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

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Summary

The MT cytoskeleton is essential for cellular organization. Interestingly, the MT cytoskeleton is very dynamic; it dramatically changes shape throughout the cell cycle. This dramatic reorganization of the MT array depends on a property of MTs termed "dynamic instability": the intrinsic ability of MTs to rapidly switch between a growing and a shrinking state. Regulation of the parameters that govern dynamic instability throughout the cell cycle, allows for flexibility in the organization of MTs. An important role of the MT array is the generation of forces needed for the proper positioning of various cellular organelles. Interactions of motor proteins with dynamic MTs are partly responsible for this force generation; however MTs themselves are also capable of generating forces, while interacting with physical barriers, such as the cell cortex.

In this thesis we study force generation by MT assembly as well as disassembly, while MTs interact with a physical barrier. We also study how these physical forces regulate MT dynamics, in combination with MT associated (motor) proteins (MAPs), which regulate MT dynamics biochemically. More on the cellular level, we study the role of MT force generation in cellular organization. As stated in the title: "Force generation at microtubule ends: An *in vitro* approach to cortical interactions", we use *in vitro* techniques. In *in vitro* experiments minimal functional modules are isolated. This is in large contrast to *in vivo* experiments where many processes occur simultaneously in a small confining space, and are often entangled.

In chapter 2 we describe the *in vitro* assays that we use to study MT-cortex interactions. We exploit microfabrication techniques to make physical barriers. These microfabricated structures are incorporated in four different assays. In the first assay MTs, with one end attached to the surface, are grown with their other end against rigid gold barriers. We use gold barriers to, via thiol-chemistry, specifically bind proteins that interact with MTs, to the barriers. In the second assay MTs are also attached to the surface at one end, but they are grown against glass barriers. This assay allows us to study the effect of MT pushing forces on MT dynamics, for example in the presence of MT associated proteins. In the third assay we use microfabricated chambers to study the role of pulling forces compared to pushing forces in cellular organization. Here, a MT aster is confined in a microfabricated chamber. Pulling forces arise from interactions between MT ends and motor proteins that are specifically attached to the chamber walls. Pushing forces result from MT assembly against the walls of the microfabricated chamber and from elastic restoring forces of the MT array. In the last

assay an optical trap is used in combination with microfabricated structures. Either a single MT or multiple MTs (depending on the specific experimental conditions) are grown from an axoneme, a rigid bundle of MTs. The axoneme is attached to a bead, held in an optical trap, and positioned in front of a microfabricated barrier. MT growth and shrinkage against the barrier results in displacement of the bead in the optical trap. The usage of an optical trap allows us to measure MT based forces quantitatively. In all four assays the microfabricated barriers function as a minimal system to mimic the cell cortex. In the case of pushing forces the essential function of the barrier is to oppose growth. In the case of pulling forces the barrier is specifically coated with proteins that connect the barrier to the shrinking MT and therefore transmit the generated pulling forces.

Several *in vitro* studies have shown that MT growth can generate pushing forces on the order of several pN [88, 94]. In cells however, MTs often function in bundles. For example in the mitotic spindle, a bundle of MTs interacts with the kinetochore. A collection of MTs is also responsible for force generation at the chromosome arms. In chapter 3 we use the above-described optical trap assay to study the dynamics and force generation of a growing MT bundle. We find that MTs that grow in a parallel bundle and are only coupled at their base can generate much higher forces than individual MTs. The maximum pushing force generated by a MT bundle scales linearly with the number of MTs in the bundle. This is in contrast to previous experiments on actin bundles that suggest that the forces generated by an actin bundle do not scale with the number of actin filaments in the bundle. Interestingly the force generated by a MT bundle couples the dynamics of the MTs in the bundle. The bundle can cooperatively switch to a shrinking state, due to a force induced coupling of the dynamic instability of single MTs. We can reproduce these cooperative switches with a simple computer simulation.

Where growing MTs generate pushing forces, shrinking MTs can generate pulling forces, as has been shown in *in vitro* experiments [99, 101]. Pulling forces are complicated because a link has to be made and maintained with a shrinking MT in order to transmit the generated force to an object. *In vivo* experiments have suggested that the motor protein dynein, located both at the cortex and at the kinetochore may play an important role in forming the link to a shrinking MT. In chapter 4 we study *in vitro* whether the motor protein dynein is sufficient to form a link between a physical barrier (mimicking the cortex or the kinetochore) and a shrinking MT, and whether this link withstands an opposing pulling force. MTs are grown against a rigid barrier specifically coated with dynein. The dynamics and the forces generated by the interaction of the MT end and the dynein-coated barrier are studied. We find that

dynein, when mechanically attached to a growth-opposing barrier, can hold on to a shrinking MT end and generate pulling forces up to ~5 pN. In addition, dynein, 'cortex'-attached dynein captures MT ends, induces catastrophes and slows down subsequent MT shrinkage. Our results provide a mechanistic explanation for observations in living cells and provide new information for theoretical models describing cellular organization by pulling forces.

In vivo experiments have shown that pulling forces play an important role in the positioning of organelles, such as the mitotic spindle, in the cell. In chapter 5 we study the role of pulling forces in positioning processes in an experimental model system. MT asters are grown in microfabricated chambers (as described above) and pulling forces are introduced by specifically attaching dynein to the chamber walls. The position of the aster is measured (a) when only pushing forces are present (no dynein at the wall), (b) when the pulling to pushing ratio is low (low dynein amounts at the wall), (c) when the pulling to pushing ratio is high (high dynein amounts at the wall). Surprisingly, and in contrast with previous theoretical speculations, pulling forces center a MT aster more reliably in a microfabricated chamber then pushing force alone. We developed a simple mathematical description for this improved centering, in which pulling forces center an aster due to an anisotropic distribution of MTs in the microfabricated chamber. The anisotropic distribution is due to MT growth-induced sliding. MTs are initially nucleated isotropically. However their increasing length forces them to slide along the microfabricated chamber wall when they grow against it. Eventually the MTs are captured by a motor protein and pulling forces are generated by the anisotropic MT array. The net force generated by this MT array reliably centers the MT aster.

So far we have mainly focused on MT force generation and the regulation of MT dynamics by these forces. However MT dynamics are also biochemically regulated by MAPs. One large class of these MAPs are the +TIPS: proteins that are known from *in vivo* observations to specifically track the growing MT plus-end. In chapter 6 we reconstitute the plus-end tracking of three +TIPs from fission yeast, Mal3, Tea2, and Tip1, *in vitro.* We find that Mal3 autonomously tracks both ends of the MT. Single molecule studies show that Mal3 very transiently binds to the MT end and therefore most likely does not end-track by copolymerization but by recognition of the MT end. Tea2, a motor-protein, and Tip1 need Mal3 and each other to track the MT end, and they specifically track the MT plus-end. Mal3 is needed to load Tea2 and Tip1 on the MT lattice. This complex then moves together processively to the MT plus-end (for which Mal3 is not needed).

In chapter 7 we present additional research directions. In the first section two new experiments are described to test the theory developed in chapter 5. In the first experiment we propose to grow MT asters in lipid droplets which should allow for very dramatic sliding. In the second experiment we propose to deform a *C. elegans* embryo. In this system the mitotic spindle is positioned by pulling forces. Deforming the embryo would allow us to evaluate how much geometry affects the positioning of the mitotic spindle in *C. elegans.* This should elucidate the biological relevance of our sliding model. In the second section we study the combined effect of physical and biochemical regulation of MT dynamics. MTs are grown in the presence of Mal3 against glass barriers. Our preliminary results show that force might enhance the destabilizing effect of Mal3 on MTs. In the third section the assay and preliminary results on MT capture by non-motor proteins are presented. *In vivo,* capture by (non-motor) proteins at the cortex is thought to play an important role in cell polarization. In the fourth section we describe the first steps in developing an *in vitro* assay to study the delivery of proteins to the cell cortex by transport at the end of MTs compared to simple diffusion of these proteins. Delivery of proteins to the cell cortex is thought to be important for cell polarization as well.

In conclusion, we have studied mechanisms of force generation by dynamic MTs. On a more cellular level we have studied the consequence of this force generation on the positioning of cellular objects in a confining space. All the experiments in this thesis have been performed *in vitro*. The next step will be to investigate how we can extrapolate the concepts we learned from *in vitro* experiments to the complexity of an *in vivo* system.