional kymograph is a particular case of a chromo-kymograph in which the sub-slices are attributed the same colour (or none).

Comet tracking

Individual comets were imaged by using the indicated EB constructs tagged with GFP. Movies with a time lapse of 2 sec were acquired on a spinning disk confocal microscope and individual comets were manually tracked using the "Manual Tracking" plugin of ImageJ. Comets were followed continuously until the fluorescent signal disappeared. The comet tracking time is defined as the continuous time a single comet track is followed (in seconds). The mean growth distance is defined as the distance the comet travelled until the signal disappeared (μm). The mean velocity is defined as the instantaneous velocity between consecutive time frames as given by the formula v=mean distance (μm)/time (sec).

Distance and mean velocity measurements

Individual cells were tracked using the "Manual Tracking" plugin of ImageJ. Cell nuclei were used as reference points. "Interphase" was defined as random 10 time frames that were at least 20 time frames separated from mitosis. All time frames were acquired with a 10 min interval. "Cumulative distance travelled by cells" was defined as the sum of all consecutive distances travelled by cells during the time-lapse. "Mean velocity" was defined as the mean distance travelled by the cells in each time frame divided by the time-lapse of each time frame.

Statistical analysis and data presentation

When data is represented as box-whisker plots, the box size represents 75% of the population and the line inside the box represents the median of the sample. The size of the bars (whiskers) represents the maximum (in the upper quartile) and the minimum (in the lower quartile) values. Statistical analysis for multiple group comparison was performed using a parametric one-way analysis of variance (ANOVA) when the samples had a normal distribution. When the sample did not have a normal distribution, multiple group comparison was done using a nonparametric ANOVA (Kruskal-Wallis). All pairwise multiple comparisons were subsequently analyzed using either post-hoc Student-Newman-Keuls (parametric) or Dunn's (nonparametric) tests. When comparing only two experimental groups, parametric *t* test was used when the sample had a normal distribution or nonparametric Mann-Whitney test was used for samples without normal distribution. All statistical analyses were performed using SigmaStat 3.5 (Systat Software, Inc.).

3 - Results

Functional analysis of EB1 and EB3 during mitosis

Given the apparent contradictions regarding human EB functions obtained from previous fixed and live-cell analyses, the lack of live-cell studies of EB3 function and the finding that EB1 and EB3 exist preferentially as an heterodimer *in vitro* and *in vivo* (De Groot et al., 2010; Komarova et al., 2009), we were interested in investigating whether EB proteins play distinct or overlapping roles throughout mitosis. For this purpose, we performed individual or simultaneous depletions of EB1 and/or EB3 in HeLa cells, followed by thorough fixed- and live-cell microscopy analyses of phenotypes. Immunofluorescence and western blot analyses confirmed that EB1 and EB3 are expressed in HeLa cells, co-localizing to microtubule plus-ends in interphase and throughout mitosis/cytokinesis, while decorating mitotic spindles, spindle poles and mid-bodies (Figure 1A). The specific localization of EB1 and/or EB3 was sensitive to RNAi-mediated depletion of each respective protein (Figure 1B, C). As opposed to individual depletions, only co-depletion of EB1 and EB3 caused a slight but significant increase in the normal mitotic index of HeLa cells (Figure 1D). This likely reflected the enrichment of double-depleted cells in prometaphase (Figure 1D). Dual-wavelength spinning-disk confocal microscopy of live RNAi-depleted HeLa cells stably co-expressing H2B-Histone-GFP/α-tubulin-mRFP to simultaneously monitor chromosome and mitotic spindle behavior revealed that the observed prometaphase accumulation resulted in a delay in anaphase onset (Figure 2A). Accordingly, HeLa cells upon control (luciferase) RNAi spent 35±13 min from nuclear envelope breakdown (NEB) to anaphase onset. When EB1 or EB3 were individually depleted, we found a marginal but not statistically significant increase of time between NEB to anaphase onset (respectively 35±14 min and 38±16 min). The only significant difference was found in EB1/EB3 co-depleted cells, which went from NEB to anaphase in 47±25 min ($p<0.05$; Figure 2A), suggesting at least some degree of functional redundancy between EB1 and EB3 in early mitosis. Curiously, we noticed that the longest observed delay in anaphase onset (54.5±25 min) upon EB1/EB3 co-depletion correlated with the presence of spindles with excessive rotation.

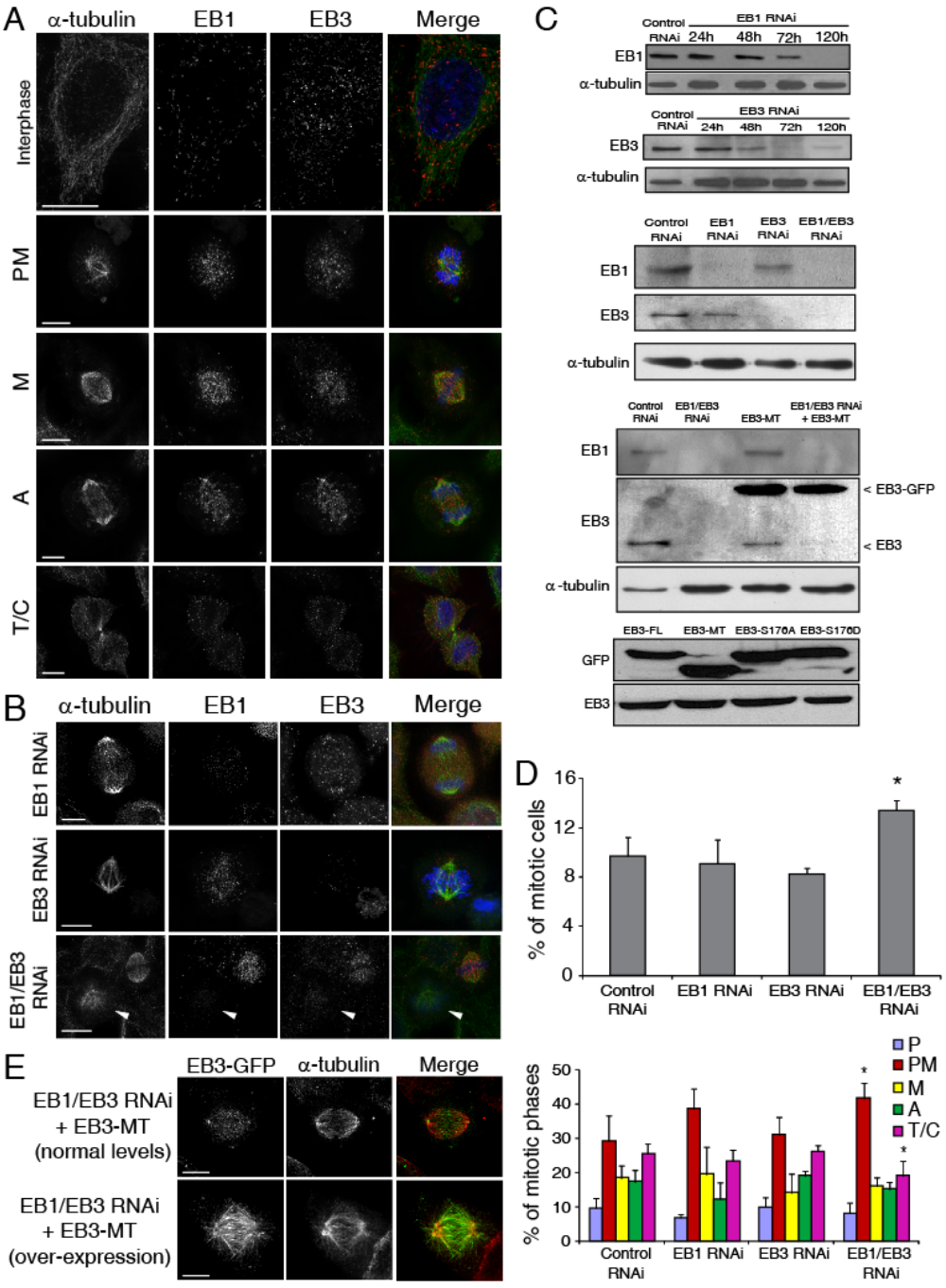

Figure 1 – Description of EB proteins localization during mitosis. (A) Immunostaining of EB1 and EB3 during the different stages of mitosis. (B) Immunolocalization of EB1 and EB3 in mitotic cells after depletion with shRNA. (C) Determination of depletion efficiency of EB proteins using Western blotting. The highest depletion efficiency was obtained after 120h of RNAi. (D) Quantification of the number of mitotic cells upon EB1 or EB3 individual depletions both proteins in tandem. Note the slight increase in the number of mitotic cells in the EB1/EB3 depletion (top panel). This increase is likely due to an accumulation of cells in prometaphase and a decrease in the number of telophases seen in the same group (bottom panel). (E) Expression of the rescue constructs in an EB1/EB3 depleted background both at normal levels (top panels) and in over-expressing cells (bottom panels). Scale bar, 10 μm; * $p<0.05$ using nonparametric ANOVA (Kruskal-Wallis) followed by post-hoc Dunn's test. All experiments were done in triplicate.

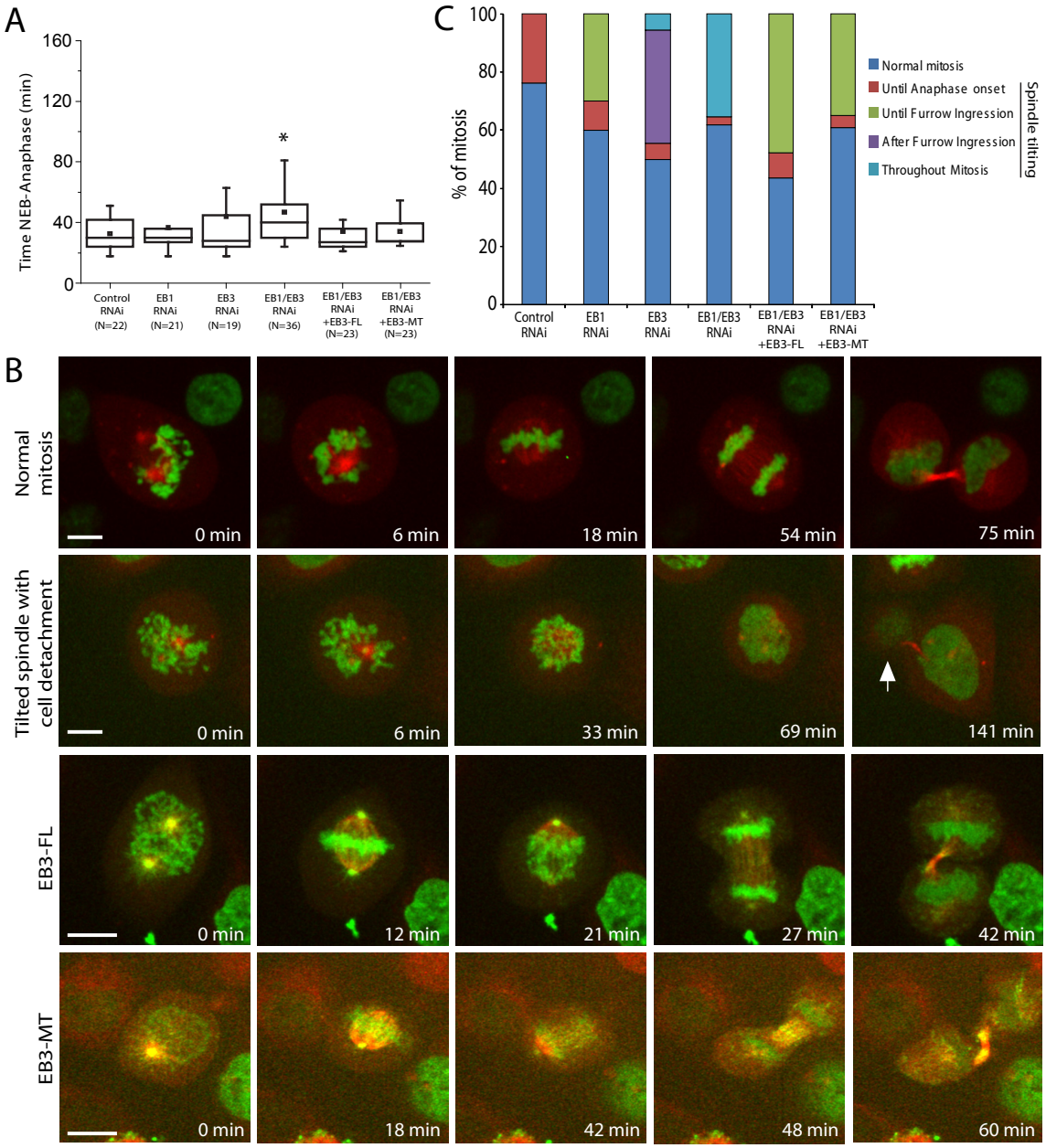

Figure 2 – Depletion of EB proteins leads to different phenotypes during mitosis. HeLa cells stably expressing H2B-GFP/α-tubulin-mRFP were depleted of EB1 and EB3 individually or in tandem and imaged by spinning disk confocal microscopy. (A) Timing from Nuclear Envelope Breakdown (NEB) to anaphase onset was quantified for each experimental condition. (B) Selected frames from time-lapse movies showing the phenotypes quantified. White arrow indicates daughter cell that failed to attach to the substrate. (C) Quantification of phenotypes in all experimental groups. EB3-FL and EB3-MT correspond to cells depleted of EB1 and EB3 in tandem and expressing full-length EB3-GFP or the microtubule binding domain of EB3, respectively. Time is in min. Scale bar, 10 □m. * $p<0.05$ using nonparametric ANOVA (Kruskal-Wallis) followed by post-hoc Dunn's test. Experiments were done in triplicate and N represents the number of cells filmed for each condition.

We further observed that EB1 depletion caused spindle tilting in about 50% of the cells, and most of these spindles did not align with the substrate until furrow ingression (Figure 2B, C). In the same period, individual EB3 depletion did not cause significant problems in spindle alignment with the substrate (Figure 2C). However, after furrow ingression, spindle tilting was observed in 50% of EB3-depleted cells (Figure 2C). Finally, EB1/EB3 co-depleted cells showed spindle tilting throughout mitosis in approximately 50% of the cells (Figure 2B, C). Overall, these observations suggest that EB1 and EB3 are important for spindle alignment relative to the substrate at distinct mitotic stages.

EB proteins intervene in a number of microtubule-dependent cellular functions, either by direct suppression of catastrophes (and thus promotion of microtubule growth) or by indirect loading of other factors to growing microtubule plus-ends (Akhmanova and Steinmetz, 2008). To distinguish between these possibilities we investigated whether the observed mitotic spindle tilting in live double-depleted cells could be rescued either by expression of full length EB3 fused with GFP (EB3-FL) or an EB3-GFP construct containing only the microtubule-binding domain (EB3-MT), which is sufficient to suppress microtubule catastrophes *in vivo* (Komarova et al., 2009), but lacking the EB partner-binding domain at the C-terminal. When EB1/EB3 co-depleted cells expressed any of the EB3-GFP rescue constructs at near endogenous levels, the duration from NEB to anaphase was indistinguishable from control or individual EB1 depletion. Additionally, the spindle tilting occurred mostly until furrow ingression which is equivalent to EB1 depletion only (Figure 1E, Figure 2B and C). This demonstrates that exogenous EB3 expression can rescue the EB3-specific but not the EB1-specific phenotype. In addition, these data demonstrate that the microtubule binding domain of EB3 is sufficient to revert spindle tilting in late mitosis.

EB1 and EB3 play temporally distinct roles in spindle orientation and post-mitotic cell adhesion to the substrate

Interaction of astral microtubules with the cell cortex is essential for spindle positioning in human cells (Thery et al., 2005). Moreover, EB1 is necessary for maintaining an intact astral microtubule array and to determine spindle position (Bruning-Richardson et al., 2011; Rogers et al., 2002; Toyoshima and Nishida, 2007).

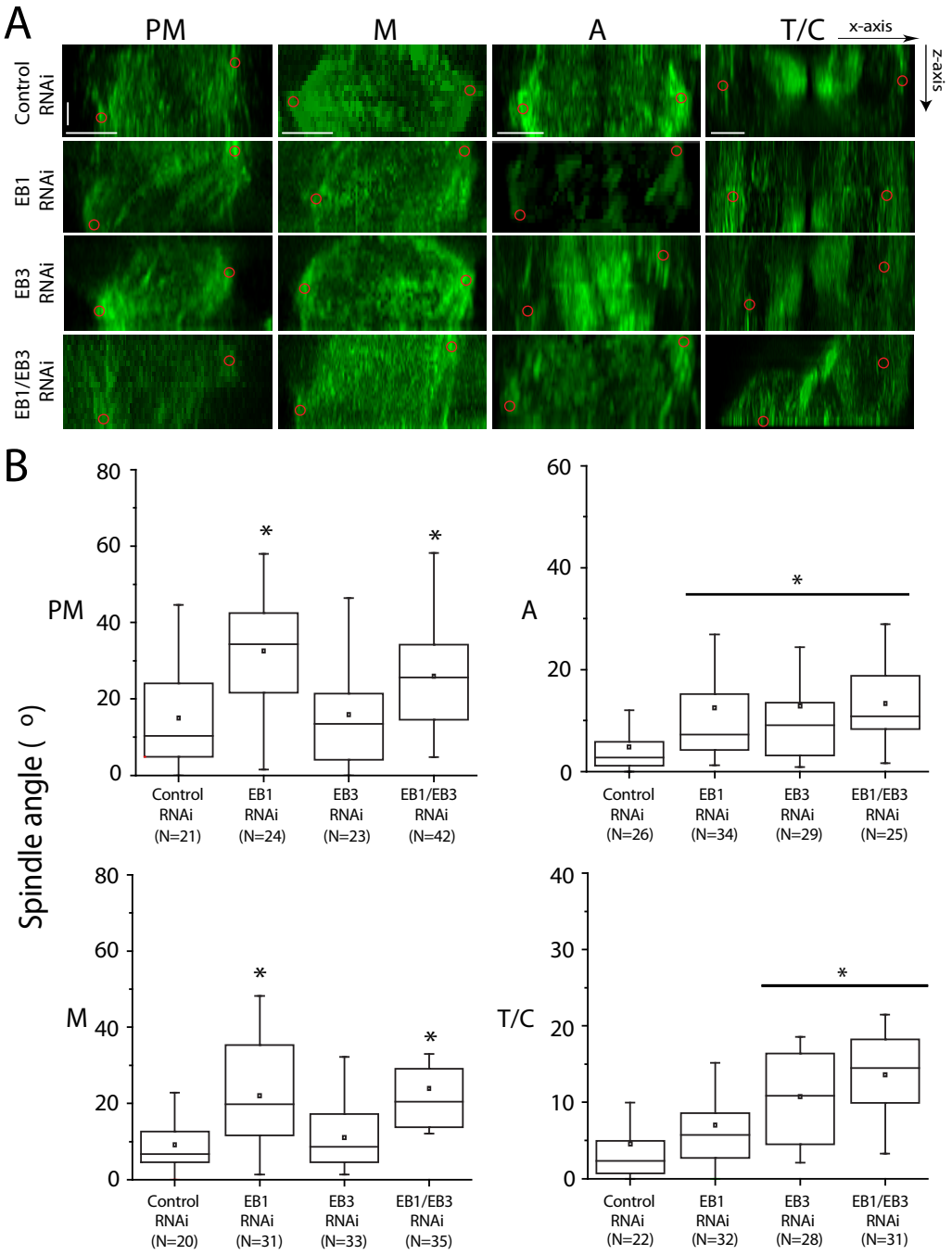

Figure 3 – Quantification of spindle alignment with the substrate. (A) HeLa cells were immunostained with an α-tubulin antibody and lateral projections of the spindle were used to quantify spindle angle in relation to the substrate. Red circles highlight the spindle poles. (B) Quantification of spindle angles in prometaphase 'PM', Metaphase 'M', Anaphase 'A' and Telophase/Cytokinesis 'T/C' for all treatment groups. Horizontal scale bar, 5 μm; vertical scale bar, 1.5 μm. * $p<0.05$ using parametric ANOVA followed by post-hoc Student-Newman-Keuls test. Experiments were done in triplicate and N represents the number of cells quantified for each condition.

To investigate in detail the nature of the observed spindle alignment defects upon depletion of EB proteins we determined the spindle angle relative to the substrate throughout mitosis after depleting EB1 and EB3, either separately or simultaneously (Figure 3A and B). During prometaphase in control cells the spindle is still aligning with the substrate and, therefore, the measured angle was between 5º and 24º in 75% of the cells. Spindle angle then gradually decreased to approximately 8º as cells reached metaphase, with minimal values from anaphase onwards, indicating nearly complete alignment relative to the substrate. EB1-depleted cells, however, were unable to align their spindles properly from prometaphase until anaphase onset. For comparison, spindle angle in metaphase upon EB1 depletion was higher than in prometaphase in control cells.

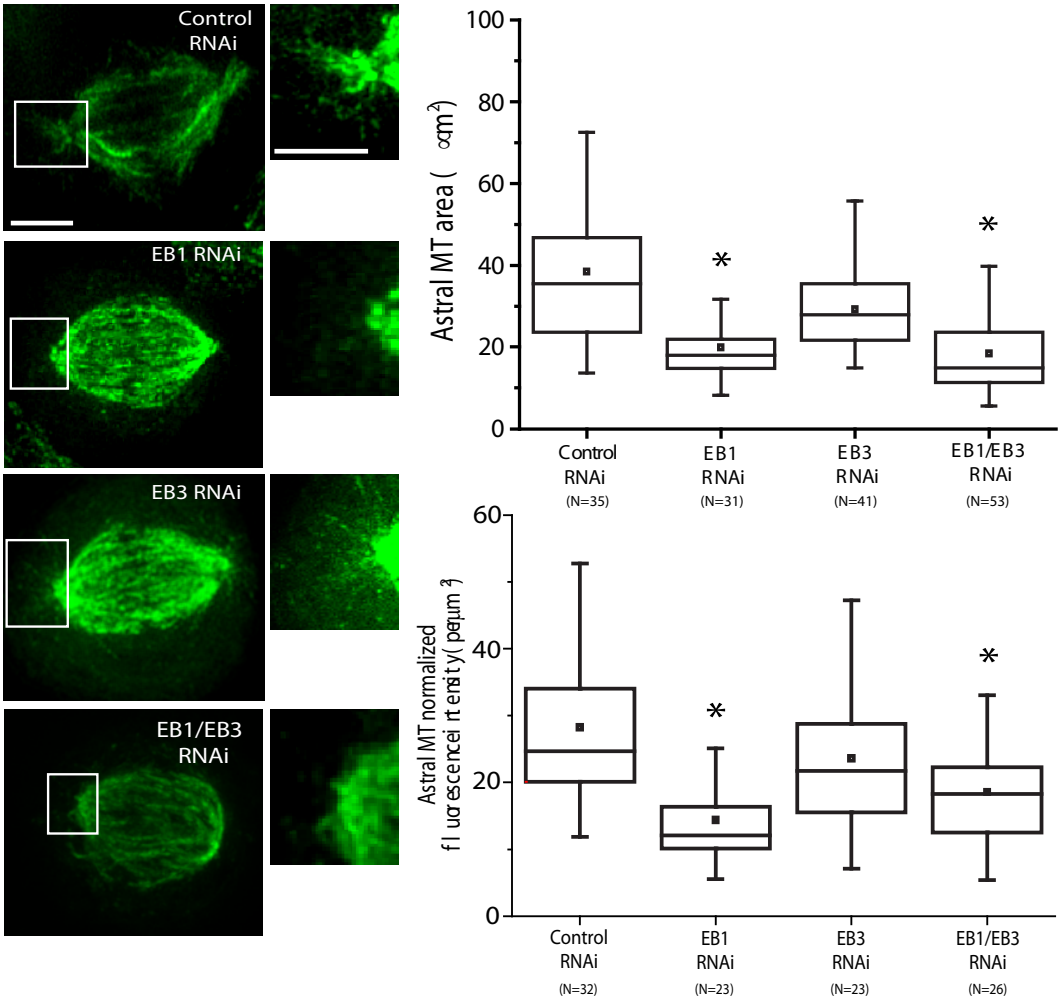

Figure 4 – Quantification of astral microtubules area and fluorescence intensity. Cells immunostained with an α-tubulin antibody were used to quantify the area occupied by astral microtubules or the fluorescence intensity of astral microtubules. Scale bar, 10 μm; * $p<0.05$ using nonparametric ANOVA (Kruskal-Wallis) followed by post-hoc Dunn's test. Experiments were done in triplicate and N represents the number of cells quantified for each condition.

This may be due to the fact that EB1-depletion induces approximately a 50% decrease in astral microtubule area and respective fluorescence intensity (Figure 4), which could compromise the capacity of spindles to interact normally with the cell cortex, in agreement with previous reports (Thery et al., 2005; Toyoshima and Nishida, 2007). Nonetheless, EB1 depleted cells were still able to maintain a low spindle angle in telophase, suggesting that they were able to establish proper attachment to the substrate upon mitotic exit. EB3 depletion, on the other hand, did not induce alterations in spindle angle in early mitosis relative to controls nor did it decrease astral microtubule area or respective fluorescence intensity (Figure 3A and B, Figure 4). However, anaphase and telophase spindles showed increased angles relative to the substrate, comparable to those observed for control cells in prometaphase (Figure 3A and B). In agreement with our live-cell observations, EB1/EB3 co-depletion resulted in an increased spindle angle throughout mitosis, consistent with a requirement for individual EB proteins at different mitotic stages (Figures 2C, 3A and B).

From our live-cell confocal microscopy analysis we noticed that the increased spindle tilting observed in late mitosis upon EB3 or EB1/EB3 depletion normally correlated with a substrate adhesion defect of one of the daughter cells as they exit from mitosis (Figure 2B). A more extensive live-cell analysis by phase-contrast microscopy revealed that EB3 or EB1/EB3, but not control or EB1 RNAi, resulted in a significant delay in adhesion and spreading between the first and second daughter cells (Figure 5A and B). Expression of any of the EB3-GFP rescue constructs in EB1/EB3 co-depleted cells also significantly reduced the observed delay in adhesion and spreading between the first and second daughter cells to levels similar to those observed in control or EB1 RNAi cells (Figure 5B).

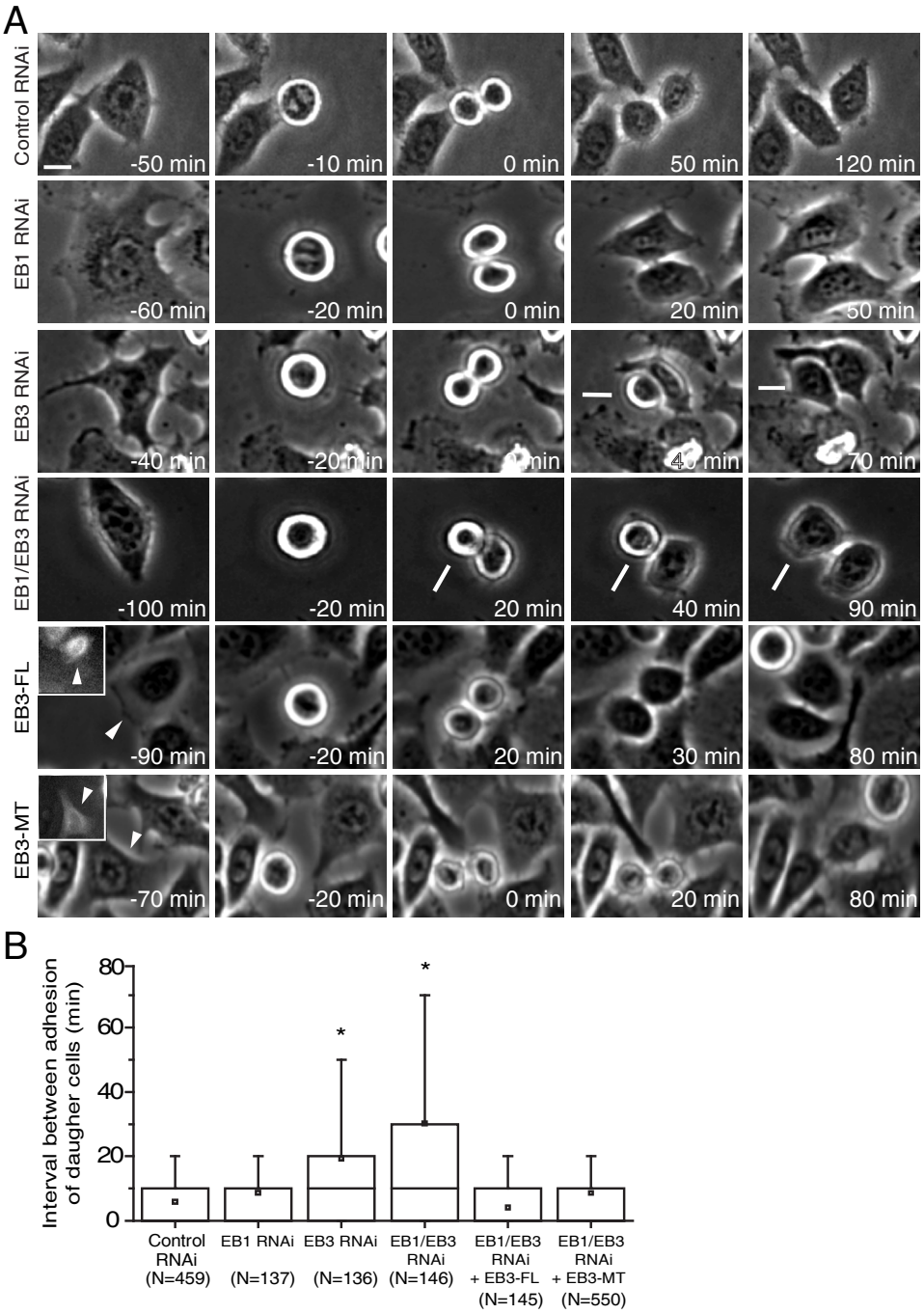

Figure 5 – Quantification of post-mitotic cell adhesion to the substrate. (A) HeLa cells were filmed using phase-contrast microscopy. Zero minutes corresponds to the first anaphase frame observed. (B) Quantification of the delay in adhesion between the first and the second daughter cells. White arrows indicate cells with an uncoordinated adhesion to the substrate. White arrowheads indicate cells expressing either the full length EB3-GFP (EB3-FL) or the microtubule binding domain of EB3 (EB3-MT). The insets represent the GFP-expressing cells that were tracked in this particular example. Time is in min. Scale bar, 10 μm. * $p<0.001$ using nonparametric ANOVA (Kruskal-Wallis) followed by post-hoc Dunn's test. Experiments were done in triplicate and N represents the number of cells quantified for each condition.

Overall, these results suggest that the observed requirement of EB3 for normal mitotic progression, post-anaphase spindle position/orientation and post-mitotic daughter cell spreading, rely essentially on its direct impact on microtubule dynamics and is largely independent on its C-terminal binding partners.

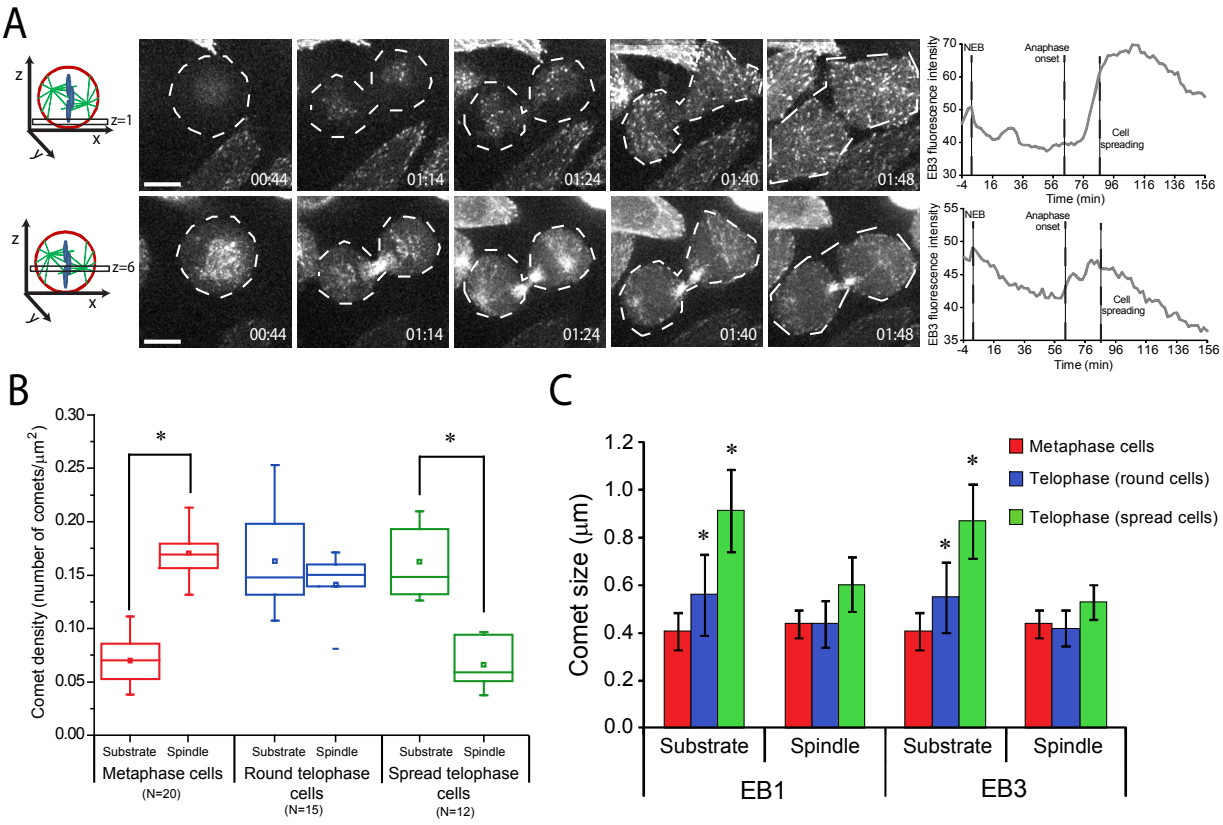

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Figure 6 – Distribution of EB1 and EB3 comets throughout mitosis. (A) Selected frames from time-lapse movie of an EB3-GFP expressing cell. Quantification of EB3-GFP fluorescence intensity was measured close to the substrate (Z=1) and higher in the spindle region (Z=6). Time is in hours: mins. Zero minutes corresponds to mitotic entry. (B) EB comet density near the substrate and in the spindle region. The number of comets was measured in each slice and normalized to the area of the respective slice. (C) Quantification of individual EB comet size in the different slices and for different stages of mitosis. Note the increase in comet size near the substrate as cells are exiting mitosis. Horizontal scale bar, 5 μm; vertical scale bar, 1.5 μm. * $p<0.001$ using a parametric *t* test. Experiments were done in triplicate and N represents the number of cells quantified for each condition.

Microtubule plus-ends have a bias towards the substrate during daughter cell spreading

To investigate how the microtubule cytoskeleton reorganizes at the exit from mitosis we tracked microtubule plus-ends in live and fixed cells. We found that upon anaphase onset microtubule plus-ends decorating spindle microtubules re-distribute and show a bias towards the substrate, which persists until daughter cell re-spreading (Figure 6A, B). Concomitantly, comet size increases specifically near the substrate (Figure 6C). These findings suggest that microtubule plus-ends respond to signalling cues from the adhesion substrate and/or re-distribution of microtubule plus-ends towards the substrate is important for cell-substrate adhesion during mitotic exit.

EB3 regulates the stabilization of focal adhesions during mitotic exit

The re-establishment of cell-substrate adhesion at the end of mitosis relies on the recruitment of FAK to focal adhesions (Pugacheva et al., 2006; Yamakita et al., 1999). By using spinning-disk live-cell microscopy analysis of HeLa cells expressing FAK-GFP during mitotic exit we determined that FAK normally accumulated in focal complexes near the substrate shortly after anaphase onset (Figure 7A and B). These structures were short-lived and showed high turnover (Figure 7C), arising in the middle of the daughter cells and progressing towards their periphery as cells changed shape (Figure 7B-D). As post-mitotic cells spread, initial focal complexes disappeared and gave way to longer-lived focal adhesions that allowed for stabilization of the cell shape (Figure 7B and C). Upon depletion of EB1 alone we did not find any alteration in the occurrence or persistency of focal adhesions during mitotic exit (Figure 7C). Moreover, all the focal adhesions identified by FAK-GFP were also positive upon immunostaining with a pFAK(Y397) antibody indicating the presence of catalytically active FAK (Figure 7D). Depletion of EB3 did not impair the formation of the high-turnover, short-lived active focal complexes that were formed upon mitotic exit similarly to control cells (Figure 7A-D). However, EB3-depleted cells that showed uncoordinated spreading and attachment problems had an asynchronous formation of focal adhesions (Figure 7B and D). Moreover, the formation of adhesion complexes in the detached cell was concomitantly delayed until its re-attachment to the substrate (Figure 7B). Importantly, lack of EB3 prevented the formation of stable, long-lived focal adhesions (Figure 7B and C). In fact, these cells were not able to stabilize their shape and keep moving constantly upon spreading, denoting a deficient interaction with the substrate.

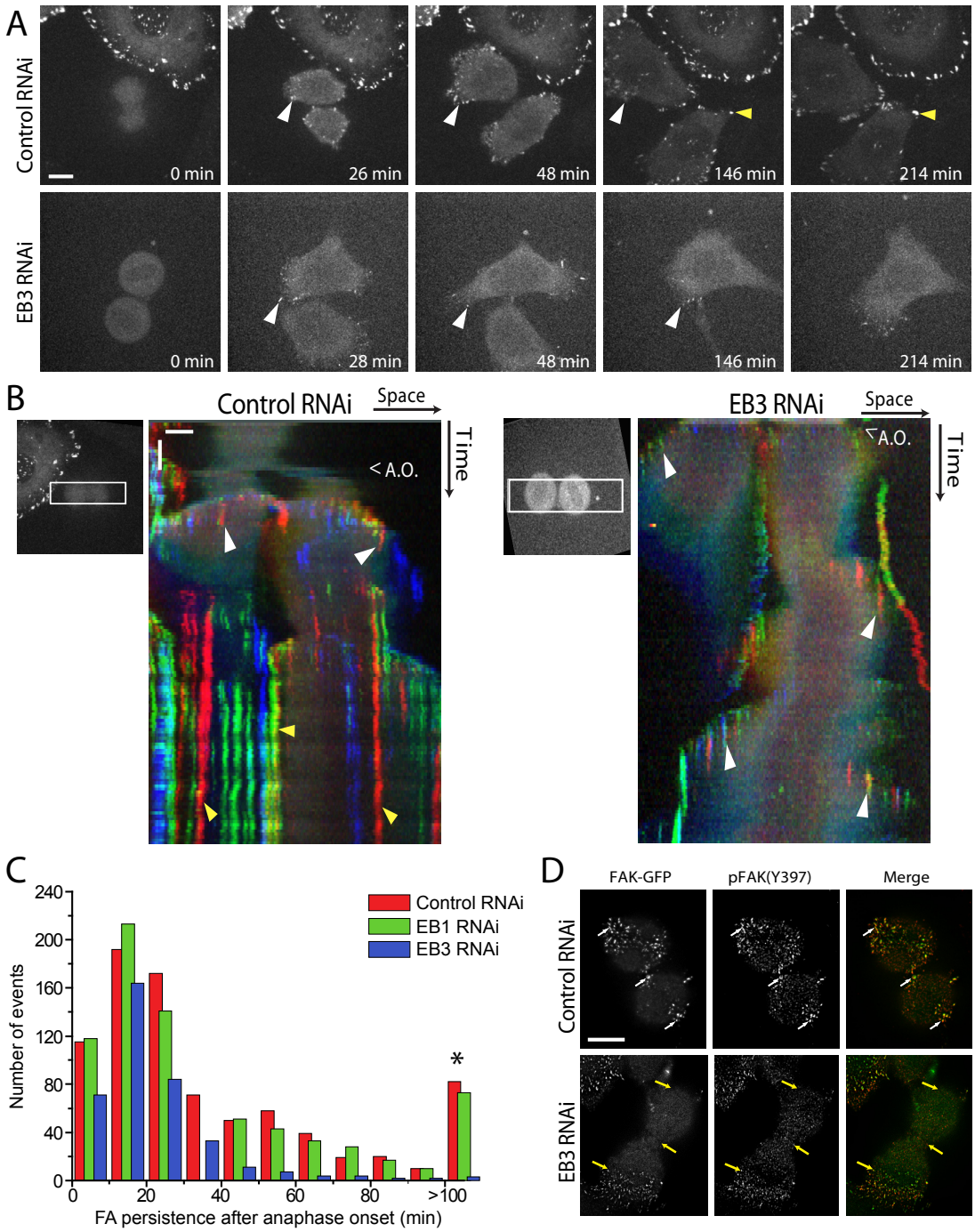

Figure 7 – Stabilization of focal adhesions upon mitotic exit. (A) HeLa cells expressing FAK-GFP were imaged by spinning disk confocal microscopy during mitotic exit. (B) Chromo-kymographs of control and EB3-depleted cells expressing FAK-GFP while exiting mitosis. To generate the chromo-kymographs, a ROI was selected that was aligned with the long axis of the cells in anaphase. (C) Quantification of focal adhesion persistency after anaphase onset, as measured by the time a FAK-GFP positive structure was maintained from the moment it appeared until it vanished. (D) Immunostaining showing the co-localization of FAK-GFP with the active, phosphorylated form of FAK (pFAK-Y397). Note the extensive co-localization of both forms of the protein. White arrowheads highlight the nascent focal adhesions formed after anaphase onset. Yellow arrowheads highlight the more stable, mature focal adhesions that appear later on as cells stabilized their shape.

Given that coordination of daughter cell spreading at the end of mitosis can be ensured by expression of EB3-MT construct, these results suggest that post-mitotic stabilization of focal adhesions relies on EB3-mediated regulation of microtubule dynamics near the substrate.

EB3 dephosphorylation upon mitotic exit is necessary for coordinated post-mitotic cell spreading

It has been reported that overall mitotic phosphorylation of EB3 at S176 by Aurora kinases decreases as cells enter in G1 (Ban et al., 2009). We hypothesized that dephosphorylation of EB3 upon mitotic exit may act as a functional switch, which is required for coordinated daughter cell spreading and adhesion to the substrate. To test this we generated EB3 mutants that carried S-A or S-D mutations at S176 to mimic either constitutively absent or persistent phosphorylation, respectively (Figure 8A). Both EB3 mutants localized normally to the plus-ends of microtubules throughout mitosis (Figure 8A). Expression of either S176A or S176D mutants on an EB1-depleted background did not affect the metaphase spindle angle typically observed upon EB1 depletion (Figure 8B and C), reinforcing the idea that positioning the metaphase spindle parallel to the substrate depends specifically on EB1. Notably, expression of the non-phosphorylatable, but not the phospho-mimetic, mutant in cells depleted of endogenous EB3 successfully restored telophase spindle angle to control values (Figure 8B). In agreement, cells depleted of endogenous EB3 which expressed EB3-S176D showed problems in late spindle alignment, which led to uncoordinated daughter cell spreading and delayed adhesion (Figure 8D and E). Overall, these data suggest that EB3 dephosphorylation on S176 is specifically required for post-mitotic daughter cell adhesion to the substrate.

Figure 7 (cont.) - In (D), white arrows show focal adhesions in control cells appearing in the middle and the top of both daughter cells. Yellow arrows show focal adhesions in an EB3-depleted cell appearing in one of the daughter cells but not in the middle or the other cell. Time is in min. A.O. – Anaphase Onset. Horizontal scale bar, 10 μm; vertical scale bar, 20 min. * $p<0.001$ using nonparametric ANOVA (Kruskal-Wallis) followed by post-hoc Dunn's test.

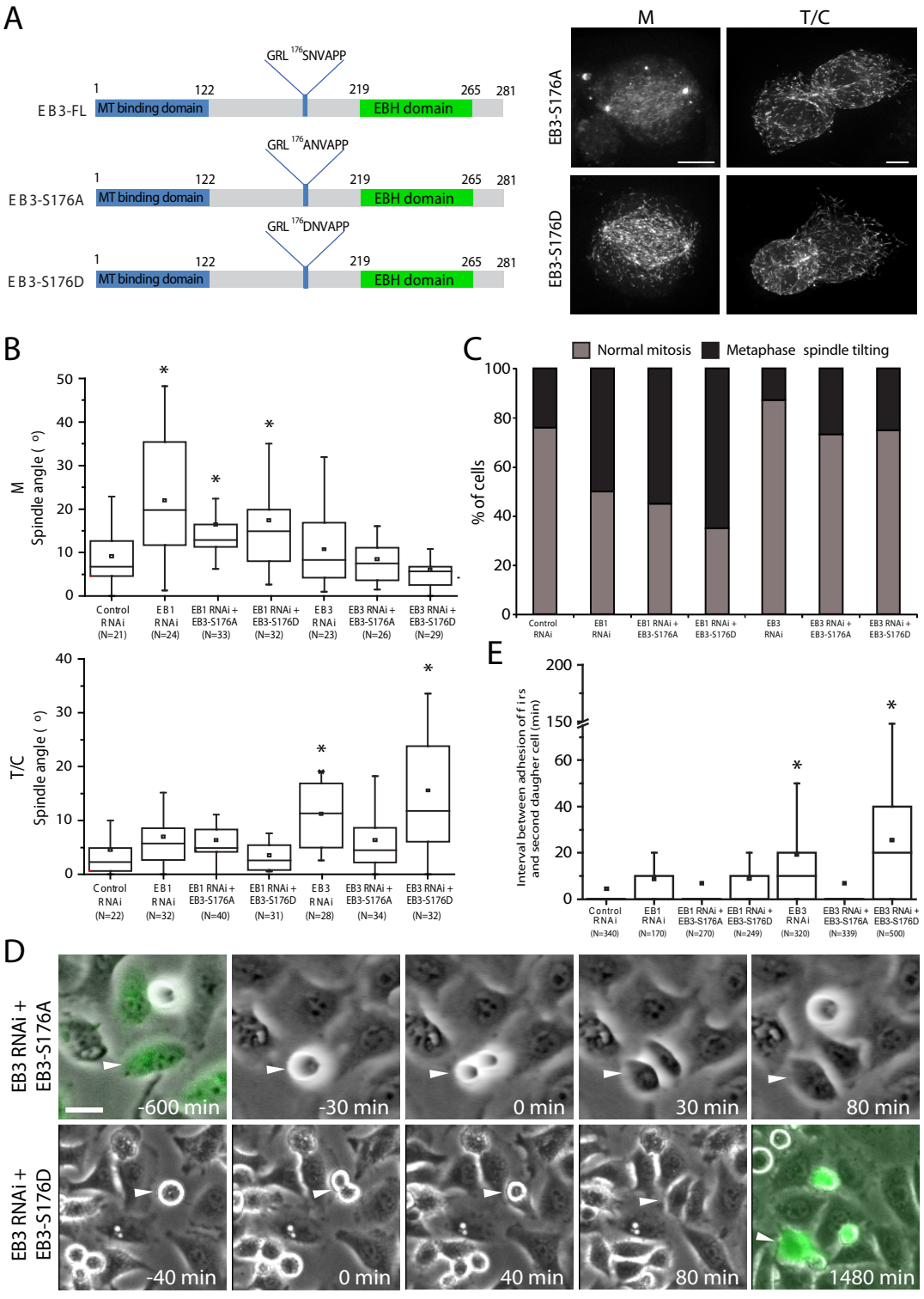

Figure 8 – Dephosphorylation of EB3 during mitotic exit is required proper attachment to the substrate. (A) Diagram of full-length EB3 and the EB3-S176A and EB3-S176D mutants (left panel). Localization of EB3-S176A and EB3-S176D mutants in metaphase 'M' and telophase 'T/C' cells that were depleted of endogenous EB3 (right panels). Note the plus-end localization of the mutants. (B) Quantification of spindle angles in metaphase and telophase/cytokinesis using fixed cells. (C) Quantification of mitotic phenotypes observed using spinning disk live cell imaging. Note that neither EB3 mutant is unable to rescue the EB1 phenotypes observed in metaphase cells.

EB3 phosphorylation status controls microtubule growth

Our previous results showed that expression of the EB3-MT construct (which includes S176), significantly re-established coordinated daughter cell spreading and adhesion, suggesting a direct role of EB3 in the control of microtubule dynamics required for this process. To test this, we determined the respective impact of expression of either EB3-S176A or EB3-S176D mutants on microtubule dynamics. For this purpose we tracked the respective EB3-decorated comets upon depletion of endogenous EB3 in adherent interphase cells and measured the comet life-time, travelled distance and growth velocity (Table I). We found that constitutive phosphorylation of EB3 on S176 significantly increases comet life-time and travelled distance, while reducing growth velocity. On the other hand, constitutive dephosphorylation of EB3 on S176 has no significant effect on comet life-time, while reducing both the distance travelled and growth velocity. Overall, these data indicate that EB3 phosphorylation status is important for normal microtubule dynamics, with phosphorylation of S176 promoting microtubule growth.

Table I. Quantification of comet tracking parameters

Parameters	EB3-FL (176/10) [a]	EB3-S176A (227/13)	EB3-S176D (385/22)
Comet life-time (s)	21.2 ± 10.0	20.1 ± 13.4	40.6 ± 22.0*
Growth distance (μm)	7.1 ± 4.2	6.6 ± 4.8*	8.8 ± 5.2*
Mean velocity (μm/s)	0.383± 0.07	0.320± 0.09*	0.249± 0.07*

[a] Numbers within parenthesis represent the number of tracked comets/number of cells in three independent experiments.

* p<0.001(nonparametric ANOVA followed by post-hoc Dunn's test)

Figure 8 (cont.) - (D) Selected frames from time-lapse movies of cells expressing either EB3-S176A or EB3-S176D. White arrowheads highlight a cell that was tracked in each of the groups. Note the simultaneous spreading of both daughter cells in the EB3-S176A treatment, in contrast with the uncoordinated spreading observed in the EB3-S176D group. (E) Quantification of the delay in adhesion of the two daughter cells. The EB3-S176A mutant is able to induce coordinated attachment of both daughter cells to the substrate, whereas the EB3-S176D fails to do so. Time is in min. In (A), scale bar, 5 μm; In (E), scale bar, 20 μm. * p<0.001 using nonparametric ANOVA (Kruskal-Wallis) followed by post-hoc Dunn's test. Experiments were done in triplicate and N represents the number of cells quantified for each condition.

EB3-dependent accumulation of integrins at the cleavage furrow is required for efficient cytokinesis

Microtubules are essential for the completion of cytokinesis (Wheatley and Wang, 1996) and EB-like proteins have previously been implicated in this process in yeast, Dictyostelium and sea urchin (King et al., 2010; Muhua et al., 1998; Strickland et al., 2005b). As so, we investigated the respective requirements of human EB1 and/or EB3 during cytokinesis upon RNAi and live-cell microscopy in HeLa cells. We found that EB3 and EB1/EB3 depletion induced cytokinesis failure in approximately 10% of attempts, whereas EB1 depletion alone had no significant impact in this process (Figure 9A and B; see also (King et al., 2010)). Moreover, and in contrast to control cells, we found that the midbody of approximately 76% of EB3-depleted cells (and similarly, EB1/EB3-depleted cells) that failed cytokinesis was unable to establish a stable position between the two daughter cells, suggesting that stabilization of the midbody is important for this process.

Cell adhesion components, such as integrins, have been implicated in the completion of cytokinesis (Pellinen et al., 2008; Reverte et al., 2006). As so, we reasoned that interfering with EB3 may have an impact on integrin function required for midbody stabilization. To test this we resorted to GE11 cells derived from integrin β1 knockout mice (Gimond et al., 1999), into which we introduced human integrin β1 tagged with GFP for its direct visualization in living cells. This construct rescues the reported cytokinesis defects in GE11 cells (Figure 10). Next, we used integrin β1-GFP expressing cells for spinning-disk confocal microscopy upon control or EB3 RNAi. We found that integrin accumulation at the cleavage furrow was significantly decreased upon EB3 depletion (Figure 9C). These results were further confirmed by immunodetection of integrin β1 in fixed material and shown to be EB3 specific (Figure 9D). Finally, expression of EB3-FL, but not the EB3-MT construct significantly rescued normal integrin accumulation at the cleavage furrow and reverted the observed cytokinesis defects upon EB3 or EB1/EB3 RNAi (Figure 9B and D). This suggests that EB3 or a yet unidentified EB3-mediated cargo interacts with integrins through the EB3 C-terminal domain. Taken together, these data support a role for EB3 in midbody stabilization during cytokinesis, by allowing local accumulation of integrins.

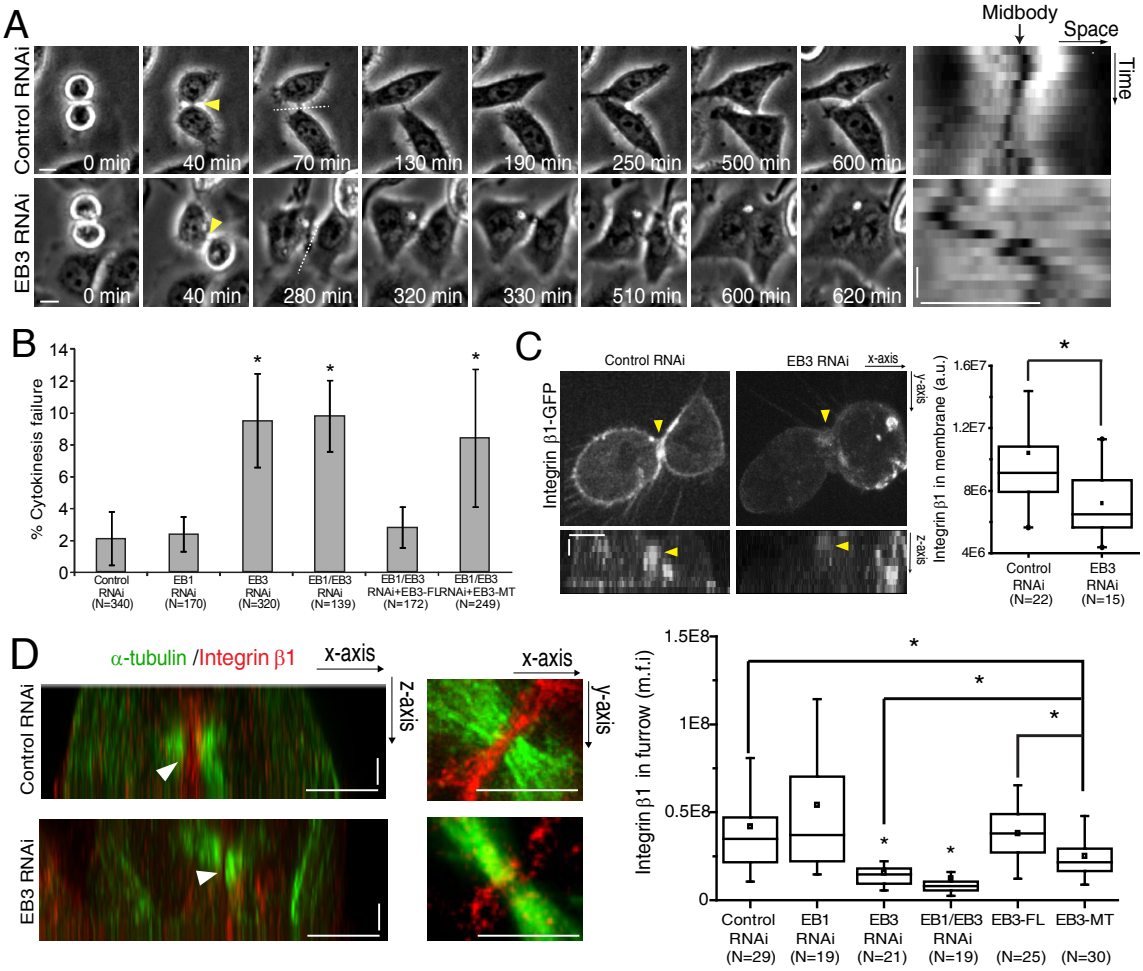

Figure 9 – EB3 is required for integrin accumulation in the furrow. (A) HeLa cells were filmed in phase contrast to determine the number of failed cytokinesis events. Yellow arrowheads indicate the first time-frame where the midbody could be observed. The dashed line indicates the region that was selected to generate the kymograph of the midbody position. Vertical scale bar, 50 min. Note the formation of a binucleated cell in an EB3-depleted background, where the midbody fails to stabilize. Zero minutes corresponds to the first anaphase frame observed. (B) Quantification of the number of failed cytokinesis per total mitoses observed. Note that EB3 (but not EB1) is required for successful completion of cytokinesis. (C) Live imaging of cells expressing integrin β1-GFP. Top (top panels) and lateral views (bottom panels) of cells during mitotic exit. These cells were used to quantify the amount of integrin accumulation in the furrow during mitotic exit. (D) Immunostaining of α–tubulin and integrin ⍰1 in the furrow region. These cells were used to quantify endogenous levels of integrin β1 in the furrow region during mitotic exit. Note that EB3 induces a decrease in the levels of integrin in the furrow region that cannot be rescued by EB3-MT. Time is in min. Horizontal scale bar, 10 μm; vertical scale bar, 3 μm. $p<0.001$ using nonparametric ANOVA (Kruskal-Wallis) followed by post-hoc Dunn's test. Experiments were done in triplicate and N represents the number of cells quantified for each condition.

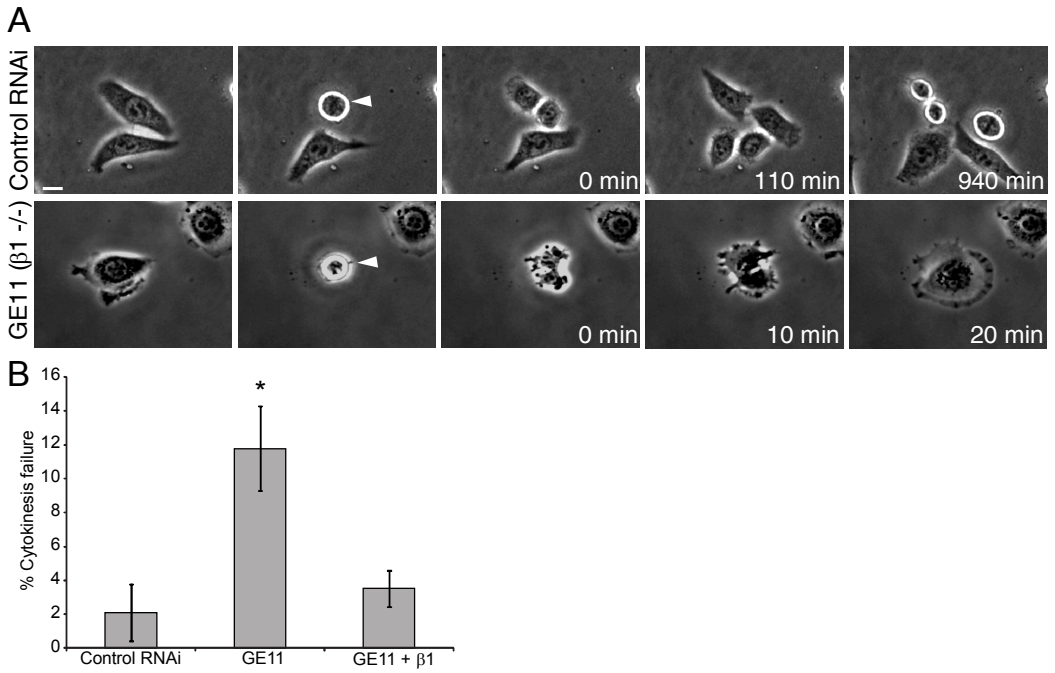

Figure 10 – Integrin is required for successful completion of cytokinesis. (A) HeLa cells transfected with control RNAi or GE11 β1-/- cells were filmed by phase-contrast microscopy to determine cytokinesis efficiency. (B) Quantification of the percentage of cytokinesis failures in HeLa cells transfected with control RNAi, GE11 β1-/- cells and GE11 β1-/- expressing human integrin β1-GFP. Scale bar, 10 μm; * $p<0.001$ using nonparametric ANOVA (Kruskal-Wallis) followed by post-hoc Dunn's test. Experiments were done in triplicate and N represents the number of cells quantified for each condition.

EB3 phosphorylation by Aurora B kinase increases its association with the midbody and is required for efficient cytokinesis

Aurora B kinase is a major regulator of cytokinesis (Ruchaud et al., 2007) that interacts with and phosphorylates EB3 on S176 (Ban et al., 2009). To investigate whether this specific phosphorylation of EB3 is required for cytokinesis we depleted cells of endogenous EB3 by RNAi and expressed RNAi-resistant EB3-FL or EB3-S176D mutant, in the presence/absence of Aurora A or B inhibitors, added upon anaphase onset. Treatment of live anaphase cells with the more specific Aurora A inhibitor MLN8054 (Manfredi et al., 2007) did not lead to any cytokinesis defects (Figure 11A and B). On the other hand, inhibition of Aurora B with ZM447439 (Ditchfield et al., 2003) in anaphase cells expressing EB3-FL induced cytokinesis failure in approximately 60% of the cells (Figure 11A and B). The observed cytokinesis failure correlated with a faster dissociation of EB3 from the midbody, without affecting EB3 localization on microtubule plus-ends in the cell body (Figure 11A and C). Remarkably, when cells expressing EB3-S176D were treated with ZM447439 there was a significant rescue of the observed cytokinesis defects, associated with an increased retention of EB3-S176D at the

midbody (Figure 11A and B). Overall, these results suggest that phosphorylation of EB3 on S176 by Aurora B kinase increases EB3 retention at the midbody and ensures normal completion of cytokinesis.

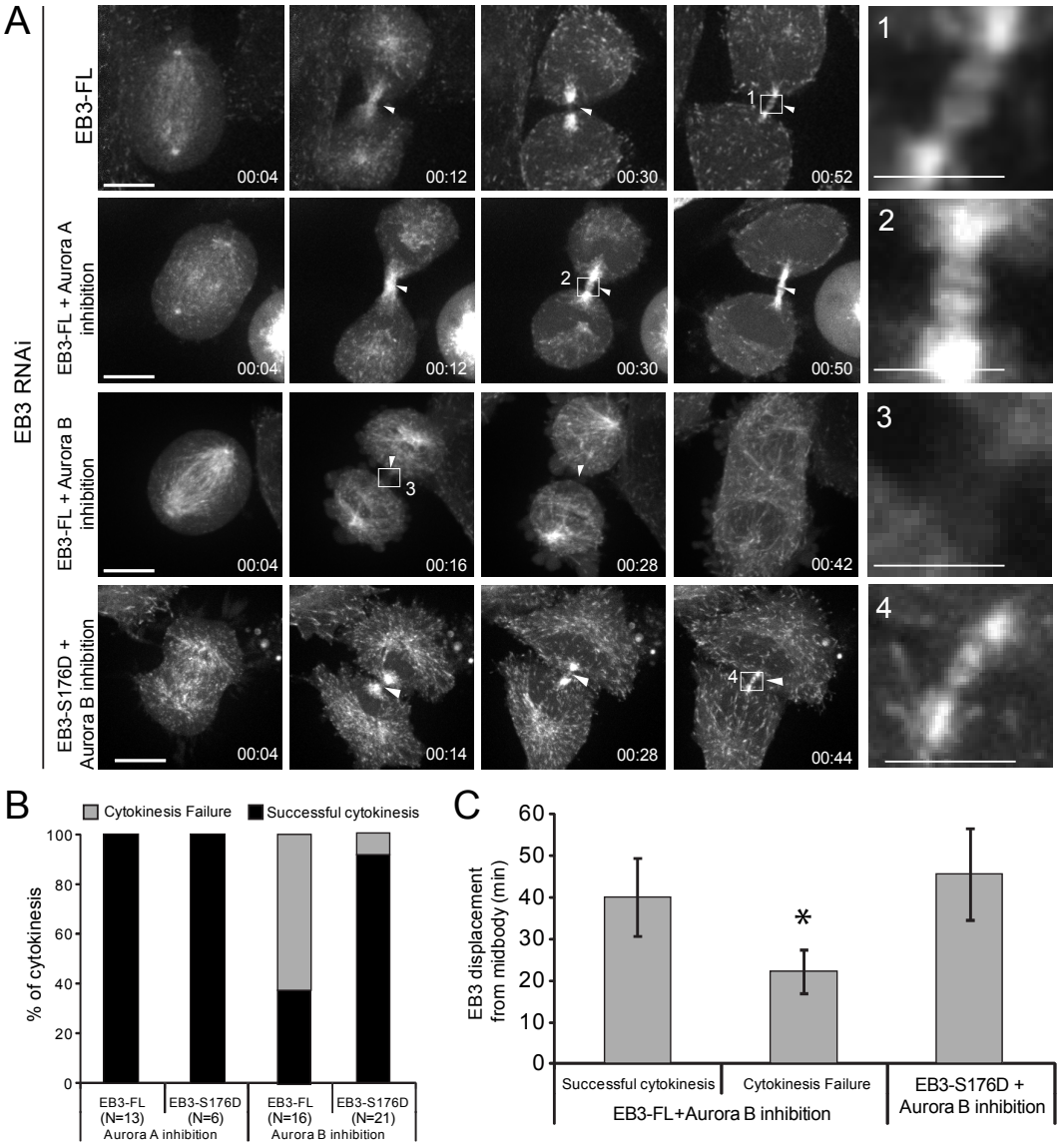

Figure 11 – Phosphorylation of EB3 by Aurora B kinase is required for cytokinesis completion. (A) Selected frames from time-lapse movies of cells expressing either EB3-FL or EB3-S176D treated with Aurora A inhibitor (MLN8054) or Aurora B inhibitor (ZM447439). EB3-S176D localizes to the midbody even when Aurora B kinase is inhibited. (B) Quantification of the percentage of cells expressing EB3-FL or EB3-S176D that fail cytokinesis after Aurora A or Aurora B inhibition. (C) Quantification of the EB3 midbody retention time after Aurora B inhibition. Note that EB3-S176D remains associated with the midbody for longer times after Aurora B inhibition when compared to cells that fail cytokinesis. White arrowhead indicates location of midbody. Time lapse is 2 minutes. Time is in hours: mins. Scale bar, 10 μm. Inset scale bar, 3 μm. $p<0.001$ using nonparametric ANOVA (Kruskal-Wallis) followed by post-hoc Dunn's test. Experiments were done in triplicate and N represents the number of cells quantified for each condition.

EB3 phosphorylation on S176 is spatially regulated by Aurora B kinase and is required for efficient cytokinesis

Our finding that coordinated daughter cell adhesion during mitotic exit and the efficiency of cytokinesis require distinct EB3 phosphorylation states on S176 suggest that different MT subpopulations close to the cell substrate and at the midbody are spatially regulated by Aurora B. Consistent with this idea, the existence of an Aurora B phosphorylation gradient from the spindle midzone throughout anaphase/telophase has been recently demonstrated (Fuller et al., 2008), but its biological significance remained unclear. Therefore, we sought to investigate whether EB3 phosphorylation on S176 was spatially regulated by Aurora B during mitotic exit/cytokinesis. For this purpose we used a phospho-specific antibody against S176 (Ban et al., 2009) and determined the respective fluorescence ratio relative to ectopic EB3-MT-GFP, which we used to enhance MT plus-end labelling. As a control, we determined the fluorescence ratio of endogenous EB1 relative to EB3-MT-GFP. These experiments confirmed our hypothesis and clearly showed enrichment of EB3 phosphorylation on S176 associated with midbody MTs (Figure 12A, B). Finally, Aurora B inhibition with ZM447439 or expression of EB3-S176A mutant in EB3-depleted cells, significantly reduced the epitope recognized by the phospho-specific antibody against S176 (Figure 12C). Taken together, these data support that EB3 phosphorylation on S176 is spatially regulated by an Aurora B activity gradient during late mitosis/cytokinesis.

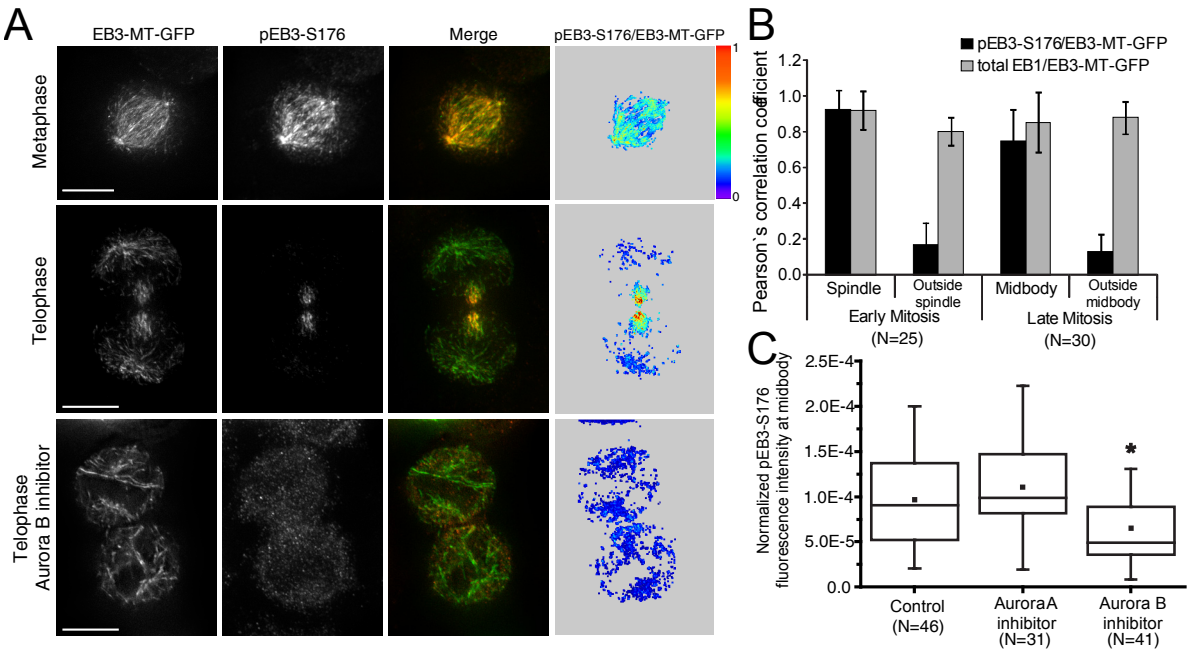

Figure 12 - Immunolocalization of endogenous pEB3-S176. (A) Cells expressing the EB3-MT-GFP construct were used to detect endogenous phosphorylation of EB3 at S176. The ratio between pEB3-S176 and EB3-MT-GFP is shown in the right panel. (B) Quantification of the sub-cellular co-localization of endogenous pEB3-S176. Note that phosphorylation of EB3 at S176 occurs throughout the spindle in early mitosis but is concentrated in the midbody in telophase. (C) Inhibition of Aurora B leads to a significant decrease in the levels of pEB3-S176 in the midbody. * $p<0.001$ using non-parametric ANOVA followed by post-hoc Dunn`s test. Experiments were done in triplicate and N represents the number of cells quantified in each condition.

4 - Discussion

There are several EB-family proteins in humans, but whether they play specific or redundant roles at the microtubule plus-ends and in particular cellular processes remained unclear. Here we investigated the respective roles and regulatory mechanisms behind EB1 and EB3 throughout mitosis, post-mitotic cell adhesion and cytokinesis.

EB1 has been shown to be involved in spindle orientation in many model organisms (Green et al., 2005; Lee et al., 2000; Rogers et al., 2002; Toyoshima and Nishida, 2007). This is thought to occur through stabilization of astral microtubules, which mediate interactions with the cell cortex (Green et al., 2005; Toyoshima and Nishida, 2007). Our experiments show that EB1 controls spindle position relative to the substrate specifically during early mitosis, while EB3 is not required for this purpose. Unlike EB1, cells depleted of EB3 still have a normal astral microtubule array highlighting the EB1-specific regulation of mitotic spindle orientation early in mitosis through its ability to stabilize astral microtubules.

Attachment of post-mitotic cells to the substrate involves the re-assembly of adhesion complexes that were previously inactivated as cells entered mitosis. This is accompanied by a change in microtubule organization and dynamics as cells acquire an interphase-like appearance. The interaction of microtubules with adhesion components has been extensively studied and it has become increasingly clear that they can influence each other's behaviour. On one hand, targeting of focal adhesions by microtubules promotes their disassembly and this event requires an intact microtubule array (Ezratty et al., 2005; Kaverina et al., 1999). On the other hand, FAK activation can induce stabilization of microtubules in migrating cells (Palazzo et al., 2004). Less clear is how dynamic microtubules influence the reformation of adhesion complexes as cells re-organize their cytoskeleton at the exit from mitosis.

Here we show that, during mitotic exit, there is a sudden enrichment of FAK at the focal adhesion sites, correlating with a functional switch of EB3 and re-distribution of microtubule plus-end towards the substrate. We also provide evidence that this functional switch is regulated by phosphorylation. In yeast, the single EB-like orthologue is phosphorylated during anaphase by Aurora B/Ipl1p, and this is required for normal spindle elongation and disassembly (Zimniak et al., 2009). In humans, Aurora B re-localizes from the chromosomes to the spindle midzone at the end of mitosis, generating a phosphorylation gradient that diffuses towards the cell periphery (Fuller et al., 2008; Tan and Kapoor, 2011).

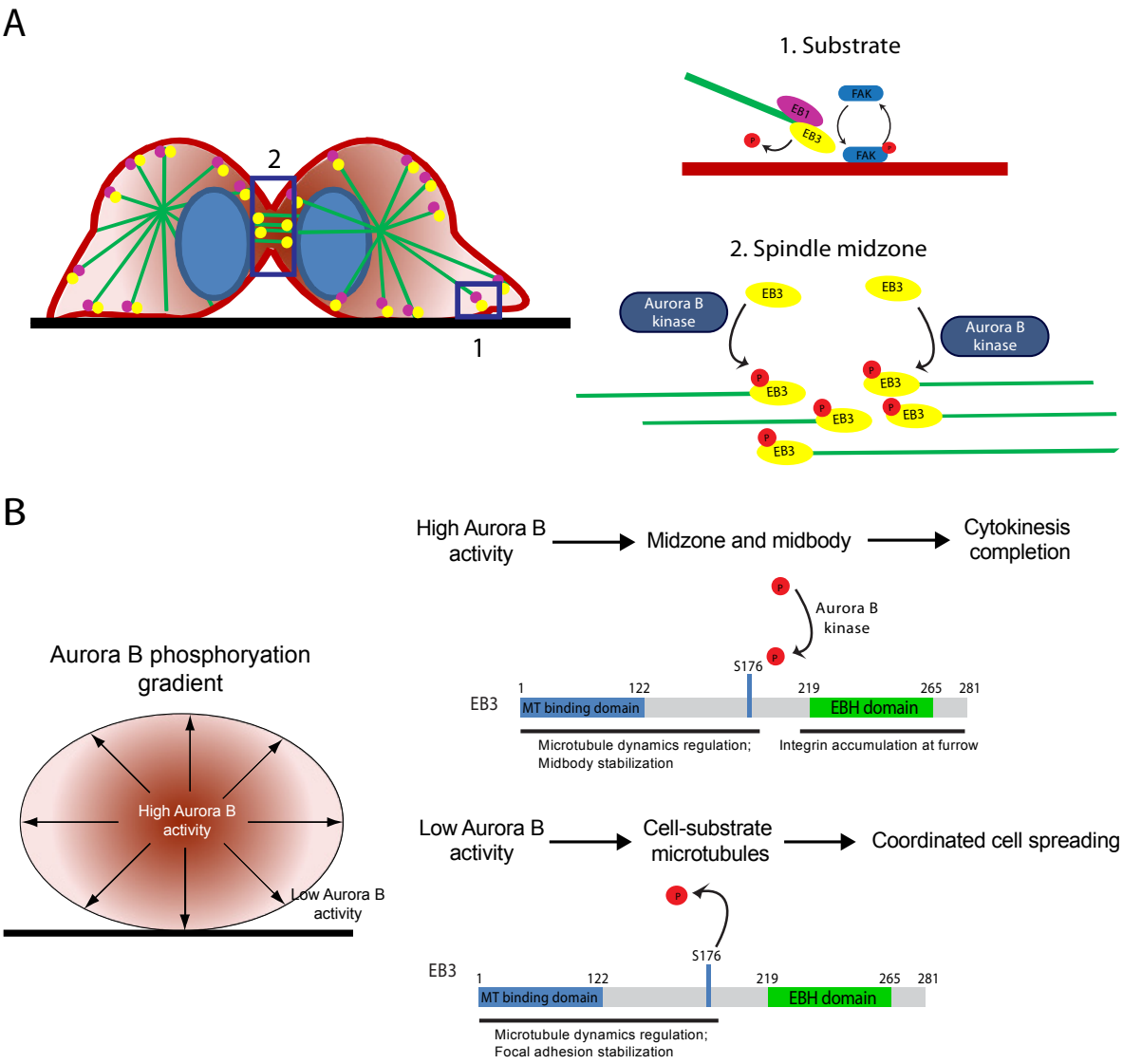

Figure 9 - Ferreira et al., 2012

Figure 13 – Proposed model for the role of EB proteins during mitotic progression and exit. (A) In late mitosis, an Aurora B phosphorylation gradient ensures that only microtubules in the furrow region remain phosphorylated. Microtubules in the vicinity of the substrate contain dephosphorylated EB3. (B) Phosphorylated EB3 remains associated with midbody microtubules in close proximity with the Aurora B phosphorylation gradient and ensures completion of cytokinesis. Dephosphorylation of EB3 occurs near the substrate and allows coordinated daughter cell spreading.

The functional significance of this phosphorylation gradient in human cells and the identity of Aurora B substrates potentially involved in microtubule reorganization at the exit from mitosis remained unknown. Our results suggest that during mitotic exit there is a shift in EB "dominance" required for spindle orientation, with EB3 becoming the key player in coordinating the re-adhesion and spreading of daughter cells to the substrate and completion of cytokinesis. On this regard, it has long been proposed in yeast that an EB-dependent cytokinesis checkpoint might be sensitive to mitotic spindle orientation (Muhua et al., 1998). Interestingly, these functions are differentially regulated by phosphorylation on S176, a previously reported Aurora B phosphorylation site on EB3 (Ban et al., 2009). Accordingly, coordinated daughter cell spreading depends on EB3 S176 dephosphorylation, while completion of cytokinesis requires EB3 S176 phosphorylation. Moreover, phosphorylated EB3 on S176 was previously found enriched in the spindle midzone/midbody during late anaphase and cytokinesis (Ban et al., 2009). Altogether, these findings imply that EB3 exists in distinct phosphorylation states during mitotic exit and are consistent with the presence of an Aurora B phosphorylation gradient diffusing from the spindle midzone/midbody regions towards the cell periphery.

In line with these data we propose that in the vicinity of the spindle midzone/midbody, phosphorylation of EB3 allows stabilization of these microtubule structures and completion of cytokinesis (Figure 13A and B). Earlier reports identified a role for EB-like proteins in cytokinesis in yeast, Dictyostelium and sea urchin eggs (King et al., 2010; Muhua et al., 1998; Strickland et al., 2005b). Importantly, we show that in human cells EB3, but not EB1, regulates this process, suggesting that systems which only have a single EB-like protein centralize different functions, which are normally mediated by distinct EB proteins in human cells. Notably, we demonstrate that successful completion of cytokinesis additionally requires the C-terminal domain of EB3, suggesting a functional dependence on a yet unidentified cargo. Integrins are potential candidates, since we show that EB3 regulates integrin accumulation at the cleavage furrow. Furthermore, integrin deposition at the cleavage furrow is essential for cytokinesis completion (Pellinen et al., 2008). While it is possible that EB3-decorated microtubules may serve as tracks for integrin delivery to the cleavage furrow, we have so far been unable to identify any direct interaction between EB3 and integrins, suggesting that, if occurring, it is either very transient or mediated by third partner(s). This remains an important issue for future investigation.

Consistent with an Aurora B phosphorylation gradient diffusing from the spindle midzone/midbody, EB3 associated with microtubule plus-ends that are biased towards the

adhesion substrate at the cell periphery will be dephosphorylated (or less phosphorylated), limiting microtubule growth. This is required for focal adhesion stabilization and coordinated daughter cell adhesion to the substrate (Figure 13A). Interestingly, induction of cell protrusions was shown to specifically require growing microtubules and to be independent of microtubule shortening or tubulin concentration (Waterman-Storer et al., 1999). This suggests that simply tipping the balance in favour of microtubule growth is sufficient to induce destabilization of post-mitotic cell adhesion factors, which might be in the basis of cancer cell dissemination and metastasis.

IV. ROLE OF EB PROTEINS IN SPINDLE POSITIONING

1 - Introduction:

Proper alignment of the mitotic spindle is a prerequisite for correct separation of sister chromatids and successful completion of mitosis. This requires that cells have evolved mechanisms to regulate spindle positioning in four dimensions.

Intrinsic and extrinsic spatial cues are thought to play a major role in positioning the spindle both in the xy-axis and in the z-axis. In cultured cells, orientation of the spindle poles parallel to the cell substrate is reminiscent of the mechanism that exists in animal epithelia. Human cells have been shown to use integrin signalling, together with EB1 and Myosin X to achieve proper positioning of the spindle (Toyoshima and Nishida, 2007), and this also requires PI3K signalling and Cdc42 (Mitsushima et al., 2009; Toyoshima et al., 2007). Recently, it was also demonstrated that a spindle pole-derived signal influences dynein localization and subsequent interaction with LGN and NuMA (Kiyomitsu and Cheeseman, 2012). In addition to intrinsic factors, spindle positioning can also be influenced by external factors. In agreement, inducing cell shape changes (O'Connell and Wang, 2000) or changing the distribution of the extracellular matrix (Thery et al., 2005) is sufficient to displace the spindle. In this latter case, it was also shown that spindle displacement requires astral microtubules, actin and the Src kinase. Notably, the extracellular matrix contacts the mitotic cell body through a set of actin-based retraction fibres (RFs) (Mitchison, 1992). Recently it was shown that actin-based RFs actively transmit extracellular forces to the spindle allowing it to position accordingly (Fink et al., 2011). This force transmission occurs through a dynamic actin wave that can influence spindle microtubules. Although it is clear that both intrinsic and extrinsic factors impact on how the spindle positions itself, the nature of the forces necessary to achieve this in time and space is still poorly understood. What remains unclear is whether microtubules can also influence this dynamic actin behaviour.

EB proteins are members of a group of MT plus-end binding proteins known as +TIPs. Two members of the family (EB1 and EB3) can exist as heterodimers and regulate microtubule growth and dynamics. We set out to determine whether dynamic microtubules can impact on actin behaviour during mitotic spindle positioning. To do so, we investigated whether EB proteins could provide a molecular link between the actin- and microtubule based-mechanisms of spindle positioning.

We show that microtubules are required to maintain a dynamic actin wave during mitosis. Moreover, we demonstrate that EB1 is necessary for correct spindle alignment in the

relation to the z-axis by regulating astral MT interaction with the cortex. On the other hand, EB3 is necessary for aligning the spindle in the xy-axis by responding to external positional cues provided by RFs. Our results support a model where dynamic MTs are required for transmitting external cues provided by RFs to the spindle, which are necessary for its correct positioning during mitosis. We propose that EB1 and EB3 form an EB-module that allows differential regulation of spindle position by sensing the whole volume of the cell.

2 - Material and Methods

Cell culture and drug treatment

All cell lines used were cultured in DMEM with 10% fetal bovine serum (FBS) and grown in a 5% CO2 atmosphere at 37ºC. HeLa Utrophin-GFP/H2B-mCherry and HeLa EB3-GFP/Lifeact-mCherry cell lines were a gift from Matthieu Piel (Institut Curie, Paris, France). For live-cell imaging experiments, cells were seeded on micropatterned coverslips [as described previously by (Fink et al., 2011)]. Microtubule depolymerization was done by incubating cells with 1 μM of nocodazole (Sigma-Aldrich).

shRNA and transfection experiments

For detailed descriptions, please refer to Chapter III, section 2 (page 113). All transfection experiments were performed on 6-well plates prior to transferring to micropatterned substrates.

Time lapse microscopy

For detailed descriptions, please refer to Chapter III, section 2 (page 114).

3 - Results

EB proteins differentially regulate spindle position by discriminating the xy- and the z-axes

Spindle positioning has to be tightly regulated in time and space. Although it has been shown that microtubules and actin can independently modulate this mechanism, it is not yet clear whether they physically interact to regulate this process. EB1 was previously shown to regulate spindle position in relation to the substrate (i.e. in the z-axis)(Draviam et al., 2006; Green et al., 2005; Toyoshima and Nishida, 2007). Strikingly, it was recently demonstrated that EB1 is not required for aligning the spindle with the long cell axis (i.e. in the xy-axis) (Bruning-Richardson et al., 2012). In order to test the role of EB proteins in spindle positioning, we turned to micropatterning techniques. More specifically, we decided to use fibronectin-coated line patterns (Figure 1). Micropatterning techniques have been extensively used to study mitotic processes and they allow constraining of cells to the predesigned pattern (Fink et al., 2011; Thery et al., 2005; Thery et al., 2006). In our context, line patterns are convenient because they allow confinement of the cells along the lines, restraining spindle positioning in one of the dimensions (the xy-axis).

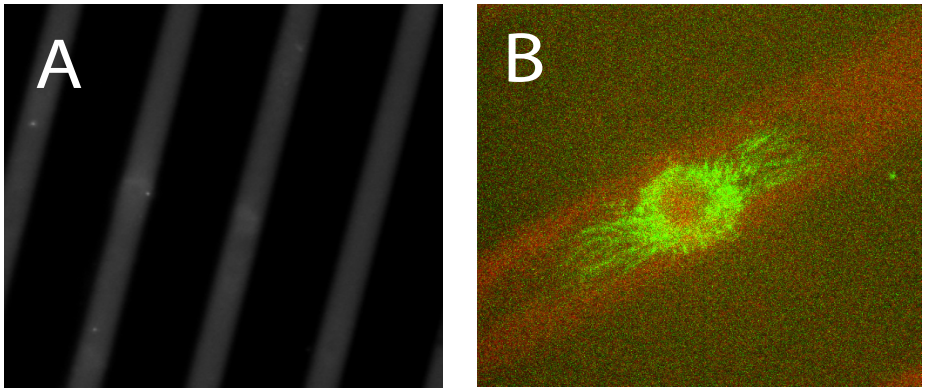

Figure 1 – Line pattern highlighted by labelling fibrinogen with Alexa-594. Coverslips are micropatterned with fibronectin and labelled fibrinogen to assess the quality of the pattern (A). Cell expressing Myrpalm-GFP seeded on a line micropattern with its retraction fibres (green) aligned with the micropattern (red) (B).

When cells enter mitosis in a line pattern, their retraction fibres will be attached specifically to the pattern, leading the spindle to become parallel with the line pattern (Figure 1 and Figure 2). As a result, cells will exit mitosis and reattach along the line. By measuring the angle formed between the line pattern and the anaphase cell position, we can determine the

spindle position at the moment of anaphase onset. Accordingly, when HeLa cells are transfected with a control RNAi they will always divide along the line and the measured angle at anaphase onset is 0^{o} (Figure 2A). With this setup, we can now ask whether EB proteins impact on spindle angle. To do this, we depleted individual EB1, EB3 or both proteins in tandem using an RNAi approach. When cells are depleted of EB1, there is an increase in the spindle angle at anaphase onset (Figure 2A). However, it should be noted that even though these cells misalign their spindle during mitosis, they enter and exit mitosis still aligned with the substrate (Figure 2B). This means that EB1-depleted cells enter normally in mitosis, but their spindle is not receiving the cues provided by the extracellular matrix, which leads to mispositioning. Even more interesting is the fact that they manage to recover the orientation when reattaching to the pattern. This observation implies that the attachments to the substrate are functional and further strengthen the idea that it is some spindle component that is not interacting with the cell cortex. Indeed, EB1 has been shown to be required for astral microtubule nucleation/function [see chapter III; Figure 2 and (Toyoshima and Nishida, 2007)] and this leads to displacement of the spindle in the z-axis. This means that EB1 is necessary to define the z-axis but ultimately does not influence xy-axis position. In accordance with this, EB1-depleted cells were reported to align their spindle with the long cell axis (Bruning-Richardson et al., 2012).

Curiously, depletion of EB3 leads to a much more severe phenotype. Many of the cells already enter mitosis without being aligned with the pattern (Figure 2A and 2B). These cells show extensive displacement of the spindle during mitosis and a significant percentage fails to re-align with the pattern after mitosis. This suggests that EB3-depleted cells are no longer capable of defining the xy-axis during mitosis and that the spindle is not aligning with the long cell axis. We have previously shown that EB3 does not interfere with astral microtubule function (see chapter III; Supplementary Figure 2) therefore, it is possible that EB3 may be interfering with spindle positioning through another mechanism. Interestingly, it is possible to revert EB3-mediated phenotypes by expressing the microtubule binding domain of EB3 (EB3-MT), which suggests that this process mainly depends on regulation of microtubule dynamics (Figure 2B).

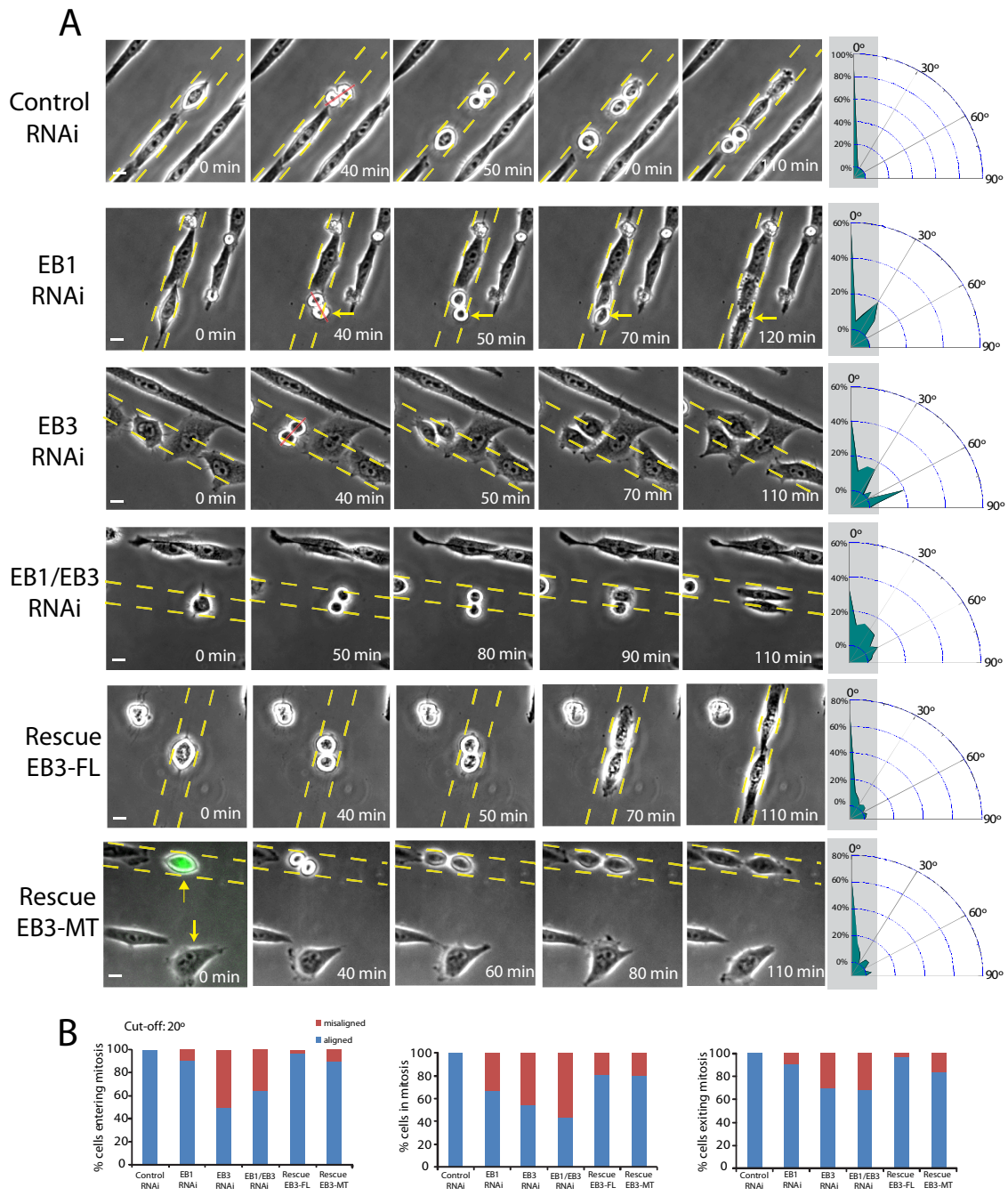

Figure 2 – Quantification of spindle angles during mitosis on a line pattern. (A) Cells were seeded in a line pattern and filmed using time-lapse phase contrast microscopy. Anaphase spindle angles were determined in relation to the line and angle values were plotted on a radar plot. When cells divided along the line, angle value was defined as 0^{o}. (B) Cells were individually tracked to determine whether they entered, progressed and exited mitosis along the line pattern. The percentage of cells that entered aligned, remained aligned and exited aligned was calculated, using a cut-off angle value of 20^{o}. Expression of EB3-MT efficiently restores spindle alignment. Yellow arrowhead highlights a cell transfected with EB3-MT dividing along the line pattern whereas the yellow arrow highlights an EB3-depleted cell outside the line boundaries. Scale bars, 10 μm. Time lapse is 10 min.

These results highlight the differential roles of EB1 and EB3 in spindle positioning. While EB1 is required for z-axis positioning but does not interfere with extracellular coupling/sensing, EB3 is required for xy-axis positioning without interfering with astral microtubule nucleation/stability.

EB3 does not interfere with retraction fibre formation during mitosis

RFs are actin-based structures that ensure mitotic cell attachment to the substrate (Mitchison, 1992). Recently it was shown that RFs respond to changes in the extracellular matrix and transmit extracellular forces to the spindle (Fink et al., 2011; Thery et al., 2005). Given that EB3 seems to regulate spindle position in the xy-axis, we wondered whether it would regulate RF formation/distribution and this would explain the phenotypes observed upon EB3 depletion. For that purpose, we imaged cells HeLa cells expressing Myrpalm-GFP/H2B-mCherry seeded on line patterns. If indeed EB3 is required to establish or maintain RFs, then EB3-depleted cells should have disorganized or absent RFs. In fact, this is not the case as EB3-depleted cells still had robust retraction fibres (Figure 3A) and these nicely co-localize with actin as can be seen with the actin marker Lifeact (Figure 3B).

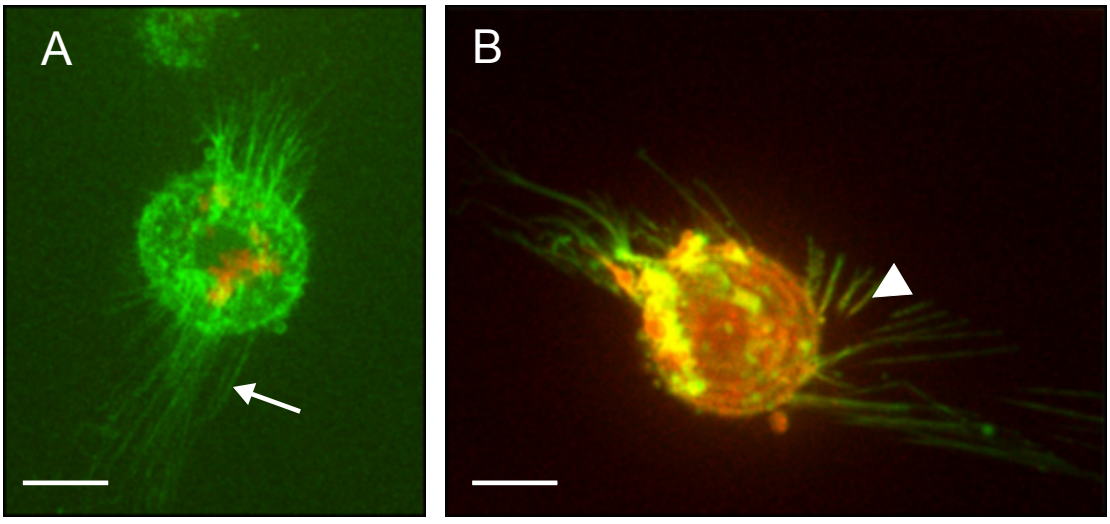

Figure 3 – Retraction fibre distribution of EB3-depleted cells on line patterns. (A) HeLa cell depleted of EB3, expressing Myrpalm-GFP/H2B-mCherry and showing robust retraction fibres that localize to the line pattern (white arrow. Note in (A) that this particular cell already entered anaphase misaligned with the axis determined by the retraction fibres. (B) Projection of HeLa cell depleted of EB3, expressing Myrpalm-GFP/Lifeact-mCherry highlighting the co-localization between both markers in the retraction fibres (white arrowhead). Scale bars, 10 μm.

Furthermore, many of the cells depleted of EB3 entered anaphase with their spindle misaligned, even though their RFs were aligned with the pattern (Figure 3A). It remains to be determined whether these retraction fibres are functional and can transmit extracellular signals to the spindle.

During metaphase microtubules preferentially target the polar cortex as opposed to the equatorial cortex

During mitosis, microtubules emanate from the centrosomes and come into contact with the cell cortex. It has been shown in many systems that contact of microtubules with the cortex is essential to position the mitotic spindle (Carminati and Stearns, 1997; Kozlowski et al., 2007; O'Connell and Wang, 2000). Given that EB proteins are essential regulators of microtubule function, we set out to determine the behaviour of EB3-labelled microtubules during metaphase. For that purpose, we seeded HeLa cells expressing EB3-GFP and Lifeact-mCherry in line micropatterns and filmed them while in mitosis.

To assess comet behaviour, we imaged cells with a time lapse of 2 seconds (Figure 4A). Afterwards, two different regions were selected that correspond to the pole region (Figure 4A; top panel) and the equator region (Figure 4A; bottom panel). Maximal projections of the entire movie were generated which allowed a visualization of the complete microtubule track (Figure 4B). As can be easily observed, microtubules preferentially contact the cortex near the pole region. Comparatively, very few microtubules contact the equatorial cortex. This suggests that some factor exists near the polar cortex that favours or directs microtubule polymerization or, inversely, some inhibiting factor exists near the equatorial cortex. This preferential localization of EB3-labelled microtubules correlates with a polarized cluster of actin filaments that appears during mitosis and was described previously [Figure 4C; (Fink et al., 2011; Mitsushima et al., 2010)]. The spatial correlation between microtubule polymerization and the actin cluster lead us to consider that microtubules and actin may interact to position the mitotic spindle.

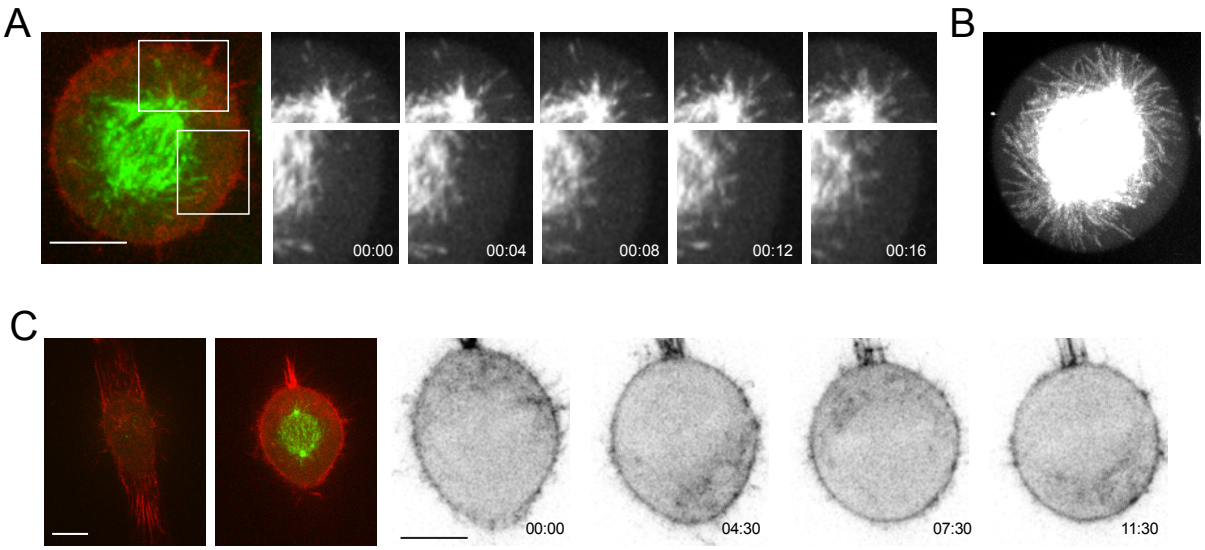

Figure 4 – Microtubule and actin behaviour during mitosis. (A) Time-lapse movie of cell expressing EB3-GFP/Lifeact-mCherry. Cells were imaged with a time-lapse of 2 seconds. Regions selected are representative of the pole region (top panel) and the equatorial region (bottom panel). (B) Maximal projection of EB3 comets from a time-lapse movie. Note the concentration of comets near the polar cortex, as opposed to the equatorial cortex. (C) Dynamic actin behaviour in mitotic cells. Cells were seeded in a line micropattern an imaged with a time-lapse of 15 seconds. During mitosis, actin exhibits dynamic polymerization behaviour, moving between the polar cortices. Scale bars, 10 μm. Time is in min:sec.

Microtubules are necessary for dynamic actin behaviour during mitosis

During mitosis, positioning of the spindle requires a dynamic wave of actin filaments that transmits extracellular forces imposed on the cell to the inside, influencing spindle movement (Fink et al., 2011; Mitsushima et al., 2010). In fact, it has been shown that the actin waves can be polarized by seeding the cells on specific fibronectin patterns and that the spindle follows these waves (Fink et al., 2011). This is based in the observation that treatment with a low dose of nocodazole (which stabilizes astral microtubules without affecting the spindle structure), will make the spindle move randomly inside the cell without affecting the actin waves. Based on these observations and in our previous results, we reasoned that microtubules might also influence dynamic actin behaviour. For that purpose, we filmed HeLa cells expressing the actin marker Utrophin tagged with GFP and H2B tagged with mCherry and assessed whether they show the same dynamic behaviour of actin (Figure 5). At that point, we treated cells with 1 μM nocodazole to induce complete depolymerization of astral and spindle microtubules and followed actin behaviour.

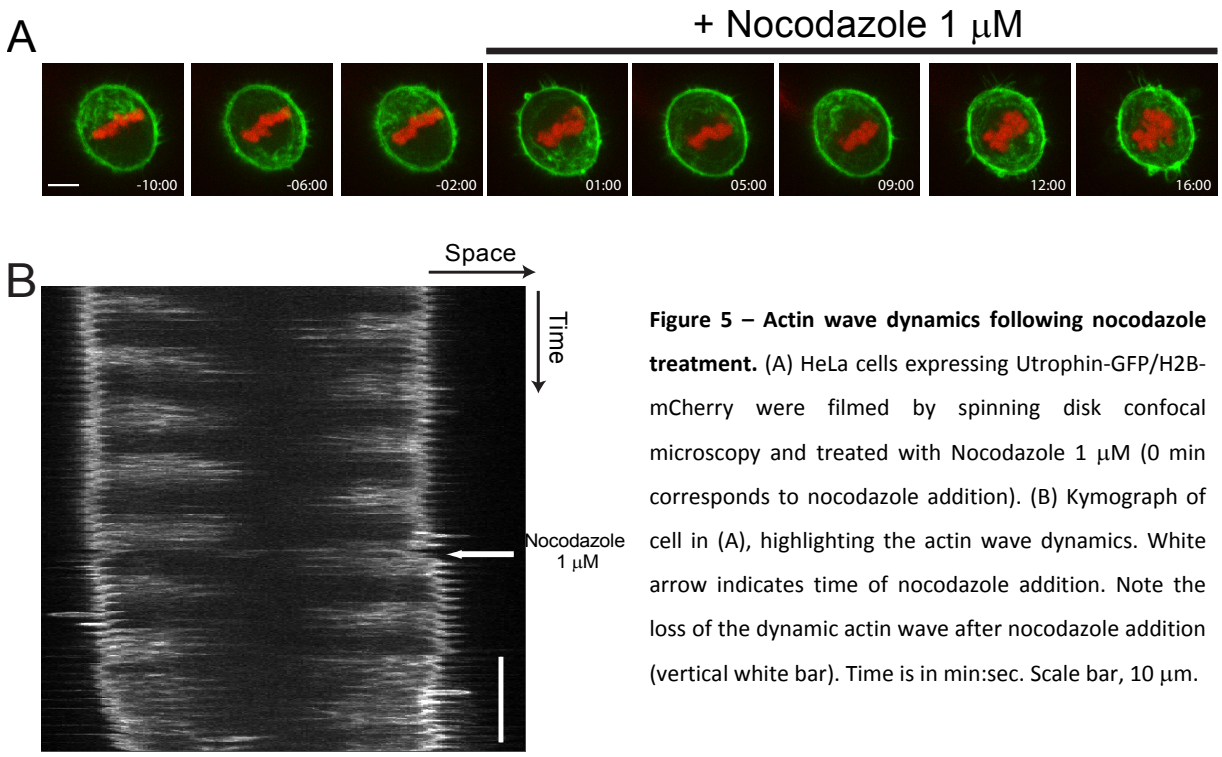

Figure 5 – Actin wave dynamics following nocodazole treatment. (A) HeLa cells expressing Utrophin-GFP/H2B-mCherry were filmed by spinning disk confocal microscopy and treated with Nocodazole 1 μM (0 min corresponds to nocodazole addition). (B) Kymograph of cell in (A), highlighting the actin wave dynamics. White arrow indicates time of nocodazole addition. Note the loss of the dynamic actin wave after nocodazole addition (vertical white bar). Time is in min:sec. Scale bar, 10 μm.

As can be observed in Figure 5, treatment with nocodazole effectively induced a loss of polarization in actin behaviour. In fact, approximately 85% of the cells that exhibited the polarized actin movement before nocodazole addition lost that same behaviour following the treatment. It should be noted that 15% of the cells did not have this polarized movement even before nocodazole addition and these did not show any changes in actin behaviour upon drug addition. Furthermore, nocodazole treatment induced extensive cortical blebbing, further suggesting that microtubules are required for actin function during mitosis.

EB3 is required for the maintenance of the dynamic actin wave

So far we have demonstrated that interfering with EB3 gives rise to spindle positioning defects in the xy-axis without perturbing RF formation. In addition, we have also shown that microtubules are important to maintain the dynamic behaviour of actin during mitosis. Therefore, we reasoned that EB3 may be involved in the generation or maintenance of this actin wave. In order to test this, we seeded HeLa cells expressing the actin marker Utrophin-GFP and histone H2B-mCherry onto line patterns and imaged them to determine whether they normally exhibited this dynamic actin behaviour (Figure 6A). As was expected, when seeded in

line patterns, control cells exhibit a polarized actin movement, with polymerization events occurring at both poles. In fact, about 68% of control cells showed this type of behaviour, with 10% showing a circular movement. In about 20% of the cells, we could not find any discernible pattern (Figure 6A and 6B). This type of behaviour can be easily visualized using a kymographic approach (Figure 6B). When cells have a bipolarized actin polymerization, we can observe actin waves appearing cyclically and jumping from one pole to the other (Figure 6B, Bipolar Movement; panel 0^{o}). When we analyse the perpendicular axis (90^{o}), no polymerization events are observed, which reinforces that actin polymerization is jumping from one pole to the other.

When the same analysis is performed in EB1-depleted cells, approximately 60% of the cells still show polarized actin behaviour, which is similar to what we observe in control cells (Figure 6B; graph panel). However, about 25% of the cells show disorganized actin polymerization (Figure 6A and 6B). It should be noted that despite this increase, EB1-depleted cells still maintain alignment with the pattern, which suggest they still have a functional attachment of RFs to the extracellular matrix. Interestingly, EB1 depletion induces spindle tilting during metaphase (see Chapter III) but this does not seem to influence actin behaviour. These tilted cells still manage to reattach to the line patterns without significant problems.

On the other hand, when EB3 is depleted, we have approximately 50% of the cells with disorganized or absent actin polymerization (Figure 6A and 6B). Strikingly, only 30% of EB3-depleted cells exhibit normal bipolar actin polymerization, indicating a severe defect in the connection between RFs and the actin cortex. This may also explain why they fail to position their spindle along the line pattern. Accordingly, cells that had defects in actin polymerization showed displacement of the spindle in the xy-axis, which meant that they eventually entered anaphase with misaligned spindles. Overall, these results highlight the crucial role that both dynamic microtubules and dynamic actin have during mitosis and identify EB proteins as potential regulators of this cross-talk.

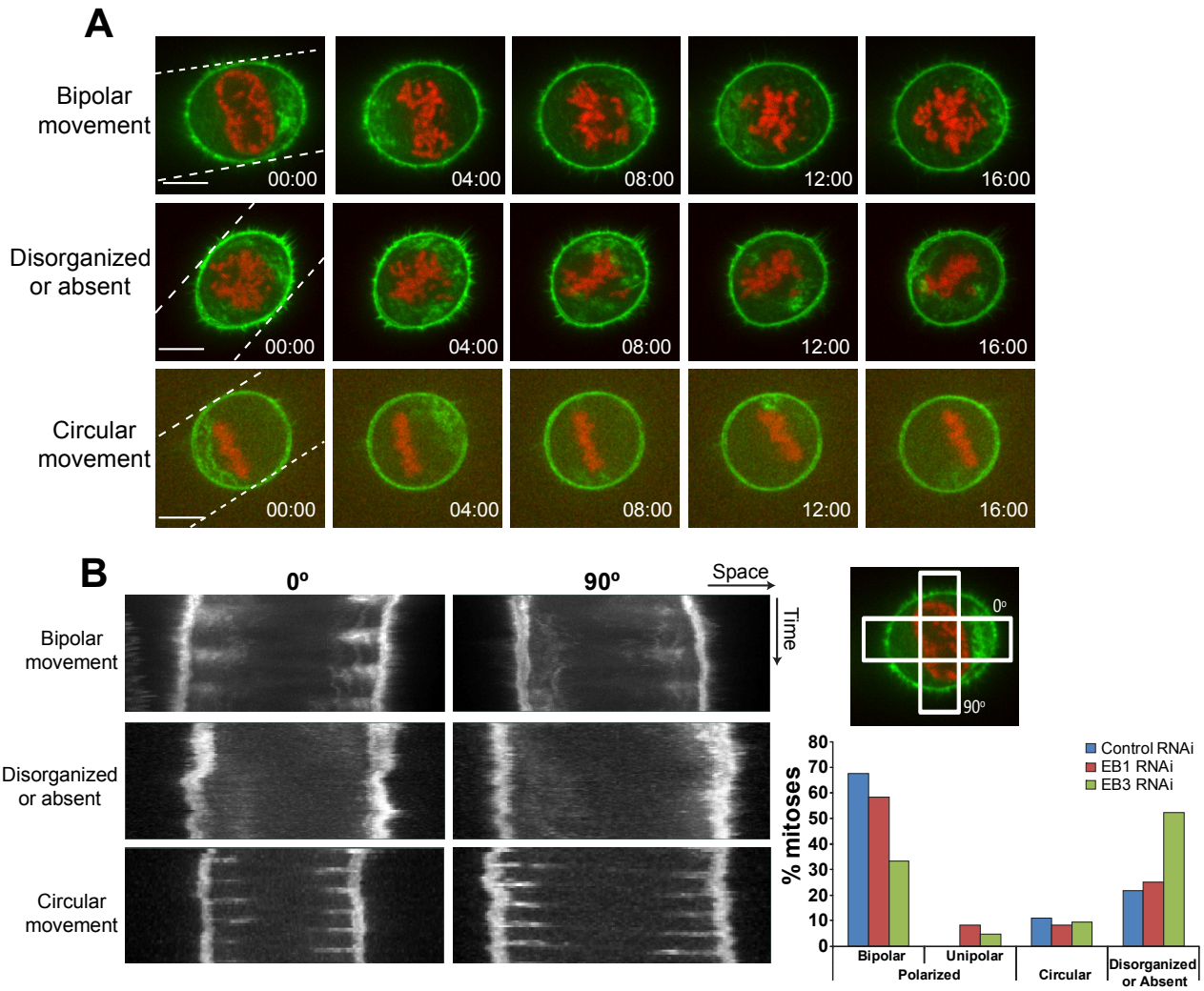

Figure 6 – Dynamic actin behaviour during mitosis. (A) HeLa cells expressing Utrophin-GFP/H2B-mCherry were seeded on line patterns and imaged during mitosis to assess the dynamic behaviour of actin. The most common behaviours are depicted and include bipolar actin movement, disorganized or absent movement and circular movement. (B) Kymographic analyses done on these cells allowed quantification of the relative proportions of actin behaviour observed in EB1- and EB3-depleted cells. Note the increase in the number of cells with disorganized or absent actin movement after EB depletion. Scale bars, 10 μm. Time lapse is 30 sec. Time is in min:sec.

4 - Discussion

Positioning of the mitotic spindle involves a cross-talk between intrinsic and extrinsic cues. Upon mitotic entry, cells will round up and decrease the attachment to the substrate in a process that involves hydrostatic pressure and increased contractibility of the cell cortex (Stewart et al., 2011). This means mitotic cells will remain attached to the extracellular matrix by a set of actin-based RFs (Fink et al., 2011; Mitchison, 1992; Thery et al., 2005). These RFs not only provide cues for positioning of the spindle in relation to the topology of the substrate (Thery et al., 2005) but they also actively participate in spindle positioning by transmitting forces applied to the cells which, in turn, influences actin dynamic behaviour (Fink et al., 2011). It has long been known that microtubules play an active role in spindle positioning (Samora et al., 2011; Toyoshima and Nishida, 2007; Wang et al., 1995). Recently, actin contribution has also been described in a number of different systems (Fink et al., 2011; Li et al., 2008; Mitsushima et al., 2010; Schuh and Ellenberg, 2008). Interfering with astral microtubule function by treating cells with low doses of nocodazole does not affect actin dynamic behaviour (Fink et al., 2011) however, our results demonstrate that complete microtubule depolymerization does affect actin behaviour. This means that spindle microtubules impact on actin behaviour and implies that some microtubule-based component is required for actin polymerization.

Given their role on microtubule function, we have studied EB proteins in the context of spindle positioning. We show that EB proteins differentially regulate spindle position by sensing the whole volume of the cell. In this context, EB1 has a role in spindle position in relation to the substrate during early mitosis [our results and (Toyoshima and Nishida, 2007)] but does not affect position of the spindle in the long cell axis (Bruning-Richardson et al., 2012). Inversely, EB3 does not influence spindle position in the z-axis (see Chapter III) but we show that it does regulate lateral spindle movements (xy-axis). It seems clear that EB1 is required for astral microtubule nucleation which, as has been shown previously, must contact the cortex to allow proper spindle position (Bruning-Richardson et al., 2012; Draviam et al., 2006; Green et al., 2005; Rogers et al., 2002; Toyoshima and Nishida, 2007). However, it should be noted that presence of astral microtubules *per se* is not sufficient to allow proper positioning of the spindle. In fact, MAP4 depletion, which does not interfere with astral microtubule nucleation, is sufficient to displace the spindle (Samora et al., 2011). This reflects the need for interaction of astral microtubules with a specific cortical component. Notably, some of these cortical components such as dynein, LGN or NuMA have been associated with

spindle positioning by allowing the capture of astral microtubules at the cell cortex (Du and Macara, 2004; Kiyomitsu and Cheeseman, 2012; O'Connell and Wang, 2000). It remains to be determined whether EB1 acts indirectly by regulating astral microtubule nucleation or directly by interacting with one of those cortical components. Whichever the mechanism, the interaction must be biased towards the polar cortices in the z-axis. Interestingly, EB3 specifically controls spindle position in the xy-axis. This nicely correlates with the dynamic actin wave observed during mitosis and implies that EB3 must interact with actin. How would EB3 regulate spindle movement? Such a mechanism would require that either EB3 directly impacts on actin dynamics or that there must be an interaction of EB3 with an actin-binding protein. Accordingly, it has been reported that the microtubule-actin crosslinking factor ACF7/MACF has a SxIP motif that specifically interacts with EB proteins (Honnappa et al., 2009; Slep et al., 2005) and EB3 associates with the F-actin binding protein Drebrin (Bazellieres et al., 2012; Geraldo et al., 2008). On the other hand, one may consider that preferential actin polymerization at the polar region could lead to astral microtubule capture and subsequent spindle movement. However, it is still unclear how EB3-labelled microtubules themselves may trigger actin polymerization. At this point, it is known that the actin movement is dependent on Arp2/3 and CDK1 (Mitsushima et al., 2010) but in the future, further work has to be done in order to determine what links EB3 to the actin cytoskeleton. Furthermore, it will be crucial to determine how this relates to transmission of forces from RFs to the spindle.

Overall, our results demonstrate that microtubules can regulate spindle position in a 3D environment by influencing actin dynamic behaviour. This is accomplished by an EB module that probes the z-axis (EB1) and the xy-axis (EB3) and further highlights the functional diversity of EB proteins during mitosis.

V. ROLE OF EB PROTEINS IN INTERPHASE

(Submitted to Journal of Cell Science)

Summary:

Crosstalk between microtubules and cell adhesion components is crucial in the regulation of cell adhesion and motility. In migrating cells, stabilization of microtubules is dependent on integrins and Focal Adhesion Kinase (FAK). On the other hand, targeting of focal adhesions (FAs) by microtubules leads to a disassembly of these structures. Overall, control of microtubule dynamics impacts turnover rate of adhesion structures, leading to changes in cell motility.

Here we show that End-Binding (EB) proteins have differential effects on cell motility and adhesion through their distinct role in the regulation of microtubule dynamics and stabilization of FAs. EB1 depletion blocks cell motility and induces a stabilization of focal adhesions while EB3 depletion induces a rapid turnover of these structures with an associated increase in cell motility. Moreover, we show that EB3 is involved in the establishment of extracellular spatial references important to maintain adhesion to pre-defined patterns. Finally, we demonstrate that cell motility is regulated by phosphorylation of EB3. Overall, EB proteins control the stability of FAs through their ability to modulate microtubule dynamics, thus regulating cell adhesion and migration.

1 - Introduction:

Regulation of cellular adhesion and motility is a multi-factorial mechanism that involves the coordinated contribution of actin, microtubules and a long list of proteins involved in the formation, maintenance and disassembly of focal adhesions. It has already been established that microtubules play an important role in directional cell migration (for review see (Kaverina and Straube, 2011). In recent years, evidence has accumulated that implicates microtubules in the remodelling of adhesion complexes. Notably, microtubules have been shown to specifically target focal adhesions and promote their turnover through kinesin-1 (Kaverina et al., 1999; Krylyshkina et al., 2002). In addition, microtubules are involved in focal adhesion disassembly in a mechanism that involves dynamin (Ezratty et al., 2005) but also clathrin-mediated integrin endocytosis (Ezratty et al., 2009). On the other hand, adhesion components themselves can also affect microtubule behaviour. In fact, integrin-based adhesion complexes have been shown to induce stabilization of microtubules through the concerted action of FAK and Rho (Palazzo et al., 2004). Moreover, paxillin (a component of focal adhesions) has been shown to directly induce microtubule catastrophes (Efimov et al., 2008).

Microtubule End-Binding (EB) proteins are part of a conserved family of microtubule plus-end tracking proteins (+TIPs) [reviewed by (Gouveia and Akhmanova, 2010)]. In humans, they consist of three closely related members, EB1, EB2 and EB3. EB1 has been the most widely studied due to its interaction with the C-terminus of Adenomatosis Polyposis Coli [APC; (Su et al., 1995)] but little is known about other family members. It is known that these proteins regulate microtubule dynamics by suppressing microtubule catastrophes (Komarova et al., 2009) and are also responsible for the loading of other proteins to the microtubule plus-end (Gouveia and Akhmanova, 2010). Interestingly, recent work has involved EB1 and APC in the stabilization of microtubules and promotion of cell migration (Wen et al., 2004). On the other hand, it was also reported that EB1 also negatively regulates cell motility (Schober et al., 2009).

Given that it is still not clear how microtubule mediated roles influence cell migration and adhesion mechanisms and taking into account that EB proteins act as master regulators of microtubule dynamics, we decided to address whether they would play a role in the regulation of cell motility.

2 - Material and Methods

Cell culture and cell lines

For detailed descriptions please refer to Chapter III, section 2 (page 113). All experiments were performed using HeLa S3, unless otherwise stated. GE11 integrin β1 -/- cell line was a gift from Reinhard Fassler (Max Planck Institute of Biochemistry, Martinsried, Germany). Myrpalm-GFP/H2B-mCherry cell line was a gift from Matthieu Piel (Institut Curie, Paris, France).

shRNA and transfection experiments

For detailed descriptions please refer to Chapter III, section 2 (page 113). pFB-Neo-Integrin β1-GFP was a gift from Dr. Martin Humphries (University of Manchester, UK). pEGFP-FAK was a gift from Dr Peter Wang (UCSD, La Jolla, USA). mRFP-FAK was a gift from Gregg Gundersen (Columbia University, USA).

Antibodies and Immunofluorescence

For detailed descriptions please refer to Chapter III, Section 2 (page 114).

Timelapse microscopy

For detailed descriptions please refer to Chapter III, Section 2 (page 114). Micropatterned coverslips (a gift from Dr Matthieu Piel, Institute Curie, Paris, France) were used as described previously (Thery and Piel, 2009).

Distance and mean velocity measurements

Individual cells were tracked using the "Manual Tracking" plugin of ImageJ. Cell nuclei were used as reference points. "Interphase" was defined as random 10 time frames that were at least 20 time frames separated from mitosis. All time frames were acquired with a 10 min interval. "Cumulative distance travelled by cells" was defined as the sum of all consecutive

distances travelled by cells during the time-lapse. “Mean velocity” was defined as the mean distance travelled by the cells in each time frame divided by the time-lapse of each time frame.

Microtubule dynamics measurement

To measure microtubule dynamics, HeLa cells expressing α-tubulin-GFP were seeded on fibronectin-coated coverslips and imaged with a time-lapse of 2 sec for a maximum of 5 min. Individual microtubules were then selected and used to generate kymographs which depict growth, catastrophe and pause events.

Quantification of number of EB comets throughout the cell volume

Quantification of the normalized fluorescence intensity of EB comets was performed by obtaining the mean intensity values for each z-slice. These were then normalized to the lower intensity value obtained for the respective cell and averaged for each treatment group. Results were plotted as mean fluorescence intensity in relation to the z-axis.

Chromo-kymographic analysis of Focal Adhesions

Analysis of FA formation and persistency were done using chromo-kymographic methods that were described previously elsewhere (Pereira and Maiato, 2010). For detailed descriptions on chromo-kymograph generation and analysis, please refer to Chapter III, Section 2 (page 116).

Statistical analysis

All statistical procedures used to analyse data are explained in Chapter III, Section 2 (page 117).

3 - Results and Discussion:

EB1 and EB3 have distinct roles in cell motility

EB proteins act as major regulators of microtubule dynamics. EB1 was recently described as a negative regulator of cell motility (Schober et al., 2009). On the other hand, we have previously shown that EB3 impacts post-mitotic cell spreading and adhesion (manuscript submitted). Given these results we tested what would be the role of EB proteins in the regulation of cell motility during interphase. To test this we tracked individual HeLa S3 cells during interphase upon control, EB1, EB3 or EB1/EB3 RNAi over a period of 15 hours (Figure 1A and Supplementary movies). Consistent with previous observations in melanoma cells (Schober et al., 2009), EB1-depletion significantly decreased cell motility relative to controls, with approximately 40% reduction in the cumulative distance travelled (Figure 1B and C). This difference in the migrated distance however, did not imply a significant change in the mean velocity of EB1-depleted cells relative to controls (Figure 1D). EB3-depleted cells, on the other hand, were more motile than control cells, with a 50% increase in the cumulative distance travelled. In addition, they also had a significantly higher mean velocity (Figure 1D). The same was true for EB1/EB3-depleted cells, pointing to a dominant role of EB3 over EB1 with respect to interphase cell adhesion and motility.

Accumulation of EB1-labelled microtubules near the basal cortex of epithelial cells was recently reported (Hotta et al., 2010) and this is important for the stabilization of microtubules near the membrane through interaction with LL5s and CLASPs. Our immunofluorescence analysis of EB1 and EB3 localization revealed that both EB proteins are enriched at microtubule ends near the substrate (Figure 1E). Together with our previous experiments, these results place EB proteins in the right spatial context to regulate cell motility and adhesion and highlight a differential role of EB proteins in these processes.

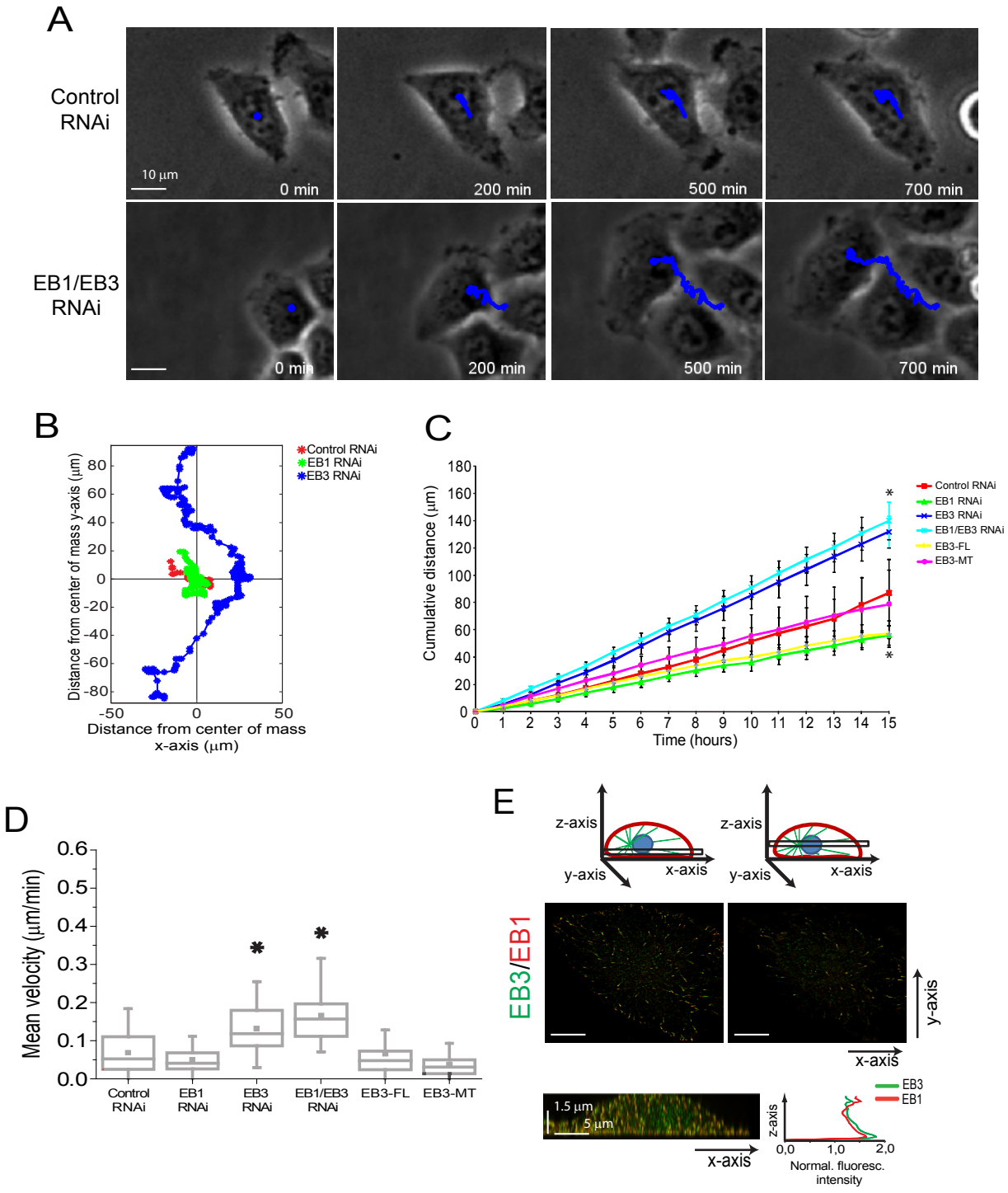

Figure 1 – EB proteins differentially regulate cell motility. (A) Cells were seeded on fibronectin coated coverslips and manually tracked. (B) Individual cell tracks representative of control cells and cells depleted of EB1 or EB3. (C) Cumulative distance travelled by cells over a period of 15 hours. Time is in hours. Note the difference between EB1- or EB3-depleted cells and control cells. (D) Mean velocity of cells (µm/min). (E) Distribution of EB1 and EB3 comets throughout the entire cell volume. Note the accumulation of EB comets near the substrate. * p<0.001 using a one-way ANOVA followed by a post-hoc Student-Newman-Keuls test.

EB proteins are composed of an N-terminal microtubule-binding domain and a C-terminal partner binding domain. To clarify whether EB3-mediated effects on cell motility are due to its ability to regulate microtubule dynamics or interact with partner proteins, we performed rescue experiments by expressing an RNAi-insensitive, N-terminal portion of EB3 tagged with GFP (EB3-MT) in an EB3-depleted background. This was sufficient to restore cell motility and mean velocity to control values (Figures 1C and 1D). Our results demonstrate that EB3-mediated regulation of microtubule dynamics is necessary for correct attachment of cells to the substrate. Interestingly, expression of the full length EB3 (EB3-FL) was more efficient in this respect, reverting motility to EB1-like values (Figure 1C). This difference also suggests that the C-terminal portion of EB3 is necessary to provide additional attachment to the substrate. Taken together, these data also explain why the EB3-MT construct is not as effective as the full length EB3 in this respect. Overall, our results suggest that EB3 controls cell motility by regulating microtubule dynamics.

EB proteins regulate a differential cellular response to defined adhesion micropatterns

From our previous single cell tracking, we noticed that EB3 and EB1/EB3 depletion induced dramatic shape changes in many cells. Given that attachment of cells to the substrate occurs through integrin-mediated adhesion complexes we decided to visualize GE11 cells expressing integrin β1-GFP by spinning-disk confocal microscopy. In accordance to our previous results, a significant number of EB3 (or EB1/EB3) depleted cells presented an abnormal morphology, with very long and dynamic protrusions that leave a thin trail of membranous material (Figure 2A). It has been shown that the architecture of extracellular matrix architecture can influence microtubule growth and stability (Thery et al., 2006). In fact, the authors demonstrate that microtubule plus-ends specifically orient towards adhesion sites when in contact with non-adhesive surfaces. To address the nature of the morphological changes exhibited by the lack of EB3, we seeded cells on defined micropatterned coverslips coated with fibronectin. Under these conditions, control cells remained confined within the predefined pattern of fibronectin and only moved along the line (Figure 2B, C and Supplementary movie).

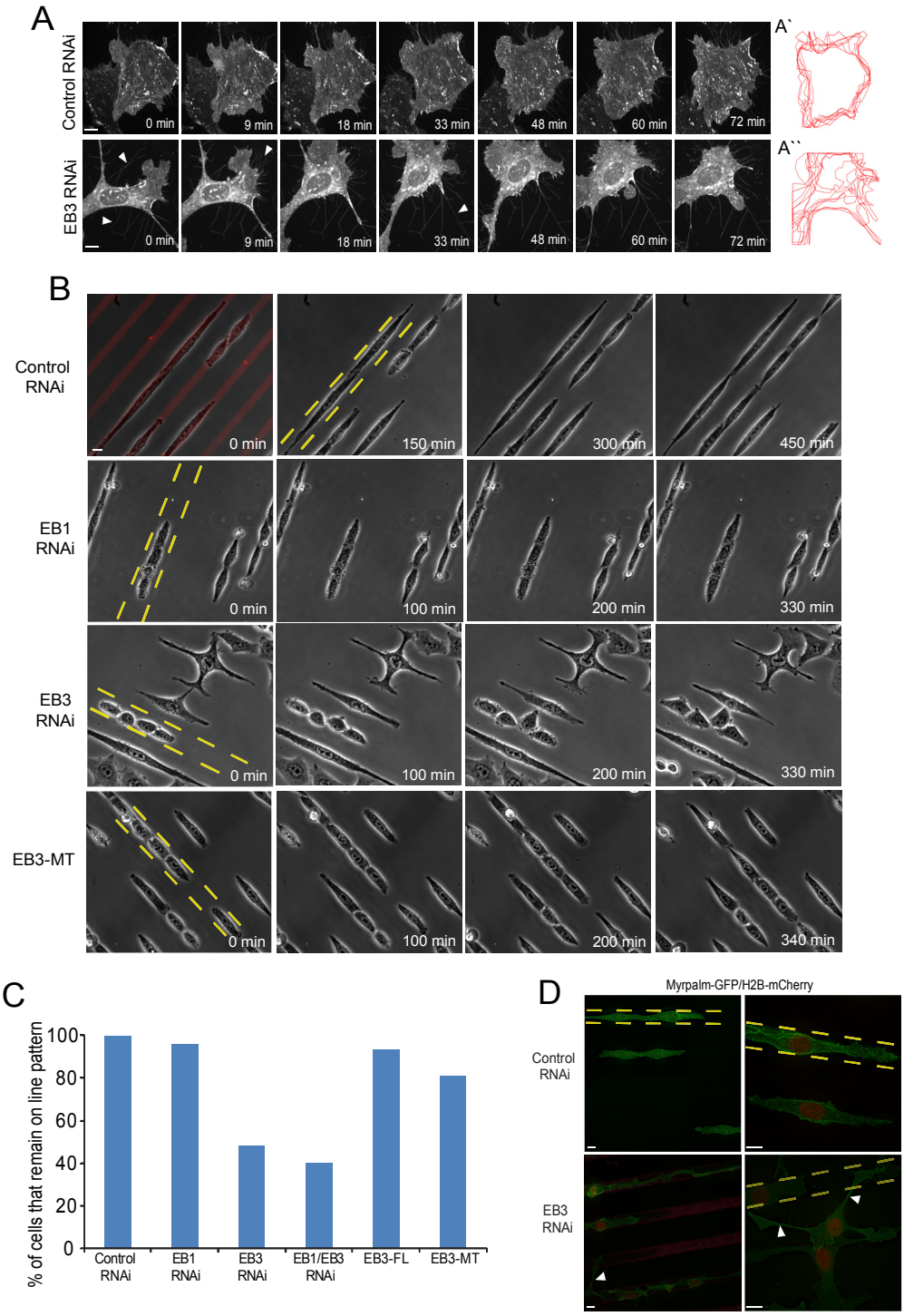

Figure 2 – EB proteins differentially regulate attachment to predefined adhesion patterns. (A) GE11 (integrin β1 -/-) cells re-expressing integrin β1-GFP and depleted of EB3 display altered shapes. (B) HeLa S3 cells were seeded in line micropatterns and filmed to determine their ability to attach to predefined adhesion patterns. (C) Quantification of the number of cells that remain on the line patterns following EB depletion and rescue experiments. (D) Cells depleted of EB3 exhibit protrusions and are able to jump from one line to the other whereas control and EB1-depleted are not. Scale bars, 10 μm. In (A), time lapse is 3 min. In (B), time lapse is 10 min. Time is in minutes.

Furthermore, in these cells the microtubule array was parallel with the patterned line and aligned with the long cell axis (results not shown).

When EB1 was depleted, cells remained mostly stationary in the pattern and only very rarely exited the boundaries defined by the fibronectin lines (Figure 2A, B). This is in agreement with our earlier observation that EB1 depletion induced a decrease in cell motility relative to control cells. Both these conditions indicate that cells are polarized along the line and are able to receive the extracellular signal provided by the extracellular matrix. On the other hand, EB3 or EB1/EB3 RNAi induced loss of polarization and leading cells to frequently leave the pattern and invade neighbour lines (Figure 2B, C). Imaging of HeLa cells stably expressing the membrane marker Myrpalm-GFP and Histone H2B-mCherry, highlighted the highly abnormal cytoplasmic morphology and the formation of cell protrusions that invaded neighbour lines (Figure 2D). This erratic behaviour and invasion capacity was significantly rescued by expression of either the EB3-FL or EB3-MT constructs (Figure 2B, C). This indicates that EB3-mediated regulation of microtubule dynamics is required for the establishment of extracellular spatial references important to respect defined adhesion patterns.

Differential regulation of interphase microtubule dynamics by EB1 and EB3

Dynamic microtubules are necessary for efficient cell attachment and motility (Hotta et al., 2010; Liao et al., 1995). Our results indicate that EB1 and EB3 impact differently on the ability of cells to attach to the substrate. Moreover, we also verified that some of these adhesion defects could be reverted using the microtubule-binding domain of EB3. To clarify these differences in behaviour between EB1 and EB3, we proceeded to measure the dynamics of individual interphase microtubules in control, EB1- or EB3-depleted cells using a HeLa GFP-tubulin cell line (Table I). Analysis of dynamic instability parameters showed that both EB1- and EB3-depleted cells have higher growth and shortening rates when compared to control cells (Table I). However, EB3-depletion induced a higher number of rescue events (transitions from shortening to growth) and catastrophe events (transitions from growth to shortening) than control or EB1-depleted cells. As expected, microtubules from EB1-depleted cells presented less rescue events than control cells. As a consequence, microtubules in EB3-depleted cells spent more time in growth and less time in pause than microtubules in control cells.

	Control RNAi	EB1 RNAi	EB3 RNAi
Rate of growth, μm/min	9.2 ± 3.0	**12.99 ± 8.5**	**11.5 ± 3.9**
Rate of shortening, μm/min	-18.9 ± 5.9	**-22.9 ± 6.1**	**-25.5 ± 6.2**
Transition frequencies			
Growth-Shortening, s^{-1}	0.0096	0.0071	**0.0240**
Growth-Pause, s^{-1}	0.0093	**0.0123**	**0.0137**
Shortening-Growth, s^{-1}	0.0115	**0.0075**	**0.0264**
Shortening-Pause, s^{-1}	0.0077	**0.0113**	0.0107
Pause-Growth, s^{-1}	0.0069	**0.0115**	**0.0106**
Pause-Shortening, s^{-1}	0.0101	0.0121	**0.0143**
Time in Growth, %	21.0	18.7	**38.1**
Time in Pause, %	68.1	72.8	**48.5**
Time in Shortening, %	10.8	8.1	12.9

Table I – Quantification of microtubule dynamic instability parameters. Individual microtubules were imaged and parameters of dynamic instability were obtained for the different experimental groups. Results in bold highlight significant differences ($p<0.001$) using a nonparametric ANOVA (Kruskal-Wallis) followed by a post-hoc Dunn`s test.

This is also in contrast to the behaviour observed in microtubules from EB1-depleted cells. Overall, the changes in dynamic instability parameters induced by EB3 depletion indicate that, under these conditions, these microtubules are less stable which may impact on their ability to interact with the adhesion complexes and helps clarify the differences observed between individual EB depletions. The role of microtubules in cell adhesion mechanisms has been addressed in a number of previous studies. Notably, targeting of focal adhesions by microtubules promotes their disassembly and this event requires an intact microtubule array (Ezratty et al., 2005; Kaverina et al., 1999). Furthermore, microtubule plus-ends have been shown to be attached to the basal cortex of the cells by interaction with LL5s (Hotta et al., 2010). We have shown that EB3 is important to regulate microtubule dynamic instability. Higher catastrophe and rescue frequencies that occur in EB3-depleted cells may lead to an increased targeting of focal adhesions by microtubules. This could, in turn, induce an increased turnover of adhesion complexes at the membrane and prevent focal adhesion stabilization following mitotic exit. Curiously, EB1 has an antagonistic effect on cell adhesion and motility

when compared to EB3. It is interesting to note that lack of EB1 leads to microtubules spending more time in the "paused" state and having less rescue events which could diminish the ability of these microtubules to target adhesion complexes, thus decreasing adhesion turnover.

EB proteins are involved in the stability of focal adhesions

Microtubules are important in the regulation of cell attachment to the substrate. In light of our previous results we were interested in determining whether EB proteins would be involved in the regulation of focal adhesion formation or stabilization. For that purpose, we performed spinning-disk confocal microscopy of HeLa cells expressing FAK-GFP. In compliance with the previous observations, we confirmed that EB3 depletion interfered with the stabilization of focal adhesions (FAs, Figure 4A-C). This rapid turnover of FAs was notorious during cell migration (Figure 4A, bottom left panel and 4C) and correlated with a significant decrease in FA persistency time of EB3-depleted cells when compared to control cells ($p<0.001$; Figure 4A and 4C). These highly dynamic structures were positive for pFAK (Y397), indicating that they were able to transmit integrin signals to the cytoplasm (Figure 4B). These results confirm that lack of EB3 leads to a loss of FA stabilization, possibly through an increased targeting of these structures by highly dynamics microtubules. Curiously, EB1-depleted cells had higher persistency times for FAs when compared to controls ($p<0.001$; Figure 4A-C), which accounts for the increased stability of adhesion and reduction in the distance travelled by these cells. In fact, these cells failed to disassemble FAs, leading to a growth of these structures (Figure 4C). This can be attributed to the more stable microtubules which are present upon EB1 depletion. These microtubules would not be able to target FAs which would lead to increased stabilization of these structures. On the other hand, we cannot exclude that EB1 itself would be necessary for the delivery of a putative "relaxing factor" to FAs, as has been suggested previously (Palazzo and Gundersen, 2002). In this situation, absence of EB1 would prevent disassembly of focal adhesions indirectly. However, given that we could revert the migration defects by re-introducing the microtubule binding domain of EB3 points towards EB-mediated microtubule dynamics as the main regulator of adhesion and migration. To further confirm this, we decided to take advantage of two mutant versions of EB3 that have a point mutation at serine 176 (EB3-S176A and EB3-S176D; Figure 4D).

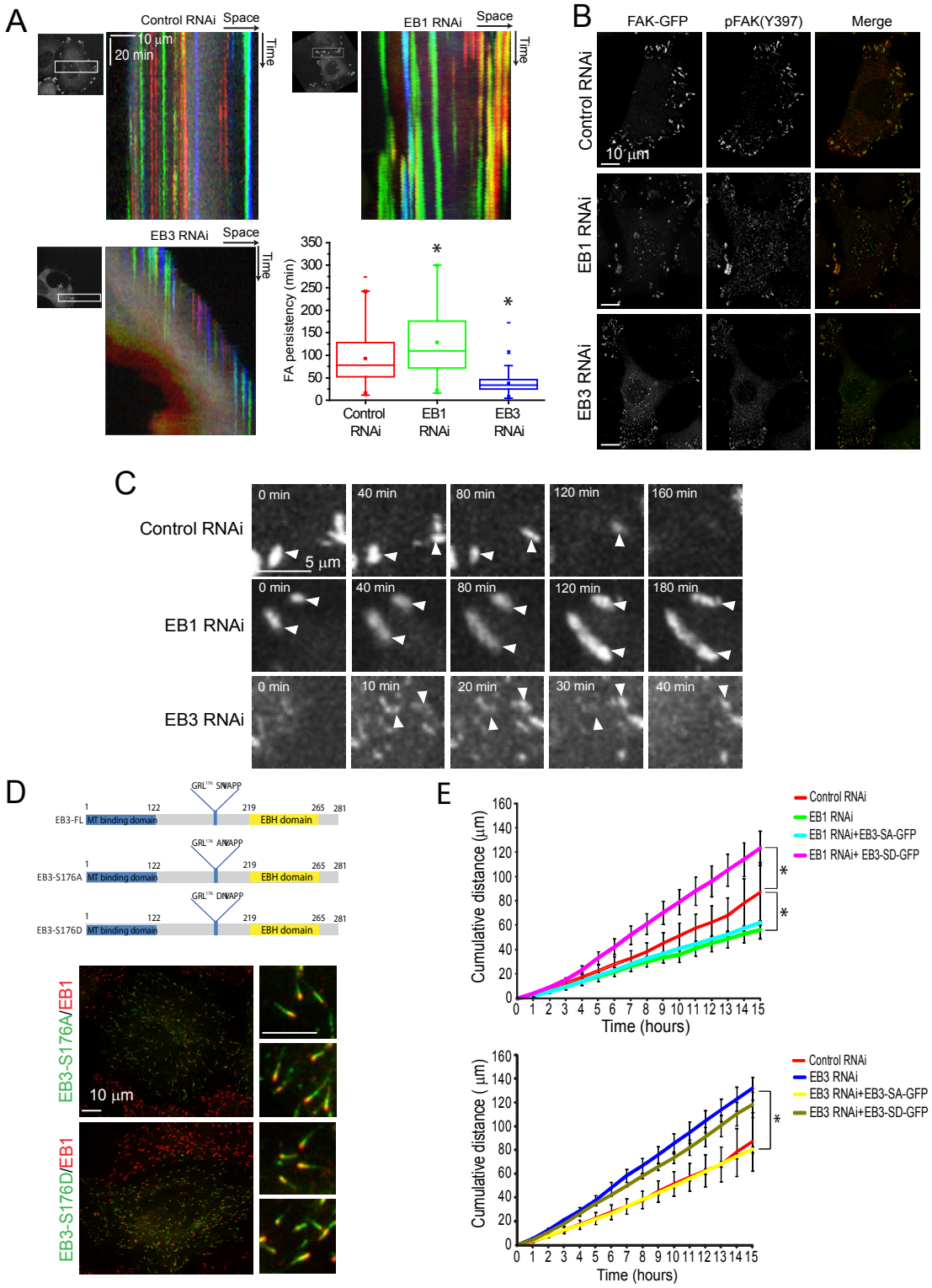

Figure 4 – Effect of EB proteins on focal adhesion stabilization. (A) Quantification of focal adhesion (FA) stability. FA stability is analysed using a chromo-kymographic approach. (B) FAK-GFP structures are positive for pFAK(Y397) even in EB3-depleted cells. (C) Focal adhesions are hyper-stabilized in EB1 RNAi and are destabilized in EB3 RNAi. (D) EB3-S176A-GFP and EB3-S176D-GFP still localize to the plus-end of microtubules. (E) Quantification of cell motility of the different EB3 mutants. Note that EB3-S176A-GFP is able to rescue the motility phenotype whereas the EB3-S176D-GFP cannot.

We have previously shown that expression of EB3-S176D-GFP upon mitotic exit is sufficient to induce changes in microtubule dynamics leading to uncoordinated post-mitotic cell spreading (Ferreira, Pereira, Akhmanova and Maiato, submitted manuscript). This mutant form of EB3 is very useful because it still has an intact C-terminal domain which may still interact with specific cargo, but induces significant alterations in microtubule dynamics. As was expected, expression of either EB3-S176A-GFP or EB3-S176D-GFP did not change their ability to interact with and accumulate at microtubule plus-ends (Figure 4D). Interestingly, expression of EB3-S176D was not sufficient to restore motility to control values (Figure 4E). On the other hand, introducing the EB3-S176A was enough the restore cell motility. This is in accordance with our observations that the S176A mutant restores normal interphase microtubule dynamics, whereas the S176D mutant does not (Ferreira, Akhmanova and Maiato, manuscript under review). In addition, the EB3-S176D mutant can act as a dominant negative, given that its expression in an EB1-depleted background significantly increases the motility of these cells (Figure 4E). This happens because expression of EB3-S176D in control cells is sufficient to displace endogenous EB1 from the plus-ends (our unpublished observation).

Overall our results show that EB proteins differentially regulate cell motility and adhesion through their ability to control microtubule dynamics. In this context, lack of EB3 (or presence of EB1 only) induces a higher dynamic instability leading to increased FA turnover and cell motility. On the other hand, lack of EB1 (or presence of EB3 only) induces more static microtubules, promoting FA stabilization and decreased cell motility.

VI. PHOSPHOREGULATION OF EB PROTEIN ASSOCIATION TO MICROTUBULE PLUS-ENDS

1 - Introduction:

Microtubule plus-end accumulation is the defining feature of a +TIP. The first evidence of tip-tracking behaviour came from studies using GFP tagged CLIP170 (Perez et al., 1999). Many other proteins were later described to have the same dynamic localization on microtubules. In order to accumulate on the distal tip of the microtubule, proteins can use several mechanisms. These include direct end binding to the microtubule tip, copolymerization with tubulin dimers or oligomers, directed transport with a kinesin motor and hitchhiking by attaching to another +TIP (Akhmanova and Steinmetz, 2008). Although the loading mechanisms of many +TIPs have already been described, it is still not completely known how EB protein association to microtubule ends is regulated. This is of utmost importance when we take into account the fact that most +TIPs that use the hitchhiking mechanism, do so by binding to EBs. In accordance, CLIPs, CLASPs and APC all accumulate at the distal part of microtubules in an EB-dependent manner (Lansbergen and Akhmanova, 2006).

Several lines of evidence have accumulated to explain how EB proteins might associate with the microtubule plus-ends. It was recently suggested that EBs might recognize the GTP cap. In fact, it is possible to mimic the EB-binding site by using GTPγS (a slowly hydrolysable form of GTP) (Maurer et al., 2011). This was also shown when a different analogue is used. Under these conditions, EB1 recognizes the GMPCPP microtubule lattice as opposed to the GDP lattice (Zanic et al., 2009). In fact, EB proteins might be able to recognize the nucleotide state of the microtubule end and this is required both for EB binding but also for microtubule stabilization (Maurer et al., 2012). One other interesting hypothesis involves electrostatic repulsive interactions between the C-terminal domain of EB1 and the microtubule lattice (Buey et al., 2011). Under these conditions, the negatively charged C-terminal domain would create a repulsive interaction with the microtubule lattice, forcing the protein to accumulate specifically at the plus-end. Finally, post-translational modifications such as phosphorylations might also be involved. In accordance, a mutation on the linker region of Mal3 (the fission yeast homologue of EB1) is sufficient to abolish interaction of the +TIP with the plus-end (Iimori et al., 2012). Moreover, phosphorylation of Bim1p (the budding yeast homologue of EB1) by Aurora/Ipl1p is sufficient to remove the +TIP from static and dynamic microtubules (Zimniak et al., 2009). This phosphorylation occurs during anaphase in vivo and is reported to be important for normal spindle elongation and disassembly of the spindle midzone. Interestingly, in human cells, Aurora B does not phosphorylate EB1, but its activity requires

interaction with the +TIP (Sun et al., 2008). EB3, on the other hand, is a substrate of Aurora kinases (Ban et al., 2009).

In this chapter, we provide evidence for a phospho-regulatory mechanism that controls EB association to the microtubule plus-end and explore how this is regulated during the cell cycle. In addition, we show that heterodimerization is required for phospho-regulated association of EBs to the microtubule tip.

2 - Materials and Methods

Cell culture, reagents and antibodies

For a detailed description of all reagents used, please refer to Chapter III, Section 2 (page 113).

Time lapse microscopy

For a detailed description please refer to Chapter III, Section 2 (114).

Drug treatments

The broad PP1 and PP2 phosphatase inhibitor Okadaic Acid (OA) was used at the indicated concentrations and for the indicated times. Aurora A inhibitor (MLN8054) was used at a concentration of 250 nM for 1h. Aurora B inhibitor (ZM447439) was used at a concentration of 2 μM for 1h. Wherever indicated, MG132 was used at a concentration of 5 μM for 2h. Nocodazole was used at a concentration of 1 μM to induce microtubule depolymerization for the times indicated. All drugs were dissolved in DMSO. Appropriate controls were performed by incubating the cells with an equivalent amount of solvent.

Quantification of comet lengths

Individual EB1 or EB3 comets were quantified using the line measurement tool in Autoquant X2.1.3. In brief, immunofluorescence images of EB1 and EB3 were acquired for interphase or mitotic cells and the individual comet fluorescence intensity was measured. An exponential curve fitting was then applied to the obtained intensity curve to determine comet length. When either EB1 or EB3 were depleted by RNAi, the comet length for the remaining EB was determined in the same manner.

Tracking of comet trajectories and life-time

Individual comets were imaged by using either full length EB3-GFP or EB1-GFP in the presence of DMSO or Okadaic acid 100nM. In addition, the point mutants EB3-S176A and EB3-S176D were used in the same experimental conditions. Movies with a time lapse of 2 sec were

acquired and individual comets were manually tracked using the “Manual Tracking” plugin of ImageJ. Come Comets were followed continuously until the fluorescent signal disappeared and did not appear in the following frame after that.

Statistical analyses

For a detailed description on statistical tests used to analyse all the experimental groups please refer to Chapter III, Section 2 (page 117).

3 - Results

Inhibition of phosphatases induces cell-cycle specific plus-end accumulation of EB proteins

It has been shown previously that yeast EB1 association to the microtubule plus-ends is regulated by phosphorylation (Iimori et al., 2012; Zimniak et al., 2009). In fact, phosphorylation of EB1 negatively regulates its association to the plus-ends. Moreover, human EB3 was shown to be phosphorylated at serine 176 and this increased the stability of the protein during mitosis (Ban et al., 2009). Here, we set out to test whether mammalian EB proteins could be regulated by phosphorylation and if so, would they be temporally regulated during the cell cycle. We started by measuring EB1 and EB3 comet size in interphase and mitosis (Figure 1).

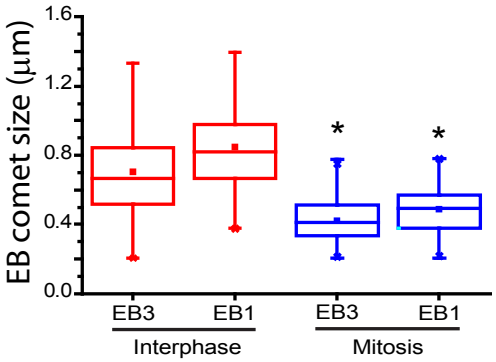

Figure 1 – EB comet size during interphase and mitosis. EB1 and EB3 comets were visualized using immunofluorescent images and comet size was quantified. Note the significant decrease in mitotic EB1 and EB3 comet size when compared to the respective interphase comets. * $p<0.001$ when using a parametric t-test.

As can be seen, there is a significant difference in comet size from interphase to mitosis (Figure 1). These results suggest a difference in EB association to the plus-ends between interphase and mitosis that could reflect either a difference in the structure of the microtubule itself or in the affinity of EBs. To elucidate this, we treated human HeLa cells with Okadaic Acid (OA), a broad inhibitor of PP1 and PP2A phosphatases. We decided to use a low dose OA treatment (10nM), which is reported to inhibit PP2A but not PP1 and a high dose OA treatment (100nM), which should inhibit both types of phosphatases. We reasoned that by blocking phosphatase activity, EB proteins would remain in their phosphorylated state. Should this lead to alterations in plus-end binding, we could detect it by immunofluorescence detection with antibodies against the endogenous protein. Analysis of EB1 and EB3 comet length in metaphase cells allowed us to conclude that phosphatase inhibition does not impact EB accumulation during mitosis (Figure2A and 2B). However, in interphase cells we could detect a significant increase in the comet size upon okadaic acid treatment (Figure 2A and 2B).

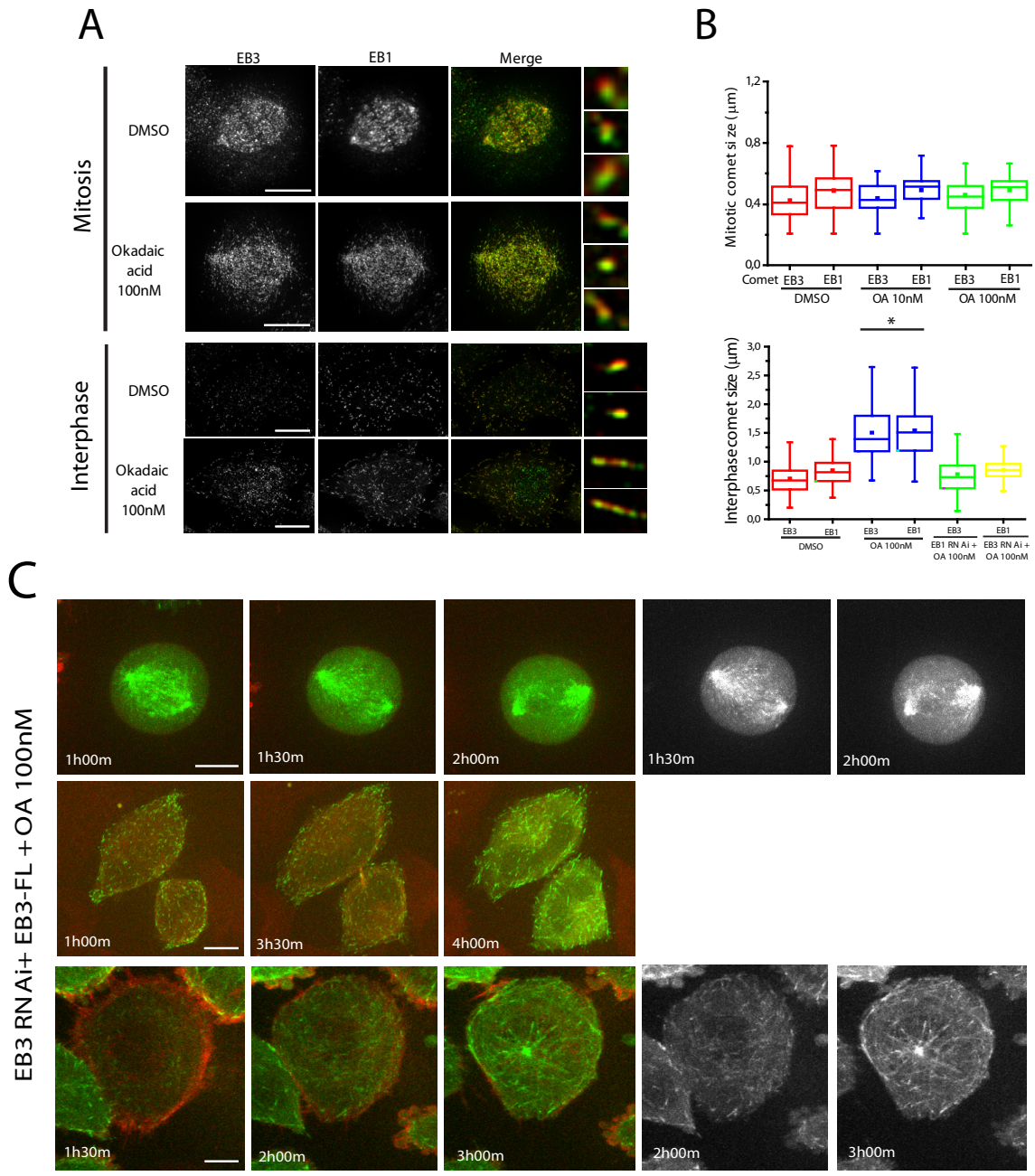

Figure 2 – Accumulation of EB proteins on microtubule plus-ends is cell cycle specific. (A) HeLa S3 cells were seeded on FBN coverslips, treated with OA and fixed for immunofluorescence. (B) EB1 and EB3 comets were measured after DMSO or OA treatment in mitotic or interphase cells. Treatment with OA induces a significant accumulation of EB1 and EB3 only in interphase cells. This accumulation is only seen when both proteins are present at the plus-end. (C) Cells expressing EB3-GFP/Lifeact-mCherry are treated with OA to check for the accumulation at the plus-ends. This accumulation occurs during mitotic exit and interphase but not in mitotic cells. Time lapse is 2 min. Time is in hours:min. Scale bars, 10 µm.

Interestingly, this accumulation no longer occurred if only EB1 or EB3 were present. When either EB1 or EB3 were depleted and phosphatases were inhibited, the interphase-specific accumulation was abolished (Figure 2A and 2B). This is a surprising result that suggests the necessity of EB1/EB3 heterodimer formation for the regulation of EB association to the plus-ends. We then decided to extend our analysis of EB accumulation by repeating our OA treatment in cells expressing full length EB3 tagged with GFP (Figure 2C). In accordance with our previous results, mitotic cells did not display any marked increase in EB3 accumulation at microtubule plus-ends (Figure 2C). It should be noted that OA treatment *per se* does induce some structural changes in the spindle (Figure 2C; top panel) as was reported before (de Pennart et al., 1993; Gliksman et al., 1992; Vandre and Wills, 1992). Normal microtubule plus-end accumulation of EB3 is still observable under these conditions, although there is no increased binding of EB3 to the microtubules. It is not surprising that OA treatment induces changes in spindle structure. In fact, it has been shown that specific inhibition of PP1 or PP2A phosphatases induces different effects on microtubule dynamics (Tournebize et al., 1997). Whereas type 1 phosphatases control microtubule dynamics into and out of mitosis, type 2A phosphatases are required to maintain the steady-state length of microtubules in mitosis. Nevertheless, in our OA experiments, mitotic EB3 comet size or behaviour did not change upon addition of the drug. On the contrary, in post-mitotic and interphase cells adding OA induced a pronounced accumulation of EB3 in the microtubule plus-ends (Figure 2C). The accumulation could be seen as soon as mitotic telophase cells began to spread onto the substrate. The fact that we can induce an increase in the post-mitotic comet size after OA treatment suggests that dephosphorylation of EBs should occur during mitotic exit to ensure normal binding to the plus-end. Interestingly, we could reproduce this same behaviour when we express a form of EB3 that contains a phospho-mimicking mutation on serine 176.

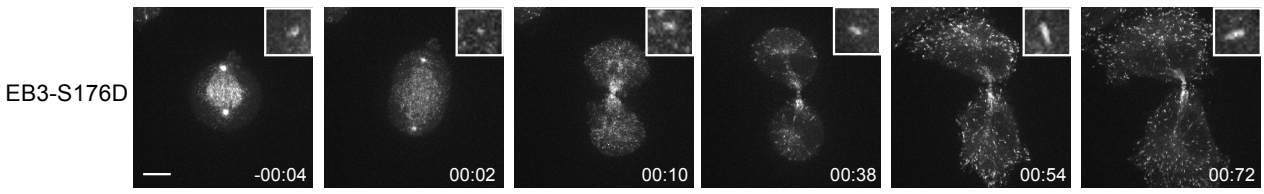

Figure 3 – Cell expressing EB3-S176D-GFP during mitosis. A cell expressing EB3-S176D-GFP (but still with endogenous EB3) was imaged by spinning disk microscopy throughout mitosis. Zero minutes corresponds to anaphase onset. High magnification insets represent an EB3 comet from the respective time frame. Notice the increase in comet size after furrow ingression. Scale bar, 10 μm. Time-lapse is two minutes. Time is in hours:min.

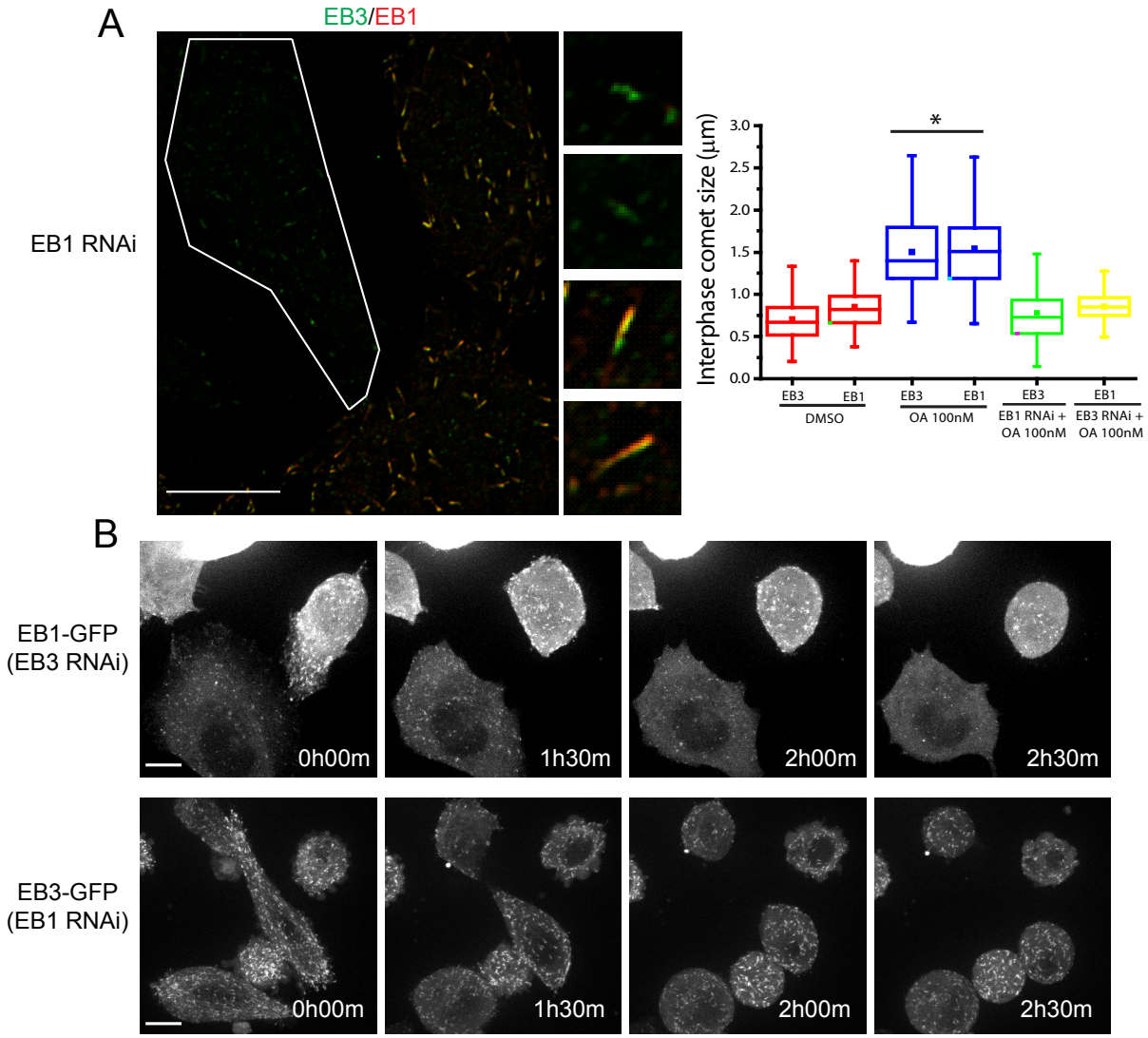

Figure 4 – Accumulation of EB proteins at the microtubule plus-ends requires heterodimerization. (A) EB3 comets in an EB1-depleted cell (white outline) and EB1 and EB3 comets in non-depleted cells treated with OA 100 nM. Depletion of either EB no longer leads to EB accumulation after OA treatment. (B) Live cell imaging of EB1-GFP expressing cells in the absence of EB3 (top panel) and EB3-GFP expressing cells in the absence of EB1 (bottom panel). Cells were treated with OA 100 nM for the time indicated. Note in the EB1-GFP panel that EB1-decorated comets decrease after OA treatment when EB3 is depleted. Scale bar, 10 µm. Time lapse is two minutes. Time is in hours:min.

This phosphorylation was previously reported to occur during mitosis (Ban et al., 2009) and we have demonstrated that dephosphorylation must occur to allow correct attachment of post-mitotic cells to the substrate (Chapter III). In fact, cells that express the EB3-S176D mutant show an increase in EB3 labelling of the plus-ends upon mitotic exit and spreading, suggesting that this process must be tightly regulated (Figure 3). Similarly to what was observed for the

endogenous proteins, this increased accumulation was lost when one of the EB proteins was depleted by RNAi (Figure 4A and 4B). In fact, some cells even showed complete displacement of EB1 or EB3 from the plus-end upon OA treatment. Taken together, these results suggest a phospho-regulatory mechanism of EB association to the plus-end that is active in the transitions between mitosis and interphase.

Towards the identification of which kinase(s) control(s) EB association to the microtubule plus-ends during mitosis

If EB protein association to the plus-end is regulated by phosphorylation in a cell-cycle dependent manner, then we postulated that one or more of the mitotic kinases might be responsible to trigger this event. We decided to begin by analysing EB protein primary sequence for potential phosphorylation sites that might have an overlap with previously reported (Figure 5). In order to do so, we obtained the aminoacid sequences of EB1 and EB3 and aligned them in Jalview2 to maximize residue conservation.

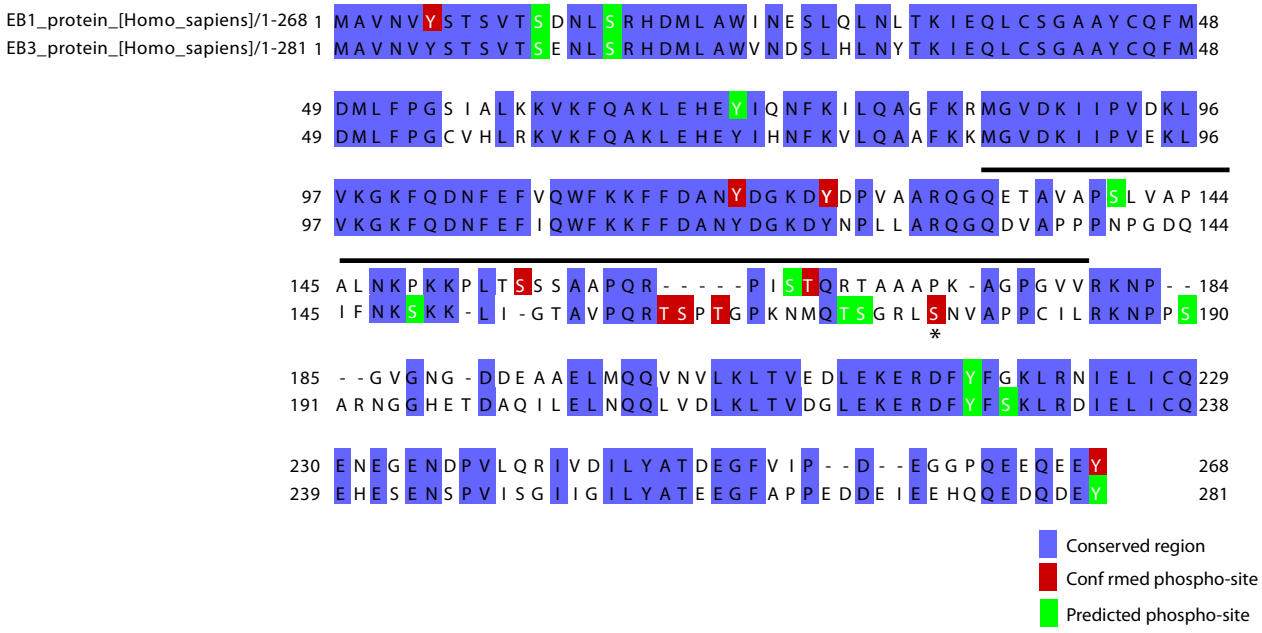

Figure 5 – Sequence alignment and phospho-site prediction for EB1 and EB3. Aminoacid sequence of human EB1 and EB3 were aligned to maximize residue conservation. These sequences were then introduced in several phosphorylation prediction algorithms. All residues that came out positive in the algorithms are highlighted in the sequence. Blue denotes a conserved residue, red denotes a confirmed phospho-site already described in the literature and green denotes a predicted phospho-site. Note the cluster of phospho-sites that accumulates in the linker region (black bar), especially in EB3. * marks the residue that was confirmed in our studies.

The sequences were then introduced in NetPhos 2.0 Server and PhosphoSitePlus to retrieve information on predicted phospho-sites (Figure 5). Unlike the yeast homologue, human EB1 does not have a cluster of phosphorylation sites in the linker region (black bar). Interestingly, EB3 has a high number of putative phosphorylation sites in the linker region, one of which (* S176; Figure 5) has already been described as a target of Aurora kinases (Ban et al., 2009) and was further studied during the course of this thesis. In addition, it should be noted that the linker region also contains an SP site at residue 162, which is a putative CDK1 consensus site. In light of these results, we sought to determine which kinase(s) could be responsible for EB phosphorylation.

We first turned our attention to Aurora kinases because they were recently identified as being responsible for EB3 phosphorylation during mitosis (Ban et al., 2009). Moreover, Ipl1p/Aurora kinase is also responsible for phosphorylation of the linker region of yeast Bim1p/EB1 and this also regulates EB1 binding to microtubules (Zimniak et al., 2009), although in human cells EB1 does not seem to be a target of Aurora B (Sun et al., 2008). For this purpose we treated cells expressing EB3-GFP with Aurora A, Aurora B or Aurora A+B inhibitors to determine whether Aurora inhibition would lead to loss of tip-tracking behaviour (Figure 6). Inhibition of Aurora A has been reported to lead to deficiencies in chromosome alignment and delays in mitosis (Hegarat et al., 2011; Hoar et al., 2007; Scutt et al., 2009) without perturbing its completion. We treated HeLa cells expressing EB3-GFP with 0.25 μM of the Aurora A inhibitor MLN8054 (Manfredi et al., 2007) and filmed them when they were in mitosis. In our experiments, we did not observe any significant impact on the overall spindle structure. Cells normally formed a bipolar spindle with no apparent alterations in the ability of EB3 to associate to the microtubule ends. When the same cell line is treated with 2 μM of the Aurora B inhibitor (ZM447439)(Ditchfield et al., 2003), two very distinct phenotypes can be observed. If the inhibitor is added very early during mitosis (i.e. prophase/prometaphase), about 64% of the cells show spindle collapse and no apparent EB3 comets are observed, apart from a very bright centrosome cluster (Figure 7A and B). This does not mean that Aurora B inhibition disassembles the spindle by displacing EB3 from the plus ends but it does suggest that Aurora B is required for normal microtubule dynamics during early mitosis. Addition of the inhibitor later during mitosis (upon anaphase onset) allows progression through mitosis, but these cells ultimately fail cytokinesis (see Chapter III). The cytokinesis failure phenotype is consistent with previous reports on the role of Aurora B during late mitotic events, which include the production of a phosphorylation gradient and the interaction with essential components of the centralspindlin complex such as MKLP1 (Fuller et al., 2008; Guse et al., 2005).

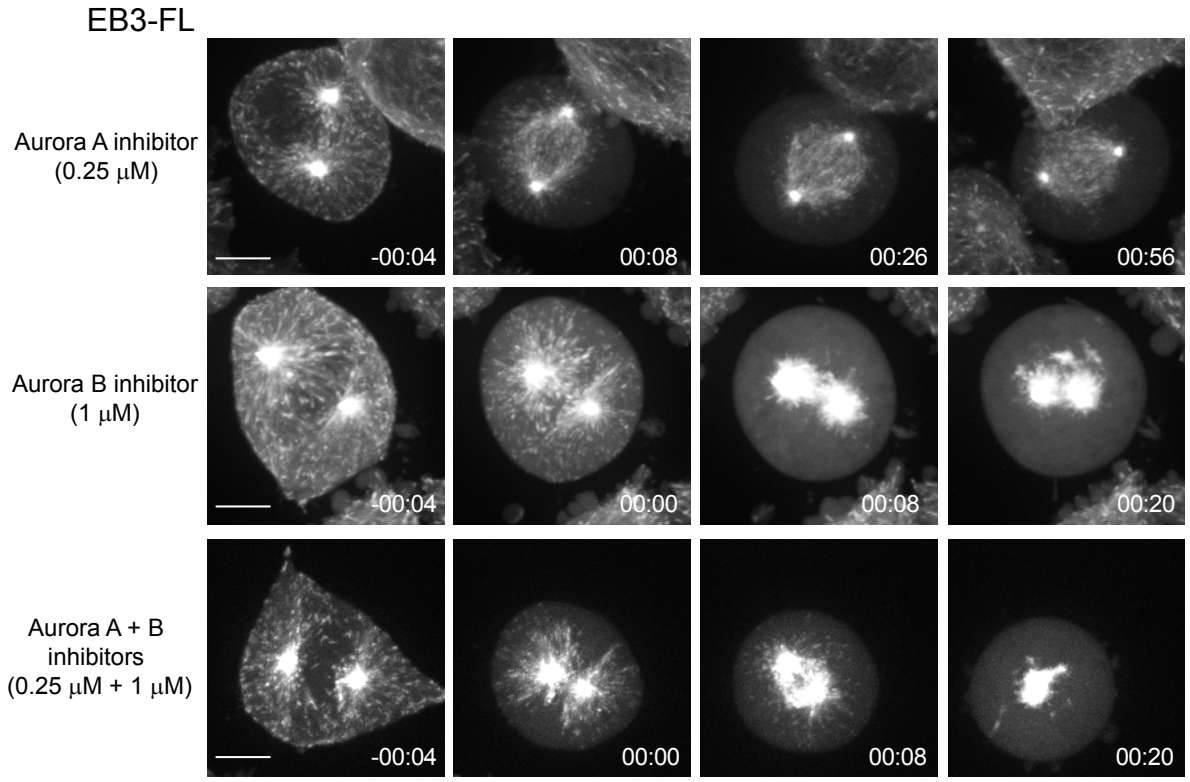

Figure 6 – Aurora B inhibition during early mitosis leads to disassembly of spindle microtubules. Cells were treated with Aurora A inhibitor (MLN8054), Aurora B inhibitor (ZM447439) or both at the same time during mitosis. Treatment of cells with Aurora B (but not Aurora A) inhibitor leads to disassembly of spindle microtubules. Scale bar, 10 μm. Time lapse is 2 min. Time is in hours:min. Zero minutes corresponds to NEB.

Inhibition of both Aurora A and Aurora B simultaneously leads to a phenotype similar to Aurora B inhibition only (Figure 6). Overall, these results indicate that Aurora B is required for regulating microtubule dynamics during early mitosis, which allows the formation of a stable bipolar spindle. Interestingly, this change in microtubule dynamics leads to displacement of EB3 from microtubule plus-ends. It remains to be determined whether Aurora B directly regulates association of EB3 to microtubule tips.

At this point, we became interested in determining whether Aurora B directly controls microtubule dynamics by phosphorylating EB3. It has been previously shown that both Aurora A and Aurora B interact with EB3 and are required for its mitotic phosphorylation at Serine 176 (Ban et al., 2009).

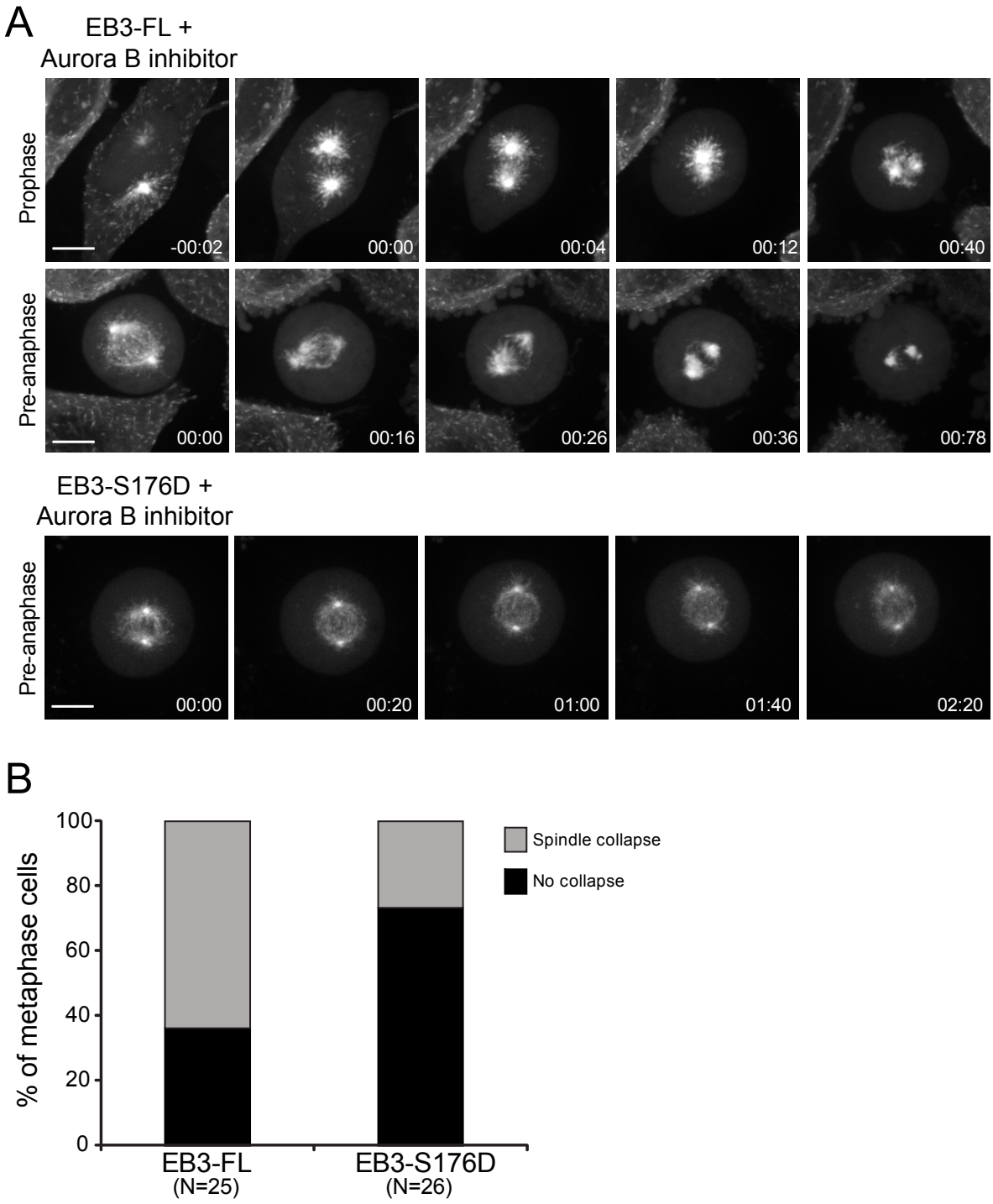

Figure 7 – Aurora B inhibition during early mitosis induces spindle collapse. (A) Cells were treated with Aurora B inhibitor (ZM447439) during early mitosis. (B) Quantification of spindle collapse upon Aurora B inhibition in cells expressing either EB3-FL-GFP or EB3-S176D-GFP. Expression of the mutant EB3-S176D-GFP together with Aurora B inhibition significantly rescues the phenotype. Scale bars, 10 μm. Time-lapse is 2 minutes. Time is in hours:min.

If this is the case, mimicking EB3 phosphorylation by expressing the EB3-S176D mutant in an Aurora B inhibition situation should revert the phenotype and allow the formation of a bipolar spindle.To do this, we depleted endogenous EB3 by RNAi and expressed the EB3-S176D-GFP mutant in HeLa cells. These were then treated with Aurora A, Aurora B or Aurora A + Aurora B inhibitor. As expected, treatment with Aurora A inhibitor alone did not produce any significant changes in EB3 tip-tracking or spindle structure (data not shown). Interestingly, when EB3-S176D-GFP expressing cells were treated with Aurora B inhibitor (or with Aurora A + Aurora B inhibitors), they managed to maintain a relatively stable spindle structure for a long period of time (96±15 min as opposed to 33±11 min in EB3-FL expressing cells) and approximately 65% of EB3-S176D cells even entered anaphase (as opposed to only 36% of the cells expressing EB3-FL; Figure 7A and B). This indicates that mimicking phosphorylation of EB3 on serine 176 is able to partially revert the process but does not completely rescue it, suggesting that either additional residues on EB3 must also be phosphorylated by Aurora B or some other yet unknown factor may also play a role.

We then proceeded to assess whether microtubules were still able to nucleate after Aurora inhibition. For that purpose, we treated cells expressing either the full length EB3 (EB3-FL), the constitutively phosphorylated EB3 (EB3-S176D) or the phospho-null EB3 (EB3-S176A) with the Aurora inhibitors for 1 hour. Following that, mitotic cells were filmed with a time-lapse of 2 seconds in order to observe microtubule nucleation events (Figure 8). Maximal projections were then generated to visualize microtubule growth tracks (Figure 8; “Maximal projection”). When cells were treated with DMSO, we observed a high number of EB3 comets which nucleate from the centrosomes towards the spindle and the cell cortex. When Aurora A was inhibited in cells expressing EB3-FL, comets were still visible but the overall density is lower. This is in agreement with a previous report that showed a defect in spindle microtubule dynamics upon Aurora A inhibition (Hegarat et al., 2011). Nevertheless, Aurora A inhibited cells could still nucleate microtubules to a high extent (Figure 8). Inversely, treatment with Aurora B inhibitor abolished the appearance of EB3 comets almost completely. This was similar to what was observed for the simultaneous inhibition of Aurora A and Aurora B and reflects the functional difference between both kinases.

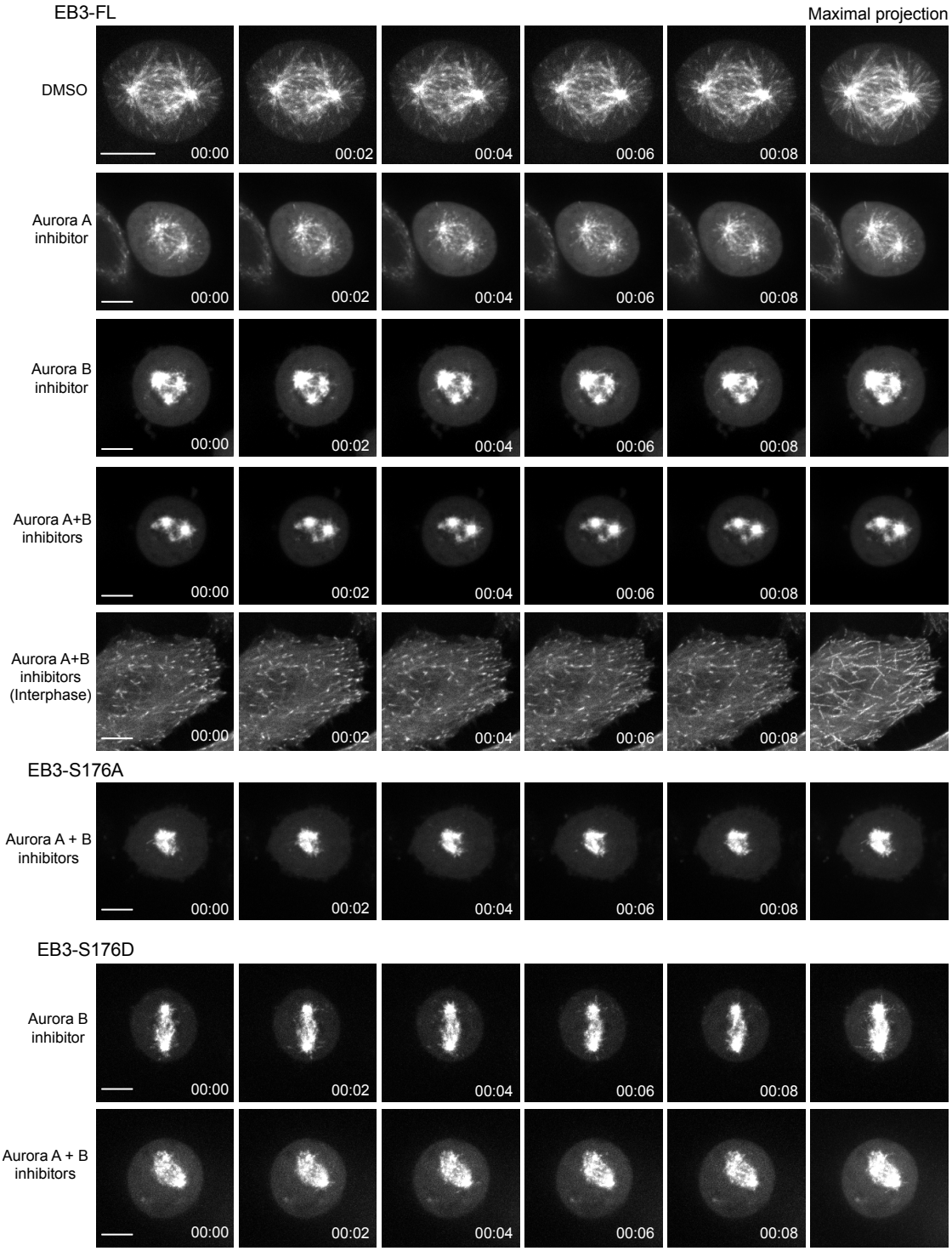

Figure 8 – Microtubule nucleation upon Aurora inhibition. Cells expressing EB3-FL, EB3-S176A or EB3-S176D were treated with Aurora A inhibitor (MLN8054), Aurora B inhibitor (ZM447439) or both at the same time for 1 hour. Following that period, cells were filmed with a time-lapse of 2 seconds. Maximal projection of 5 consecutive frames is represented on the right panels. Note that Aurora A inhibition does not affect microtubule growth capacity. Inhibition of Aurora B blocks microtubule nucleation capacity which can be partially rescued by expression of EB3-S176D. Interphase cells are not affected by Aurora A or Aurora B inhibition. Scale bars, 10 μm. Time lapse is 2 min. Time is in min:sec.

Strikingly, kinase inhibition did not affect microtubule growth in interphase cells to a great extent, as they exhibited apparently normal EB3 tracks. Expression of the EB3-S176A mutant was not able to rescue the effects of Aurora A and Aurora B inhibition. In fact, no tracks were distinguishable after inhibition of the kinases, indicating that this phosphorylation is indeed required to maintain microtubule growth. On the contrary, when Aurora B inhibition was applied to cells expressing EB3-S176D, the overall spindle structure was similar to control cells and some EB3 comets could still be observed (Figure 8). However, the number of EB3 comets was clearly reduced when compared to controls. As expected simultaneous inhibition of Aurora A and Aurora B in cells expressing EB3-S176D resembled the single Aurora B inhibition (Figure 8). Again, it must be noted that prolonged incubation with the drug eventually led to spindle disassembly and complete loss of EB3 tracks. Taken together, our observations demonstrate that phosphorylation of EB3 at serine 176 is required to maintain a stable bipolar spindle structure, but probably other residues must also be phosphorylated by Aurora B in order to maintain normal microtubule nucleation. In this context, it will be interesting to determine whether the other residues in the linker region that were predicted to be phosphorylated have any effect on EB tip tracking behaviour (Figure 5).

Inhibition of phosphatases or imposing EB3 phosphorylation increases EB3 affinity for the plus-end and affects microtubule dynamics

Given the observed changes in EB association to the microtubules upon kinase/phosphatase inhibition, we set out to determine whether this would have an impact on overall microtubule dynamic behaviour. For that purpose, we filmed EB3-GFP cells incubated with or without 100 nM OA for 2h, after which we added 1 μM nocodazole. In control DMSO-treated cells, disappearance of EB3 comets was almost immediate upon nocodazole addition (2 min; Figure 8A and 8B). In addition, a set of stable EB3-labelled microtubules was evident which remained unchanged for the rest of the movie (Figure 9A). Inversely, when cells were pre-incubated with OA 100nM, nocodazole-induced microtubule depolymerization was significantly delayed (8 min; Figure 9A and 9B). In this situation, EB3 comets could still be observed throughout the entire cell and the population of stable microtubules that was visible in DMSO-treated cells was no longer present (Figure 9A). Overall this indicates that phosphatase inhibition increases

the affinity of EB3 for the microtubule tip and, as a consequence, microtubules are more dynamic.

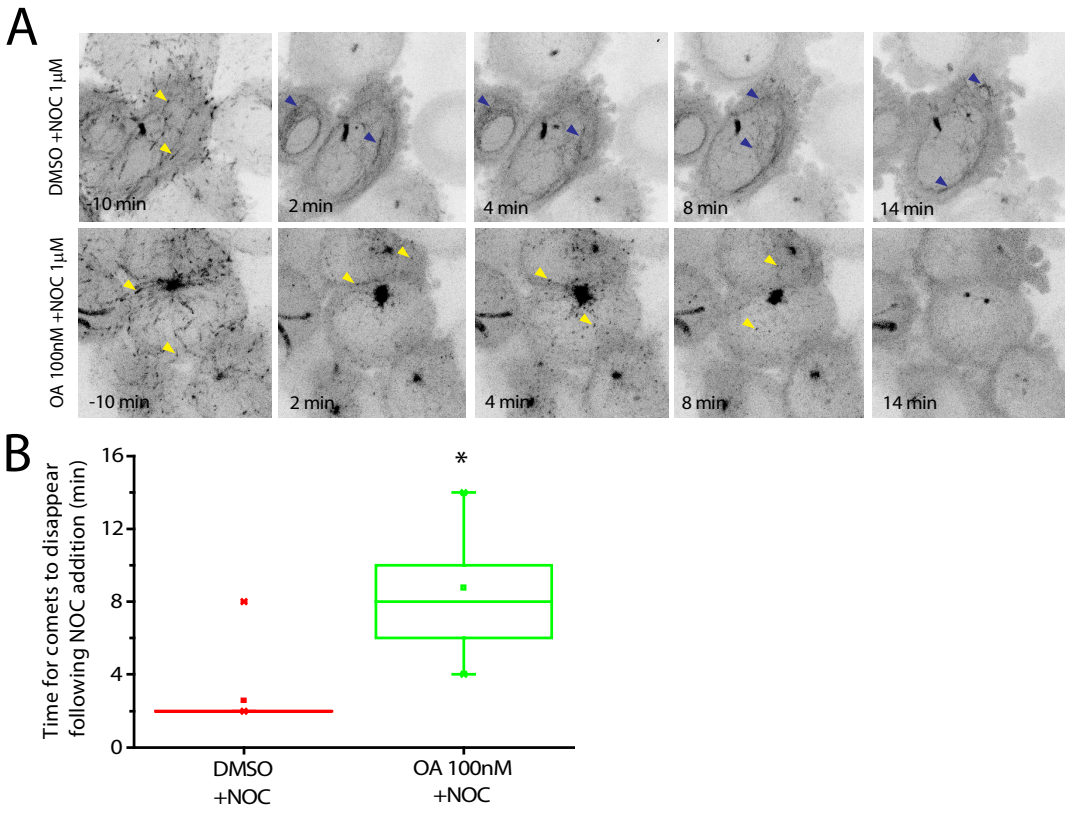

Figure 9 – Treatment with OA increases the affinity for the plus-ends. (A) Cells expressing EB3-GFP are treated with OA 100 nM for 2 hours followed by Nocodazole 1 µM for the time indicated. (B) Comets (yellow arrowheads) are tracked and the time it takes for them to disappear is quantified. Note the stable, nocodazole resistant microtubules in DMSO treated (blue arrowheads), but not OA treated cells. Time lapse is 2 minutes. Time is in min.

If microtubules are indeed more dynamic, then this should be observable by analysing individual EB3 comet behaviour. For that purpose we quantified comet growth time, distance and velocity (Figure 10). As was expected, treatment with 100 nM OA induced an increase in both comet distance and travelled time when compared to control cells (Figure 10A and 10B). When the same treatment was applied to cells expressing the EB3-S176A mutant, comets travelled shorter times but over similar distances to the OA treatment (Figure 10A and 10B). This suggests that inhibiting phosphorylation of EB3 may lead to a decreased binding of EB3 to the plus-end and would explain the shorter tracks observed upon EB3-S176A expression. On the other hand, when we express the EB3-S176D mutant, comets moved for longer distances

than controls but not as long as the 100nM OA treatment with “normal” EB3. Overall this indicates that phosphorylation at the S176 site regulates the binding time of EB3 to the microtubule tip but that other phosphorylation sites must also be involved in tip binding.

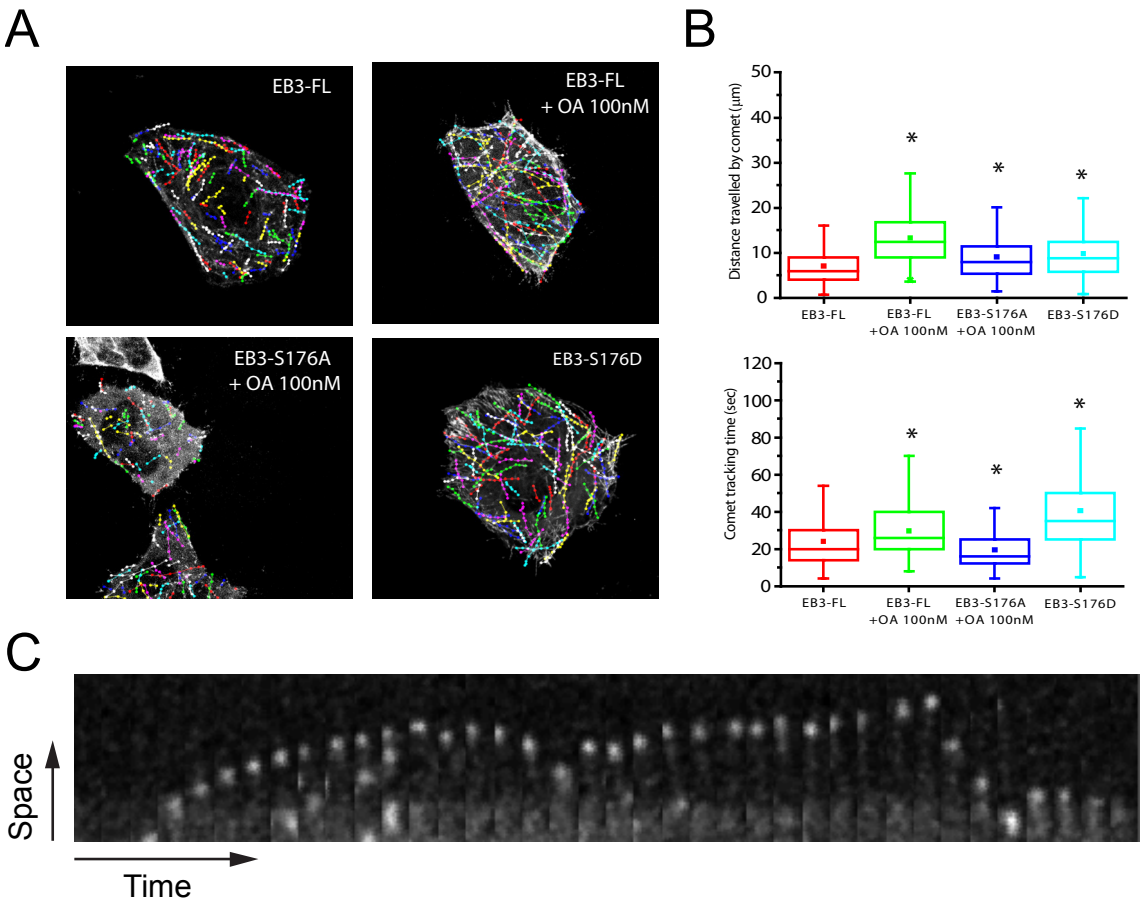

Figure 10 – Treatment with OA induces changes in microtubule dynamics. (A) Cells expressing EB3-GFP are treated with OA 100 nM for 2 hours and individual comets manually tracked. (B) Dynamic parameters measured include the mean distance travelled by the comets (in µm) and the mean comet growth time (in seconds). Note the increase in the distance and the time travelled by the comets after treatment with OA 100 nM. (C) Kymograph of an EB3-S176D labelled comet. Note that EB3 remains associated even when the microtubule is shrinking.

This can be further confirmed by analysing individual EB3-S176D comets. In fact, the constitutively phosphorylated form of EB3 remains associated to the plus-end even when the microtubule is depolymerising, which further supports the hypothesis that phosphorylation of EB3 increases affinity for the plus-end (Figure 10C). At this point, we cannot rule out that other residues in the linker region may also contribute for EB association, which will have to be addressed in the future.

4 - Discussion

The mechanism of EB protein association to the plus-ends of microtubules remains an open question. Many recent reports demonstrate that EBs specifically recognize the nucleotide state of tubulin and this favours accumulation of the proteins in the GTP cap (Maurer et al., 2011; Maurer et al., 2012; Zanic et al., 2009). Interestingly, the C-terminal domain of EB1 was also shown to be required for efficient tip-tracking behaviour by providing long-range electrostatic repulsive interactions that would drive the EBs towards the plus-ends of microtubules (Buey et al., 2011). While appealing, these results do not take into account the post-translational modifications that affect EB behaviour. Namely, phosphorylation of yeast EB1 homologue by Aurora kinase (Zimniak et al., 2009) and of human EB3 by Aurora kinases A and B (Ban et al., 2009), which affect the ability of EBs to associate to the microtubule and influence its dynamics.

Here we provide evidence for a cell-cycle regulated mechanism that controls EB association to the microtubule plus-end. Phosphorylation of EB3 must occur upon mitotic entry and this is regulated by Aurora kinase B. This will ensure that EB proteins are in a “high affinity” state for the microtubule, which favours microtubule dynamic instability. Then the question remains of why mitotic comets are smaller than interphase ones? One possible explanation is that phosphorylated EB3 has a higher turnover at the microtubule tip, which means the protein would associate to and dissociate from the microtubule faster than dephosphorylated EB3. How would then EB3 be forced to dissociate from the plus-end during mitosis? It has already been shown that in interphase, individual +TIPs bind to the microtubule tip with low affinity. Under these conditions both CLIP170 and EB3 exhibit rapid turnover behaviour on plus-ends as shown by FRAP experiments (Dragestein et al., 2008), with a continuous exchange of EBs with the cytoplasmic pool (Dixit et al., 2009; Dragestein et al., 2008). Overall, this means that EB turnover is much higher than binding site turnover and implies that the formation of comets depends on the exponential decay of EB binding sites in the microtubule structure. If this is so, then we would expect that a higher rate of decay of EB binding sites during mitosis would force the dissociation of EB3 from the microtubule, even if EB3 was in its “high affinity”, phosphorylated form. It would be interesting to determine whether there are any differences in the GTP cap size or GTP-tubulin distribution during mitosis, as EBs recognize the GTP state of tubulin (Maurer et al., 2012), which can easily be done using a specific antibody against GTP-tubulin (Dimitrov et al., 2008). In addition to this, if EBs associate and dissociate more rapidly from the microtubule tip during mitosis, it would

explain the increase in microtubule dynamics that is observed upon mitotic entry (Belmont et al., 1990; Rusan et al., 2001). This increase is mainly due to a higher microtubule catastrophe rate (Belmont et al., 1990; Rusan et al., 2001), implying that microtubule stabilizers have to be inactivated or displaced. Assessing whether phosphorylation of EB3 affects its interaction with other potential microtubule stabilizers is also necessary to elucidate these differences.

Interestingly, we also demonstrate that the increase in affinity depends on heterodimerization between EB proteins. It was recently reported that *in vitro* heterotypic association between EB1 and EB3 is preferred over association with EB2 (De Groot et al., 2009). Taken together with our results, this suggests that EB1 and EB3 heterodimerization is required to increase affinity for the microtubule and provides a functional explanation for the requirement of heterodimer formation *in vivo*. Therefore, it is possible that in human cells phosphorylation of EB3 may affect the behaviour of the EB1/EB3 heterodimer and regulate its affinity for microtubules. In accordance, phosphatase inhibition after EB3 depletion does not induce EB1 accumulation at the plus-ends of microtubules. This further confirms the necessity of heterodimer formation and phosphorylation in stabilization of EB binding to the microtubule. Phosphorylation of EBs by Aurora kinases has already been reported. In yeast, EB1 was described as a direct target of Aurora and phosphorylation of EB1 leads to its displacement from microtubules (Zimniak et al., 2009). In human cells, EB3 (but not EB1) is a substrate of Aurora kinase B (Ban et al., 2009; Sun et al., 2008). However, it was not clear how EB3 phosphorylation by Aurora kinases might influence EB or microtubule behaviour. While it safe to assume that phosphorylation at serine 176 is required for increasing the affinity towards microtubules, it is possible that additional residues are also necessary. Accordingly, others have shown that multiple Aurora-mediated phosphorylations occur in EB1 that regulate its behaviour (Zimniak et al., 2009) and we have demonstrated that expression of the EB3-S176D mutant does not fully rescue Aurora B inhibition. This may also explain the differences observed in terms of microtubule dynamics between OA treatment of the normal EB3 and expression of the EB3-S176D mutant.

VII. GENERAL CONCLUSIONS

Microtubules are highly dynamic structures whose function is tightly regulated by a series of MAPs. These will associate with the microtubule and by stabilizing or destabilizing it, will induce changes in its behaviour and ultimately determine its fate. Within the larger family of MAPs, there is a specific group which associates with the distal part of the microtubule and is thus termed plus-end tracking proteins (Akhmanova and Steinmetz, 2008). These +TIPs have become the principal regulators of microtubule function. At the core of this group, we can find the EB family of proteins. These are autonomous tracking proteins that recognize the microtubule tip through their conserved calponin homology (CH) domain (Bu and Su, 2003; Slep, 2010). While they do not require interaction with other +TIPs to associate with microtubules, they are essential in recruiting other +TIPs (Akhmanova and Steinmetz, 2008; Lansbergen and Akhmanova, 2006). Furthermore, they are essential in the regulation of microtubule dynamics by promoting microtubule polymerization (Komarova et al., 2009).

Identification of the mitotic functions of EB proteins

During the course of this thesis we defined some of the cellular functions of human EB proteins during mitosis. Given their high homology and similar role in the regulation of microtubule dynamics, it was assumed that they would have at least a partially redundant function during mitosis. This is hardly surprising as other +TIPs such as CLASPs are also partially redundant (Mimori-Kiyosue et al., 2006; Pereira et al., 2006) and much of the work has been performed using EB1 as a model for general EB behaviour. Surprisingly, we identified differential roles for EB1 and EB3 during mitotic progression and exit. In mitosis, there is a coordinated action of EB1 and EB3 to properly position the mitotic spindle. It was previously demonstrated that EB1 is important for mitotic spindle positioning by regulating astral microtubule function (Bruning-Richardson et al., 2012; Draviam et al., 2006; Green et al., 2005; Toyoshima and Nishida, 2007). We confirmed these results and further demonstrated that EB1 is necessary to confine the spindle in the z-axis, presumably by interacting with specific polar cortical components (Chapter III and Chapter IV). It remains to be determined which component(s) could interact with EB1 to regulate this process, although the dynein/dynactin complex is a good candidate. We also found that EB3 does not affect mitotic progression in the same way. It does not seem to regulate astral microtubule nucleation (Chapter III), but is required to maintain actin dynamic behaviour (Chapter IV). As a consequence, EB3 is required to regulate spindle position in the xy-axis. How does EB3 regulate discriminate between the xy- and the z-axes? It was already demonstrated that actin dynamics could influence spindle

behaviour (Fink et al., 2011), here we show that dynamic microtubules are also required for the formation and propagation of the mitotic actin wave and this requires EB3. By influencing the behaviour of a dynamic actin wave, EB3 ensures that the spindle aligns with the long cell axis and with the force-generating RFs. In light of this, we propose that cells assemble an "EB module" that allows cells to fine tune spindle positioning in 3D. They do so by responding to specific spatial cues transmitted by extracellular, actin-based forces which are connected to EB3 and EB1, but also intrinsic forces, be it from the spindle or the chromosomes as has been shown recently (Kiyomitsu and Cheeseman, 2012). How EB1 and EB3 can couple the spindle to specific force-transmitting components will be an interesting subject of study in the near future.

Regulation of post-mitotic cell adhesion by EB proteins

Taking into account the differential effects of EB1 and EB3 during mitosis, we proceeded to characterize the post-mitotic roles of EBs. We demonstrated that EB3 is required for coordinated daughter cell attachment and stabilization FAs (Chapter III and Chapter V). On the other hand, EB1 is required for disassembly of FAs, as its depletion leads to hyper-stabilization of these structures and faster attachment of the cells to the substrate (Chapter V). Therefore, it is not surprising that similar effects are also observed in interphase cells and demonstrates the relevance of EBs throughout the cell cycle. One interesting question arises of how EB1 and EB3 differentially regulate attachment to the substrate. Our results highlight the functional diversity of EB proteins in the regulation of cell attachment and motility and this can be easily observed in Chapters III and V, when expression of the several EB3 constructs used fail to rescue EB1-specific phenotypes. It is known that microtubules play an important role in the turnover of cell-substrate adhesions, as microtubule targeting assists in the disassembly of FAs (Kaverina et al., 1999) and cell migration critically depends on dynamic microtubules (Liao et al., 1995). This means that the overall dynamic state of microtubules may impact on adhesion turnover, because it regulates the dynamics of cell-substrate attachments. In agreement, we demonstrate that EB3 acts as a microtubule "pause-promoting" factor, which allows FAs to stabilize (Chapter III and Chapter V). Interestingly, this process is dependent on a phospho-regulatory mechanism. While in mitosis, EB3 is kept phosphorylated and this maintains the protein in a different functional state (Chapter III). At the transition from mitosis to interphase, EB3 dephosphorylation is essential to switch the dynamic state of microtubules. Accordingly, we have shown that by imposing EB3 phosphorylation at serine 176 during

interphase, we maintain microtubules in a growth-prone state which correlates with defects in daughter cell attachment and higher cell motility. Curiously, induction of lamellipodial cell protrusions required for cell migration was shown to specifically require growing microtubules and to be independent of microtubule shortening or tubulin concentration (Waterman-Storer et al., 1999). On the contrary, EB1 is required for microtubule rescues and in its absence, microtubules tend to exhibit more transitions to the pause state (Chapter V). We and others have shown that EB1 positively regulates cell adhesion and migration [Chapter V; (Schober et al., 2009)]. Overall, this indicates that when EB1 is absent, microtubules will tend to pause more frequently and cells will migrate less and attach more stably to the substrate. Taken together, it is conceivable that EB1 and EB3 differentially regulate cell attachment and migration due to their respective impacts on microtubule dynamics. However, this does not rule out that interaction of EBs with other proteins through their EBH domain may also influence daughter cell attachment and cell motility. Accordingly, by expressing the microtubule binding domain of EB3 we successfully rescue cell adhesion and motility (Chapter III and Chapter V) but fail to accumulate integrin at the furrow, leading to cytokinesis failure (Chapter III). In the future, it would be interesting to determine whether the changes in microtubule dynamics imposed by EB1 and EB3 have a general effect on cell attachment and motility or in alternative, if there are interacting targets that could explain the specificity of EB proteins. In addition, it is still not clear to what extent the C-terminal domain of EB proteins contributes to the regulation of post-mitotic cell attachment.

What defines the association of EB proteins to the plus-ends

One of the major outstanding issues in +TIP biology is determining the molecular basis which defines tip-tracking behaviour both spatially and temporally. While many +TIPs use co-polymerization, motor-based transport or hitchhiking, EB proteins use a direct end-recognition mechanism. This means that EB proteins must recognize and distinguish between the microtubule plus-end and the more mature microtubule lattice. It is known that EB proteins quickly exchange at the plus-end. This means that EBs associate to their binding sites on the plus-end with high affinity while binding with lower affinity to the microtubule lattice. Recent efforts clarified that EB proteins bind with higher affinity to GMPCPP tubulin and GTPγS, which resemble GTP-tubulin (Maurer et al., 2011; Zanic et al., 2009). In addition, structural data demonstrated that EBs specifically bind close to the GTP-binding site, which allow them to recognize the nucleotide state of tubulin (Maurer et al., 2012). These *in vitro* observations help

define the fundamental structure recognized by EB proteins but further *in vivo* confirmation is still lacking. A distinct level of EB regulation occurs on a temporal scale which may involve phosphorylation of EB proteins by Aurora kinases (Ban et al., 2009; Zimniak et al., 2009). Nevertheless, it is not clear how these phosphorylations affect EB protein behaviour and, more importantly, how it may affect EB association to the plus-ends.

Here we demonstrate that association of EB proteins to the plus-end is temporally regulated by Aurora kinase B-mediated phosphorylation (Chapter VI). Upon mitotic entry, Aurora kinase B is required for proper assembly and dynamics of spindle microtubules and EB accumulation at the plus-ends. At least partly, this occurs through the phosphorylation of EB3 at serine 176 but it remains to be determined which other residues may be regulated in this manner. More specifically, whether other residues in the linker region of EB3 (or even in EB1), also contribute to plus-end association. Moreover, we also demonstrate that accumulation of EBs specifically requires heterodimerization between EB1 and EB3, which correlates with recent findings that describe heterotypic association between EB1 and EB3 (De Groot et al., 2010) and provides the first *in vivo* functional data for the relevance of heterodimer formation (Chapter VI). One standing issue relates to the differences observed between mitotic and interphase comet size. If indeed EB proteins recognize the GTP state of tubulin, it will be important to determine whether the plus-end accumulation observed in interphase upon phosphatase inhibition correlates with an increase in the GTP cap size or simply reflects a higher association of EBs to the microtubule. In addition, it will be relevant to determine why, during mitosis, phosphorylation of EB3 does not result in increased plus-end accumulation. Again, this might be due to structural changes on the microtubule or to the affinity of EB proteins towards the plus-end. Overall, these changes highlight the dynamic nature of EB association to the microtubule and demonstrate that EBs depend on other factors other than the nucleotide state of tubulin for this process. This highlights the need for further studies combining *in vitro* approaches with functional studies in cells.

Abbreviations:

+TIP – Plus-end tracking protein

Å – Angstrom

aMTOC – Acentriolar Microtubule Organizing Centre

APC – Adenomatous Polyposis Coli

ATP – Adenosine triphosphate

CAP-Gly – Cytoskeleton-associated protein-glycine-rich

CDK – Cyclin-Dependent Kinase

CH – Calponin Homology

CLASP – CLIP-Associating Protein

CLIP – Cytoplasmic Linker Protein

DAPI – 4', 6-diamidino-2'-phenylindole dihydrochloride

EB – End Binding

EBH – End Binding Homology

FA – Focal Adhesion

FAK – Focal Adhesion Kinase

FRAP – Fluorescence Recovery After Photobleaching

FSM – Fluorescent Speckle Microscopy

GDP – Guanosine diphosphate

GFP – Green fluorescent protein

GTP – Guanosine-5'-triphosphate

HeLa – Human adenocarcinoma cell line obtained from Henrietta Lacks

KDa – Kilodalton

MAP – Microtubule Associated Protein

MCAK – Mitotic Centromere-Associated Kinesin

min – minutes

MtLS – Microtubule Tip Localization Signal

MTOC – Microtubule Organizing Centre

NEB – Nuclear Envelope Breakdown

nm – nanometre

OA – Okadaic acid

PAGE – Polyacrylamide Gel Electrophoresis

PH – Pleckstrin Homology

RFP – Red fluorescent protein

RNA – Ribonucleic Acid

RNAi – RNA interference

sec – seconds

SEM – Standard error of the mean

SD – Standard deviation

SDS – Sodium Dodecyl Sulphate

shRNA – short hairpin RNA

TAT – Tubulin Acetyl Transferase

TOG – Tumour Overexpressed Gene

TTL –Tubulin Tyrosine Ligase

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