

BRIEF REPORT



CAP-Gly proteins contribute to microtubule-dependent trafficking via interactions with the C-terminal aromatic residue of α -tubulin

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SUMMARY

In mammals, the C-terminal tyrosine residue of α -tubulin is subjected to removal/re-addition cycles resulting in tyrosinated microtubules and detyrosinated Glu-microtubules. CLIP170 and its yeast ortholog (Bik1) interact weakly with Glu-microtubules. Recently, we described a Microtubule- Rho1- and Bik1-dependent mechanism involved in Snc1 routing. Here, we further show a contribution of the yeast p150Glued ortholog (Nip100) in Snc1 trafficking. Both CLIP170 and p150Glued are CAP-Gly-containing proteins that belong to the microtubule +end-tracking protein family (known as +Tips). We discuss the +Tips-dependent role of microtubules in trafficking, the role of CAP-Gly proteins as possible molecular links between microtubules and vesicles, as well as the contribution of the Rho1-GTPase to the regulation of the +Tips repertoire and the partners associated with microtubules.

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Introduction

Microtubules are one of the 3 main types of cytoskeletal network found in all eukaryotic cells. They are involved in a large array of key cellular functions including mitotic spindle formation and positioning, intracellular trafficking and cell polarization. Microtubules are assembled from α -tubulin/ β -tubulin dimers, which interact to form hollow tubes.¹ Because tubulin dimers are polarized and they assemble in a head-to-tail fashion, the resulting tube is also polarized, with so-called plus (+) and minus (–) ends.² In many cell types, the –ends of microtubules are embedded in microtubule-organizing centers near the nucleus.³ Microtubule +ends, in contrast, radiate into the cytoplasm and alternate between growing and shrinking phases.⁴ Thus, microtubules possess intrinsic dynamic properties,⁵ tuned by a wide repertoire of cellular effectors, underlying their versatile architectures in cells.² Some microtubule modulators bind directly to microtubules (motors, microtubule-associated proteins),⁶ while others bind to tubulin dimers (stathmin),⁷ sever microtubules (spastin, katanin)⁸ or chemically modify tubulin subunits (kinases, phosphatases, tubulin-modifying enzymes).⁹ Members of the family of tubulin-modifying enzymes catalyze reversible post-translational modifications including detyrosination,

polyglutamylation, glycylation and acetylation.^{9,10} In the tyrosination/detyrosination cycle, the C-terminal tyrosine of α -tubulin is removed from the peptide chain by an as-yet unidentified carboxypeptidase; tyrosine is added back onto the chain by a tubulin tyrosine ligase (TTL). This cycle generates 2 pools of tubulin, tyrosinated α -tubulin (Tyr-tubulin) and detyrosinated α -tubulin which has an exposed glutamate at its C-terminus, and is thus known as deTyr-tubulin or Glu-tubulin.¹¹ Most α -tubulins are initially synthesized as tyrosinated tubulin, and the tyrosine residue is cleaved on long-lived microtubules.¹² As the TTL uses only soluble tubulin dimers as substrates, detyrosinated microtubules must first be disassembled for re-tyrosination of their dimers before their re-assembly.^{13,14} At the molecular level, tubulin tyrosination-detyrosination is a well-known characteristic of microtubule dynamics.^{15,16} Stable/long-lasting microtubules with a low dynamicity often harbor detyrosinated tubulin, whereas tyrosinated tubulin is mostly found in dynamic microtubules. This difference in stability between Tyr- and Glu-tubulin enriched microtubules is not due to alterations to their intrinsic properties, but rather to the recruitment of regulators specific for the tyrosinated form of tubulin at

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microtubule +ends.^{17,18} The tubulin tyrosination cycle is involved in several physio-pathological mechanisms. Indeed, loss of TTL activity, which results in the accumulation of Glu-tubulin, confers a selective advantage on cancer cells during tumor growth,^{19,20} and TTL suppression in mice leads to a lethal disorganization of neuronal circuits.²¹

Tyrosinated tubulin-dependent effectors were first identified in studies using a *Saccharomyces cerevisiae* strain exclusively expressing an α -tubulin lacking its terminal aromatic residue (*tub1-Glu* strain) to model detyrosinated, Glu-tubulin.²² This work established that Bik1, the yeast ortholog of the mammalian microtubule-associated protein CLIP-170, is sensitive to the tyrosination status of the α -tubulin at microtubule +ends and that it is present in lower amounts at the microtubule extremity in Glu-microtubules.²² This property was later shown to be shared by the mammalian ortholog, CLIP170, and other proteins containing a CAP-Gly domain, such as p150Glued.²³ The CAP-Gly proteins, CLIP170 and p150Glued, are members of the microtubule +end-tracking protein family (known as +Tips). Strikingly, the 2 other proteins shown to be sensitive to microtubule tyrosination, the microtubule depolymerizing motor MCAK^{18,24} and the Centromere-associated protein E (CENP-E)/Kinesin-7,²⁵ also belong to the microtubule +end-tracking family. Tyrosination of microtubule +ends is therefore likely to define the repertoire of microtubule +end-associated (+Tip) proteins, ensuring spatio-temporal functions in the microtubular network.

Yeast CLIP170 ortholog, a key role in linking microtubule +ends to membrane trafficking

In a recent report, the *tub1-Glu* strain was once again used to investigate the role of the α -tubulin C-terminal aromatic residue, and we revealed that deletion of the C-terminal aromatic residue of α -tubulin induced defects previously associated with impaired endocytic trafficking.²⁶ First, the actin-binding protein Abp1, which normally labels forming endosomes, accumulated in comet tail structures. Second, the V-SNARE protein Snc1 was abnormally distributed within the cell.²⁶ Snc1 plays a major role in the secretory pathway as it promotes the fusion of vesicles with the plasma membrane during exocytosis.²⁷ Endocytosis-mediated recovery of Snc1 from the plasma membrane allows its re-use in subsequent rounds of membrane fusion.²⁸ The defect associated with the *tub1-Glu* mutation was particularly apparent during budding.²⁶ At this stage, Snc1 is normally enriched at the bud plasma membrane as a consequence of highly polarized trafficking in the direction of the bud and limited diffusion to the mother cell's

plasma membrane. This strong polarity is partly attributed to active endocytosis in the bud area allowing efficient recycling of the vesicular fusion machinery.²⁹ In *tub1-Glu* cells, the polarized localization of GFP-Snc1 is lost and the protein distributes homogeneously across the mother and bud plasma membranes.²⁶ Interestingly, other membrane cargoes transiting through the endocytic pathway are not noticeably affected, thus the mutation does not have a general effect on the internalization process *per se*.²⁶ The apparent specificity toward Snc1 may point toward cargo- or site-specific mechanisms. Given that a similar phenotype was observed with *ts*-mutants of tubulin, which affected microtubule polymerization and dynamics, we proposed that the C-terminal aromatic residue of α -tubulin plays a role in the context of dynamic microtubules.²⁶ Currently, the precise function of microtubules in this trafficking process is incompletely known. Using null mutants, we excluded a role in Snc1 trafficking for the dynein protein *DYN1* and for the 2 major kinesins *KIP2* and *KIP3*, but found that the +Tip protein Bik1 was involved.²⁶ Although the phenotype was less marked than with the *tub1-Glu* mutation, disruption of *bik1* led to loss of GFP-Snc1 polarized distribution.²⁶ Interestingly, expression of a constitutively active form of Rho1 that restored enrichment of Bik1 at the +end of *tub1-Glu* microtubules also complemented the *tub1-glu* phenotype with regard to Snc1 routing,²⁶ thus further supporting the key role played by the CAP-Gly protein in this microtubule tyrosinated +end-dependent mechanism (Fig. 1). Several results, mainly produced with systematic yeast 2-hybrid screens indicated that Bik1 is a partner of a large panel of trafficking-associated proteins (Table 1). Among these proteins, Bik1 interacts with End3, Ent2, Scd5 and Sla1, all of which are involved in the early steps of endocytosis. Taken together, these data are strongly indicative of a functional link between microtubules, Bik1 and actors of the endocytic machinery for efficient Snc1 trafficking. The chronology of interactions and the precise molecular mechanism (s) through which microtubules and Bik1 contribute to trafficking remain to be elucidated. Interestingly, CLIP170 was initially identified in mammals as “a link between endocytic vesicles and microtubules” by Pierre et al. (1992),³⁰ but since then experimental data regarding the function of CLIP170 in endosomal trafficking have remained sparse. In the vicinity of the bud plasma membrane, membrane remodeling and cell growth are sustained by active trafficking. The dynamic nature of microtubule +ends could allow Bik1-mediated recruitment of trafficking effectors from the environment, thus driving their enrichment at the bud tip and promoting membrane/cargo uptake in this specific area. These effectors could include endocytic adaptors as well as proteins

Table 1. Physical interaction of Bik1p and Nip100p with the endosomal, endocytic, and endocytosis GO features. Proteins are listed in α betical order. For Yeast 2 hybrid interactions (Y2H) entries are noted as BD (binding domain) or AD (activation domain) to signify the direction of the 2-hybrid interaction. Number refers to amino-acid used to do the Y2H test. 1. Y. Wang et al., Coiled-coil networking shapes cell molecular machinery. *Molecular biology of the cell* **23**, 3911 (Oct, 2012). 2. J. Ptacek et al., Global analysis of protein phosphorylation in yeast. *Nature* **438**, 679 (Dec 1, 2005). 3. B. L. Drees et al., A protein interaction map for cell polarity development. *The Journal of cell biology* **154**, 549 (Aug 6, 2001).

Bik1 interactors			
Gene	Type	This study	Description
END3	Y2H Bik1 (309–397) BD, End3 (310–349) AD (1)	Y2H Bik1(1–440) BD, End3 (1–349) AD	EH domain-containing protein involved in endocytosis, actin cytoskeletal organization and cell wall morphogenesis
ENT2	Y2H Bik1 (309–397) BD, Ent2 (213–245) AD (1)	Y2H Bik1 (1–440) BD, Ent2 (1–257) AD	Epsin-like protein required for endocytosis and actin patch assembly; contains clathrin-binding motif at C-terminus
SCD5	Y2H Bik1 (309–397) BD, Scd5 (840–869) AD (1)	N.D.	Protein required for normal actin organization and endocytosis; multicopy suppressor of clathrin deficiency
SLA1	Y2H Bik1 (309–397), Sla1 (587–620) AD (1)	N.D.	Protein required for assembly of the cortical actin cytoskeleton; interacts with proteins regulating actin dynamics and proteins required for endocytosis.
SNF7	Y2H Bik1 (309–397) BD, Snf7 (60–109) AD (1)	N.D.	One subunits of the ESCRT-III complex; involved in the sorting of transmembrane proteins into the multivesicular body (MVB) pathway.
STV1	Y2H Bik1 (309–397) BD, Stv1 (112–156) AD (1)	Y2H Bik1 (1–440) BD, Stv1 (1–196) AD	Subunit a of the vacuolar-ATPase V0 domain; Stv1p is located in V-ATPase complexes of the Golgi and endosomes
YCK1	Biochemical Activity (2)	N.D.	Palmitoylated plasma membrane-bound casein kinase I isoform; involved in morphogenesis, proper septin assembly, endocytic trafficking.
Nip100 interactors			
Gene	Type	This study	Description
DID4	Y2H Nip100 (198–375), Did4 (15–56) AD (1)	N.D.	Protein of the ESCRT-III complex, required for sorting of membrane proteins into MVB vesicles, and for delivery of vacuolar enzymes to the vacuole
END3	Y2H Nip100 (101–185) BD, End3 (310–349) AD (1)	Y2H Nip100(1–868) BD, End3 (1–349) AD	EH domain-containing protein involved in endocytosis, actin cytoskeletal organization and cell wall morphogenesis
IMH1	Y2H Imh1 (131–278) BD, Nip100 (101–185) AD (1)	N.D.	Protein involved in vesicular transport, mediates transport between an endosomal compartment and the Golgi
RHO1	Y2H Rho1 BD, Nip100 AD (3)	N.D.	GTP-binding protein required to activate the PKC1 pathway and β -1,3 glucan synthase, member of the rho subfamily of ras-like proteins, GTP-bound form
SNX4	Y2H Nip100 (198–375) BD, Snx4 (217–261) AD (1)	Y2H Nip100(1–868) BD, Snx4 (1–423) AD	Sorting nexin, involved in retrieval of late-Golgi SNAREs from post-Golgi endosomes to the trans-Golgi network and in cytoplasm to vacuole transport
TLG1	Y2H Nip100 (198–375) BD, Tlg1 (62–97) AD (1)	N.D.	Essential t-SNARE that mediates fusion of endosome-derived vesicles with the late Golgi
VAB2	Y2H Nip100 (198–375) BD, Vab2 (111–151) AD (1)	N.D.	Protein with a potential role in vacuolar function; likely member of BLOC complex involved in endosomal cargo sorting
VFA1	Y2H Nip100 (198–375) BD, Vfa1 (121–164) AD (1)	N.D.	Protein that interacts with Vps4p and has a role in vacuolar sorting; localizes to endosomes in a Vps4-dependent manner
VPS20	Y2H Nip100 (198–375) BD, Vps20 (141–170) AD (1)	N.D.	Myristoylated subunit of ESCRTIII, the endosomal sorting complex required for transport of transmembrane proteins into the MBV pathway to the vacuole
VPS60	Y2H Nip100 (198–375) BD, Vps60 (15–63) AD (1)	Y2H Nip100(1–868) BD, Vps60 (1–229) AD	Cytoplasmic and vacuolar membrane protein involved in late endosome to vacuole transport
DSC3 YOR223W	Y2H Nip100 (198–375) BD, YOR223W (164–195) AD (1)	Y2H Nip100(1–868) BD, DSC3 (1–292) AD	Subunit of the DSC ubiquitin ligase complex. Protein found in the ER and vacuole lumen; overexpression of DSC3 affects endocytic protein trafficking

controlling the addition of post-translational modifications used as internalization/sorting signals (Fig. 1). At this stage, it would be interesting to know whether trafficking is globally facilitated in the presence of Bik1 and Tyr-tubulin in the area of the bud or whether *bik1* disruption or the *tub1-Glu* mutation specifically impairs Snc1 uptake. In addition, several of the Bik1 partners identified are implicated in

cytoskeletal actin organization, which would allow local cross-talk between microtubule- and actin-dependent processes. In addition, or alternatively, Bik1 could be involved in tethering endocytic vesicles and facilitating their formation/movement along microtubules by translating the mechanical properties and dynamicity of microtubules (Fig. 1).

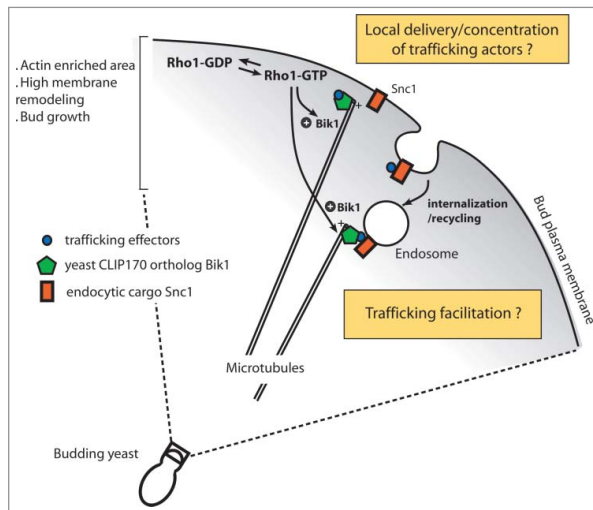


Figure 1. Possible roles for microtubules, yeast CLIP170 ortholog and Rho1 in trafficking. The bud is a site where membranes are highly dynamic and actin patches are concentrated. In this area, microtubules are oriented with their +end directed toward the distal region of the plasma membrane. The yeast CLIP170 ortholog, Bik1, preferentially accumulates at this extremity of microtubules, through a mechanism involving the active form of Rho1. Bik1-dependent linking of microtubules to the trafficking machinery could facilitate Snc1 routing by tethering vesicles, thus facilitating their locomotion or the local enrichment of endocytic actors at sites of cargo uptake.

The yeast p150Glued ortholog, another player in the game?

The fact that the *bik1*-null mutation had a diminished effect compared with that of the *tub1-Glu* mutation²⁶ led us to investigate a possible role for other members of the family of CAP-Gly-containing +end-tracking proteins. As shown for Bik1 in 2-hybrid experiments,²⁶ the yeast p150Glued homolog Nip100 interacts weakly with Glu-tubulin; this contrasts with Stu2 (yeast XMAP215), a +Tips protein that is devoid of CAP-Gly domains, which binds equally well to *wt* and Glu-tubulin (Fig. 2A). In *nip100*Δ mutant cells, GFP-Snc1p localization was abnormal, with less than 45% of cells displaying polarized distribution concentrated in the bud (Fig. 2B, C). Instead, and in contrast to the effect of the *bik1*Δ mutation, GFP-Snc1p accumulated on vesicles in the mother and bud cytoplasm (20% in *nip100*Δ cells vs. 8% in the parent; $p < 0.05$, Fisher's exact test), as well as in the vacuole (36.4% in *nip100*Δ cells vs. 5.4% in the parent; $p < 0.0001$, Fisher's exact test). Accumulation of GFP-Snc1 in the vacuole lumen would suggest that in the *nip100*Δ background the protein is not correctly transported and rather addressed to the vacuole where it gets

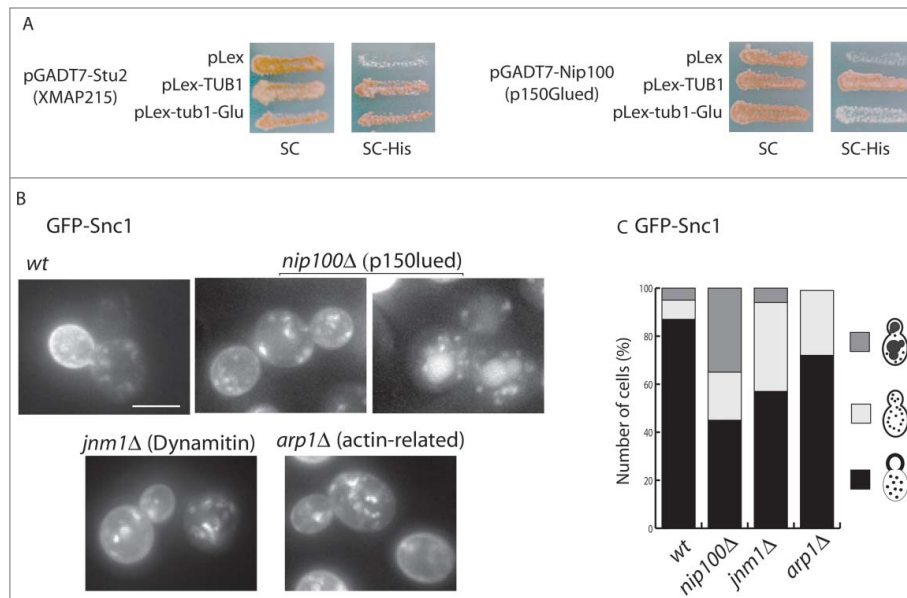


Figure 2. Nip100 contribution to Snc1 trafficking A) Analysis of the interaction between wild-type or mutated α -tubulin and the microtubule +end tracking proteins Nip100 (yeast p150Glued ortholog) and Stu2 (yeast XMAP215 ortholog) using the 2-hybrid system and selective medium (viability of yeast cells (SC) and positive interactions (SC-His). Impaired growth resulted in small white colonies; normal growth produced red colonies. B) Localization of GFP-tagged Snc1 expressed from a low-copy plasmid in *wt*, *nip100*Δ, *jnm1*Δ and *arp1*Δ strains. The cells were grown exponentially at 30°C and imaged as described.²⁶ C) Quantification of GFP-Snc1 signal in *wt*, *nip100*Δ, *jnm1*Δ and *arp1*Δ strains. The protein was detected either i) on cytoplasmic dots and at the bud plasma membrane (black), ii) on cytoplasmic dots homogeneously distributed throughout the mother and bud cells (light gray) or iii) on cytoplasmic dots and in the vacuole (dark gray). The percentage of cells in each category is indicated (*wt* $n = 62$, *nip100*Δ $n = 57$, *jnm1*Δ $n = 35$, *arp1*Δ $n = 37$ cells).

degraded. Nip100 is a subunit of the Dynactin protein complex, together with dynamitin (Jnm1) and the actin-related protein, Arp1. *jnm1Δ* and *arp1Δ* mutants showed similarly impaired GFP-Snc1 trafficking (Fig. 2B, C), indicating that Nip100 probably functions as part of the Dynactin complex in Snc1 routing. The phenotype observed in the *nip100Δ* strain is quite different to that observed in the *tub1-Glu* and *bik1Δ* mutants, suggesting that the roles played by Nip100 and Bik1 intervene at distinct steps in trafficking. This distinction may also reflect functions for Nip100 which may be partially independent of its interaction with the C-terminus of α -tubulin. Indeed, in mammals, a microtubule-independent role for p150-Glued at ER exit sites was described by Verissimo et al. (2015).³¹ Nevertheless, and very interestingly, Nip100 can also bind to actors in the endocytic pathway, mostly distinct from those interacting with Nip100 (Table 1). Indeed, Nip100 interacts with Snx4, Imh1, Tlg1, Vps20, and Vps60, which are known to be involved in trafficking between endosomes and late Golgi or late Golgi and the vacuole. Notably, and in striking contrast to Bik1 and Nip100, the +end-tracking protein Bim1, the yeast EB1 ortholog, which is insensitive to the tyrosination status of microtubules,^{22,23,32} has not been reported to interact with endosomal proteins. In line with this difference in interaction partners, *BIM1* deletion caused no alterations to Snc1 distribution.²⁶ Thus, even though the contribution of Nip100 to Snc1 trafficking requires further analysis, it is tempting to propose specific roles for CAP-Gly-containing proteins of the +Tips family in these trafficking events.

Rho1 spatially regulates the yeast CLIP170 ortholog at microtubule + ends

We recently discovered a role for Rho1 in controlling the localization of Bik1 at the +end of microtubules.²⁶ A constitutively active form of Rho1 relocalizes Bik1 to the +ends of Glu-microtubules (i.e., microtubules lacking the C-terminal aromatic residue of α -tubulin). This active form of Rho1 also enhanced Bik1 localization at microtubule +ends in a wild-type strain and induced a preferential accumulation of the protein at microtubule +ends within the bud.²⁶ Rho1 therefore functions as a spatial regulator of Bik1 localization.²⁶ Rho1 is the yeast ortholog of the mammalian GTP-binding protein RhoA and plays a key role in several processes underlying polarized cell growth.³³ Growth in yeast is limited by the presence of a cell wall, the cell therefore needs to organize regions with a weakened wall to allow the incorporation of new membranous material.³⁴ Coordinated regulation of cell wall biosynthetic

enzymes, vesicular trafficking and/or membrane delivery and actin organization is therefore necessary to sustain polarized growth. Rho1 is localized in the area of polarized growth³⁵ where it can directly regulate the activity of the (1,3)- β -glucan synthase (Fks1), which produces the linear polymer (1,3)- β -D-glucan, the main structural component responsible for the rigidity and strength of the cell wall.³⁵ Rho1 is also implicated in activation of the atypical protein kinase C, Pkc1, which regulates cell wall repair via effects on the mitogen-activated protein kinase Mpk1/Slt2 1 and the transcription factor Skn7.³³ More recently, a role for Rho1 in endocytosis was demonstrated, this role depends on at least 2 processes: a clathrin-dependent pathway involving the target of rapamycin complex 2 (TORC2), the Rho1 guanine exchange factor (GEF) Rom2, and the β -glucan synthase Fks1;³⁶ and a clathrin-independent pathway involving the formin Bni1 and the Rho1 GEF Rom1/2.^{37,38} Rho1 is also involved in polarized secretion through the exocyst component Sec3.³⁹ Finally, Rho1 controls actin organization through activation of the actin cable-assembling formin Bni1.³³ Our results suggest a new role for Rho1 in enhancing the amount of Bik1 at microtubule +ends, thus it could contribute to Bik1- and microtubule +end-dependent processes.²⁶ Through the regulation of Bik1 localization, Rho1 could coordinate additional events which are essential for efficient polarized growth, such as microtubule assembly in the environment of the bud tip, modulation of trafficking and thereby membrane remodeling, targeting of dynein to the cortex and subsequent positioning of the spindle between mother and daughter cells. How Rho1 controls Bik1 localization at microtubule +ends is currently unclear; no direct interaction between Bik1 and the constitutively active form of Rho1 was observed in 2-hybrid assays (data not shown). The GTP-bound form of Rho GTPases binds a variety of partners including kinases and scaffolding proteins. Both Bik1 and CLIP170 are phosphoproteins, and phosphorylation of CLIP170 has been shown to control its association with microtubule +ends.⁴⁰⁻⁴² In addition, in yeast, Bik1 is actively transported to the microtubule +ends by the kinesin Kip2, which facilitates its targeted localization.^{32,43} Recently, Kip2 phosphorylation by the yeast kinase GSK3 β was demonstrated to tune its association with microtubules.⁴⁴ A simple hypothesis is that Rho1 modulates Bik1s association with microtubules through the recruitment of specific kinase(s)/phosphatase(s) which control Bik1 phosphorylation or the phosphorylation state of other partners in its interaction with microtubules. The +end-tracking protein Bim1 is known to contribute to Bik1 +end localization in the *tub1-Glu* strain,³² but whether Rho1 affects Bim1 localization at microtubule +ends or its interaction with Bik1 has yet to be investigated.^{32,45,46} Rho1 has been demonstrated to interact with Nip100⁴⁷ (Table 1),

suggesting that it might regulate this CAP-Gly protein. A detailed analysis of Rho1s contribution to the recruitment and affinity of all microtubule +end-tracking proteins will need to be performed to gain a more comprehensive view of the role played by this GTPase on +Tips proteins and a possible specificity in the regulation of CAP-Gly proteins.

CAP-Gly protein-dependent role(s) of dynamic tyrosinated microtubules in membrane trafficking in other cellular systems

In mammals, the participation of microtubules in vesicular trafficking is well documented. In neurons, a network of stable detyrosinated microtubules ensures long-range transport along the axon and dendrites through motor-dependent mechanisms. Dynein is required for retrograde transport from the periphery to the cell body, whereas kinesins are responsible for trafficking in the opposite, anterograde, direction. Recent work from Nirschl and colleagues (2016)³³ established a role for CLIP170 in the growth cone in the initiation of dynein-driven transport. In view of our results from the yeast model indicating a Bik1-dependent dynein-independent role for microtubules, we propose that CLIP170 and possibly p150Glued also participate in trafficking through a mechanism not requiring dynein, but relying on their specific binding to the tyrosinated +end extremity. The distal region of growth cones is an area with active membrane/cargo trafficking where microtubule + extremities are present in large numbers. Their enrichment in CLIP170 might similarly favor vesicular trafficking through the delivery of appropriate trafficking regulators or vesicle tethering. A CLIP170-mediated link between microtubules and vesicles may also confine newly internalized vesicles to this particular area where +end tyrosinated extremities are enriched, and restrain their trafficking to this area. Such a role could contribute to the maintenance of growth cone polarity/growth. Along this line, in TTL-deficient neurons, where the amount of tyrosinated dynamic microtubules is dramatically reduced, severe axonal growth defects have been observed *in vivo*.^{23,48} Whether RhoA plays a regulatory role in this CAP-Gly-mediated function remains to be determined; indeed, a role for RhoA activity in growth cone extension and development in the nervous system seems to be dependent of the neuronal cell type.^{49,50}

In conclusion, the role of microtubules in endocytosis and related trafficking aspects in yeast has been poorly documented up to now.⁵¹⁻⁵⁴ Our work, initially based on an unbiased yeast genetic screen²⁶ shed light on the contribution of microtubules to yeast trafficking and further shows the specific contribution of

microtubule +ends and CAP-Gly proteins to this process.

Methods

Yeast strains and plasmids

Strains used in this study were obtained from euroscarf (<http://www.euroscarf.de>). The genetic background was BY4741 (*MAT α* , *his3 Δ* , *leu2 Δ 0*, *lys2 Δ 0*, *ura3 Δ 0*). Cells were grown in yeast extract/peptone/glucose (YPD) rich medium, or in synthetic complete (SC) medium containing 2% (w/v) glucose. The GFP-Snc1 construct and methods for observation of its phenotype were described previously.²⁶ For the 2-hybrid experiments the strain provided by Promega was L40 (*MAT α* , *his3- Δ 200*, *trp1-901*, *leu2-3,112*, *ade2 lys2-801am LYS2::(*lexAop*)4-HIS3, URA3::(*lexAop*)8-lacZ GAL4*). The *TUB1*, *tub1-Glu* genes, from pRB539 and pRB539Glu,²² *BIK1* (full length) and *NIP100* (full length) from genomic DNA were cloned in the pLexA vector (addgene) as a fusion construct with the DNA-binding domain of LexA. *NIP100* (full length), *STU2* (full length), *END3* (full length), *ENT2* (1-257), *STV1* (1-196), *SNX4* (full length), *VPS60* (full length), *DSC3* (full length) from genomic DNA were cloned into the pGADT7 vector (Invitrogen) in fusion with the GAL4 activating domain.

Microscopy and image analysis

Cell imaging was performed on a Zeiss Axiovert microscope equipped with a Cool Snap ES CCD camera (Roper Scientific). Images were captured using 2×2 binning and 12 sequential z-slices were collected at 0.3- μ m step intervals with an exposure time of 200 ms. All image manipulations and montages were performed using Image J.⁵⁵

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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