Force generation at microtubule ends : An in vitro approach to cortical interactions.

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Chapter VII:

Concluding remarks and additional research directions

We have characterized how dynein, attached to a growth-opposing barrier, can regulate and pull on dynamic MT ends, mimicking a specific interaction at the cell cortex in chapter 4. Interestingly the cell exploits the cell boundary in many more ways for cellular organization. This chapter starts with concluding remarks about follow-up experiments, mainly focused on chapter 5 (section 7.1). Subsequently three mechanisms involving interactions of MTs with the cell cortex and the (preliminary) experiments that address them are described. In section 7.2 the influence of force on the regulation of MT dynamics by plus-end binding proteins is discussed. In vivo it has been suggested that MAPs (MT Associated Proteins) can act as a cell boundary sensor for MTs, by rapidly inducing catastrophe as soon as the MT grows against the cortex [64]. We show preliminary results on the effect of force on the regulation of MT dynamics by Mal3 (see also chapter 6). In section 7.3 the capture of dynamic MT ends by non-motor proteins is studied, which is thought to play an important role in cellular polarization [73, 74]. In vivo data suggest that the interaction of two proteins, IQGAP1, which is localized at the cortex, and CLIP170, which tracks the growing MT end, can capture and stabilize dynamic MTs [80, 205]. In section 7.4 we exploit our in vitro assay to study this interaction. The first steps towards reconstituting delivery of proteins at the tip of MTs to the cell cortex are described, a mechanism present in many different cells [46].

7.1 Positioning processes based on microtubule pulling forces

7.1.1 Studying the role of geometry in cellular organization in lipid droplets

In chapter 5 we have studied the role of pulling forces in positioning processes. In this experiment motor proteins are stably attached to a rigid microfabricated chamber wall. This is an appropriate model system for cells with a hard boundary where cortical anchors are embedded in the actin cortex and cannot move. In many cells however cortical attachment sites are not firmly bound, but can move through the membrane, for example in budding yeast cells [206]. In this situation the sliding mechanisms we presented in chapter 5 will be more complex because captured MTs will also reposition. It will be exciting to investigate what the effect of mobile capturing sites is on MT-based positioning processes. A possible approach to this question will be to confine MT asters in lipid droplets with, for example, dynein molecules specifically bound to the inside of the lipid layer. Droplets are small containers encapsulating aqueous solution, separated by a lipid monolayer from the surrounding oil. It has been shown that it is possible to encapsulate and anchor active proteins in lipid droplets [207]. By a well-designed micro fluidics system biotinylated lipid droplets can be merged [208], allowing for sequential introduction of, for example, streptavidin, biotinylated dynein and centrosomes with tubulin. By confining the lipid droplets in narrow micro fluidic channels the geometry of the droplet can then be manipulated [208]. This experiment may be the logical next step in understanding the role of pulling forces in MT positioning processes.

7.1.2 The role of geometry in spindle positioning in first cell stage *C. elegans* embryos

We have shown in chapter 5 that, pulling forces can center a MT aster in a microfabricated chamber when coupled to MT sliding. It is hard to anticipate how relevant this mechanism is *in vivo*. It would be fascinating to physically deform the *C. elegans* embryo and study the positioning of the mitotic spindle in this deformed embryo, similar to recent experiments with *S. pombe* cells, where the cells were physically forced to grow in a bent shape, and thereby reorganized their MT array [209, 210]. It is hard to predict how difficult or simple it will be to physically deform the *C. elegans* embryo, but it would be very interesting to study the role of geometry on the

MT organization in these more complex cells, and compare it to both *in vitro* experiments and theoretical models that describe MT based positioning processes.

7.2 Regulation of microtubule dynamics by a combination of force and +TIPs

7.2.1 Introduction

Recent studies in *S. pombe* cells in interphase showed that interaction with the cell cortex, particularly at the cell ends, regulates MT dynamics [64, 70]. There is increasing evidence that this regulation is partly due to compressive forces generated by MTs growing against the cell end [64, 113]. The compressive forces reduce the growth velocity, which possibly directly reduces the catastrophe rate, as observed *in vitro* [72]. In addition there are proteins, like the kinesin-8, KLP5/6, that regulate the MT dynamics at the cell cortex [64]. It is suggested that KLP5/6, which is a catastrophe-inducing motor protein, can sense the MT growth velocity. It accumulates at the MT plus-end of slower growing MTs and in this way enhances the catastrophe rate at the cell ends.

Our goal is to study the combined effect of force and +TIPs on the regulation of MT dynamics. We hope to unravel mechanisms that are relevant for many different cell types that exploit physical barriers and +TIPs to locally regulate MT dynamics [46, 211]. We use an *in vitro* assay where MTs are grown against glass barriers in the presence of one or more +TIPs [72, 88]. We are interested in the effect of force on the regulation of MT dynamics by Mal3, Tea2 or Tip1. Tip1 is an especially interesting candidate. *In vivo* studies suggest that Tip1 stabilizes MTs in the cytoplasm, but rapidly dissociates from the MT end when the MT contacts the cell end. After Tip1 dissociation the MT immediately undergoes catastrophe, giving rise to the speculation that Tip1 might act as a cell boundary sensor [70].

In this section the assay and preliminary results on the effect of force in the presence of Mal3 are presented.

7.2.2 Experiments

The experiment to measure the influence of force on MT dynamics in the presence of +TIPs is similar to experiments previously described [72, 88]. Guanylyl-(alpha, beta)-methylene-diphosphonate (GMPCPP [31]) stabilized biotinylated MT seeds are attached via biotin-streptavidin linkage to a surface coated with biotinylated-PLL-PEG [212]. Dynamic MTs are grown from this seeds against glass barriers made as

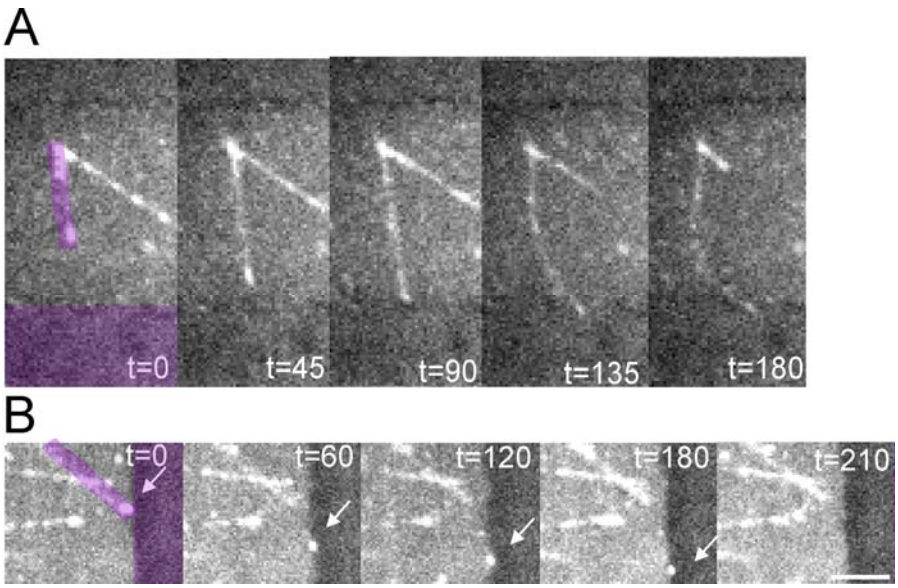

Figure 7.1

Time sequence of MT growing against glass barriers in the presence of Mal3-alexa488 (A) or Mal3, Tea2 and Tip1-GFP (B) against a glass barrier. The MTs of interest and the barrier are highlighted in the first image of the sequence. Scale bar indicates 2 μm.

described in section 2.1.1. Experiments are performed at 15 μM tubulin and at 25 ^{o}C. To prevent MTs from growing over the barriers, a crowding agent, methyl-cellulose, is added. We have performed experiments to verify that the end of the MT can be detected while in contact with the barrier. Now we can correlate the dynamics of the +TIPs at the plus-tip with MT catastrophes. Indeed both when Mal3-Alexa488 is imaged (Fig. 7.1A) as well as when Tip1-GFP is imaged in the presence of Mal3 and Tea2 (Fig. 7.1B), the plus-end of the MT can be monitored while the MT grows against the barrier.

Subsequently we started with studying the effect of force on the regulation of MT dynamics by Mal3 in more detail, since Mal3 is essential for the tip-tracking of Tip1 and Tea2. We focus our analysis on MTs that stall in a straight conformation against the microfabricated barrier, in contrast to the image sequence in figure 7.1. We compare the average contact time, $T_{contact}$ (the time that the MT stalls at the barrier until a catastrophe), in the presence and absence of Mal3 (Fig. 7.2). The contact time decreases from 100 ± 30 s in the absence of Mal3 to 15 ± 3 s at a 200 nM Mal3 concentration, a 7 ± 4 fold decrease. The decrease of the catastrophe time of freely growing MTs in the presence of 200 nM Mal3 was only 3 ± 1 under the same experimental conditions (measured in our group [41]).

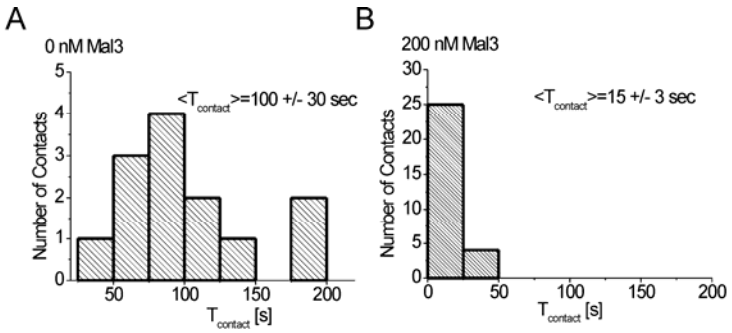

Figure 7.2

Contact times in the absence (A) and presence (B) of 200 nM Mal3.

7.2.3. Conclusion/Discussion

The fact that the difference in the reduction in catastrophe time is not the same for both situations mentioned above is intriguing. Recent high resolution electron microscopy and optical tweezers data suggest that Mal3 changes the MT end structure, so it is tempting to speculate that this modified structure is more prone to undergo catastrophe due to force [41, 213, 214]. Nevertheless more experiments are necessary

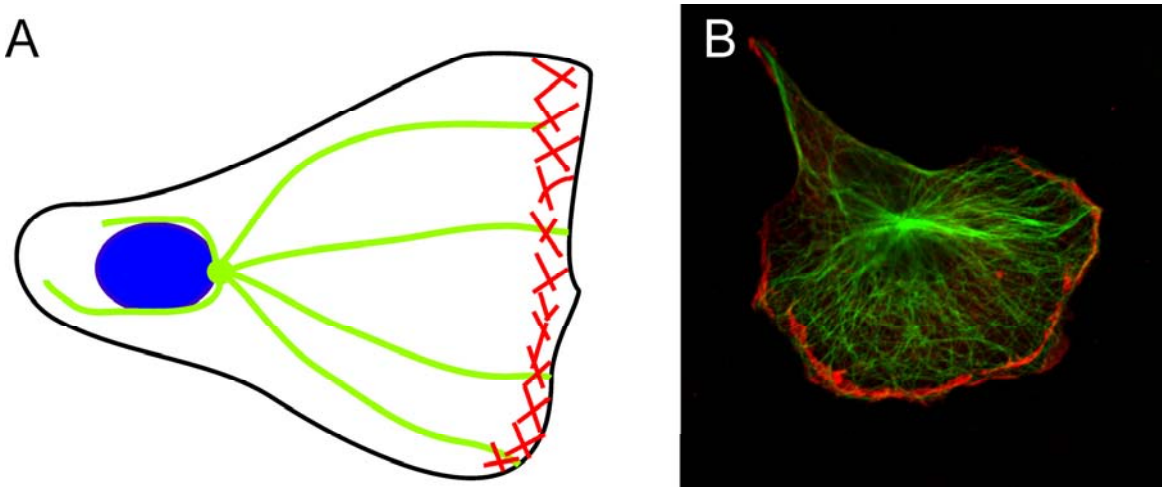

Figure 7.3

MT organization in a migrating cell. (A) Cartoon of the MT array (green) in migrating cells. MTs interact with actin (red) at the cortex of the leading edge, where they are locally stabilized, resulting in a polarized MT array. (B) Fluorescent microscopy picture of a polarized fibroblast. The MTs are stained in green, the cortical actin-binding protein IQGAP1 is stained in red. Image made by T. Watanabe, Nagoya University.

to show the significance of the effect of force.

Furthermore, since we can monitor the MT end in contact with the barrier, we would like to correlate the process of catastrophe at the barrier with the intensity of the +TIP at the MT end. Do all +TIPs simultaneously disappear from the plus-end before a catastrophe, or is there a typical sequence of events that precedes a catastrophe? In dual color experiments, Mal3 and Tea2 or Tip1 can be simultaneously measured, allowing for correlating the dynamics of Tea2, Tip1 and Mal3. Information about the sequence of events will shed light on the end-tracking mechanisms of Mal3, Tea2, Tip1, and thereby on end-tracking mechanisms in general. In addition we can obtain information about the MT catastrophe itself. As said above Mal3, and its homolog EB1 may alter the growing MT end structure, but maybe they also recognize this structure [41, 213, 214]. If this is true, the dynamics of Mal3 may reveal the dynamics of the MT end structure before a catastrophe, which could shed new light on the molecular mechanism underlying catastrophe.

7.3 Microtubule capture by non-motor proteins

7.3.1 Introduction

MT polymerization dynamics are important for cellular polarization [74, 215]. Dynamic instability allows MTs to search three dimensional space [116], so that they may find specific target sites within the cell [17]. If the sites are not homogenously distributed, capture of MTs at these target sites can generate an asymmetric MT array. For example, in migrating cells, MT plus-ends are thought to be stabilized and captured at specific cortical regions on the leading edge, leading to an asymmetric distribution of MTs in the cell, essential for directed cell migration (Fig. 7.3A) [73, 78].

In vivo studies in motile fibroblasts suggest that MTs are captured by the interaction of a plus-end tracking protein, CLIP170 [45], and an actin binding protein, IQGAP1, that is localized at the cortex and does not bind directly to MTs [77, 80]. These experiments show that IQGAP1 binds to CLIP170, and partially colocalizes with MTs at the leading edge [80] (Fig. 7.3B). However these *in vivo* experiments do

not show whether CLIP170 and IQGAP1 are sufficient for MT capture. Moreover they do not reveal the molecular mechanisms of this interaction. We have designed an *in vitro* reconstituted system to address these questions. Through our experiments we hope to learn about the capturing of MTs by CLIP170 and IQGAP1, however we also hope to shed more light on MT capturing mechanisms in general. Can non-motor proteins also capture MTs, and is the mechanism similar to what we have observed for dynein (chapter 4)? In this section we show our progress in developing this *in vitro* assay.

7.3.2 CLIP170 tracks growing microtubule ends in the presence of EB1

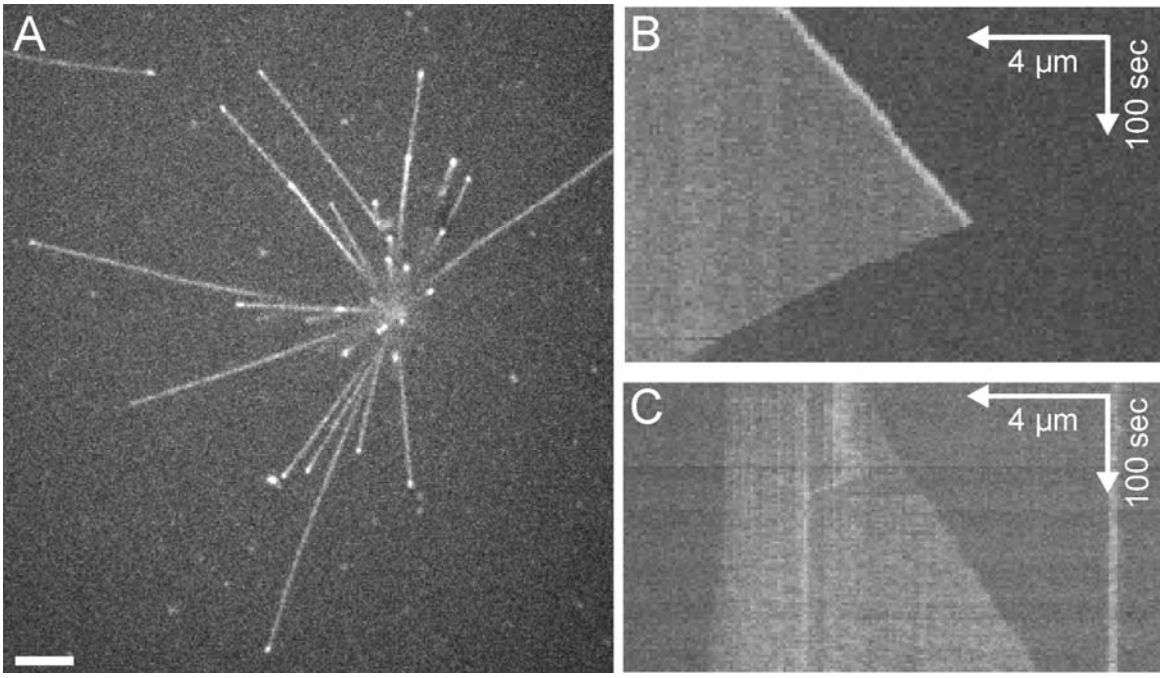

Figure 7.4

EB1 (in contrast to CLIP170) can autonomously track dynamic MT ends. (A) EB1-GFP localizes faintly on the MT lattice but is strongly enhanced on the tips of MTs growing from a centrosome. Scale bar indicates 3 µm. (B) Kymograph of a growing and shrinking MT in the presence of EB1-GFP. Clearly EB1-GFP tracks the growing but not the shrinking MT end. (C) Kymograph of a MT growing in the presence of CLIP170-GFP. CLIP170-GFP localizes on the MT lattice but is not autonomously enhanced at the growing MT end. Images taken with spinning disk confocal microscopy.

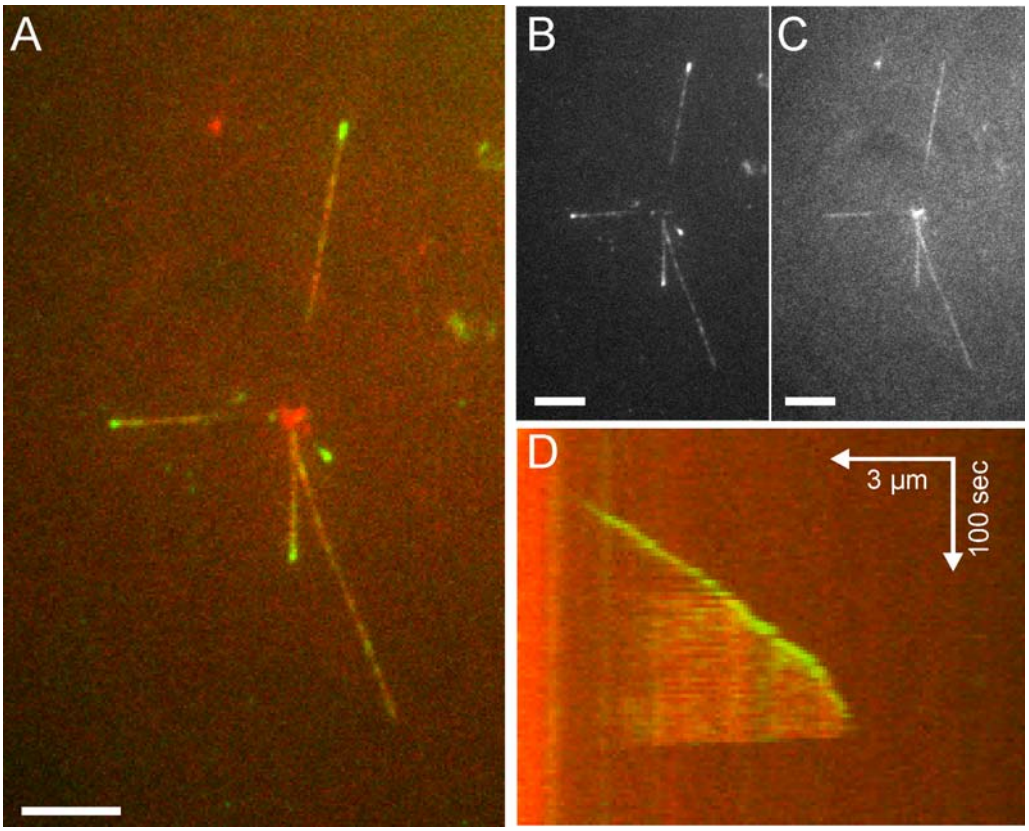

Figure 7.5
CLIP170 tracks growing MT ends in the presence of EB1. (A) Color merge of a TIRF image of the MTs in red and CLIP170 in green. (B) TIRF image of the CLIP170 (C) TIRF image of the MTs. (D) Kymograph showing that CLIP170 tracks the growing end but not the shrinking end of dynamic MTs.

We first reconstitute the end-tracking of CLIP170 to dynamic MT ends. It was previously shown that CLIP170 needs EB1 for end tracking [55, 58], so we exploit EB1 (full length), with and without GFP, in our assays. For CLIP170 we use a truncated construct, CLIP170-608, which is the H2 fragment [55, 58, 216] with an additional part of the coiled coil domain, with optionally a GFP attached (herein referred to as CLIP170 (–GFP)).

The plus-end tracking behavior of EB1 and CLIP170 is studied in the following *in vitro* experiments. Centrosomes are non-specifically attached to the glass surface of a flowcell, and subsequently the surface is blocked with 0.2 mg/ml PLL-PEG (SurfaceSolutions, Switzerland) and 1mg/ml κ-casein. Afterwards the solution

containing tubulin and optionally the +TIPs are added (30 μM tubulin, 0.2 mg/ml κ-casein, 1 mM GTP, 0.1 % methyl cellulose, an oxygen scavenger system, 100 mM KCl, in MRB80 with optionally 1.5 μM rhodamine tubulin, and/or 100 nM EB1, and/or 50 nM CLIP170). The sample is sealed and imaged at 25 ^{o}C with Spinning Disk Confocal Microscopy on an inverted Leica microscope equipped with a spinning disk from Yokogawa, or with TIRF microscopy, using a NIKON-Roper TIRF system.

Figure 7.4A and B show that similar to Mal3 [56], EB1 autonomously tracks MT plus-ends, and faintly stains the lattice [55-58]. CLIP170 in contrast does not autonomously track the growing plus-end but faintly stains the complete MT lattice (Fig. 7.4C). However in the presence of EB1, CLIP170 tracks the growing plus-end. It does not track the shrinking end and only faintly stains the MT lattice (Fig. 7.5).

7.3.3 IQGAP1 binds specifically to gold barriers

IQGAP1 is attached to a gold barrier as described in chapter 2.2. We use a truncated version of IQGAP1 in this assay, Strep-MBP-IQGAP1-CT-His, which is similar to the construct described in Ref. [80] as MBP-IQGAP1-CT, but extended with a strep-tag (Strep) and a his-tag (His). The strep-tag allows for specific binding to streptavidin

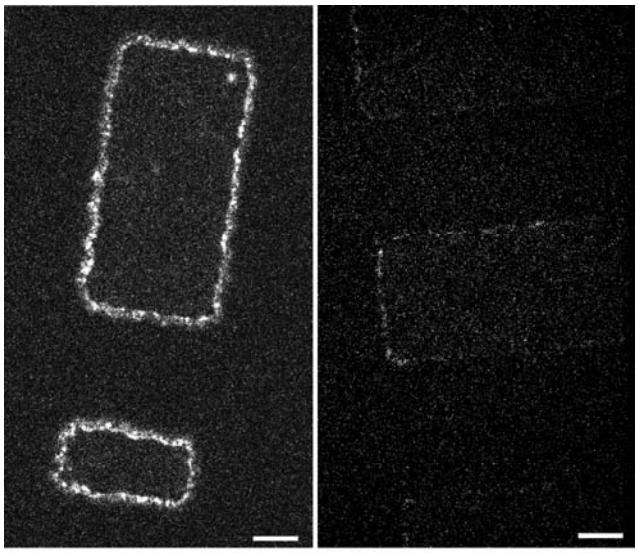

Figure 7.6

Strep-MBP-IQGAP1-CT-His specifically binds to a gold layer. A FITC antibody against IQGAP1 only localizes to the gold layer of a microfabricated chamber, where Strep-MBP-IQGAP1-CT-His is attached (left image). It does not localize to Strep-MBP-His which is similarly attached to the gold layer (right image).

[217], and the his-tag improves the purification, resulting in a more pure protein. We verified that this construct indeed localizes to gold barriers coated with biotin via gold-specific chemistry. First the IQGAP1 construct is attached to the activated gold barrier. Subsequently the presence of the IQGAP1 construct is detected by incubating the sample with FITC-antibodies against IQGAP1. Figure 7.6 (left image) shows that the antibodies specifically localize at the edges of the microfabricated chambers. In a negative control sample we coat the gold layer with strep-MBP-his and then incubate the sample with the FITC-antibodies against IQGAP1. In this case almost no binding of the antibodies to the gold layers can be detected (Fig. 7.6, right image). We conclude that we can specifically coat the gold barriers with Strep-MBP-IQGAP1-CT-His.

7.3.4 Preliminary results on microtubule capture by IQGAP1 and CLIP170 *in vitro*

We performed preliminary experiments where dynamic MT were grown from centrosomes in the presence of EB1 and CLIP170-GFP against IQGAP1-coated gold barriers, as described in section 2.3.1. In our first experiments we did not observe obvious capture of MTs by IQGAP1 and CLIP170, as observed in the presence of dynein (chapter 4). MTs that were in contact with the barrier shrank away from the barrier within a time that at first sight was comparable to the time MTs spend against a MBP-coated barrier (data not shown). A quantitative analysis and more statistics are however needed to investigate the precise effect of the IQGAP1-coated-barrier on MT dynamics. As a side remark: We sometimes observe MT nucleation from the barrier,

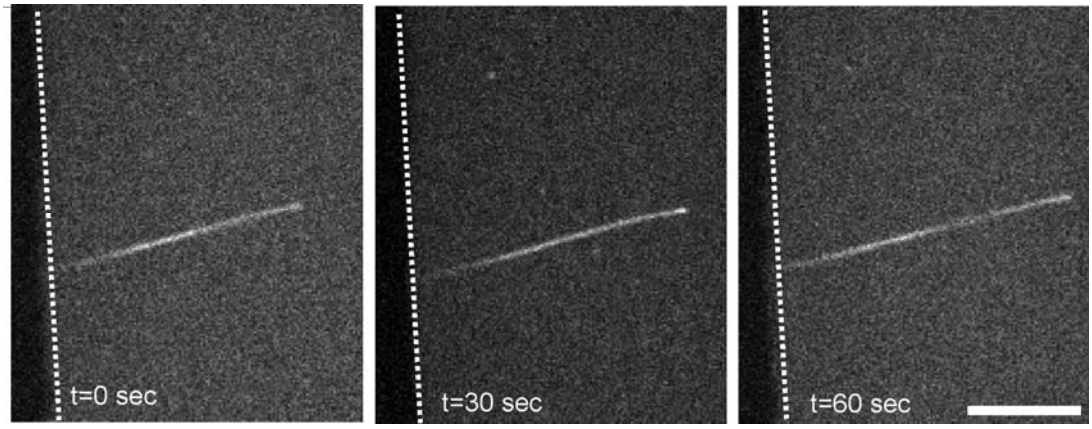

Figure 7.7
An IQGAP1-coated barrier nucleates MTs in the presence of EB1 and CLIP170-GFP. Scale bar indicates 5 μm. The white dotted line indicates the edge of the gold-barrier.

which we never observe in the absence of IQGAP1, confirming that IQGAP1 is present and active at the barrier in our experiments (Fig 7.7).

7.3.5 Conclusion/Discussion

We made the first steps towards reconstituting the capture of MTs by the interaction of non-motor proteins, CLIP170 and IQGAP1. More experiments are necessary to be able to draw conclusions about this potential capturing mechanism. In addition, in the literature a thorough analysis of the regulation of MT dynamics by CLIP170 is missing which is essential for interpreting the contact times at the barrier in the presence of CLIP170 and IQGAP1. Therefore it is recommendable to study also the effect of CLIP170 on free MT dynamics, which is an interesting study on its own.

From a more biophysical perspective it will be interesting to study how specific MT capture is to a particular protein. What is the minimal requirement for MT capture? Can any protein that is attached to a barrier and interacts with the MT end induce MT capture? How much does MT capture depend on the affinity of the capturing protein for the MT end? We can speculate that dynein binds strongly to the MT end, resulting in prolonged capture. In contrast CLIP170 rapidly exchanges on the MT tip [55, 58] and does not show prolonged capture in our preliminary experiments. The role of the binding affinity in MT capture can be studied by employing several biotinylated proteins that bind MTs with a different affinity in our *in vitro* assay.

7.4 Towards the role of microtubule-based transport in cortical pattern formation

7.4.1 Introduction

Many different cell types, from mobile fibroblasts [218] to fission yeast cells [68] (Fig. 7.8A-B), display non-homogenous protein patterns on their cell cortex. These patterns are important for cell morphogenesis: in fibroblasts the small G-protein Cdc42 localizes at the leading edge where motility is also initiated [218]. In fission yeast the cell-end marker protein Tea1 that among others is responsible for recruiting the actin-dependent cell-growth machinery, is specifically located at the cell ends [68] (Fig. 7.7A). MTs contribute to these non-homogenous protein patterns [219]. Tea1 travels to the cell ends at the tip of growing MTs, where it is deposited at the cortex (Fig. 7.8AB)[4]. The dynamics and organization of MTs are important for this protein delivery. If the MTs are too short they do not reach the cell end, if they are too long

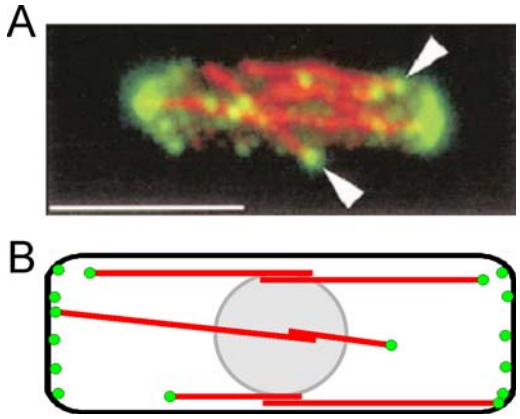

Figure 7.8

Tea1 forms a non-homogeneous protein pattern on the cell cortex of an *S. pombe* cell, and is localized at the tip of growing MTs. (A) Fluorescence image showing MTs in red and Tea1 in green, taken from [4]. (B) Schematic picture of protein patterning by MTs.

they buckle along the cell end. In both cases they do not deliver their cargo at the correct position. Interestingly, the proteins that are involved in transporting cell end markers are also involved in regulating MT dynamics [4, 70, 189].

Why are proteins transported on the tip of dynamic MTs? What is the advantage of this system over simple diffusion? Diffusion based processes can also generate non-homogenous protein patterns, as has been shown for example in *E. coli*. In *E. coli* Min proteins oscillate between the cell poles to select the cell center as the division site [220]. This non-homogeneous pattern has been reconstituted *in vitro*, and depends purely on reaction-diffusion processes [221]. Our aim is to study the role of MT based transport versus diffusive transport in cortical protein pattern formation. For this purpose we would like to set up a minimal *in vitro* system. This model system should allow us to perturb MT based transport and diffusion independently and evaluate the resulting protein patterns. We hope to learn what the advantages are of both strategies in terms of precision, speed and flexibility. In this section a possible future experiment is discussed. First results are shown and recommendations for continuation of these experiments are given.

7.4.2 Experimental plan

In the final *in vitro* experiments we would like to reconstitute non-homogenous protein patterns by MT-based delivery in microfabricated chambers. By growing MTs from a centrosome, and confining them in an elongated microfabricated chamber, we plan to

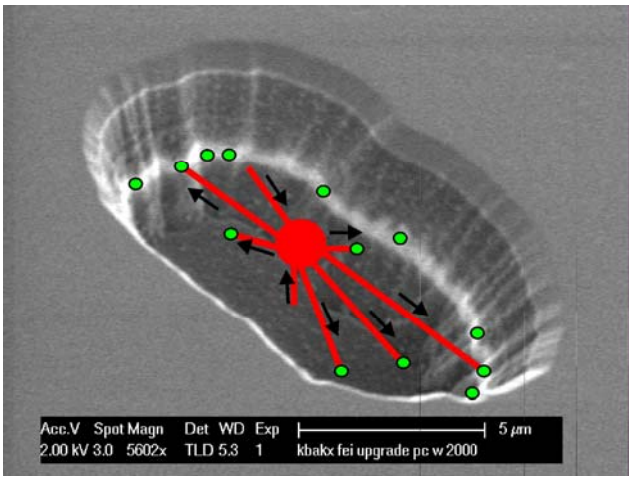

Figure 7.9

SEM image of a microfabricated chamber. A schematic MT array and the patterning proteins at the MT tips and walls are drawn in the microfabricated chamber.

manipulate the MT array to grow along the long axis of the chamber (Fig. 7.9). By varying the concentration of the patterning proteins in solution we plan to control the ratio of diffusing over transported protein. By exploiting surface chemistry and attaching protein tags to the patterning proteins we plan to control binding of the proteins to the microfabricated chamber walls. In this section the design considerations for the experiments are discussed step by step.

7.4.3 +TIPs travel with the end of growing microtubules and regulate their dynamics

One of the first hurdles in setting up these *in vitro* experiments consists of reconstituting transport at the tip of growing MTs *in vitro.* Fortunately this first hurdle has been recently taken, as shown in chapter 6. The plus-end tracking system that we use consists of three components, Mal3, Tea2 and Tip1. This allows for quite sophisticated manipulation of MT dynamics as well as patterning protein concentration. If, for example, Tip1 is the patterning protein, the concentration of Tip1 at the MT tip relative to the concentration in solution will depend on the Tea2 concentration. However, by varying the Tea2 concentration most likely the MT dynamics will change, but this effect potentially can be counteracted by varying the Mal3 or the tubulin concentration. Unfortunately at this point the regulation of MT dynamics by the combination of the three +TIPs is not yet well understood. Regulation of MT dynamics by Mal3 has been studied in depth [41], however a detailed study of

the effect of Tea2 and Tip1 is lacking. Therefore it is advisable to explore the effect of Tea2 and Tip1 more extensively, beyond the initial characterization in chapter 6.

7.4.4 Confining microtubules and patterning proteins in microfabricated chambers

In the experiments with microfabricated chambers performed so far, we ensured that the sealing prevents MTs from growing out of the microfabricated chamber. However in the ultimate patterning experiments the sealing of the microfabricated chamber should also inhibit diffusion of patterning proteins between different microfabricated chambers. Only then the total number of patterning proteins in the microfabricated chamber conserved. Thus are the diffusing fraction and the tip-tracking fraction of patterning proteins coupled. This is particularly important because one possible role of MT based transport is to effectively deplete proteins from the environment. In this case MTs act as "sticky tubes" that collect proteins from the environment and prevent them from binding at random positions, bringing the proteins to the proper position at the cell cortex.

So far we did not test whether the sealing of our microfabricated chambers inhibits diffusion. By performing FRAP (Fluorescence Recovery After Photobleaching) experiments the exchange of proteins between the microfabricated chamber and the environment can however easily be tested [61].

7.4.5 Protein binding with tunable affinities to microfabricated chamber walls

An important parameter in this experiment is the binding affinity of the patterning protein for the microfabricated chamber walls. The chambers will be quite small, 5-20 μm in length, on the order of a fission yeast cell. The time it will take for a typical protein to diffuse from the middle of the microfabricated chamber to the boundary can easily be calculated. The theoretical diffusion constant of a typical ~50 kD protein is on the order of 5.10^{-11} m^2/s. Therefore it will take such a protein ~1 sec to reach the chamber boundary. If the protein has a high affinity for the walls all the binding sites on the chamber wall will be occupied by the diffusing proteins, immediately after the experiment starts. In this case one may expect that it is no longer possible to form a non-homogeneous protein pattern. In the other extreme case, if the binding affinity is very low, it may not be possible at all to bind proteins to the chamber walls. We therefore aim to bind proteins to the microfabricated chamber walls via a tag of which the affinity can be tuned. We chose the polyhistidine-tag, consisting of 6 histidines

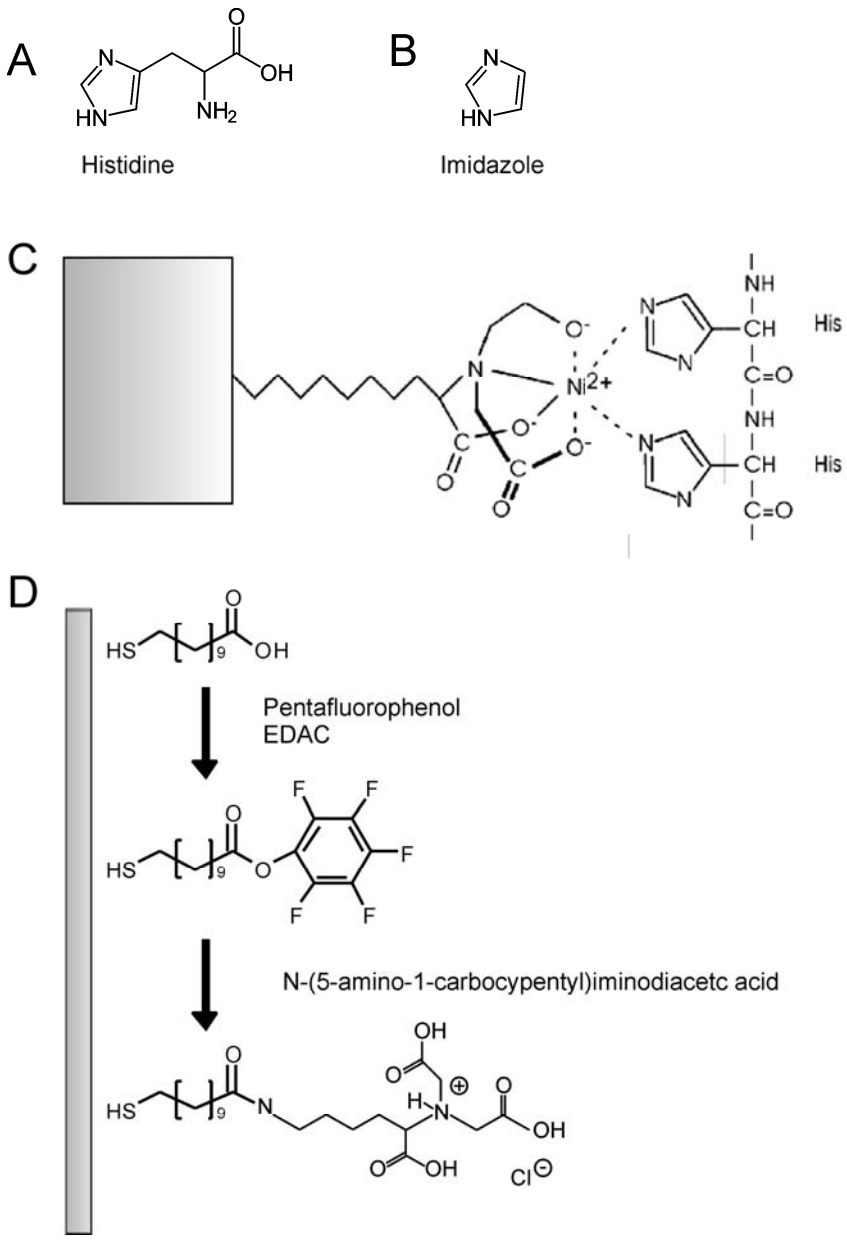

Figure 7.10

Activation of the gold surface with NiNTA. (A) molecular structure of histidine (B) molecular structure of imidazole (C) semi 3-d structure of histidine-tags bound to Ni-NTA. (C) Chemical reaction to bind Ni-NTA to a gold surface.

(Fig. 7.10A) usually referred to as His-tag, which specifically binds to Ni^{2+}-nitrilotriacetic acid (Ni-NTA) (Fig. 7.10C) [210]. The affinity can be tuned by adding imidazole in solution (Fig. 7.10C), which reduces the affinity by competing for

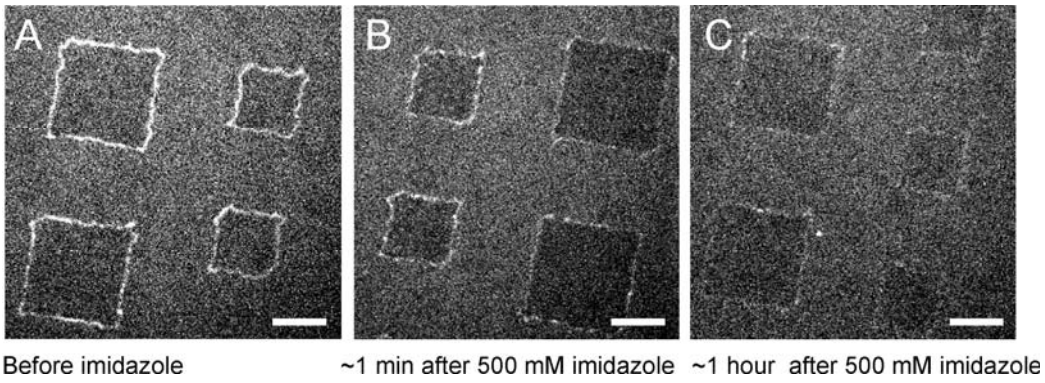

Figure 7.11

Specific association and dissociation of Tip1-GFP to gold barriers. (A) Tip1-GFP associates specifically to Ni-NTA that is attached to gold barriers. After imidazole is added the signal significantly reduces after one minute (B) and is almost completely removed after one hour (C).

binding sites. The Ni-NTA is attached specifically to the gold barriers in the microfabricated chambers via thiol-chemistry, as described for biotin (section 2.2.1, Fig. 7.10D) [222, 223].

Preliminary experiments in figure 7.11 show that Ni-NTA can be bound via thiol-chemistry to a gold barrier and that a His-tagged protein, in this case Tip1-GFP, associates with it. The affinity of Tip1-GFP to the Ni-NTA can strongly be reduced by adding imidazole in a high concentration (Fig. 7.11). After one minute the intensity of

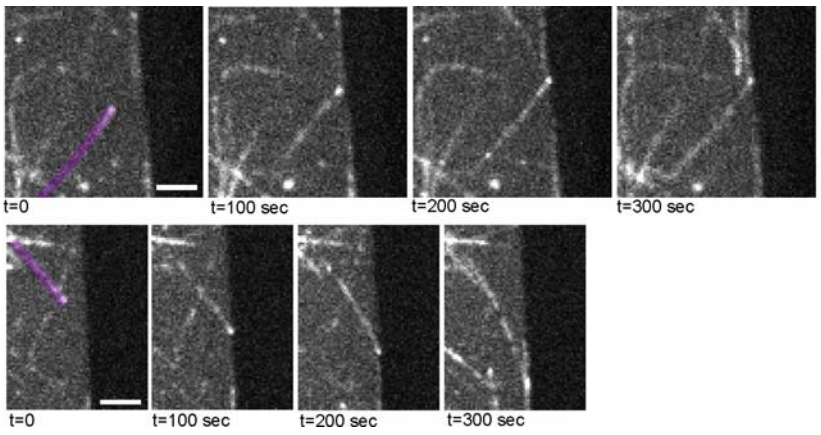

Figure 7.12

MT growing against gold barriers in the presence of Mal3, Tea2 and Tip1-GFP. Time sequences show that the end accumulation of Tip1-GFP is clearly visible when the MT grows against the gold barrier. The MT of interest is highlighted in the first image of the sequence. Scale bars indicate 2 µm.

the Tip1-GFP on the chamber walls has already decreased, and after one hour almost all Tip1-GFP has dissociated from the walls. We attribute the remaining fluorescent signal at the walls to non-specific absorption of proteins to the chamber walls, possibly enhanced by initial specific binding.

Considering the unbinding rates in the literature, the fast dissociation (visible after 1 minute) is probably unbinding of the His-tag from the Ni-NTA, the slow unbinding (on the time scale of one hour) is probably unbinding of non-specifically bound protein. Nevertheless, the clear decrease in intensity confirms that the affinity of the his-tag to the Ni-NTA can be regulated by imidazole, as has been shown previously [222, 223]. Control experiments in the absence of imidazole did not show this clear decrease. In the described experiments, Tip1-GFP was not present in solution and therefore there was no rebinding to the wall after imidazole addition. The next step in evaluating this interaction will be to measure the on- and off-rate of Tip1-GFP from the microfabricated chamber walls, with Tip1-GFP in solution and in the presence of different imidazole concentration. This can be measured by performing FRAP experiments on the chamber walls.

7.4.6 Conclusion/Discussion

We have jumped the first hurdles in reconstituting protein pattern formation *in vitro*. The big challenge will be to combine all the different aspects in one experiment. We think the next milestone towards this experiment will be to form protein patterns on microfabricated barriers instead of in microfabricated chambers. MTs should be grown in the presence of the +TIPs Mal3, Tea2 and Tip1-GFP, from surface attached nucleation sides against microfabricated gold barriers that are activated with Ni-NTA (an assay similar to section 2.3.1). When using barriers instead of chambers, the patterning protein concentration in solution can not be depleted, however this simplified experiment allows for extensive testing of the optimal binding affinities and the proper MT dynamics. As a first step we show that the end of the MT is visible if it grows against a (in this case bare) microfabricated gold barrier, (Fig. 7.12) so that if any patterns form, they should be detectable in these experiments.

7.5 Acknowledgements

The work on the effect of force on MT dynamics has been performed together with Inga Maria Tharun, and E. Laura Munteanu. The experiments on the *in vitro* reconstitution of MT capture by IQGAP1 and CLIP170 were performed in collaboration with Takashi Watanabe and Kozo Kaibuchi (Nagoya University, Japan). I would like to acknowledge Chris Rétif and Guillaume Romet-Lemonne for their help with the microfabrication, Joost te Riet for his advice on the attachment of Ni-NTA to a gold surface, and Martin Loose for discussions about studying positioning processes in lipid droplets.