Dynamics and structural features of the microtubule plus-ends in interphase mouse fibroblasts

Zovko, S.

Citation

Zovko, S. (2010, June 22). *Dynamics and structural features of the microtubule plus-ends in interphase mouse fibroblasts*. Retrieved from https://hdl.handle.net/1887/15711

Version:	Corrected Publisher's Version
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/15711

Note: To cite this publication please use the final published version (if applicable).

CHAPTER VI

General discussion and Summary

General discussion and Summary

In the past few decades scientific research has revealed that MTs are major players in a variety of vital cellular processes, including intracellular transport, cell division, motility, cell adhesion, organelle anchorage, transmission of receptor signaling and neurite outgrowth. Moreover, there is a body of evidence that neurodegenerative diseases like Alzheimer's disease, Parkinson, developmental disease like Williams Syndrome, as well as cancer progression and metastasis, may be linked to MT malfunction due to differential expression of the regulators of the MT dynamics and/or differential expression of the tubulin isoforms. Cancer therapy nowadays is based on MT disruption using agents like taxol, colchicine and vincristine, which block cell division in a nonspecific manner. This everything-but-elegant approach leads to targeting of non-malignant cells as well, causing a surplus of adverse side effects in the patient.

Within a cell, MTs are regulated by a variety of MT binding and signaling molecules. This goes for both interphase MTs and kinetochore-associated MTs. Insight in fundamental processes like intrinsic plus-end dynamics, which are linked to the structural properties of MT plus-ends, and the mechanisms of action of the proteins influencing these features might eventually lead to the discovery of 'druggable' targets of this dynamic system, allowing for specific targeting of diseased cells.

All the different faces of the plus-ends

Two major objectives of the present study were to i) visualize MT plus-end structures of MTs present in the periphery of interphase mouse fibroblasts and ii) gain more insight into the relationship between the plus-end and its structure. To accomplish the first objective we have studied dynamically instable MT plus-ends in interphase mouse fibroblasts using EM, ET and fluorescence microscopy. Given the small dimensions of the MT plus-end, EM was the method of choice for the proper visualization of the plus-end structure. Using this approach we were able to observe nine distinct interchangeable plus-end conformations. Furthermore, ET enabled us to visualize plus-end structures in 3D space (three-dimensional volume) and thus validate the existence of all nine plus-end conformations: sheet-straight, sheet-frayed 1 and 2, forked, flared, early-frayed, frayed, blunt open and blunt closed morphology. Except for sheet-frayed 2 ends, these plus-end conformations had previously already been observed on the tips of *in vitro*-assembled MTs and *in situ* kinetochore MTs (kMTs), confirming the relevance of earlier *in vitro* studies. Therefore, one can conclude that the interphase MTs at the cell periphery of mouse fibroblasts resemble in vitro-assembled MTs in many aspects and can be used to understand and interpret the different dynamic states of cellular MTs.

In order to minimize fixation and staining artifacts, we have developed a whole-mount cryo-electron tomography approach to visualize the MT plus-ends in their close-to-native state (Koning *et al.*, 2008) (also see Chapter V). This approach proved to be very suitable for visualizing various cellular structures, including MT plus-ends, in the periphery of very thin cells like fibroblasts. In addition, whole-mount cryo-EM on fibroblasts (or other very thin cells) allows the study of MT plus-ends in other cells than those that form neurite-like protrusions.

It should be noted, however, that the analysis of the MT plus-ends from tomograms and electron micrographs still requires manual tracing. This requires precision and may not be fully objective, even when utilizing a number of preset criteria. The development of automated MT protofilament or MT wall tracking software would contribute to a more objective and probably more accurate analysis and classification of these structures. Although some efforts have been made in this field, for instance in the lab of dr. Bruce McEwan, automation of segmentation of the protofilaments in tomograms/ micrographs remains a challenge. An interesting advance is the development of automated protofilament curvature analysis software developed in the laboratory of Dick McIntosh (McIntosh *et al.*, 2008).

To correlate the MT plus-end conformation to the actual dynamic state of an MT, we shifted the balance of dynamic instability toward shrinkage by exposing cells to a MT depolymerizing agent, nocodazole. In this nocodazole-induced “dynamic” depolymerization system we followed the distributions of each of the nine characteristic plus-end conformations in time. Each of these conformations exhibited a different behavior in time as a result of sequestering of tubulin by nocodazole, which is indicative of a proper plus-end classification. By integrating behaviors of each plus-end conformation we proposed a kinetic model which connects the dynamic state of MT plus-ends with their conformation. This model comprises eight morphologically distinct,

though interchanging conformations of the plus-end that are able to switch from one state to another during the process of MT depolymerization. As a future step it would be important to study the transitions of the plus-end conformations on *in vitro* grown MTs upon MT depolymerization by nocodazole. Moreover, wash-out experiments after nocodazole treatment could provide insight into the transitions of the plus-end conformations during MT re-growth. Also time-course experiments in which cells are exposed to taxol or LPA (lysophosphatidic acid; induces MT stabilization at the leading edge through Rho/mDia pathway) (Palazzo *et al.*, 2004) might be useful to investigate plus-end conformations during the process of stabilization of the MTs and plus-end capturing at the leading edge, respectively.

Since EM doesn't allow tracking dynamic processes in time, we used an indirect approach by inducing depolymerization with nocodazole. In order to directly correlate a particular MT plus-end conformation to its dynamic state one could use a number of approaches:

a) Application of immunogold labeling of the endogenous +TIPs (or GFP-tagged +TIPs) and study the conformation of the plus-end decorated with these proteins in EM. This enables direct identification of the plus-end conformation exhibited by a growing MT plus-end. The problem with this approach is that that only epitopes exposed at the surface of the section are labeled. Moreover, the traditional procedure used for immunogold labeling of thin sections is not suitable for detailed morphological studies and, at least in our hands, did not allow proper detection of the morphology of the plus-end.

b) Saponin-induced permeabilization of cells followed by incubation with nanogold linked antibodies directed against, for example, GFP-tagged +TIPs in cells expressing the tagged protein. Cells are then chemically fixed or cryo-plunged, freeze-substituted, epon-embedded, sectioned and analyzed using EM. This approach, currently being optimized in our lab, would provide new insight in the conformation of +TIPs decorated plus-ends.

c) The correlation between dynamics and structure of the plus-end may be studied using cells expressing fluorescently tagged plus-end binding proteins by a fluorescence microscope, which, during live-cell imaging, are cryo-immobilized at a particular time point, and further investigated with an EM/ET microscope. This would enable visualization and localization of the MT plus-end of interest at the light-microscopy resolution level and subsequent in-depth investigation of the structure of the particular plus-end in high-resolution EM/ET mode. This correlative (cryo) light- microscopy and (cryo) electron-microscopy approach is currently being developed and utilized in various labs (Agronskaia *et al.*, 2008; Plitzko *et al.*, 2009).

One of the major challenges in the MT plus-end field is the identification of the specific features at the plus-end that are recognized by +TIPs. Our published work as well as that of others is consistent with the hypothesis that the plus-end tracking by +TIPs occurs by their ability to recognize structural feature(s) of the plus-end, rather than through co-polymerization with tubulin. An important study on the *in vitro* reconstituted

plus-end tracking system involving CLIP-170 and EB1 proteins showed that use of a slowly hydrolyzable analogue of GTP, GMPCPP, caused these +TIPs to bind over the whole length of MT by diffusion-based sliding rather than specifically at the plus-end (Dixit *et al.*, 2009). This suggests that EB1 and CLIP-170 selectively target growing MT plus-ends because of their ability to recognize a structural and/or chemical feature generated by GTP-hydrolysis.

Recently an antibody recognizing GTP-tubulin has been generated (Dimitrov *et al.*, 2008). This development will allow investigation of the structural nature of the 'GTP-cap' using a correlative (fluorescence + EM/ET) approach on *in vitro*-assembled MTs as well as on cellular MTs. This method might lead to solving the question which plus-end conformations are targeted by +TIPs, as well as whether a +TIP recognizes the GTP-induced conformation or a particular structural feature specific for the plus-end, or both.

Our data on the distribution of plus-end conformations over two zones within the cell periphery suggest that shrinking plus-ends might be captured/ stabilized by cellular factors associated with the cellular periphery (this thesis, Chapter III). These factors might include cortical actin filaments, cell-cell junctions and cell-extracellular matrix adhesions. Given that the nocodazole treatment was relatively short (up to 3 minutes), it was not possible to determine whether these captured MTs were transiently stabilized MTs or were long-lived, posttranslationally modified MTs. Nevertheless, one may speculate that, since the stabilized plus-ends exhibit the frayed conformation, i) they represent the fraction of MTs which started shrinking due to nocodazole exposure and which were then transiently captured by, for example, cell-matrix adhesions (Kaverina *et al.*, 1998) or ii) that these frayed ends have already been stabilized/captured prior to the nocodazole-treatment. The latter would suggest that the physical properties of the plus-end might be of importance for establishing a proper connection between a particular MT and a capturing factor. It is possible that a particular plus-end conformation is essential in order to allow capturing of a MT and its functioning as a machine in the processes that involve pulling (eg. chromosomes during cell division, (Kwon *et al.*, 2008) and/or pushing (against the membranes (Meunier *et al.*, 2009). Each of these two processes might relay on the presence of a different plus-end conformation and/or +TIPs.

CLIPs and MT plus-end morphology

Within the scope of this thesis, we aimed to elucidate whether CLIP-170 and CLIP-115, +TIPs known to modify the plus-end dynamics in cells, also modify and influence plus-end morphology itself. Moreover, we investigated which plus-end conformation is associated with the CLIP170/115-mediated plus-end rescue-phase. For this purpose we examined and scored plus-ends in the peripheral area of WT MEFs and compared them to CLIP170/115-deficient MEFs. We hypothesized that the absence of the CLIPs might result in a shift in frequency, or even a malformation, of a particular plus-end conformation(s). The observation that all nine plus-end conformations that could be detected in 3T3 cells (Chapter III) and in wild type (WT) MEFs (Chapter V), were also found to be present in double-knock out (DKO) MEFs (MEFs derived from CLIP-170 and

CLIP-115 KO mice) indicates that CLIPs are not required for the design or modulation of the plus-end conformation/morphology as such. This suggests either that this is an intrinsic property of the plus-end, which is supported by previous *in vitro* studies, or that proteins other than CLIPs are involved in the formation of these plus-end conformations.

In addition, we have observed a significant reduction in the frequency of sheet-straight ends and a much less pronounced reduction in the frequency of forked (not significant reduction) and flared (not significant reduction) ends in DKO vs WT MEFS. It is difficult to say to what extent the observed reduction was due to the decrease of growing MTs in the periphery as a consequence of reduced rescue frequency which typifies these DKO cell. With other words, it is possible that, at least partly, the observed reduction in frequency of growth-associated plus-end conformations is due to the lack of binding of CLIPs to these conformations and their subsequent stabilization. Besides CLIPs association with growing plus-ends adopting forked and sheet-straight conformations (representing the comet-tail structures of the growing MTs), CLIPs might be involved in stabilization of the flared ends too. A reduced frequency of flared ends, characterized by slightly outward curved protofilaments which presumably have lost their connection with each other and thus are not growing, in CLIP-170/115-deficient cells, implies that CLIPs might stabilize this conformation, preventing it from collapsing. In other words, the flared end might be a transition conformation to which CLIPs could bind with higher affinity, and rescue it from its conversion to the frayed end (Zovko *et al.*, 2008). This is supported by the finding that the N-terminal part of CLIP-170, which contains the MT binding domains, binds to curved tubulin oligomers in assembly condition *in vitro* (Arnal *et al.*, 2004). This indicates that CLIPs could have two modes of action: one would be the tracking of the growing plus-end (sheet-straight and forked), the other mode of action would then entail the stabilization and rescue of the flared conformation from transiting into a frayed end. The latter could perhaps involve CLIP-170 as a dimer which would provide a connection between two protofilaments, with one CLIP-170 molecule binding to one and the second CLIP-170 binding to the adjacent flared protofilament, pulling them together to form a blunt end, which can then transit into a growing one. This is supported by the findings that mutant CLIP-170 and CLIP-115 that cannot form homodimers, are able to track growing plus-ends (Perez *et al.*, 1999; Hoogenraad *et al.*, 2000). Similar dual mode of action of +TIPs has recently been reported for EB1 protein: dimerization is not required for the tracking behavior of EB, but it is for its anti-catastrophe activity in cells (Komarova *et al.*, 2009). Interestingly, the plus-end targeting by CLIP-170 is dependent on the presence of EB1 (Bieling *et al.*, 2008; Dixit *et al.*, 2009).

In summary, we have shown a number of morphologically distinct, though interchangeable, MT plus-end intermediates characterizing the process of MT dynamic instability, more specifically process of MT depolymerization. Insight in the fundamental aspects of this process is a prerequisite for better understanding of the complex interaction between MT plus-ends and cellular factors like +TIPs. Our findings indicate that MTs can be stabilized by unidentified factors in the periphery of the interphase mammalian cell, and that this stabilization might be dependent on the conformation of the plus-end and possibly on the recognition of the specific plus-end conformation by these cellular factors. Thus, in the right place, at the right time a particular conformation

of the MT plus-end can be utilized by various cellular factors, whether they are the +TIPs or focal adhesion-associated proteins or others, to perform work, stabilize the cellular shape or to regulate other structures. Of note, many of the plus-end binding factors which are present in the periphery of an interphase cell are also present on the plus-ends of kinetochore MTs, indicating the possibility that although in different context, these factors might exhibit similar regulatory behavior during e.g. mitosis (Tanenbaum *et al.*, 2006). Hence, further research in the fundamental aspects of MT plus-end structures/dynamics and its regulation might lead to identification of druggable targets for e.g. control of cell division of specifically diseased cells.

References

Agronskaia, A.V., Valentijn, J.A., van Driel, L.F., Schneijdenberg, C.T.W.M., Humbel, B.M., van Bergen en Henegouwen, P.M.P., Verkleij, A.J., Koster, A.J., and Gerritsen, H.C. (2008). Integrated fluorescence and transmission electron microscopy. Journal of Structural Biology *164*, 183-189.

Arnal, I., Heichette, C., Diamantopoulos, G.S., and Chrétien, D. (2004). CLIP-170/Tubulin-Curved Oligomers Coassemble at Microtubule Ends and Promote Rescues. Current Biology *14*, 2086-2095.

Bieling, P., Kandels-Lewis, S., Telley, I.A., van Dijk, J., Janke, C., and Surrey, T. (2008). CLIP-170 tracks growing microtubule ends by dynamically recognizing composite EB1/tubulin-binding sites. J. Cell Biol. *183*, 1223-1233.

Dimitrov, A., Quesnoit, M., Moutel, S., Cantaloube, I., Pous, C., and Perez, F. (2008). Detection of GTP-Tubulin Conformation in Vivo Reveals a Role for GTP Remnants in Microtubule Rescues. Science *322*, 1353-1356.

Dixit, R., Barnett, B., Lazarus, J.E., Tokito, M., Goldman, Y.E., and Holzbaur, E.L.F. (2009). Microtubule plus-end tracking by CLIP-170 requires EB1. Proceedings of the National Academy of Sciences *106*, 492-497.

Hoogenraad, C.C., Akhmanova, A., Grosveld, F., De Zeeuw, C.I., and Galjart, N. (2000). Functional analysis of CLIP-115 and its binding to microtubules. J Cell Sci *113*, 2285-2297.

Kaverina, I., Rottner, K., and Small, J.V. (1998). Targeting, Capture, and Stabilization of Microtubules at Early Focal Adhesions. J. Cell Biol. *142*, 181-190.

Komarova, Y., De Groot, C.O., Grigoriev, I., Gouveia, S.M., Munteanu, E.L., Schober, J.M., Honnappa, S., Buey, R.M., Hoogenraad, C.C., Dogterom, M., Borisy, G.G., Steinmetz, M.O., and Akhmanova, A. (2009). Mammalian end binding proteins control persistent microtubule growth. J. Cell Biol. *184*, 691-706.

Koning, R.I., Zovko, S., Bárcena, M., Oostergetel, G.T., Koerten, H.K., Galjart, N., Koster, A.J., and Mieke Mommaas, A. (2008). Cryo electron tomography of vitrified fibroblasts: Microtubule plus ends in situ. Journal of Structural Biology *161*, 459-468.

Kwon, M., Godinho, S., Chandhok, N., Ganem, N., Azioune, A., Thery, M., and Pellman, D. (2008). Mechanisms to suppress multipolar divisions in cancer cells with extra centrosomes. Genes & Development *22*, 15.

McIntosh, J.R., Grishchuk, E.L., Morphew, M.K., Efremov, A.K., Zhudenkov, K., Volkov, V.A., Cheeseman, I.M., Desai, A., Mastronarde, D.N., and Ataullakhanov, F.I. (2008). Fibrils Connect Microtubule Tips with Kinetochores: A Mechanism to Couple Tubulin Dynamics to Chromosome Motion. Cell *135*, 322-333.

Meunier, B., Quaranta, M., Daviet, L., Hatzoglou, A., and Leprince, C. (2009). The membrane-tubulating potential of amphiphysin 2/BIN1 is dependent on the microtubule-binding cytoplasmic linker

protein 170 (CLIP-170). European Journal of Cell Biology *88*, 91-102.

Palazzo, A.F., Eng, C.H., Schlaepfer, D.D., Marcantonio, E.E., and Gundersen, G.G. (2004). Localized Stabilization of Microtubules by Integrin- and FAK-Facilitated Rho Signaling. Science *303*, 836-839.

Perez, F., Diamantopoulos, G.S., Stalder, R., and Kreis, T.E. (1999). CLIP-170 Highlights Growing Microtubule Ends In Vivo. Cell *96*, 517-527.

Plitzko, J.M., Rigort, A., and Leis, A. (2009). Correlative cryo-light microscopy and cryo-electron tomography: from cellular territories to molecular landscapes. Current Opinion in Biotechnology *20*, 83-89.

Tanenbaum, M., Galjart, N., van Vugt, M., and Medema, R. (2006). CLIP-170 facilitates the formation of kinetochore–microtubule attachments. EMBO journal *25*, 13.

Zovko, S., Abrahams, J.P., Koster, A.J., Galjart, N., and Mommaas, A.M. (2008). Microtubule Plus-End Conformations and Dynamics in the Periphery of Interphase Mouse Fibroblasts. Mol. Biol. Cell, E07-07-0681.