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FUNKČNÍ CHARAKTERIZACE VYBRANÝCH PROTEINŮ REGULUJÍCÍCH MIKROTUBULY

FUNCTIONAL CHARACTERIZATION OF SELECTED MICROTUBULE REGULATORY PROTEINS

Ing. Stanislav Vinopal

Školitel/Supervisor:

Doc. RNDr. Pavel Dráber, CSc.

Oddělení biologie cytoskeletu/Department of Biology of Cytoskeleton
Ústav molekulární genetiky AV ČR, v.v.i./
Institute of Molecular Genetics of the AS CR, v.v.i.

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SOUHRN

Mikrotubuly (MTs) hrají zásadní roli ve vnitřní organizaci buňky a vnitrobuněčném transportu, v regulaci polarity, pohyblivosti a přenosu signálů i v buněčném dělení a diferenciaci. MTs vytvářejí složité struktury, které jsou díky jejich dynamické povaze schopné rychlé reorganizace dle potřeb buňky. Dynamika, stabilita a prostorové uspořádání MTs jsou regulovány mnoha faktory. Jedním z nich jsou proteiny regulující MTs. V této práci jsme se zabývali funkční charakterizací tří proteinů regulujících MTs: Ca^{2+}-senzoru STIM1, spastinu, proteinu štěpícího MTs, a γ-tubulinu, který je zásadní pro nukleaci MTs.

Zjistili jsme, že aktivace žírných buněk kostní dřeně (BMMCs) vede ke tvorbě výběžků cytoplasmatické mebrány, které obsahují MTs. Tvorba těchto MT výběžků je závislá na transportu extracelulárních Ca^{2+} iontů do buňky, který je řízen proteinem STIM1 lokalizovaným na endoplasmatickém retikulu. STIM1 se váže na MTs a potlačení jeho exprese zabraňuje tvorbě MT výběžků. To naznačuje, že Ca^{2+} ionty mohou hrát roli při regulaci MTs. Jelikož snížení exprese STIM1 způsobuje také poruchy chemotaxe, popsané MT výběžky mohou být hypoteticky důležité pro vnímání vnějších signálů.

Glioblastoma multiforme je nejčastější a nejagresivnější maligní primární nádor mozku u člověka, jehož možnosti léčby jsou dosud velmi omezené. Ukázali jsme, že koncentrace proteinu spastinu, který katalyzuje štěpení MTs, je zvýšená u gliomů a glioblastomových buněčných linií a že jeho množství se zvyšuje se vzrůstající malignitou nádoru. Snížení exprese spastinu v glioblastomové linii vede k významnému snížení jejich pohyblivosti a zároveň ke zvýšené proliferaci. Ovlivnění funkce spastinu v buněčné migraci a proliferaci může potenciálně vést k zavedení nových přístupů k léčbě glioblastomů.

Jako první jsme ukázali, že klíčový protein pro nukleaci MTs γ-tubulin je přítomen v jadérkách různých typů buněk. Pomocí hmotnostní spektrometrie jsme v buněčném jádře identifikovali protein C53, který interaguje s γ-tubulinem. Zjistili jsme, že γ-tubulin může modulovat funkci C53 v G2/M kontrolním bodu po jeho aktivaci poškozením DNA. Dále jsme ukázali, že savčí γ-tubulin 2 je schopen nukleovat MTs a nahradit tak γ-tubulin 1. γ-Tubuliny jsou také rozdílně exprimované v průběhu časné myší embryogeneze i ve tkáních dospělce. Z našich výsledků vyplývá, že savčí γ-tubuliny jsou při nukleaci MTs funkčně ekvivalentní.

ABSTRACT

Microtubules (MTs) play crucial roles in intracellular organization and transport, cell polarity, motility, signalling, division and differentiation. MTs form complex arrays, which are, due to their highly dynamic nature, capable of rapid reorganization in response to cellular requirements. Dynamics, stability and spatial organization of MTs are regulated by many factors including MT regulatory proteins. In the presented study we functionally characterized three selected MT regulatory proteins: Ca^{2+}-sensor STIM1, MT severing protein spastin and γ-tubulin that is essential for MT nucleation.

We found out that activation of bone marrow mast cells (BMMCs) leads to the formation of plasma membrane protrusions containing MTs. Formation of these MT protrusions is dependent on an influx of extracellular Ca^{2+} regulated by protein STIM1, located in endoplasmic reticulum. STIM1 associates with MTs and its depletion prevents formation of MT protrusions. This indicates that Ca^{2+} ions might be involved in MT regulation. Since STIM1 depletion also causes defects in chemotaxis, we propose that MT protrusions might be involved in sensing of external signals recognized by BMMCs.

Glioblastoma multiforme is the most common and most aggressive malignant primary brain tumor in humans. We demonstrated that MT severing protein spastin is overexpressed in glioma and glioblastoma cell lines and that its expression level increases with tumor malignancy. Glioblastoma cells depleted of spastin exhibit significantly lower motility and an increased proliferation rate. Modulation of these spastin functions in cell migration and proliferation has a potential to become a part of novel approaches to treatment of invasive gliomas.

We showed for the first time that γ-tubulin is present in the nucleoli of various cell types. We identified new γ-tubulin interacting protein C53 in the nucleus using mass spectrometry and found out that γ-tubulin can modulate C53 function in G2/M checkpoint activation after DNA damage. Furthermore, we showed that mammalian γ-tubulin 2 is able to nucleate MTs and substitute for γ-tubulin 1 in cultured cells and that these γ-tubulins are differentially expressed in mouse early embryogenesis and in adult tissues. Based on our results we propose that mammalian γ-tubulins are functionally equivalent with respect to their MT nucleation activity.

I. INTRODUCTION

I.1 Microtubules

I.1.1 Microtubule organization and functions

MTs are critically important for spatial and temporal organization of eukaryotic cells. They play crucial roles in intracellular transport, organelle positioning, establishment of cell polarity and shape, motility, signalling and cell division. In order to perform all of their functions, MTs are arranged in complex arrays that can be rapidly reorganized in response to cellular requirements. Such rapid rearrangements are possible due to the highly dynamic nature of MTs.

In the majority of interphase animal cells MTs form radial arrays emanating primarily from the centrosome, a microtubule-organizing centre (MTOC) localized near the nucleus. MTs explore cytoplasm with their free ends and reach the cell periphery. After onset of cell division, they radically reorganize to form mitotic or meiotic spindle, very dynamic and distinct structure necessary for precise separation and delivery of sister chromatids or homologous chromosomes, respectively, to future daughter cells. However, MT organization can be completely different in specialized cells. [1, 2].

MTs serve as tracks for transport of RNA, proteins and mebraneous vesicles mediated by microtubule motor proteins. By the action of molecular motors and other microtubule-associated proteins (MAPs) MTs organize intracellular space by precise positioning of organelles such as mitochondria, Golgi apparatus (GA) and endoplasmic reticulum (ER). MTs and actin microfilaments (MFs) are required for establishment and maintenance of cellular polarity in migrating cells [2].

Besides described dynamic arrays, MTs form also highly stable and long-lived structures such as centrioles. A pair of centrioles constitutes the structural core of animal centrosomes. Centrioles are surrounded by a complicated layer of unknown ultrastructure named the pericentriolar material (PCM) that is composed of fibrillar proteins and MTs emanating from the centrosome are anchored at the PCM [3]. Centrosomes serve as an important MTOC by nucleating and anchoring MTs [4] and as a hub for many signalling molecules including those involved in cell cycle progression [5].

I.1.2 Microtubule structure and dynamic instability

MTs are hollow tubes of about 25 nm in diameter composed of α- and β-tubulin heterodimers. Soluble tubulin heterodimers are abundant in the cytoplasm and can polymerize in a head-to-tail fashion into polarized protofilaments, which in turn associate laterally to form a cylindrical MT lattice. The majority of MTs grown *in vivo* consist of 13 protofilaments. 13-protofilament MTs have unique geometry; their protofilaments run straight along the MT length and do not twist around the MT [6].

As a consequence of head-to-tail polymerization of tubulin heterodimer and a unidirectional arrangement of protofilaments, MTs are intrinsically polar. The end with exposed β-tubulin is called the plus (+) end, the other end with exposed α-tubulin is called the minus (-) end. Stochastical switching between periods of growth and rapid shortening of (+) ends, occurring both *in vitro* and *in vivo,* is termed „dynamic instability“ [7]. Historically, the transition between growth to shrinkage is called *catastrophe*, the opposite transition is called *rescue.*

Each tubulin monomer contains one binding site for a guanine nucleotide. Since GTP bound to α-tubulin is physically traped at the dimer interface, it cannot be exchanged and hydrolyzed. On the other hand, β-tubulin-bound GTP can be hydrolyzed and resulting GDP can be exchanged for GTP. Tubulin heterodimers with bound GTP (GTP-tubulin) are essential for MT

growth. GTP-tubulin is incorporated into growing MTs and GTP is hydrolyzed to GDP few moments after polymerization yielding GDP-tubulin forming the majority of the MT lattice.

Depolymerizing protofilaments are frayed and curved reflecting the bent conformation of GDP-tubulin [8]. While GDP-tubulin increases the curvature of protofilaments putting strain on the lattice, fluctuating layers of GTP-tubulin on the (+) end, termed „GTP cap", stabilize the MT due to the strong interactions among GTP-tubulin subunits. If GTP cap is lost or a crack develops between protofilaments at the end, they peel apart and curl outward, finally releasing the stored free energy from GTP hydrolysis [9, 10]. Curled protofilaments subsequently depolymerize, GDP is exchanged for GTP in free tubulin dimers and GTP-tubulin can take part in the next round of MT polymerization.

In addition to above described α- and β-tubulin, tubulin superfamily comprises γ, δ, ε, ζ, η, θ, ι and κ-tubulin. They account together for less than 1% of the total tubulin content in the cell. While α-, β- and γ-tubulin are found in all eukaryotes and are highly conserved across species, the other tubulins are not as ubiquitous. It seems that the primary function of these less ubiquitous tubulins is the regulation of centriole and basal body stability and duplication; only α- and β-tubulin are capable of polymerizing into MTs [11]. γ-Tubulin will be discussed in greater detail in the chapter I.2.

I.1.3 Regulation of microtubule dynamics and functions

Dynamics, stability and spatial organization of MTs are regulated by many factors including different tubulin isotypes, tubulin post-translational modifications (PTMs), microtubule regulatory proteins such as MAPs, MT severing proteins and MT motor proteins. Small ligands like specific tubulin drugs and mechanical forces also affect MT stability. Since MTs can be regulated by an astonishing number of interacting proteins, we focus here only on several protein groups, which have a direct relationship to the presented publications.

An exciting hypothesis have been proposed postulating that tubulin PTMs have the potential to generate complex molecular signals that can serve as a readable code on MTs [12]. MAPs, MT severing enzymes and motor proteins are the best known downstream effectors of this code. The majority of tubulin PTMs indirectly stabilizes MTs. Patterns of PTMs are not uniform and have various subcellular and submicrotubular distribution [13]. The most characterized tubulin PTM are tubulin detyrosination, generation of Δ2-tubulin, polyglutamylation, polyglycylation and acetylation. These PTMs regulate MT dynamics and intracellular transport by recruiting or repulsing different MT regulatory proteins.

Katanin, spastin, fidgetin and VPS4 are members of AAA (ATPases associated with various cellular activities) protein family and use energy from ATP hydrolysis to sever MT lattice [14]. Katanin and spastin are crucial for neuronal development and maintenance, because their MT severing activities induce growth and branching of neurites [15]. Spastin influences mainly axon branching, whereas katanin plays a more important role in neurite outgrowth [16]. In non-neuronal cells, katanin regulates cortical MT (+) end and cell migration [17]. During mitosis, katanin controls mitotic and meiotic spindle length [18]. It has been shown that tubulin glutamylation, particularly long polyglutamate chains, attracts spastin and promotes MT severing [19].

Mutations in spastin are the most common cause of hereditary spastic paraplegia (HSP). This neurodegenerative disorder affects axons of corticospinal neurons and cause progressive spastic paralysis of the legs [20]. Mammalian spastin has 4 isoforms, longer M1 isoform, shorter M87 isoform lacking the first 86 amino acids present in M1 and truncated versions of each M1 and M87, both lacking 32 amino acids encoded by exon 4 [21].

All spastin isoforms possess C-terminal AAA ATPase domain, MT-binding domain (MTB) and a domain present in MT interacting and trafficking proteins (MIT), that is involved in

interactions with charged multivesicular body protein (CHMP) family of proteins. CHMP family members form a highly conserved complex termed endosomal sorting complex required for transport-III (ESCRT-III) that is involved in membrane scission in a variety of membrane modelling processes. The ubiquitously expressed M87 variant is found in cytoplasm, partially co-localizes with MTs, and can be recruited to endosomes and to the cytokinetic midbody during cell division in via ESCRT-III [21]. The M87 isoform has also been reported in the nucleus [21], where it acts as a transcriptional co-repressor of HOXA10 [22].

Only the M1 variants have a N-terminal hydrophobic domain (HD) that targets these spastin isoforms to the ER, early secretory pathway, and possibly also to the very early ER-to-GA intermediate compartment. Strikingly, ER proteins REEP1, atlastin-1 and RTN2 interacting with M1 spastin are also mutated in HSP. These findings indicate that HSP might be coupled with M1 spastin and support the hypothesis that abnormal ER morphogenesis is the pathogenic mechanism in HSP [21, 23]. A recent study showed that M1 has higher expression in axons of corticospinal neurons [24], however, since M87 variant is also present, precise mechanisms of HSP remain to be elucidated.

Another distinct group of MAPs are MT (+) end tracking proteins (+TIPs) that preferentially accumulate at growing MT (+) ends. +TIPs link MTs to various intracellular structures such as vesicles, cell membrane, focal adhesions, MFs and IFs cytoskeletons and kinetochores and regulate MT dynamics. +TIPs are structurally and functionally diverse, use different (+) end tracking mechanisms and are regulated by various signalling pathways [1, 25].

End binding (EB) protein family (EB1, EB2, EB3) can track MT (+) end autonomously by binding directly to tubulin. The C-terminal domain of EBs specifically recognizes a conserved SxIP motif present in many +TIPs. The ultimate C-terminal amino acids of EBs form the EEY/F motif found also on tyrosinated α-tubulin subunits and serve as a recruitment signal for proteins carrying CAP-Gly motifs [25]. Binding of many +TIPs to MT (+) ends is dependent solely on EBs. They all contain short SxIP motif that is required for interaction with EBs. Among the +TIPs with the SxIP motif is also an ER membrane protein STIM1 [25].

I.2 γ-Tubulin

γ-Tubulin is a highly conserved member of tubulin superfamily essential for nucleation of MTs in eukaryotes [6]. The γ-tubulin-coding gene is duplicated in many organisms, however, its duplication in mammals was independent of the others according to phylogenetic analyses [26, 27]. Mammalian γ-tubulin genes *TUBG1* and *TUBG2* are located on the same chromosome in tandem and their protein products are almost identical (>97% protein sequence identity in human). Although TUBG1 and TUBG2 were initially assumed to be functionally equivalent [28], Yuba-Kubo *et al.* performed a gene knock-out analysis of *Tubg1* and *Tubg2* in mice suggesting that they might have different functions. While TUBG1 was expressed ubiquitously, TUBG2 was primarily detected in brain. *Tubg1*$^{-/-}$ embryos stopped their development at the morula/blastocyst stage because of severe mitotic defects. In contrast, *Tubg2*$^{-/-}$ mice developed normally and had fertile offsprings, but adults exhibited behavioral changes including abnormalities in circadian rhythm and reaction to painful stimulations [26]. The authors concluded that TUBG1 is a conventional γ-tubulin, whereas TUBG2, which was not able to substitute for TUBG1 in *Tubg1*$^{-/-}$ blastocysts, might have some unknown function(s) in the brain. However, the molecular basis of the suggested functional difference has been unknown.

γ-Tubulin is concentrated at various MTOCs in cells, but its majority is soluble in the cytoplasm [29]. At the centrosome, γ-tubulin is associated with PCM and also with the core of centrioles. γ-Tubulin was detected at many other MTOCs including GA, apical and basal membranes of epithelial cells, midbody, basal body, along MTs in mitotic and meiotic spindles,

on condensed mitotic chromosomes and on nuclear membranes in myotubes [4]. Noticeably, various patterns of γ-tubulin distribution along interphase MTs were observed in *Drosophila* [30] and in human cancer cells [31]. In addition, we and others described γ-tubulin localization in the nucleus and nucleolus [31-34].

γ-Tubulin assembles together with other conserved proteins, named Gamma-tubulin complex proteins (GCP) in human, into γ-tubulin complexes (γTuCs). The 2.2 MDa γ-Tubulin ring complex (γTuRC) consists of γ-tubulin, GCP2, GCP3, GCP4, GCP5 and GCP6 and looks like a ring structure when observed using electron microscopy. Two molecules of γ-tubulin and one molecule each of GCP2 and GCP3 constitute γ-Tubulin small complex (γTuSC), which is a basic subunit of γTuRC. γ-Tubulin, GCP2 and GCP3 are present in all eukaryots, GCP4 and GCP5 are in most eukaryots with the exception of yeasts and GCP6 is found only in animals and fungi. A new refined template model of MT nucleation was proposed in which seven γTuSCs assemble to give rise a γTuRC with a half-subunit overlap between the first and the seventh. The resulting γTuRC exhibits 13-fold symmetry matching perfectly the MT lattice. Some special γTuSCs presumably exist containing GCP4, GCP5 or GCP6 [6].

Many proteins participate in the activation and targeting of γTuRC to MTOCs. Some of them co-purify with γTuRC such as GCP-WD/NEDD1, others are fibrillar PCM proteins like pericentrin, ninein, CDK5RAP2 or AKAP450/CG-NAP. Targeting of γ-tubulin to non-centrosomal MTOCs and its regulation is facilitated by specialized proteins. For example, a multiprotein complex augmin connects γTuRC via GCP-WD to the mitotic spindle MTs [6].

Besides MT nucleation and their anchoring at MTOCs, γ-tubulin is important also for capping of MT (-) ends, protecting them from depolymerization [35]. Further, γ-tubulin is involved in centriole biogenesis, presumably as a nucleator of centriolar MTs [36]. γ-Tubulin also modulates MT (+) end dynamics in yeasts [37, 38]. Interestingly, *Drosophila* γTuRC was detected along interphase MTs and its presence coincided with switching from a shortening or a growing phase to a pause indicating γTuRC-specific role as a catastrophe and rescue stopper. [30]. By interacting with (-) end-directed kinesin-14 Pkl1, γ-tubulin regulates bipolar spindle assembly in fission yeast [39]. Several studies suggested that γ-tubulin regulates cell cycle progression independently of MT nucleation [33, 40, 41] and that it plays a role in DNA damage signalling [42, 43].

γ-Tubulin function and timely localization is regulated by PTMs including ubiquitinylation [44, 45] and phosphorylation [46-50]. Many kinases such as phosphatidylinositol 3-kinase or Src family kinases Lyn, Syk, Fyn are associated with γTuRC and/or affect MT nucleation [48, 51-55]. Interactions with recruitment factors and MTOC localization play an important role in regulation of γTuRC function. PTMs such as phosphorylation of other γTuRC components and its recruitment factors are critical for proper regulation of MT nucleation [56-64].

Another level of control is modulation of γTuRC structural stability and stability of its individual components. A recent study suggested that (i) coregulation and stabilization of γTuSC proteins is independent of γTuRC assembly, (ii) generally, proteins incorporated in γTuRC are stabilized and protected from degradation [30]. A nucleolar and centrosomal protein HCA66 was shown to affect the stability of γTuSC *in vivo* [65].

I.3 Important model systems used in the study

I.3.1 Mast cells

Mast cells are derived from hematopoietic stem cells. In vertebrates, mast cells are widely distributed throughout the body, but they are especially located in tissues surrounding blood vessels, nerves and tissues in contact with external environment such as skin, respiratory and gastrointestinal tract [66, 67]. Mast cells play important roles in many physiological and

pathophysiological processes. They participate in wound healing, angiogenesis and in both innate and adaptive immunity [68]. From a pathological perspective, mast cells are involved in allergic disorders, autoimmune diseases, defects in adaptive and innate immunity [66, 67] and play a complex and in many aspects unknown role in modulating tumour microenvironment [69]. Various stimuli can activate mast cells to release a wide variety of biologically active products including histamine, proteases and other enzymes, cytokines, chemokines, growth factors, arachidonic acid metabolites and reactive oxygen and nitrogen species, many of which can potentially mediate physiological or pathophysiological functions of mast cells [68].

The most known mechanism of mast cell activation is through antigen- and IgE-dependent aggregation of the high-affinity IgE receptor (FcεRI). An antigen crosslinks IgE antibodies connected to the FcεRI on the plasma membrane, which starts a complex signalling cascade leading to Ca^{2+} efflux from ER stores. STIM1 is an ER membrane protein sensing intralumenal Ca^{2+} concentration. When ER Ca^{2+} stores are depleted, STIM1 changes conformation leading to its oligomerization and aggregation. Aggregates of STIM1 in the close proximity of plasma membrane associate with highly selective calcium release–activated calcium channels (CRAC) such as Orai1 and induce prolonged influx of extracellular Ca^{2+}. This extracellular Ca^{2+} influx is essential for mast cell activation and release of inflammatory mediators stored in cytoplasmic granules (termed degranulation) [70, 71]. STIM1 also represents a special type of a +TIP. It contains SxIP motif and binds to growing MT (+) ends via EB1. However, its MT-dependent movement is restricted to diffusion in the ER membrane [72, 73]

I.3.2 Glioblastoma cells

Gliomas are central nervous system (CNS) tumors arising from glial cells. They are the most frequent group of primary brain tumors [74]. According to the original cell type there are astrocytomas, oligodenrogliomas and ependymomas. Gliomas are categorized according to the level of malignancy. Low grade gliomas (WHO grade I-II) are still not anaplastic and carry a better prognosis for patients. High-grade gliomas (WHO grade III-IV) are anaplastic, malignant, diffuse and highly invasive multifocal tumors that have a very poor prognosis. Glioblastoma multiforme (GBM) arises from astrocytes and it is the most common grade IV tumor in adults and also the deadliest primary brain tumor in human. Glioblastomas are incurable by conventional therapies, since they are radioresistant, chemoresistant, and tend to recur in a local fashion despite surgical resection [75]. GBM form tendrils extending several centimeters away from the main tumor mass which makes their complete surgical excision impossible [76].

Highly motile and invasive glioblastoma cells specifically use the mesenchymal mode of migration and invasion [77]. Several signalling pathways involved in regulation of cell migration and closely related cytoskeleton remodelling such as integrin signalling and phosphatidylinositol-3 kinase (PI3-K) pathway are aberrant in glioblastoma [77, 78]. The regulation of cytoskeleton in glioblastoma cells is largely unknown. Some glioblastoma cell lines exhibit unusual regulation of cytoskeletal filaments, e.g. glioblastoma cells U87MG remain motile even in the absence of MFs and create MT-based protrusions. On the other hand, depolymerization of MTs stops their migration completely [79]. Furthermore, βIII-tubulin and γ-tubulin are highly overexpressed in glioma and glioblastoma cell lines. Moreover, γ-tubulin forms aggregates, which accumulate in the cytoplasm and at the cell periphery [80, 81]. Why particular tubulins are overexpressed and how MTs are regulated in glioblastoma cells remains to be elucidated.

II. AIMS OF THE STUDY

1) Development of new tools for measurement of MT dynamics and cell migration

2) Analysis of MT reorganization after mast cell activation and the role of Ca^{2+}-sensor STIM1 in this process.

3) Functional characterization of MT severing protein spastin in glioblastoma

4) Investigation of potential novel functions of mammalian gamma-tubulins

III. COMMENTS ON PRESENTED PUBLICATIONS

III.1 STIM1-directed reorganization of microtubules in activated mast cells

Hájková Z., Bugajev V., Dráberová E., **Vinopal S.**, Dráberová L., Janáček J., Dráber Pe., Dráber Pa. (2011). STIM1-directed reorganization of microtubules in activated mast cells. J Immunol. *186*, 913-923.

MTs are involved in processes leading to degranulation of mast cells [82-84]. We and others have previously shown that activation of mast cells either via specific aggregation of FcεRI or by unspecific activation with pervanadate leads to significant changes in MT organization [54, 85]. However, the precise role of MTs in degranulation is still incompletely understood.

In this work, we showed that activation of BMMCs induced by FcεRI aggregation or treatment with pervanadate or thapsigargin results in generation of plasma membrane protrusions containing MTs (MT protrusions). Quantification of MT (+) end dynamics after activation revealed increased density of MT tracks near the cell periphery. Formation of MT protrusions and changes in MT (+) end dynamics were dependent on extracellular Ca^{2+} influx, as they could be induced also by thapsigargin, an inhibitor of sarco/endoplasmic reticulum Ca^{2+} ATPases. Thapsigargin treatment causes depletion of Ca^{2+} from ER stores, which in turn induce an influx of extracellular Ca^{2+} controlled by store-operated Ca^{2+} entry (SOCE) [86]. It is well established that SOCE is essential for mast cell activation both *in vitro* and *in vivo* [87, 88] and can be triggered by FcεRI aggregation. Knock-down (KD) of STIM1, the key regulator of SOCE, impaired Ca^{2+} signalling, formation of MT protrusions and prevented redistribution of growing MTs after activation. Expression of siRNA-resistant STIM1 in STIM1-depleted BMMCs rescued the wild-type phenotype.

In accordance with previous studies [72, 73], we observed colocalization of STIM1 with a +TIP protein EB1 in resting BMMCs. Interestingly, we noticed that typical submembrane STIM1 puncta, which form due to STIM1 oligomerization and aggregation during the first minute after activation, are associated with MTs in the protrusions. This led us to hypothesize that MTs might be important for relocation of STIM1 oligomers to the plasma membrane, where they trigger opening of Orai channels and influx of extracellular Ca^{2+}. However, using TIRF microscopy-based live cell imaging, we directly showed that MTs are dispensable for relocation of STIM1 oligomers to the plasma membrane in BMMCs. While depolymerization of MTs had a significant impact on degranulation measured by β-glucuronidase release, Ca^{2+} uptake was only slightly affected, which correlated with our live cell imaging data.

STIM1-depleted cells exhibited defective chemotaxis toward Ag. Although we still do not know exactly, what is the function of the MT protrusions in activated BMMCs, the defective chemotaxis indicates a possible role of MT protrusions in sensing external chemotactic gradients of Ag or other signals recognized by mast cells. MT protrusions also significantly enlarge the cell surface. That might facilitate directional sorting of the membrane receptors and enhance the specific responsiveness of mast cells to various stimuli, since mast cells can participate in multiple cycles of activation and can be differentially activated to release distinct patterns of mediators, depending on the type and strength of the activating stimuli.

Taken together, we have shown that STIM1-induced SOCE and following enhanced levels of free cytoplasmic Ca^{2+} concentration are vital for degranulation, chemotactic response and MT reorganization in BMMCs. This reorganization of MTs results in the formation of MT protrusions considerably enlarging the surface of activated cells. We have also demonstrated, that the early relocation of STIM1 oligomers to the plasma membrane after activation is MT-

independent process in BMMCs. Our findings open new area for investigation of novel rational approaches to treatment of inflammatory and allergic diseases. Research on the role of Ca^{2+} signalling in MT growth and nucleation continues in our laboratory.

III.2 Microtubule-severing ATPase spastin in glioblastoma: increased expression in human glioblastoma cell lines and inverse roles in cell motility and proliferation

Dráberová E., **Vinopal S**., Morfini G., Liu P.S., Sládková V., Sulimenko T., Burns M.R., Solowska J., Kulandaivel K., de Chadarévian J.P., Legido A., Mörk S.J., Janáček J., Baas P.W., Dráber P., Katsetos C.D. (2011). Microtubule-severing ATPase spastin in glioblastoma: increased expression in human glioblastoma cell lines and inverse roles in cell motility and proliferation. J Neuropathol Exp Neurol. *70*, 811-826.

Previously, we described overexpression and aberrant patterns of noncentrosomal γ-tubulin in high-grade gliomas and showed that its expression level increases with the tumor grade [81]. βIII-tubulin is also abberantly expressed in glioma [89] and forms complexes with soluble γ-tubulin [80]. Since components of MT cytoskeleton are overexpressed in high-grade glioma, we started investigating glioma-specific expression and function of other proteins involved in MT regulation.

In this work we studied the expression and distribution of the MT severing protein spastin in 3 human glioblastoma cell lines (U87MG, U138MG, and T98G) and in clinical tissue samples representative of all grades of diffuse astrocytic gliomas. Although spastin has four isoforms in human, M87 variant was the most prevalent in the studied cells. Spastin expression was increased in neoplastic glial phenotypes, especially in glioblastoma when compared with lower grade glioma and with normal mature brain tissues. RT-qPCR and immunoblotting experiments revealed increased levels of spastin mRNA and protein expression in the glioblastoma cell lines versus normal human astrocytes.

Interestingly, we observed enrichment of spastin in the leading edges of cells in T98G glioblastoma cell cultures and in neoplastic cell populations in tumor specimens. This prompted us to test whether spastin contributes to increased motility of glioblastoma cells. We depleted spastin by siRNA (KD) and measured free migration of control and KD T98G cells. By quantification of cell motility we showed that depletion of spastin leads to a highly significant reduction of T98G motility. Surprisingly, we noticed that cells depleted of spastin proliferate significantly faster than control cells. These inverse roles of spastin in cell motility and proliferation are intriguing, especially its role in proliferation. Our findings indicate that (i) spastin might have an unknown MT-independent function in regulation of cell proliferation or (ii) that disruption of MT regulation resulting in decreased motility might activate cellular programs switching the cell to the proliferative mode.

Further investigation of spastin function in glioma will be important to elucidate mechanisms that could be potentially used for reprogramming of glioblastoma cells to the proliferative mode, which might increase their susceptibility to chemo- and radiotherapy.

In conclusion, we showed that spastin is overexpressed in glioma and its expression level rises with the grade of tumor. We also demonstrated for the first time that spastin is involved in regulation of cell motility and that its expression contributes to high motility and relatively reduced proliferation of T98G glioblastoma cells. Our work suggests new possible directions in research on MT arrangement and functions in glioblastoma cells. Currently, other MT severing proteins katanin and fidgetin are studied in respect of their role in cell motility and proliferation of glioblastoma cells in our laboratory.

III.3 Nuclear γ-tubulin associates with nucleoli and interacts with tumor suppressor protein C53

Hořejší B., **Vinopal S.**, Sládková V., Dráberová E., Sulimenko V., Sulimenko T., Vosecká V., Philimonenko A., Hozák P., Katsetos C. D., Dráber P. (2012). Nuclear γ-tubulin associates with nucleoli and interacts with tumor suppressor protein C53. J Cell Physiol. *227*, 367-382.

γ-Tubulin is a typical centrosomal protein, however, it can be found also in the cytoplasm, on cell membranes, condensed mitotic chromosomes, midbodies and along MTs in mitotic spindles [4]. In addition, γ-tubulin presence in the nucleus was reported in plants [32] and several studies suggested that γ-tubulin is a nuclear protein in animals as well [42, 43].

In this paper, we showed for the first time that γ-tubulin is present in the nucleolus. During our previous work on human glioblastoma cell lines exhibiting high expression of γ-tubulin, we repeatedly observed γ-tubulin immunostaining in the nucleus/nucleolus. The intensity of staining was dependent on used fixation procedures. We have found out that γ-tubulin can be reproducibly visualized in the nucleolus after methanol-acetic acid fixation or prolonged wash-out after methanol fixation. Immunostaining with antibodies against γ-tubulin and markers of nucleoli (UBF, nucleolin, fibrillarin) revealed γ-tubulin nucleolar localization not only in glioblastoma cells but also in other cell lines including normal human astrocytes, representing a non-transformed primary cell line.

We further confirmed γ-tubulin nucleolar localization by different anti-γ-tubulin antibodies, by expressing exogenous FLAG-tagged γ-tubulin in cultured cells and by depletion of γ-tubulin using siRNA. Immunoelectron microscopy revealed γ-tubulin localization outside fibrillar centres where transcription of ribosomal DNA takes place. In addition, γ-tubulin was detected using immunoblotting and immunofluorescence on isolated nucleoli.

Proteins can get into the nucleus during mitosis when the nuclear envelope is broken-down. A similar mechanism of nuclear entry was proposed for βII-tubulin [90]. By analysis of γ-tubulin localization during mitosis using confocal microscopy, we showed that γ-tubulin remains associated with nucleolar remnants suggesting that γ-tubulin can enter the nucleus/nucleolus during mitosis. γ-Tubulin contains putative nuclear localization sequence (NLS) and nuclear export sequence (NES); NLS has been recently experimentally verified [31, 33]. We tested, whether γ-tubulin is actively transported into the nucleus during interphase by blocking nuclear export via nuclear pores using nuclear export inhibitor leptomycin B. No significant accumulation of γ-tubulin in the nucleus was observed after leptomycin B treatment indicating that γ-tubulin is not transported to the nucleus by fast nuclear import mechanisms.

Additional experiments with γ-tubulin fused to a photoconvertible protein DendraII and heterokaryon assays also excluded fast nuclear import during interphase. However, faint staining of γ-tubulin-FLAG in the nuclei of untransfected mouse NIH-3T3 cells, forming heterokaryons with human U2OS cells transfected with γ-tubulin-FLAG, was detected 6 hours after the initiation of the experiment. It indicates that slow nuclear import of γ-tubulin occurs also in interphase.

Immunoprecipitation with anti-γ-tubulin antibody from nuclear extracts combined with mass spectrometry led to a discovery of a novel γ-tubulin interacting protein C53, also known as CDK5 regulatory subunit associated protein 3 (CDK5RAP3) or LXXLL/leucine-zipper-containing ARF-binding protein (LZAP). C53 is located at multiple subcellular compartments including nucleoli and participates in many cellular processes including NF-κB [91], G1/S [92, 93] and G2/M checkpoint signalling [94]. Overexpression of C53 in cultured cells weakens DNA damage induced G2/M checkpoint activation. Interestingly, co-expression of γ-tubulin with C53 neutralizes the inhibitory effect of C53 on DNA damage G2/M checkpoint activation.

These results suggest that γ-tubulin regulates C53 function and participates in G2/M checkpoint signalling.

Recently, the nucleolar localization of γ-tubulin has been confirmed independently by another research group [31]. A detailed description of γ-tubulin translocation to the nucleus and its novel nuclear function in regulation of S-phase progression have been also reported [33].

Collectively taken, we showed for the first time that γ-tubulin is present in the nucleolus. We identified a novel γ-tubulin interacting protein C53 and showed that, via its interaction with C53, γ-tubulin participates in the regulation of G2/M checkpoint. Our results support the increasingly accepted picture of γ-tubulin as a multifunctional protein whose role is not restricted only to MT (-) ends. Characterization of molecular mechanisms of γ-tubulin interaction with C53 and their functional consequences is underway in our laboratory.

III.4 γ-Tubulin 2 nucleates microtubules and is downregulated in mouse early embryogenesis

Vinopal S., Černohorská M., Sulimenko V., Sulimenko T., Vosecká V., Flemr M., Dráberová E., Dráber P. (2012). γ-Tubulin 2 nucleates microtubules and is downregulated in mouse early embryogenesis. PLoS ONE *7*: e29919.

In this work, we wanted to identify and characterize molecular mechanisms underlying functional differences between TUBG1 and TUBG2 suggested by Yuba-Kubo *et al.* [26]. We prepared FLAG-tagged human and mouse TUBG1 and TUBG2 and examined their subcellular localization and interactions with GCP2 and GCP4. All recombinant proteins localized properly to MTOCs such as centrosomes and mitotic spindle MTs and formed immunocomplexes with GCP2 and GCP4 being indistinguishable from each other.

Next, we tested whether TUBG2 can substitute for TUBG1 *in vivo*. We depleted endogenous TUBG1 in U2OS cells using siRNA and performed phenotypic rescue experiments by expressing siRNA-resistant mouse TUBG1-FLAG, mouse TUBG2-FLAG or human TUBG2-FLAG in these cells. The used siRNA was TUBG1-specific and immunobloting with anti-γ-tubulin antibody recognizing both TUBG1 and TUBG2 revealed that TUBG1 is a predominant γ-tubulin in U2OS cells. Depletion of TUBG1 resulted in severe mitotic spindle defects including monopolar and collapsed spindles, very similar to those observed in *Tubg1*$^{-/-}$ blastocysts [26], and induced accumulation of TUBG1-depleted cells in metaphase. As expected, mouse TUBG1-FLAG rescued wild-type phenotype and normal mitotic progression. Importantly, the same was true for both mouse and human TUBG2-FLAG indicating that TUBG2 is able to replace TUBG1 *in vivo*.

The above results indirectly implicated that TUBG2 is able to nucleate MTs. To prove it directly, we used the same experimental set-up and examined MT nucleating capability of TUBG2 by MT regrowth experiments. We focused on mitotic cells, because γ-tubulin is enriched on prophase and metaphase centrosomes [95] and we could expect a prominent effect on MT nucleation. In accordance with our previous results, mouse TUBG1-FLAG as a positive control, mouse TUBG2-FLAG and human TUBG2-FLAG rescued MT aster formation in TUBG1-depleted cells. These findings suggested that mammalian TUBG2 is capable of centrosomal MT nucleation in mitotic cells.

To strengthen the evidence of MT nucleation capability of TUBG2, we quantified MT formation by the tracking of MT (+) ends marked by EB1-GFP in interphase U2OS cells. For live cell imaging we prepared mouse TUBG1 and human TUBG2 tagged with a red fluorescent protein TagRFP and use shRNA-based system. MT formation was quantified in negative control cells, TUBG1-depleted cells and in TUBG1-depleted cells expressing either mouse TUBG1-

TagRFP or human TUBG2-TagRFP. We observed a significant reduction in the number of MT (+) end tracks in TUBG1-depleted cells. Both exogenous TUBG1 and TUBG2 rescued MT formation indicating that TUBG2 can take the place of TUBG1 also in interphase cells.

We showed by several methods that TUBG2 was able to nucleate MTs and substitute for TUBG1 in cultured cells. The inability of TUBG2 to do so in TUBG1-deficient blastocysts, where TUBG2 should have been present [26], was intriguing. Therefore we analyzed the expression of TUBG2 in wild-type mouse blastocysts in greater detail. Using RT-qPCR, we quantified mRNA levels of *Tubg1*, *Tubg2*, *Tubgcp2* and *Tubgcp5* in mouse oocytes, 2-cell stage embryos, 8-cell stage embryos and blastocysts. Surprisingly, *Tubg2* mRNA level decreased dramatically in the course of mouse preimplantation development, unlike mRNA levels of *Tubg1*, *Tubgcp2* and *Tubgcp5,* resulting in a very low amount of *Tubg2* transcript in the blastocyst. Although there had been no antibodies available distinguishing specifically between mammalian γ-tubulins, we were able to separate TUBG1 and TUBG2 using 2D-PAGE and identify their specific positions on the gel. By this method, we found out that while TUBG1 was abundant, there was a very low level of TUBG2 protein in the wild-type blastocyst, which was in a good agreement with our RT-qPCR data. In line with previous studies [26, 27], we also detected high levels of TUBG2 mRNA and protein in the brain.

Based on our data, we proposed an alternative interpretation of *Tubg1*$^{-/-}$ and *Tubg2*$^{-/-}$ phenotypes previously described in mice [26]. Endogenous TUBG2 could not replace missing TUBG1 in *Tubg1*$^{-/-}$ blastocyst, even though it can nucleate MTs, because it was not present in a sufficient amount. Behavioural abnormalities of *Tubg2*$^{-/-}$ mice do not necessarily imply just unknown function of TUBG2. They might also reflect the reduction of total γ-tubulin in the brain of *Tubg2*$^{-/-}$ mice, as TUBG2 is highly expressed in the brain [26, 27]. Yet, we cannot directly exclude the possibility that TUBG2 has some additional still unknown function(s).

In conclusion, we demonstrated that TUBG2 is able to nucleate MTs and substitute for TUBG1 *in vivo*. Further, we showed at both mRNA and protein level that TUBG2 expression is dramatically reduced in blastocysts in contrast to TUBG1. Our data indicate that TUBG2 cannot rescue TUBG1 deficiency in *Tubg1*$^{-/-}$ blastocysts, because it is not present in a sufficient amount. We propose that mammalian γ-tubulins are functionally redundant with respect to their MT nucleation activity.

IV. CONCLUSIONS

1. We developed a new method for quantification of MT (+) end dynamics in living cells. The method is based on semi-automatic tracking of MT (+) ends marked by a +TIP EB1-GFP. To quantify migration of glioblastoma cells, we developed a method for measuring of free cell migration by tracking their nuclei visualized by a vital DNA dye. These methods were successfully used in the presented publications and are now routinely used in our laboratory.

2. We found out that activation of BMMCs leads to the formation of plasma membrane protrusions containing MTs. These MT protrusions do not form in the absence of STIM1-induced SOCE suggesting that Ca^{2+} signalling might modulate MTs in activated BMMCs. Although STIM1 is associated with MTs in both resting and activated BMMCs, MTs are not required for STIM1-regulated SOCE. STIM1 depletion prevents not only formation of MT protrusion, but also causes defects in chemotaxis. We propose that MT protrusions might be involved in sensing external chemotactic gradients of antigen or other signals in mast cells.

3. We found out that spastin is overexpressed in glioma and glioblastoma cell lines and its overexpression can be linked to a trend toward high-grade malignancy. Glioblastoma cells depleted of spastin exhibit significantly lower motility but at the same time an increased proliferation rate. These novel and inverse roles of spastin in cell migration and proliferation need to be further investigated, as therapeutic targeting of spastin might be a novel approach to treatment of invasive gliomas.

4. We showed for the first time that γ-tubulin is present in the nucleoli of various cell lines of different tissue and species origin. γ-Tubulin can get into the nucleus during mitosis and interphase as well, however its interphase nuclear transport is very slow. We identified the multifunctional protein C53 as a new γ-tubulin interacting protein in the nucleus. We found out that γ-tubulin antagonizes the inhibitory effect of C53 on DNA damage G2/M checkpoint activation. Our results suggest that γ-tubulin has also other functions in addition to MT nucleation.

5. We demonstrated that mammalian TUBG2 is able to nucleate MTs and substitute for TUBG1 in cultured cells. Further, we found out that almost identical mammalian γ-tubulins can be reliably discriminated according to their electrophoretic properties by 2D-PAGE. We showed at both mRNA and protein level that, unlike TUBG1, TUBG2 expression is dramatically downregulated in mouse preimplantation development, which results in very low amount of TUBG2 in blastocysts. Our data indicate that TUBG2 cannot rescue TUBG1 deficiency in *Tubg1*$^{-/-}$ blastocysts, because it is not present in a sufficient amount. Based on our results we propose that mammalian γ-tubulins are functionally equivalent with respect to their MT nucleation activity.

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VI. LIST OF PUBLICATIONS

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