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MICROTUBULE AND SPINDLE POLE BODY REGULATION IN THE BUDDING YEAST MITOTIC SPINDLE

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Microtubule and Spindle Pole Body regulation in the budding yeast mitotic spindle

Thesis for Doctoral Degree (Ph.D.)

By

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To my late father

&

My beloved family

"Research is to see what everybody else has seen, and to think what nobody else has thought."

Albert Szent-Györgyi

Popular science summary of the thesis

As humans, we have many shared genes with other living organisms. Due to this, scientists use simple model organisms to discover genetics and molecular mechanism in humans. One of these organisms is baking yeast or *Saccharomyces cerevisiae*. Although yeast and human separated one billion years ago during evolution, they possess genes with the same function. Therefore, studies on simple organisms like yeast help us to understand biological processes related to humans.

One of the organelles that have been studied extensively in yeast is the centrosome (called Spindle Pole Body in yeast), a multiprotein complex. During cell division, genetic information must be transmitted from one generation to another. Chromosomes are structures made of DNA and proteins which carry genetic information. The centrosome is key for accurately segregating the genetic material between mother and daughter cells, using long chains of cylindrical structures termed microtubules. There are three types of microtubules with different functions in yeast, kinetochore microtubules, interpolar microtubules, and cytoplasmic microtubules. Several proteins must bind to each type of microtubule to regulate microtubule dynamics.

In each cell division, the centrosome duplicates, and the DNA replicates to form two identical copies of each chromosome (sister chromatids) that then divide equally between the two cells. A single kinetochore microtubule connects with one of the chromosomes throughout a multiprotein complex called kinetochore and, in coordination with the interpolar and cytoplasmic microtubules, align them in the proper direction and divide them between two dividing cells. Overall, microtubules, kinetochore, and chromosomes form a structure called mitotic spindle to segregate duplicated chromosomes. At the end of the cell division, each cell inherits one centrosome and one set of genetic information. Interestingly the two inherited centrosomes are different in age, one is old (from the previous cell division) and another is newly formed.

In this thesis, we used budding yeast as a model organism to discover mechanisms related to microtubule and Spindle pole body regulation.

In Study **I**, a technique was developed in our lab to separate the aged and the young Spindle Pole Body proteins to monitor their post-translational modifications, which are biochemical changes that occur after the protein is synthesized and alter the protein behavior or function.

It was previously reported the old and new Spindle Pole Bodies have different post-translational modifications essential for their proper inheritance. Applying our technique to a yeast Spindle Pole Body protein (Spc110), we found three phosphorylation (attachment of a phosphate group) sites specific to old Spc110. To understand the function of the identified phosphorylations, the cells were modified genetically to prevent phosphorylation from occurring. The results indicated that the detected phosphorylation sites are important for the timely progression of cell division and proper regulation of microtubule dynamics.

In study **II**, we focused on a protein that binds and regulates microtubules, Bik1. Bik1 is located on two types of microtubules, kinetochore microtubules and cytoplasmic microtubules. We used a system to kick off Bik1 from the kinetochore microtubule and investigated its function. Without Bik1 at kinetochore microtubules, cell cycle progression was delayed, and cells had an atypical kinetochore positioning. Furthermore, using biochemical and genetic experiments, we found that Bik1 cooperates with two motor proteins (proteins that can "walk" along the microtubules): Kip1 and Cin8. Thus, the study revealed a novel function of Bik1 and its association with the cell cycle.

In Study **III**, we investigated the stability of the Spindle Pole Body protein, Spc110. Previous research has shown the old Spc110 in the Spindle Pole Body is exchanged with the new Spc110 during the cell cycle. We observed that Spc110 degradation is very fast. Using yeast mutants, we showed that autophagy – a cellular pathway responsible for protein and organelle degradation – was not necessary for Spc110 degradation. On the other hand, we identified a distinct region on Spc110 that might potentially bind to a protease, a protein that can break its protein substrates into two peptides, which can undergo degradation upon cleavage. We genetically modified the region to inhibit protease binding and observed a relative stabilization of Spc110, indicating a possible involvement of protease in Spc110 degradation.

The thesis proposes new molecular mechanisms involved in microtubule dynamics, yeast Spindle Pole Body regulation, and cell division.

Abstract

The microtubule organizing center (MTOC) is a specialized structure with a main function in microtubule (MT) nucleation and organization. In higher eukaryotes, the main MTOC is known as the centrosome, and its functional equivalent in yeast is the spindle pole body (SPB). During mitosis, the cells build the mitotic spindle, an MT-based structure that mediates accurate chromosome segregation, which is essential to prevent aneuploidy and cancer. In yeast, the assembly and function of the mitotic spindle depend on the SPB, microtubule-associated proteins (MAPs), and motor proteins. In this thesis, we aim to advance our understanding of the general principles that regulate the SPBs, and spindle MTs in budding yeast.

In Paper **I**, we developed a method to separate the old (from the previous cell cycle) and newly synthesized SPB component Spc110 and identified age-specific phosphorylation residues in Spc110. We combined Recombination Induced Tag Exchanged – a genetic method to label old and new proteins differentially- with affinity purification. Using mass spectrometry analyses, we identified two phosphosites, S11 and S36, in old Spc110. We explore the function of these two phosphosites in non-phosphorylatable Spc110 mutants. Cells expressing $Spc110^{S11A}$ showed a distinct spindle phenotype, where tubulin intensity is higher and distributed asymmetrically. Furthermore, the double mutant $Spc110^{S11AS36A}$ was slightly delayed in cell cycle progression and re-entry in G1. Thus, we propose Spc110 phosphorylation at S11 regulates MT dynamics, whereas together with S36 regulates timely cell cycle progression in budding yeast.

In Paper **II**, we explored the role of the MT plus-end tracking protein (+TIP) Bik1 in the nucleus of budding yeast. Bik1 has a nuclear and a cytoplasmic pool, and we found that nuclear Bik1 localizes to the kinetochores in a cell cycle-dependent manner, peaking at metaphase. To explore the role of Bik1 at kinetochores, we added a Nuclear Export Signal (NES) to generate a Bik1-NES mutant that excludes Bik1 from the nucleus without disrupting its cytoplasmic pool. The Bik1-NES mutant had a slower cell-cycle progression, characterized by prolonged metaphase. Furthermore, we demonstrated that the kinetochores in Bik1-NES cells are frequently unclustered and mispositioned towards the spindle midzone in metaphase. By applying proximity-dependent methods, we identified kinesins Cin8 and Kip1 as Bik1 interactors. Bik1 and Cin8 cooperate to regulate kinetochore-MT dynamics for chromosome

congression. Hence, the study uncovers a novel role of kinetochore Bik1 in cell cycle progression and chromosome congression.

In Paper **III**, we examined the turnover of Spc110 using cycloheximide (CHX) chase experiments. The results indicated that Spc110 has a very short half-life. Performing CHX-chase experiments with the autophagy-defective mutant atg1Δ, we showed that autophagy is not essential for Spc110 degradation. On the other hand, Spc110 contains a potential Esp1 recognition site (the yeast homolog of Separase). Mutation of this Esp1 recognition site on Spc110 results in partial Spc110 stabilization. Hence, we speculate that Esp1 might be involved in Spc110 degradation.

List of scientific papers

I. Phosphosites of the yeast centrosome component Spc110 contribute to cell cycle progression and mitotic exit. **Marjan Abbasi**, Alexander Julner, Yang Tim Lim, Tianyun Zhao, Radoslaw Mikolaj Sobota, Victoria Menéndez-Benito. Biology Open. 2022, 11(11): bio059565

II. The microtubule plus-end tracking protein Bik1 is required for chromosome congression. Alexander Julner, **Marjan Abbasi**, Victoria Menéndez-Benito. Molecular Biology of the Cell. 2022, 33(5), br7

III. Studies on Spc110 turnover. **Marjan Abbasi** and Victoria Menéndez-Benito. Manuscript

Contents

List of abbreviations

+TIP	Plus-End Tracking protein
aMT	Astral Microtubule
APC/C	Anaphae Promoting Complex/Cyclosome
ATG	Autophagy Gene
BiFC	Bimolecular Fluorescence Complementation
BioID	Proximity-Dependent Biotin Identification
CA	Centrosome Amplification
CDK	Cyclin-Dependent Kinase
CHX	Cycloheximide
CMD	Calmodulin
CP	Central Plaque
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeat
Cvt	Cytoplasm-to-Vacuole targeting
DP	Duplication Plaque
DUB	Deubiquitin Enzymes
EM	Electron Microscopy
FEAR	Fourteen Cdc Early Anaphase Release
IL1	Intermediate Layer 1
IL2	Intermediate Layer 2
IP	Inner Plaque
ipMT	Interpolar Microtubules
kMT	Kinetochore Microtubule
KT	Kinetochore
MAP	Microtubule-Associated Protein
MEN	Mitotic Exit Network

MT	Microtubule
MTOC	Microtubule-Organizing Center
NE	Nuclear Envelope
NEBD	Nuclear Envelope Break Down
NES	Nuclear Export Signal
Ni-NTA	Nitriloacetic Acid
NPC	Nuclear Pore Complex
OP	Outer Plaque
PAM	Protospacer Adjacent Motif
PAS	Phogophore Assembly Site
PCM	Pericentriolar Material
PTM	Post-Translational Modification
RITE	Recombination Induced Tag Exchange
RP	Regulatory Particle
S	Serine
SAC	Spindle Assembly Checkpoint
S. cerevisiae	*Saccharomyces cerevisiae*
SPB	Spindle Pole Body
SPOC	Spindle Position Checkpoint
Thr	Threonine
Tio2	Titanium Dioxide
TOR	Target of Rapamycin
Ub	Ubiquitin
UPS	Proteasome-Ubiquitin System
WT	Wild Type
γ-TuSC	Gamma Tubulin Small Complex

1. LITERATURE REVIEW

1.1. Spindle pole body

The term centrosome was introduced by Theodor Bovari in 1887, and ever since, there has been extensive progress in our knowledge about this organelle. The centrosome is the main organelle serves as microtubule-organizing center (MTOC) in animal cells. It is composed of one pair of microtubule (MT)-based cylinders called centrioles surrounded by a matrix of proteins known as pericentriolar material (PCM). The centrosome has a highly ordered structure, and its biogenesis is tightly tethered to the cell cycle. Centrosome facilities many cellular activities, including mitotic spindle assembly, cell polarity and migration, neurogenesis, cell-cycle progression, and accurate chromosome segregation, and works as a signaling platform for numerous signaling molecules. Notably, centrosomal abnormalities lead to many diseases; for instance, centrosome amplification (CA) has been reported in several cancer types (Schatten, 2008; reviewed in M. Lin et al., 2022). Accordingly, discovering new centrosome regulation mechanisms would improve our understanding of this organelle and might guide us to developing novel therapeutic strategies for cancer treatment.

In yeast, the spindle pole body (SPB) is a structure functionally equivalent to the centrosome. The SPB is the only MTOC in the budding yeast *Saccharomyces cerevisiae* and is involved in different functions, including chromosome segregation during mitosis and meiosis, cell cycle progression, cytokinesis, and intracellular trafficking (Cavanaugh & Jaspersen, 2017). Although centrosomes and SPBs are different in size, complexity, and structure, they share the primary role of MT nucleation. Prior research has thoroughly investigated several components localized on centrosomes and SPBs involved in mitotic progression and cytokinesis (Gromley et al., 2003; M. Lin et al., 2022). Furthermore, SPBs and centrosomes contain proteins with the same function, including scaffold proteins (Spc110/pericentrin and Nud1/centriolin), proteins involved in SPB and centrosomal duplication (Sfi1/Cdc31/centrin), and MT nucleation (γ-tubulin complex) (Table 1) (Cavanaugh & Jaspersen, 2017).

Our knowledge in centrosome regulation has been gained quickly using yeast as the model organism to decipher SPB structure, function, and regulation. The study on SPB improved our understanding of centrosome duplication and its involvement in cell cycle division.

1.1.1. SPB structure

During mitosis, MTs are critical to segregate chromosomes accurately. The yeast cells undergo closed mitosis in which the nuclear envelope (NE) remains intact through the yeast life cycle. The SPB must be embedded in the NE and nucleate MTs in the nucleus (nuclear MTs) and the cytoplasm (cytoplasmic MTs). Cytoplasmic MTs drive nuclear positioning and spindle orientation, and nuclear MTs play a key role in accurate chromosome segregation (reviewed in Cavanaugh & Jaspersen, 2017).

The structure of the budding yeast *S. cerevisiae* SPB was first described by electron microscopy (EM) in 1966 as a cylindrical organelle embedded in NE (Robinow & Marak, 1966). This study showed that the SPB consists of three layers: an outer plaque (OP), an inner plaque (IP), and a central plaque (CP). The OP and IP face the cytoplasm and the nucleus, respectively, and are essential for MT nucleation. The CP serves as an anchoring site to the NE. Later studies using cryo-EM and electron tomography showed that the SPBs contain additional layers, named intermediate layers 1 and 2 (IL1 and IL2), located between the OP and the CP (Figure 1) (Byers & Goetsch, 1975; Bullitt et al., 1997; reviewed in Jaspersen & Winey, 2004). EM studies also identified an electron-dense region that extends from one side of the CP called the half-bridge. The half-bridge main function is to provide a platform for SPB duplication. Four proteins form the half-bridge: Kar1, Sfi1, and Cdc13, localized to the cytoplasmic side of the NE, and Mps3, localized to the nuclear side (Figure 1) (Baum et al., 1986; Spang et al., 1995; Jaspersen et al., 2002; Kilmartin, 2003). Kar1 and Mps3 are integral membrane proteins that anchor the half-bridge to NE. In early G1, the half-bridge elongates and creates a full-bridge. Then, a precursor of the new SPB, the satellite, seeds on the distal part of the bridge, grows and matures to form a new SPB (Byers & Goetsch, 1975; Adams & Kilmartin, 1999). The detailed SPB duplication is explained in section 1.1.4.

Furthermore, careful measurements of the SPB structure showed that the SPB size changes in diameter from G1 to mitosis (80 nm to 110 nm), while its height (the distance between the inner and outer plaque) remains constant (Byers & Goetsch, 1975; reviewed in Jaspersen & Winey, 2004). The increase in SPB size indicates that it has a dynamic structure, as a further study revealed in terms of the exchange of SPB components in and out of the complex (Yoder et al., 2003). Specifically, this study showed that fluorescently tagged Spc110 is added to the SPB late in the cell cycle (at late G2 and M), remains stable in G2, and is exchanged with newly synthesized Spc110 in G1/S. Moreover, Spc110 and Spc42 have been monitored using

recombination-induced tag exchange (RITE), a system to differentially label preexisting and newly synthesized proteins upon Cre-recombinase switching of epitope tags (Verzijlbergen et al., 2010) (for more details about RITE system, see section 3.2). Studies using RITE showed that during cell division, newly synthesized SPB proteins, Spc110 and Spc42, were incorporated into both old and new SPBs, indicating a dynamic exchange (Menendez-Benito et al., 2013; Lengefeld et al., 2018). However, little is known about how the SPB dynamics are regulated.

1.1.2. SPB Components

The estimated size for diploid SPB, including MTs and microtubule-associated proteins (MAPs), is around 1-1.5 GDa. In total, 18 proteins form the SPB (Figure 1), 16 out of 18 subunits are encoded by essential genes, and two of them, Spc72 and Cnm67, are encoded by genes that are non-essential in some genetic backgrounds (reviewed in Cavanaugh & Jaspersen, 2017). The function of each subunit is listed in table 1.

Table 1. *S. cerevisiae* SPB components and their homologs in humans. Among the 18 identified SPB proteins, 12 have homologous components in humans.

S. cerevisiae	H. sapiens	Biological process in yeast
Tub4	GCP1	Microtubule nucleation
Spc97	GCP2	Microtubule nucleation
Spc98	GCP3	Microtubule nucleation
Spc110	Pericentrin	Links γ-tubulin complex to SPB
Spc72	CDK5RAP1	Links γ-tubulin complex to SPB
Spc42	--	Satellite/central plaque structure
Spc29	--	Satellite/central plaque structure
Cnm67	--	Satellite/central plaque structure
Cmd1	Calmodulin	Binds to Spc110
Nud1	Centriolin	Signaling
Ndc1	Ndc1	Nascent SPB insertion
Bbp1	--	Nascent SPB insertion
Nbp1	--	Nascent SPB insertion
Mps2	KASH	Nascent SPB insertion
Sfi1	Sfi1	SPB duplication/cytoplasmic side of half bridge
Cdc31	Centrin 3	SPB duplication/ cytoplasmic side of half bridge
Kar1	--	SPB duplication/half bridge structure
Mps3	Sun1/2	SPB duplication/ half bridge structure

Diverse approaches have been used to identify SPB components, including monoclonal antibodies against SPB subunits (Rout & Kilmartin, 1990), mass spectrometry analysis of enriched SPB samples (Wigge et al., 1998), genetic screens, and co-purification with known SPB proteins (reviewed in Jaspersen & Winey, 2004). Besides, the localization of the identified proteins to the SPB was proven by immunofluorescence microscopy, immune-EM, and yeast two-hybrid analyses. Several SPB components contain one coiled-coil domain involved in protein-protein interactions (Wigge et al., 1998; Jaspersen & Winey, 2004; Winey & Bloom, 2012).

The CP, IL1, and IL2 form the SPB core that contains Spc110, Cmd1, Spc42, Spc29 and Cnm67 (Figure 1). Spc42 is a protein with an N-terminal dimeric coiled-coil that scaffolds the SPB. Spc42 binds to Spc110 and Spc29 at the nuclear side, while its C-terminus extends from the CP to the IL2 and binds to Cnm67. The Spc42 is involved in SPB formation and duplication (Donaldson & Kilmartin, 1996; Adams & Kilmartin, 1999; Theos et al., 2005). Cnm67 is another coiled-coil protein that connects the SPB OP and CP. Both the N- and C-terminal domains of Cnm67 are essential for OP formation (Schaerer et al., 2001). Cnm67 interacts with Spc42 at its C-terminus while binding to Nud1 at its N-terminus. Nud1 is an OP protein that plays a role in mitotic exit and, by binding to Spc72, organizes cytoplasmic MTs (Gruneberg et al., 2000b).

Another essential coiled-coil protein is Spc110. The C-terminus of Spc110 associates with the CP via binding to Spc29, Spc42, and calmodulin (Cmd1) through its conserved PACT domain (pericentrin-AKAP450 centrosomal targeting). Disrupting the interaction between Spc110 and Cmd1 results in an aberrant SPB assembly (Alonso et al., 2020). On the nuclear side, the N-terminal of Spc110 binds to Spc98/Spc97 (Sundberg et al., 1996; M. Knop, 1997). In the OP, Spc72 binds to Nud1, and its N-terminus binds to Spc97/Spc98 (Gruneberg et al., 2000b). Temperature-sensitive spc72 mutant cells lack proper spindle elongation and chromosome segregation (X. P. Chen et al., 1998).

γ-tubulin is the most conserved SPB protein and is required for MT nucleation. In budding yeast, two copies of γ-tubulin (Tub4) form a stable complex with Spc97 and Spc98, referred to as the γ-tubulin small complex (γ-TuSC). The γ-TuSC assembles in the cytoplasm and is then anchored to both the OP and IP via two receptors, Spc72 and Spc110, to nucleate MTs at both cytoplasmic and nuclear sides (Michael Knop & Schiebel, 1998; Whitfield et al., 2002).

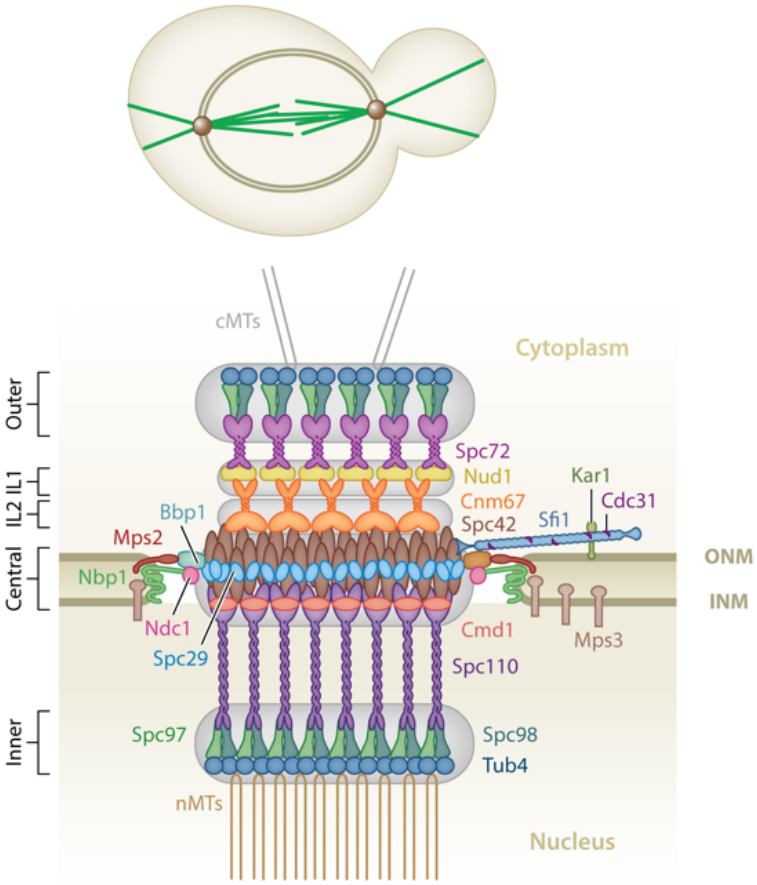

Figure 1. The structure of the SPB in budding yeast. Top: Budding yeast SPBs (brown spheres) nucleate MTs (green strands) inside the nucleus and cytoplasm. Bottom: The SPB is composed of five layers in budding yeast. cMTs: cytoplasmic microtubule, nMTs: nuclear microtubules, IL1: intermediate layer 1, IL2: intermediate layer 2. The figure reprinted with permission from the publisher (Cavanaugh & Jaspersen, 2017).

1.1.3. The yeast cell cycle

As in any other eukaryotes, the cell cycle of yeast consists of four phases: G1 (the "gap" between the end of the last division and DNA replication, in which the cell grows), S (DNA synthesis phase), G2 (the "gap" between S and M) and M (mitosis phase, in which the chromosomes are segregated, and mother and daughter cells are separated) (Figure 2).

S. cerevisiae divides asymmetrically, with a large mother cell budding a small daughter cell. The cells grow in a polarized manner, and the bud site determines the division plane (Figure 2). Therefore, the mitotic spindle must be positioned along the mother-daughter polarity axis, for accurate chromosomal segregation. As the sole MTOC in yeast, the SPB has a major role in chromosome segregation.

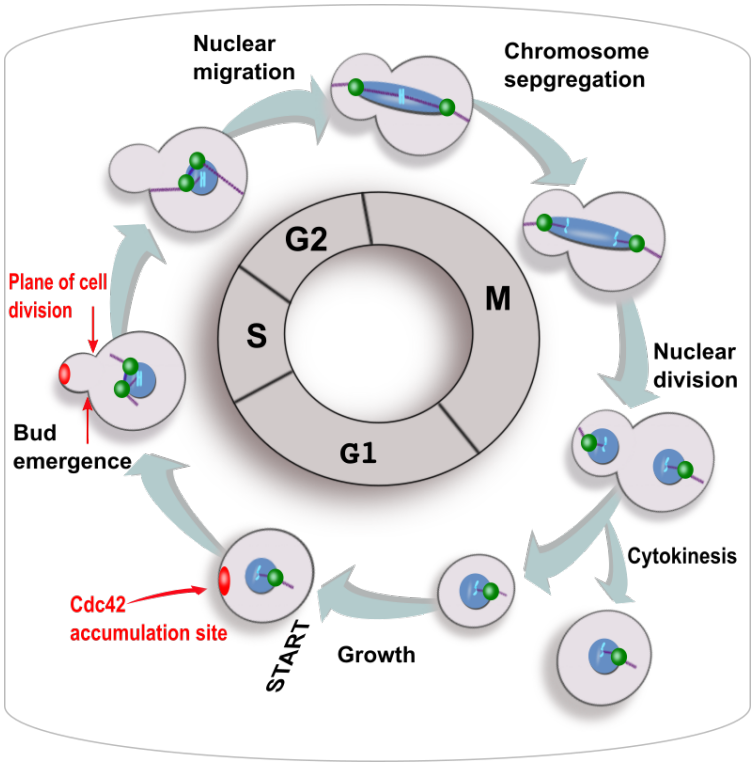

Figure 2. Diagram of Budding yeast cell division. The four cell division phases are represented in the inner ring (G1, S, G2, and M). Outside the ring, important events during yeast cell cycle progression are depicted, gray cells with blue nucleus. Green sphere: spindle pole body, dark purple strands: microtubules, light blue strand in the nucleus: chromosome.

In budding yeast, a specified site is established on the mother cell cortex by the accumulation of Cdc42, a small Rho-family GTPase, at G1 (K. E. Miller et al., 2020). The bud will emerge at this region at the beginning of the S phase which determines the polarity axis and the division plane and marks the site for future cell divisions in haploid cells (Figure 2). The septin- and actin-cytoskeletons and other polarity proteins accumulate at the bud site, and the bud initiates to grow from the tip (apical growth) (Pruyne et al., 2004). As the bud emerges from the mother cell, DNA replication and SPB duplication are initiated to prepare the cells for mitosis. In G2, the daughter cell grows evenly in all directions, a process termed isotropic growth. At G2/M, the nucleus migrates towards the mother-bud neck and orients itself along the polarity axis in a way that one SPB is positioned near the mother-bud neck and the other one is in the mother cell. In the M phase, each set of duplicated chromosomes is dragged toward the opposite poles and cells undergo mitotic exit and, subsequently, cytokinesis (Pruyne et al., 2004; Adler et al., 2022).

The transition between cell cycle phases is controlled with various checkpoints in which an incomplete or faulty phase causes a cell cycle arrest until the requirements are met. The first checkpoint occurs in mid-G1 and is called START because it controls the irreversible decision of committing to cell division. START monitors that the cell has reached a critical size, a

function of nutrient conditions and growth rate. Once past START, DNA replication, SPB duplication, and growth of the daughter cells begin (reviewed in Pruyne et al., 2004).

The core regulators of the cell cycle are cyclins that bind and activate cyclin-dependent kinases (CDKs). *S. cerevisiae* has five CDKs, named Cdc28/Cdk1, Pho85, Kin28, Ssn3, and Ctk1. Cdc28/Cdk1 is the best-studied CDK and the central coordinator of cell cycle progression (Beach et al., 1982; Mendenhall & Hodge, 1998). Budding yeast cyclins that bind to Cdc28/Cdk1 are categorized into three groups: G1 cyclins (Cln1, Cln2, and Cln3), S-phase cyclins (Clb5 and Clb6), and G2/M cyclins (Clb1-Clb4) (Pines, 1995; Murray, 1995; Mendenhall & Hodge, 1998).

In early G1, the transcriptional repressor Whi5 binds and inactivates Swi4-Swi6 (SBF) and Mbp1-Swi6 (MBF) transcription factor complexes. At START, the cyclin Cln3 forms a complex with Cdc28/Cdk1 to phosphorylate Whi5, which excludes Whi5 from the nucleus. This drives the activation of SBF and MBF to activate almost 200 genes, including the G1/S cyclin genes CLN1 and CLN2 (positive feedback loop), and genes required for DNA replication, SPB duplication, and budding of a new cell (Spellman et al., 1998; Nash et al., 2001). DNA replication is key to genome integrity and is also regulated by cyclins. The B-type cyclins Clb5/6 associate with Cdc28/Cdk1 to drive DNA replication in the S phase (reviewed in Caydasi et al., 2010). Once DNA replication is completed, the MTs emanating from each SPB, form the intranuclear mitotic spindle to capture one set of the duplicated chromosomes and align each set in the spindle equator (chromosome congression) to prepare for chromosome segregation.

Chromosome segregation is mediated by the Anaphase-promoting complex or Cyclosome (APC/C). The APC/C is a ubiquitin ligase (for more information about ubiquitin ligases, see section 1.3.1) that interacts with two substrate-specific activators: Cdc20 and Cdh1. APC/C^{Cdc20} targets securin (Pds1 in yeast) and S phase cyclin B for degradation. Pds1 inhibits a caspase-family cysteine protease, separase (Esp1 in yeast), that is inactive until sister chromatids are attached to opposite poles of the mitotic spindle. Upon Pds1 degradation, Esp1 is activated. The main Esp1 target in anaphase is Mdc1(Scc1), a subunit of cohesin, the protein complex that holds sister chromatids together. As Esp1 cleaves Mdc1, sister chromatids are separated and drawn to the opposite poles via mitotic spindle (Shirayama et al., 1999; Hauf et al., 2001). The spindle-assembly checkpoint (SAC) is in charge of monitoring MT attachment to chromosomes. The SAC negatively regulates Cdc20 to prevent securin and

cyclin B degradation until all chromosomes attach to MTs (reviewed in Musacchio & Salmon, 2007). Once SAC is shut off, the cells can enter anaphase. Finally, if the spindle is positioned properly, the growth machinery is distributed at the mother-bud neck (repolarization) to prepare the cell for mitotic exit and cytokinesis.

The proper spindle alignment along the polarity axis is essential for cell cycle progression and cytokinesis and is monitored by the spindle positioning checkpoint (SPOC). The SPOC relies on the GTPase-activating protein (GAP) complex Bfa1-Bub2 that locates in the SPB. When the anaphase spindle is misaligned, Bfa1-Bub2 inhibits the activation of GTPase Tem1, thereby blocking the mitotic exit network (MEN) (Caydasi et al., 2010; Merlini & Piatti, 2011; Chang & Walker, 2017; Adler et al., 2022). Once the cells pass SPOC, the MEN pathway activates by cyclin degradation. The Cdc28/Cdk1 activity is reduced first by the partial degradation of Clb cyclins by APC/C^{Cdc20}. Then, the activation of the phosphatase Cdc14 results in the dephosphorylation of Cdc28/Cdk1-target proteins, including Cdh1, which activates APC/C to complete the degradation of Clb cyclins (Chang & Walker, 2017). Mitotic exit is explained in further detail in section 1.1.8.

1.1.4. SPB duplication

The SPB duplication was first analyzed by EM in 1975 (Byers & Goetsch, 1975). Byers et al. observed the sequential events during SPB duplication. Since then, our knowledge about SPB duplication gradually has broadened as molecular and genetic methods, super-resolution microscopy (Burns et al., 2015), and biochemical techniques were applied in this field.

The SPB duplicates once and only once per cell cycle. SPB duplication occurs in a series of discrete steps involving the ordered assembly of SPB components. In G1, budding yeast has one SPB bearing a half-bridge. The SPB duplication starts with the elongation of the half-bridge to form a full-bridge. As mentioned before, the half-bridge is composed of four proteins: Kar1, Sfi1, Cdc31, and Mps3. The elongation of the half-bridge is driven by Sfi1 a long filamentous protein, oriented with the N-terminus adjacent to the mother SPB and the C-terminus at the distal side of the half-bridge, which forms antiparallel C-to-C dimers in G1. After full-bridge formation, the free N termini of the newly synthesized Sfi1 works as a platform for the assembly of the satellite, the precursor of the new SPB (Elserafy et al., 2014). The core components Spc42 and Spc29, and Bbp1and Mps2, which connect the SPB to the NE (Schramm et al., 2000; Burns et al., 2015) assemble on the distal cytoplasmic tip of the full-

bridge, to form the satellite at early G1 (prior to START). As the cells traverse START in late G1, the satellite matures by the addition of Nud1, Cnm67, and Ndc1, and grows to form the so-called duplication plaque (DP) that structurally is similar to the cytoplasmic side of the SPB. The DP is inserted into the NE at the same time as it grows. The duplication finishes with the assembly of the inner plaque to form the two SPBs with a side-by-side configuration, connected by a full-bridge and embedded in the NE. Finally, the bridge must be cleaved to allow SPB separation and bipolar spindle formation (Burns et al., 2015).

What restricts SPB duplication to once per cell cycle? The Sfi1 C-terminal region contains phosphorylation sites for Cdc28/Cdk1 and the polo kinase Cdc5. Importantly, phosphorylation of Sfi1 is necessary for SPB separation and also prevents extension of the bridge, and thus inhibits reduplication of SPB in the S phase and mitosis (Avena et al., 2014; Elserafy et al., 2014; Rüthnick & Schiebel, 2016). At the mitotic exit, the phosphatase Cdc14 dephosphorylates Sfi1, allowing oligomerization of Sfi1 and SPB duplication in the upcoming G1. Therefore, appropriate phosphorylation and dephosphorylation of Sfi1 by Cdk1 and Cdc14, respectively, is the key regulator of SPB duplication once per cell cycle.

Cyclin-dependent kinases are crucial for centrosome duplication in higher eukaryotes as well. A study on Xenopus laevis egg showed centrosome duplication in vivo is dependent on cyclin E and Cdk2 activity in which Cyclin E/Cdk2 inhibition prevents centrosome re-duplication (Lacey et al., 1999). Another example is a CDK substrate, CP110 in the human cell. The CP110 is a centrosomal protein that phosphorylated with CDK to promote centrosome duplication and separation (Z. Chen et al., 2002).

1.1.5. Spindle orientation

After SPB duplication and separation, one of the SPBs needs to migrate to the bud while the other one remains in the mother cell to achieve successful chromosome segregation. Spindle alignment is crucial for the equal distribution of chromosomes between mother and daughter cells. The cytoplasmic MTs interact with the cortex of the mother cell and bud to align the mitotic spindle along the mother-bud axis. Two sequential and partially redundant mechanisms control the process: the Kar9- (early) and the dynein- (late) pathways.

The Kar9 pathway acts primarily before anaphase. It is an actin-dependent mechanism that uses the class V myosin, Myo2, to transport the cytoplasmic MTs towards the bud neck through the polarized cortical actin. Myo2 is first recruited to the plus end of cytoplasmic MTs

by Kar9, a linker protein that, in turn, binds to the plus-end tip tracking protein (+TIP), Bim1 (R. K. Miller & Rose, 1998). As Myo2 walks along the cortical actin, it transports Kar9 and Bim1 in association with the plus end of cytoplasmic MTs, which results in the 'pivoting' of the spindle toward the bud neck and proper positioning of the SPBs. The alignment relies on Ka9 asymmetric recruitment towards cytoplasmic MTs of the old SPB. Kar9 phosphorylation by Cdc28/Cdk1 associated with Clb4 and Clb3 has been proposed to regulate Kar9 asymmetric loading on one SPB (the one moved to the bud), and non-phosphorylatable mutant Kar9 at position S197 and S496 is symmetrically distributed on both SPBs and leads to spindle misorientation (R. K. Miller & Rose, 1998; Liakopoulos et al., 2003; Meziane et al., 2021). One study showed that Cdk1/Clb4 phosphorylation of Kar9 prevents it from binding to the new SPB (Liakopoulos et al., 2003). By contrast, another work has shown that Cdk1/Clb5 activity promotes Kar9 localization at the MT plus end (Maekawa et al., 2003). In addition to phosphorylation, Kar9 sumoylation prevents the accumulation of Kar9 on the SPB bound to the mother cell (Leisner et al., 2008).

The late pathway acts predominantly at anaphase. It uses the minus-directed motor dynein to move the spindle across the mother-bud neck. Dynein interacts with Pac1 (Lis1 homolog), which binds Bik1 (CLIP-170 homolog), connecting the complex to the plus ends of cytoplasmic MTs. The cytoplasmic MTs grow and eventually reach the cortex, which results in the capture of dynein by the cortical protein Num1. The dynein-Num1 binding allows dynein to walk towards the minus end of the cytoplasmic MTs to generate pulling force and push the spindle toward the bud (Carminati & Stearns, 1997; Moore J.K, et al., 2009).

1.1.6. SPB inheritance

Following correct spindle orientation through the Kar9- and dynein- pathways, one SPB moves to the bud while the other one stays in the mother. The two SPBs segregate in a non-random manner, such that the old SPB is the one that migrates to the bud in virtually all cell divisions while the new one remains in the mother cell (Pereira et al., 2001). Interestingly, a recent study has revealed that this pattern of SPB inheritance is important to maintain the replicative lifespan in budding yeast (Manzano-López et al., 2019). Furthermore, many stem cells that divide asymmetrically follow non-random centrosome segregation at mitosis. For instance, in Drosophila neuroblast (NBs) and Drosophila female germline stem cells (GSCs), the renewing stem cell inherits the new centrosome while the differentiating cell keeps the old centrosome (Januschke et al., 2011; Salzmann et al., 2014). The mouse radial glial

progenitor cells from the developing neocortex and Drosophila male germline stem cells inverse this orientation (Xiaoqun Wang et al., 2009; Salzmann et al., 2014; C. Chen & Yamashita, 2021).

How do cells distinguish between the old and the new SPBs? Two models have been proposed so far. The first model is based on differences in maturation and nucleation capacity between the old and new SPBs. This model suggests that the pre-existing SPB can organize cytoplasmic MTs before the new one, and the key player in this process is Spc72. The Spc72 recruitment is delayed in the new SPB, resulting in an asymmetric distribution of Spc72 in old and new SPBs (Juanes et al., 2013). Thus, the old SPB can nucleate cytoplasmic MTs earlier than the new SPB, which drives the recruitment of Kar9 on the pre-existing SPB and promotes its orientation and movement towards the bud. Artificial forcing of Spc72 symmetric distribution between the two SPBs perturbed Kar9 polarization and randomized SPB inheritance (Hoepfner et al., 2002; Juanes et al., 2013).

The second model proposes that cell cycle stage-specific PTMs of the SPB provide age-determining cues for the spindle orientation pathways. The study supporting this model has shown that the deletion of two kinases, Swe1 (Wee1) and Kin3, and one acetyltransferase NuA4 randomize the pattern of the SPB segregation (Lengefeld et al., 2017). Furthermore, the data from this work suggested that Swe1 phosphorylates the SPB outer component, Nud1, at G1, marking the old SPB for the subsequent division before the new SPB assembly. Kin3 is required to maintain the identity of the old SPBs in the second division, while NuA4 is needed to maintain the identity of old and new SPBs (Lengefeld & Barral, 2018). Furthermore, Cdc5, the only polo-like kinase in budding yeast, regulates the recruitment of Spc72 and Kar9 to the SPB and is required for the asymmetric SPB inheritance (Matellán et al., 2020). These studies highlight the role of kinases in SPB phosphorylation to regulate asymmetric SPB inheritance.

1.1.7. SPB phosphorylation

Phosphorylation is a widespread PTM that regulates most cellular processes. The budding yeast phospho-proteome in DNA-damaged or arrested cells in distinct cell cycle stages identified over 46,000 phosphosites (Albuquerque et al., 2008; Swaney et al., 2013). Furthermore, two studies have mapped the phospho-proteome of budding yeast SPBs by mass spectrometry and showed that the SPB is highly phosphorylated (Keck et al., 2013; Fong et al., 2018). In the first study, the SPB was co-purified with TAP-tagged Mlp2, a nuclear pore

complex (NPC) protein that binds to SPB. Then, phospho-peptides were enriched with a titanium dioxide (Tio2) column, and the samples were analyzed by mass spectrometry. In total, 297 phosphorylation sites in SPB proteins were discovered. The authors also compared the phosphorylation profile of SPBs from asynchronous cells and cells arrested in G1 and mitosis. These analyses identified 54 sites unique for G1, 110 sites unique to mitosis, and the rest was shared between both phases (Keck et al., 2013). In the second study, the SPB was co-purified with TAP-tagged Spc97, a component of the γ-TuSC (Fong et al., 2016; Fong et al., 2018). This purification was followed by a second enrichment with velocity sedimentation in sucrose gradients to eliminate contaminants. The authors mapped the phosphosites of SPB purified from asynchronous cells, cells arrested in G1/S, and mitotic cells. In total, the study identified 212 phosphorylation sites, from which 26 were observed in asynchronous cells, 24 were specific for G1/S, 60 for mitosis, and 102 sites were shared between two/three conditions. Both studies confirmed that SPB is extensively phosphorylated in a cell-cycle-dependent manner.

Various kinases essential for cell cycle regulation, Cdk1, Mps1, and Cdc5, are also involved in SPB regulation and duplication (Castillo et al., 2002; Deshaies & Ferrell, 2001; Avena et al., 2014). Two of the best-characterized phosphorylated SPB proteins are Tub4 and Spc110. Tub4 phosphorylation is required for spindle assembly, MT nucleation, and MT organization (Vogel et al., 2001; T. C. Lin et al., 2011). Spc110 has Cdc28/Cdk1 and Mps1 phosphosites at its N-terminus (Friedman D.B et al, 2001) that activate γ-TuSC oligomerization at the SPB and MT nucleation (Huisman et al., 2007; T. C. Lin et al., 2014).

Apart from Tub4 and Spc110, the function of several SPB phosphosites was defined in recent decades. For instance, Spc42 phosphorylation by Cdk1 and Mps1 is essential to induce Spc42 assembly and SPB duplication (Jaspersen et al., 2004). The phosphorylation of Spc29, a component of the central plaque, at two sites: T240 and T18, is required for its interaction with Spc42 and to link the SPB to the NE, respectively (Jones et al., 2018). A single phosphorylation event of Spc29 at Thr240 is necessary for SPB duplication and mitosis progression (Holinger et al., 2009). Even though many functional studies on SPB phosphosites have been published, further research is required to understand the biological roles of the SPB phosphoproteome. An open question is whether SPB phosphosites are involved in additional mechanisms aside from SPB duplication and MT nucleation. As described in the previous section, the age-dependent phosphorylation of Nud1 regulates the SPB inheritance

(Lengefeld et al., 2017), although the presence and regulatory roles of other age-dependent SPB phosphorylations remain to be explored.

1.1.8. Mitotic exit

Apart from their roles in MT nucleation, the centrosome and SPBs have been the subject of attention for serving as signaling platforms to promote cell cycle progression (M. Lin et al., 2022). Maniotis et al. carried out laser microsurgical centrosome removal in African green monkey BSC-1 kidney cells and showed that the absence of centrosomes resulted in cell cycle arrest at G2 (Maniotis & Schliwa, 1991). Follow-up studies showed that the removal of centrosome components in different species caused cell cycle arrest in G1 to S transition (Hinchcliffe et al., 2001; Khodjakov & Rieder, 2001; Piel et al., 2001). Along with cell cycle progression, centrosome depletion leads to defects in cytokinesis (Khodjakov & Rieder, 2001; Piel et al., 2001). Furthermore, Centriolin (Nud1 homolog) silencing drives cytokinesis failure (Gromley et al., 2003; reviewed in M. Lin et al., 2022). Hence, these studies support the idea of centrosome involvement in controlling multiple phases of the cell cycle.

The SPB is a hub for cell cycle regulators involved in spindle positioning and mitotic exit (reviewed in Cuschieri et al., 2007; Ian W. Campbell et al., 2020). One well-defined example is the localization of mitotic exit network (MEN) components on the SPB (Gruneberg et al., 2000b). Mitotic exit and cytokinesis occur after chromosome segregation is completed to ensure the mother and daughter cells only receive one copy of each chromosome. Cdc28/Cdk1 must be inactivated to exit mitosis and transition into the next G1. Cdc28/Cdk1 inhibition occurs in two steps in *S. cerevisiae*. Prior to anaphase, APC/C^{Cdc20} decreases Cdc28/Cdk1 activity by degrading the S phase cyclins and part of the mitotic cyclins, although a pool of mitotic cyclins remains protected from degradation. Hence, APC/C activity is not only essential for chromosome segregation (as described in section 1.1.3) but is also required for mitotic exit. During mitotic exit, Cdh1, another coactivator of APC/C, undergoes dephosphorylation, resulting in its binding to APC/C. The active APC/C^{Cdh1} targets the remaining mitotic cyclins (Clb) for degradation, leading to Cdc28/Cdk1 inactivation (Jaspersen et al., 1998; reviewed in Peters, 2002; reviewed in Stegmeier & Amon, 2004)). APC/C^{Cdh1} also targets other important mitotic activators, including polo kinase Cdc5 (C. Visintin et al., 2008), the spindle components Ase1 (Nobes et al., 1997), Cin8 (E. R. Hildebrandt & Hoyt, 2001), and Cdc20 (Prinz et al., 1998).

Cdc28/Cdk1 inactivation is fundamental to cell cycle progression, otherwise, cells arrest in late anaphase/telophase with an extended mitotic spindle (reviewed in Morgan, 1999). The complete inactivation of Cdc28/Cdk1 is driven by Cdc14, a highly conserved phosphatase that dephosphorylates Cdc28/Cdk1 substrates (R. Visintin et al., 1998; Murray, 1995). Cdc14 inactivates Cdc28/Cdk1 via two pathways: (1) Cdc14 dephosphorylates Cdh1, which results in APC/C^{Cdh1} activation and subsequent degradation of mitotic cyclins and other mitotic proteins. (2) Cdc14 dephosphorylates Swi5, a transcription factor, which induces expression of the Cdc28/Cdk1 inhibitor Sic1 (R. Visintin et al., 1998; Ian Winsten Campbell et al., 2019). Furthermore, Cdc14 dephosphorylates Sic1, protecting it from degradation.

Cdc14 activity is regulated during the cell cycle to trigger mitotic exit at the proper time. Otherwise, the checkpoints SAC and SPOC delay cell cycle progression until their requirements are met. The SAC restrains metaphase-to-anaphase transition until all chromosomes are attached to the mitotic spindle. On the other hand, the SPOC ensures the coupling of nuclear position and mitotic exit during anaphase.

Cdc14 is sequestered in the nucleolus from G1 prior to anaphase and released from its inhibitor, Cfi1/Net1, at anaphase onset. Cdc14 release is controlled by two pathways: the Cdc fourteen early anaphase release (FEAR) network and the mitotic exit network (MEN) (reviewed in Caydasi et al., 2010). In the FEAR network, Esp1 interacts with the kinetochore-associated protein Slk9, which leads to Cfi1/Net1 phosphorylation. Likewise, polo-kinase Cdc5 phosphorylates Net1 (Shou et al., 2002). Phosphorylated Net1 results in its low affinity towards Cdc14 and induces a partial release of Cdc14 from the nucleolus in an early stage of anaphase. However, the Cdc14 release by the FEAR network is insufficient to initiate mitotic exit. Activation of the second signaling network or MEN, induces sustained Cdc14 release in the cytoplasm, SPBs, and bud neck (Yoshida et al., 2002; Stegmeier et al., 2002; Valerio-Santiago & Monje-Casas, 2011).

MEN is a GTPase-driven signaling pathway that promotes mitotic exit and consists of four protein kinases (Cdc5, Cdc15, Dbf2, and Dbf20), a protein phosphatase (Cdc14), a GTPase (Tem1), two components GTPase-activating factor (GAP) Bub1-Bfa1, a GTP exchange factor (Lte1), and a Dfb2- binding protein (Mob1) (Figure 3) (reviewed in McCollum & Gould, 2001). The core components of MEN that function upstream of Cdc14 are Tem1, the hippo-like kinase Cdc15, the polo kinase Cdc5, the Dfb2-Mob1 kinase complex, and the SPB scaffold protein Nud1 (Stegmeier & Amon, 2004, Rock & Amon, 2011). MEN components coordinate

the mitotic exit with nuclear migration to ensure that chromosomes have segregated between mother and bud for proper genome integrity. When the nucleus translocation fails during anaphase, Cdc14 is kept inactive, and cells are arrested (R. Visintin et al., 1998). The asymmetric distribution of the MEN activators (in the bud) and inhibitors (in the mother) is key to coupling nuclear migration with mitotic exit. When SPB enters the bud, MEN proteins that localize on SPB sense the environment change to initiate the MEN pathway.

Tem1 is inactive during most of the cell cycle by the GAP complex, Bfa1-Bub2. Tem1 and Bfa1-Bub2 are located at both SPBs in metaphase but accumulate at the old SPB in anaphase when the spindle is properly aligned (Molk et al., 2004; reviewed in Caydasi et al., 2010). The protein kinase Kin4, a SPOC component, localizes on the mother cell cortex and the SPB that remains in the mother cell. Kin4 activates the Bub2-Bfa1 complex to inhibit Tem1 when the SAC or SPOC is active (Pereira & Schiebel, 2005). Bfa1 can be phosphorylated either by only Kin4 or only Cdc5. Its phosphorylation with kin4 inhibits MEN, whereas phosphorylation with Cdc5 triggers the MEN pathway. In the presence of a mispositioned spindle, Kin4 phosphorylates Bfa1 on residues S150 and S180 to prevent its phosphorylation with Cdc5 (Maekawa et al., 2007). Once the Tem1-bearing SPB enters the bud where Kin4 has not resided, Tem1 comes into contact with the MEN-activating factor, Lte1, a protein that localizes at the bud cortex, and induces Tem1 alteration from GDP-Tem1 to GTP-Tem1 (active form) (Piatti et al., 2006; Falk et al., 2011; Rock & Amon, 2011). The GTP-Tem1 will then initiate a kinase cascade to release Cdc14. Tem1 and the polo kinase Cdc5 recruit Cdc15 kinase to the SPB to phosphorylate Nud1 (S63 and T78) on the SPB outer plaque. Phosphorylated Nud1 creates SPB phospho-docking sites for Mob1-Dfb2 that can be phosphorylated by Cdc15. The phosphorylated Mob1-Dfb2 then translocate into the nucleus to activate Cdc14 and release it from the nucleolus to the cytoplasm (Valerio-Santiago & Monje-Casas, 2011; reviewed in Hergovich & Hemmings, 2012; Vannini et al., 2022).

Involvement of another outer plaque component, Spc72 was identified in the MEN pathway. Spc72 interaction with Bfa1 is important for Bfa1 phosphorylation to trigger MEN (Gryaznova et al., 2016). Until now, Nud1 and Spc72 are the only identified SPB components that function in MEN. A critical unanswered question is whether other SPB components are involved in mitotic exit.

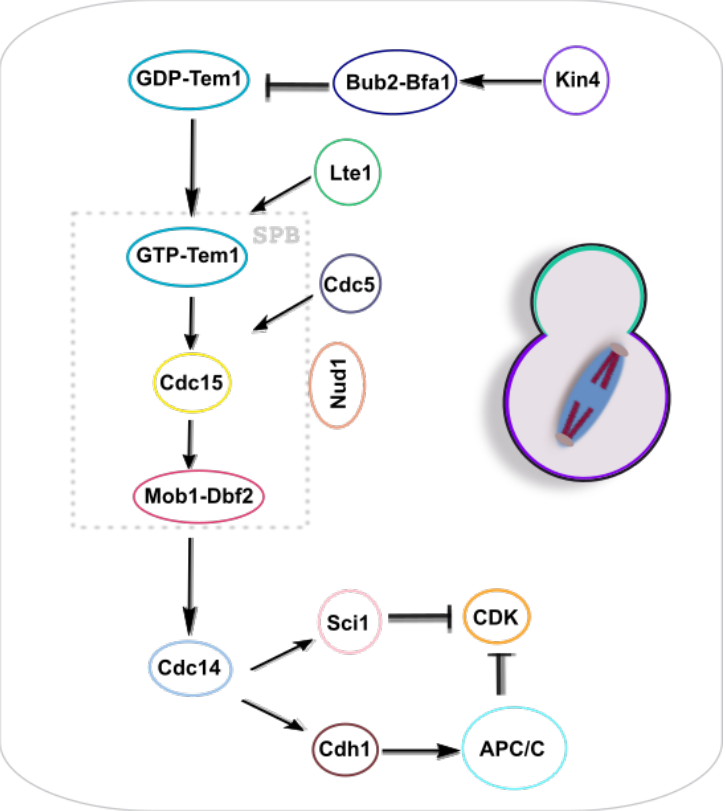

Figure 3. MEN pathway in *S. cerevisiae*. The MEN regulators are distributed in the mother and bud. Kin4 (purple), located in the mother cell cortex, activates Bub2-Bfa1, which keeps Tem1 in an inactive form (GDP-Tem1). Once the old SPB moves to the bud, Let1, a MEN-activator, induces GTP-Tem1. Then, GTP-Tem1 with Cdc5 activates the kinase cascade (Cdc15) to phosphorylate Nud1 on the SPB. Phosphorylated Nud1 creates a SPB docking site for Mob1-Dbf2. Active Mob1-Dbf2 translocates into the nucleolus to liberate Cdc14 from its inhibitor and release it into the cytoplasm. Cdc14 then inactivates Cdc28/Cdk1 by dephosphorylating Sci1 and Cdh1.

1.2. Microtubules

The accuracy in cell division depends on the mitotic spindle´s ability to segregate the replicated chromosomes between the dividing cells equally. In higher eukaryotes, a distinct chromosome region, termed the centromere, binds to MTs that position chromosomes to the spindle pole equator, forming a metaphase plate. When all sister chromatids are aligned in the metaphase plate, their linkage is dissolved by separase. Then, MTs bound to chromosomes are shortened, and each set of chromosomes moves toward the spindle pole (anaphase A). Subsequently, the overlapping antiparallel MTs elongate to segregate the spindle poles (anaphase B). Budding yeast lacks a metaphase plate, but the chromosomes are aligned forming two foci in the spindle equator (chromosome congression). As in higher eukaryotes, the yeast separase (Esp1) separates sister chromatids and chromosomes are pulled towards opposite poles. Anaphase A contributes barely to yeast chromosome segregation. Indeed, most chromosome segregation takes place in anaphase B when the spindle elongates five folds in length (Straight et al., 1997; reviewed in Emily R. Hildebrandt & Hoyt, 2000).

1.2.1. Microtubule structure

The cytoskeletal filaments consist of actin, intermediate filaments, and microtubules (MTs). MTs are cytoskeletal ”tracks” required for chromosomal segregation in mitosis and meiosis, cell shape maintenance, cell division, and intracellular transport (reviewed in Goodson & Jonasson, 2018). Structurally, MTs are hollow tubes made of repeating heterodimers of α- and β-tubulins and are the largest structures in the cytoskeleton. Multiple α- and β-tubulin dimers bind together in a head to tail-configuration to form linear chains (protofilaments) that arrange into a cylindrical pattern to shape an MT (Ledbetter & Porter, 1964; Tilney et al., 1973). Typically, thirteen protofilaments (PFs) bundle parallel to each other to form a fast-growing plus end and a slow-growing minus end MTs. At the plus end, β-tubulin is exposed, while at the minus end, only α-tubulin is exposed. The minus end is organized proximal to the SPB, whereas the plus end is at the distal side (Bergen & Borisy, 1980; Mitchison.T & Kirschner.M, 1984). Budding yeast encodes a sole β-tubulin, TUB2 (Neff et al., 1983), two α-tubulins, TUB1 and TUB3 (Schatz et al., 1986), and one γ-tubulin, TUB4. TUB4 is not assembled into the MT structure but is associated with the SPB to organize MT nucleation (see section 1.1.3).

Unlike metazoans, budding yeast undergoes mitosis without nuclear envelope breakdown (NEBD). The budding yeast SPB is embedded in the NE throughout cell division, and nuclear spindles are formed in the nucleus between two SPBs to segregate the chromosomes without NEBD. The NE barrier establishes the formation of both nuclear and cytoplasmic MT (Byers & Goetsch, 1975). The interpolar MTs (ipMTs) and the kinetochore MTs (kMTs) reside on the nuclear side and the cytoplasmic MTs in the cytoplasm. The cytoplasmic MTs radiate toward the cell cortex to mediate spindle orientation and nuclear migration. Compared to cytoplasmic MTs, kMTs are necessary for chromosome segregation and ipMTs stabilize the spindle (reviewed in Cavanaugh & Jaspersen, 2017). The kinetochore (KT) is a multiprotein complex that connects kMTs to a unique region of the chromosome (centromere) almost the entire cell cycle, except for a short pause during the S phase (Kitamura et al., 2007). In budding yeast, each kinetochore binds to a single kMT (Winey et al., 1995), and each ipMT interdigit to an ipMT from the opposite SPB. The site where the ipMTs overlap in anaphase is called the spindle midzone and stabilizes the spindles (reviewed in Winey & O’Toole, 2001; Westermann et al., 2007; Biggins, 2013). In metaphase, the number of MTs nucleated from a SPB at the nuclear side is around 20 in haploid cells and doubled in diploid cells (Peterson & Ris, 1976).

Electron microscopy revealed that sixteen kMTs bind to the sixteen chromosomes of haploid budding yeast. The number of cytoplasmic MTs varies between four to six for each pole (reviewed in Westermann et al., 2007).

MTs exhibit a unique property of stochastically switching between polymerization and depolymerization, a behavior termed dynamic instability, discovered by Mitchison and Kirschner in 1984 (Mitchison.T & Kirschner.M, 1984). Using EM and *in vitro* experiments, they observed that a subset of MTs declined in length while others continued to grow longer. Based on this observation, they suggested that MTs can transit between growth and shortening. The transition from growth to shortening is termed catastrophe, and *vice versa* is called rescue. MTs dynamic instability is caused by the hydrolysis of GTP bound to β-tubulins at their GTP-exchangeable site (E site). MTs polymerize by adding α-β tubulin dimers with GTP-bound β-tubulin, resulting in a growing tip capped by GTP (GTP-tubulin cap) (David-Pfeuty et al., 1977; Mitchiso T.J., 1993). As GTP-bound tubulin is incorporated at the MT end, it is hydrolyzed to GDP. GTP-tubulin molecules interact with each other to hold the molecules together in protofilaments, whereas the GTP hydrolysis (GDP-tubulin) induces a conformational change in protofilaments, and MTs curve outward. MTs with GDP-tubulin exposed at their plus ends undergo depolymerization or catastrophe. When the tubulin end is stabilized by a new cap of GTP-tubulin, MT switches to the rescue phase (Bond, 1987; David N. Drechsel & Kirschner, 1994; H. W. Wang & Nogales, 2005).

Dynamic instability of MTs has been investigated both in vitro and in vivo. Surprisingly, the rate of growth and shortening frequency is much higher in vivo, indicating that additional factors are involved in the MT dynamics (reviewed in Desai & Mitchison, 1997). Two classes of proteins interact with MTs to regulate their dynamics: MT-associated proteins (MAPs) and MT-motor proteins.

1.2.2. Microtubule-associated proteins

MAPs were first identified as high molecular weight proteins co-purifying with tubulin in the mammalian brain: MAP1 (300-350 KD) and MAP2 (270 KD). Another co-purified protein was Tau (55-68 KD) (Dentler et al., 1975; Murphy & Borisy, 1975; Cleveland et al., 1977). Follow-up in vitro studies revealed that MAP2 and Tau increase MT polymerization (Pryer et al., 1992; D Drechsel et al., 1992). Further research confirmed that the main function of MAPs is

regulating MT dynamics by polymerizing or depolymerizing MTs (reviewed in Goodson & Jonasson, 2018; Bodakuntla et al., 2019).

Since the plus end of MTs undergoes dynamic instability, it is not surprising that MAPs accumulate on the plus ends. A class of MAPs termed 'Plus-end-tracking proteins', or +TIP, localize to the plus ends of MTs to regulate their dynamics and mediate their interaction with cellular organelles or protein complexes. +TIP proteins are categorized into three protein families: Cytoplasmic linker proteins (CLIPs), end binding (EBs) proteins, and CLIP-associating proteins (CLASPs). The CLIPs and EBs are the most prominent +TIPs conserved in all eukaryotes (reviewed in Schuyler & Pellman, 2001).

The first identified +TIP was CLIP170 (Pierre et al., 1992). It was first shown that CLIP170 mediates the interaction of endocytic vesicles with MTs in an in vitro assay. Later, Perez. F et al. visualized GFP-tagged CLIP-170 dynamic properties in vivo (Perez et al., 1999). This work showed that CLIP-170 had treadmilling behavior in which it binds to the polymerizing end of the MT, remains immobile after binding, and then dissociates from the growing MT plus end. In addition, CLIP-170 contributes to the metaphase chromosome alignment (Dujardin et al., 1998). Follow-up studies observed treadmilling behavior in other +TIPs, including EB1(reviewed in Carvalho et al., 2003). Subsequent studies focused on understanding the role of CLIP-170 in MT dynamics regulation. The budding yeast CLIP-170 (Bik1) has an MT-stabilizing function (Carvalho et al., 2004), and fission yeast CLIP-170 (Tip1) acts as an anti-catastrophe factor to ensure cytoplasmic MTs localization to their target zone (Brunner & Nurse, 2000).

In budding yeast, +TIPs are implicated in mitotic spindle orientation in the Kar9 and dynein pathways (see section 1.1.5). Bik1 in yeast is a key player that interacts with proteins of both pathways. Bik1 directly binds to Kar9 to restrict its asymmetric loading on the old SPB, which leads to an age-dependent SPB inheritance (Miller K.E et al., 2006). Bik1 also forms a complex with the budding yeast EB1, Bim1, that regulates MTs dynamics. Blake-Hodek et al. showed that Bik1 and Bim1 have opposing activities in vitro. Bik1 inhibits MT assembly, promotes catastrophe frequency, and decreases growth rate, while Bim1 shows the opposite effect by suppressing the catastrophe (Blake-Hodek et al., 2010). The study also showed that the Bim1-Bik1 complex has the same effect as Bim1 in vitro. In addition, Bik1 plays a role in MT assembly and nuclear segregation in mitosis, and nuclear fusion in the mating process (Berlin et al., 1990).

Bik1 exists in two different pools: one is nuclear, and the other is cytoplasmic. Using fluorescence recovery after photobleaching (FRAP) experiments, Bik1 was shown to be dynamically associated with the cytoplasmic MTs, while nuclear Bik1 was immobile (Carvalho et al., 2004). The same study also showed that Bik1, unlike its human ortholog, required a kinesin-dependent transport mechanism (Kip2) to be loaded to the plus-ends. Bik1 delivery to the plus ends via Kip2 controls MT dynamics and dynein-dependent spindle orientation. Another study has shown that nuclear Bik1 in combination with Bim1 is involved in the kinetochore capture by rescuing MTs (Tanaka et al., 2005).

Another important +TIP in yeast is Stu2, which acts as a MT polymerase by increasing MT growth and decreasing catastrophe rates (Podolski et al., 2014). In addition, Stu2 is important for kinetochore and cytoplasmic MT dynamics, mitotic spindle elongation during anaphase, and proper spindle orientation (Kosco et al., 2001; Severin et al., 2001). Recent research has shown that Stu2 interacts with Spc72 to form a complex to promote MT aster formation in vitro and plays a crucial role in nucleating cytoplasmic MTs in vivo (Gunzelmann et al., 2018).

1.2.3. Microtubule-motor proteins

The second class of tubulin interactors is MT-motor proteins that mainly contribute to cargo motility throughout the MTs and act as MT dynamics regulators. The first detailed study identifying a MAP was performed in the unicellular eukaryote Tetrahymena in 1965 (Gibbons, I.R. & Rowe, A.J. 1965). The study identified a protein that they named dynein (after the unit of force), and that was selectively bound and dissociated from cilia MTs.

Two major groups of MT motors are dynein and kinesins. In budding yeast, there are six kinesin-motor proteins (Kar3p, Kip1p, Kip2p, Kip3p, Cin8, Smy1p) and a single dynein motor (Dhc1p) (reviewed in Emily R. Hildebrandt & Hoyt, 2000). Sym1p is the only one not involved in spindle function and regulates polarized cell growth (Lillie & Brown, 1998). Each kinesin-motor protein belongs to a different kinesin subfamily with a distinct function. Most kinesins consist of two motor domains that form a head capable of moving along the MTs, followed by a coiled-coil region, termed stalk, and a tail responsible for cargo attachment. Kinesin classification is based on sequence homology and structure and divides the proteins into eight subfamilies (reviewed in Hildebrandt et al., 2000). Here I briefly explain kinesin-motor protein functions relevant to the mitotic spindle.

The kinesin-5 family (BimC) members are plus-end directed motility motors required for mitotic spindle assembly and SPB separation (Hoyt et al., 1992; Saunders & Andrew, 1992; Kashina et al., 1997). Budding yeast encodes two kinesin-5 family genes: CIN8 and KIP1, with overlapping functions in spindle assembly, chromosome congression, and spindle elongation (Tytell & Sorger, 2006; Gardner et al., 2008). However, Cin8 acts primarily in the early anaphase (fast spindle elongation phase) and Kip1 is most important in the late anaphase (slow spindle elongation phase) (Straight et al., 1998). It was reported that Cin8 phosphorylation in mid to late anaphase promotes its detachment from the spindle MTs to maintain the spindle morphology (Avunie-Masala et al., 2011). Although the BimC kinesin family is considered to be directed to the plus-end, purified Cin8 behaves as a bidirectional kinesin in vitro (Roostalu et al., 2011). In this study, individual Cin8 motors move towards the minus-end on single MTs, while switching to the plus-end on antiparallel MTs (Roostalu et al., 2011). The Cin8 lacking its tail domain losses its directional preference (Gerson-Gurwitz et al., 2011).

Another well-defined plus-end directed kinesin is Kip2, which participates in the Kar9 and dynein pathways by transporting +TIPs towards the cytoplasmic MT plus-ends. In the Kar9 pathway, Kip2 is necessary for efficient Kar9 accumulation on cytoplasmic MTs (Maekawa et al., 2003). In the dynein pathway, Kip2 transports dynein to the MTs plus-end. While Lis1 and Bik1 mediate the interaction between dynein and Kip2, Bik1 and Bim1 act as Kip2 processivity factors (reviewed in Michael A. Cianfrocco et al., 2016). In addition, in vitro and in vivo studies demonstrated that Kip2 is a MT polymerase and catastrophe inhibitor (Hibbel et al., 2015).

Kip3 is a member of the kinesin-8 family of MT-motor proteins. Kip3 is a unique kinesin with dual functions; it acts as a plus-end motor kinesin and a MT depolymerase. In vivo experiments revealed that Kip3 is at the plus end of MTs and promotes MT depolymerization (Gupta et al., 2006). Moreover, Kip3 is required for nucleus migration to the bud neck (DeZwaan et al., 1997). Deletion of Kip3 results in mispositioning of metaphase kinetochores and defects in chromosome congression (Wargacki et al., 2010).

Budding yeast has a single kinesin-14, Kar3, that promotes MT depolymerization (Endow et al., 1994). Kar3 acts antagonistically with other kinesin-motor proteins. The loss of Kar3 in double mutated Cin8 and Kip1 cells suppresses the spindle collapse, while Kar3 overexpression inhibits spindle elongation in the same cells, suggesting that Kar3 opposes Cin8 and Kip1 to regulate spindle length (Saunders et al., 1997). Furthermore, Kar3 null

mutant cells have long cytoplasmic MTs, while loss of Kip2 reduces MT numbers (Huyett et al., 1998). This study also showed that loss of both Kar3 and Kip2 has an intermediate phenotype, suggesting that Kar3 and Kip2 function antagonistically to control cytoplasmic MT number and length (Huyett et al., 1998).

1.2.4. Spindle disassembly

After chromosome segregation, the mitotic spindle is disassembled. In budding yeast, spindle disassembly happens from the plus-end at the midzone once the spindle length reaches ~ 10μm (Maddox et al., 2000). The process can be described in three stages: spindle splitting, arrest of spindle elongation, and MT depolymerization. In order to elucidate the spindle disassembly mechanism, a study combined genetic analysis with microscopy to identify regulators of this process, revealing at least three pathways (Woodruff et al., 2012). In the first pathway, Cdc14 dephosphorylates Cdh1, resulting in APC/C^{Cdh1} activation. Then, APC/C^{Cdh1} stimulates the degradation of Cin8 and Ase1 at the spindle midzone, destabilizing the MTs and separating the spindle halves (Nobes et al., 1997; E. R. Hildebrandt & Hoyt, 2001). In the second pathway, the Aurora protein kinase family member, Ipl1, re-localizes to the spindle midzone, where it regulates the spindle disassembly (Buvelot et al., 2003). Ipl1 phosphorylates and inactivates the MT-growth promoter Bim1 and activates the spindle destabilizing factor She1 (Zimniak et al., 2009; Woodruff et al., 2010). In the last pathway, the MT depolymerase Kip3 destabilizes the ipMTs and leads to spindle halves shrinkage. Notably, complete spindle disassembly in the mother cell is required to generate a tubulin pool that is incorporated in the MTs of the daughter cell (Woodruff et al., 2012).

1.3. Protein degradation

Proteostasis is the process that maintains the cellular proteome in balance by regulating the synthesis of new proteins and the degradation of unfolded and aggregated proteins. Two major pathways are responsible for protein and organelle degradation in eukaryote cells, the ubiquitin-proteasome system (UPS) and autophagy. The UPS primarily eliminates short-lived proteins, whereas autophagy is responsible to degrade long-lived and aggregated proteins and even organelles. Furthermore, proteases in the lysosome/vacuole degrade proteins non-specifically. By contrast, some proteases can target specific proteins and cleaved them at distinct regions. Here, I explain the main concepts of each pathway and their involvement in MTOC regulation.

1.3.1. Proteasome-ubiquitin system (UPS)

The UPS consists of ubiquitin (Ub), a multi-enzymatic system, and the multi-subunit protease, proteasome. Ub is a conserved protein comprised of 76 amino acids that can be covalently attached to other proteins by an isopeptide bond. Protein modification by Ub (ubiquitylation) regulates numerous cellular functions such as cell-cycle progression, gene transcription, endocytic trafficking, and protein stability (reviewed in Haglund & Dikic, 2005).

Proteins are ubiquitylated through three consecutive reactions, as shown in figure 4. First, the formation of a linkage between Ub and E1 (Ub-activating enzyme) triggers the ubiquitylation process. Activated Ub is then transferred to an E2 (Ub-conjugating enzymes). Finally, a substrate-specific E3 ligase binds coordinately to the E2 enzyme and the substrate to mediate substrate ubiquitylation (Figure 4A) (Scheffner et al., 1995).

Budding yeast has only one E1 (Uba1), which is an essential gene (McGrath et al., 1991), and thirteen different E2 enzymes (Ubc1-8, Ubc-9, Ubc10-11, and Ubc12-13) have been identified. Ubc9 and Ubc12 are not conjugated to Ub but bound to the Ub-like protein Smt3 (the yeast homolog of human SUMO-1) (E. S. Johnson & Blobel, 1997). E3s are the largest group of enzymes in the ubiquitylation pathway. So far, 60-100 E3 enzymes have been identified in the yeast (Reviewed in Nath & Shadan, 2009; R. J. Braun, 2015; Zattas & Hochstrasser et al., 2018). As described earlier, APC/C is one of the E3 ligases involved in chromosome segregation and mitotic exit.

There are different types of protein ubiquitylation depending on the length of the Ub oligomer and the configuration of the Ub-Ub linkage. The simplest form is the attachment between a single Ub molecule and lysine (Lys) in the substrate protein (i.e., monoubiquitylation), which is primarily involved in endocytosis, DNA repair, and nuclear export (Haglund et al., 2003). Alternatively, Ub molecules can be bound to several individual Lys residues in the substrate (i.e., multiubiquitylation). The final form is polyubiquitylation, which is the formation of a chain of Ub molecules attached to one or more Lys residues in the substrate (Figure 4B) (reviewed in Pickart & Fushman, 2004). Ubiquitylation is a reversible reaction, and deubiquitylating enzymes (DUBs) can remove the Ub from the substrate and release free ubiquitin within the cell. The budding yeast encodes 22 DUBs, categorized into different groups according to their structure (reviewed in Amerik & Hochstrasser, 2004).

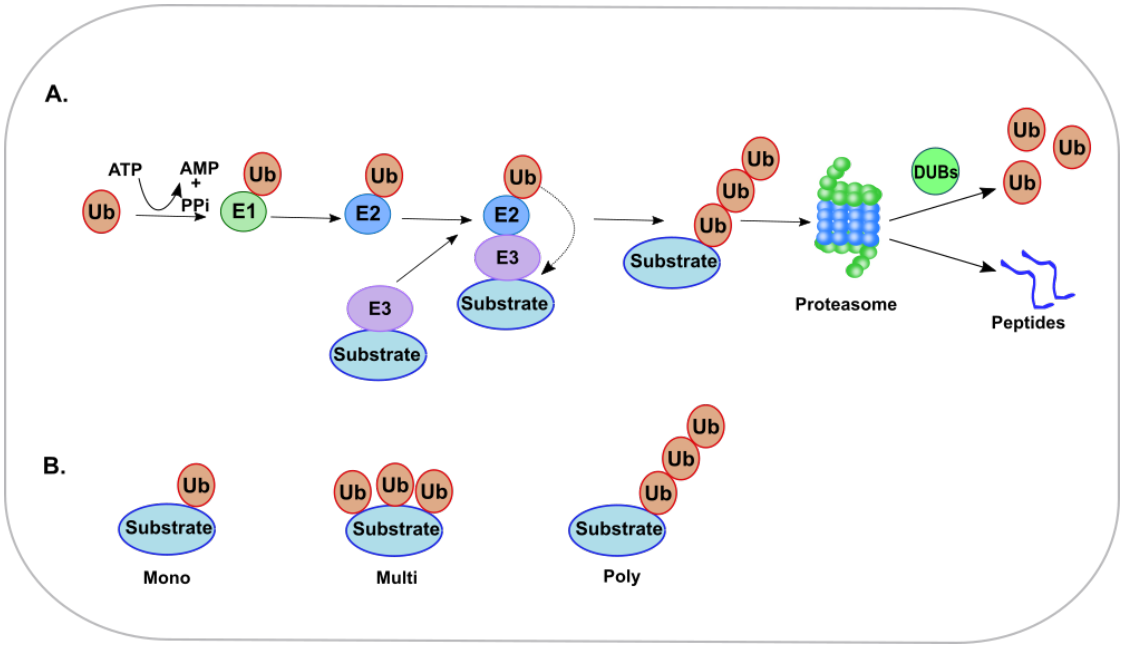

Figure 4. Simplified scheme of ubiquitylation. (A) Ub binds to E1 in an ATP-dependent process. Then, Ub is transferred to the E2 enzyme. An E3 ligase facilitates the transfer of Ub to the substrate. Poly ubiquitylated substrate is transferred to the proteasome to be degraded and digest the protein to the peptides. The ubiquitylation can be reversed with the DUB enzyme. (B) Depending on the ubiquitin attachment, the substrate could be mono-, multi-, or polyubiquitylated. Ub: Ubiquitin, DUBs: deubiquitin enzymes.

Substrates that contain poly-Ub chains are often delivered to the 26S proteasome, where they are unfolded and degraded. The proteasome is composed of a barrel-shaped 20S core particle (CP) capped with one or two 19S regulatory particles (RPs). The 19S RP recognizes the ubiquitylated proteins, unfolds, and translocates them into the CP in an ATPase-dependent process. Finally, the 20S proteasome cleaves the unfolded protein into small peptides (Baumeister et al., 1998; McClellan et al., 2019).

Several shreds of evidence indicate the UPS role in centrosome and SPB regulation (Strnad et al., 2007; Didier et al., 2007; Greenland et al., 2010; Tamborrini et al., 2018). Greenland et al. showed that deletion of the E2 enzyme Ubc4 led to increased fluorescent intensity of GFP labeled Spc110 and Spc42, suggesting that Ubc4 is (directly or indirectly) required to maintain the SPB size (Greenland et al., 2010). Further studies identified E3 ligases involved in SPB subunits degradation. In *S. cerevisiae*, APC/C^{Cdh1} targets Mps3, the SPB component required for its insertion in the NE, for the proteasomal degradation (Koch et al., 2019). Another example of E3 ligase is Dma2, which targets Nud1 for ubiquitylation in late mitosis and G1

(Tamborrini et al., 2018). In higher eukaryotes, the UPS functions in centrosome regulation have also been reported. In humans, APC/C^{Cdh1} targets SAS-6, a protein necessary for procentriole formation, for degradation to ensure a proper centrosome duplication cycle (Strnad et al., 2007). Furthermore, it was determined that proteasome inhibition results in the accumulation of pericentriolar proteins, including γ-tubulin, pericentrin, and PCM-1, suggesting a global role of the UPS in controlling centrosome homeostasis (Didier et al., 2007).

1.3.2. Autophagy

In 1963, autophagy was described for the first time by Christian de Duve, who discovered autophagosomes delivering intracellular compartments to the lysosome (Duve C.D, 1963). The autophagy refers to a process by which the cellular contents are sequestered in double-membrane vesicles and delivered to the lysosome (vacuole in yeast) for degradation. Later, Ohsumi et al. observed spherical bodies in yeast vacuoles after nutrition depletion. These spherical bodies, termed autophagic bodies, contained cytoplasmic ribosomes, glycogen granules, lipid granules, and mitochondria (Takeshige et al., 1992). This was the first report indicating a correlation between nutrition depletion and autophagy in yeast.

Autophagy is categorized into three types: microautophagy, macroautophagy, and chaperone-mediated autophagy (CMA). Microautophagy refers to a process where the protein is directedly uptaken by the lysosome/vacuole membrane. Macroautophagy is the formation of a double membrane vesicle, the autophagosome, which sequesters the cargo protein away from the lysosome/vacuole, fuses with the lysosome/vacuole membrane, and releases the inner vesicle (autophagic body) inside the lysosome/vacuole. In CMA, a chaperone binds to a target protein, unfolds it, and directedly translocates it across the lysosome membrane. CMA hasn't been identified in yeast so far. Independently of the delivery pathway, cargo proteins delivered to the lysosome/vacuole undergo degradation (reviewed in W. W. Li et al., 2012; Demine et al., 2012).

Eighteen essential autophagy-related (ATG) have been identified so far (C. W. Wang & Klionsky, 2003). Autophagy is a dynamic process that can be broken down into discrete sequential steps, as described below.

Autophagy Induction: Nutrition starvation is the key stimulator of autophagy initiation. One of the vital regulatory components of autophagy is the protein kinase TOR (Target of Rapamycin), which negatively regulates autophagy in nutrient-rich conditions by

phosphorylating Atg13. Upon starvation, Atg13 is dephosphorylated and can bind to Atg1, the sole Atg kinase, to initiate autophagy (Schmelzle & Hall, 2000; Scott et al., 2000).

Cargo selection: Two types of cargo selection by autophagy have been defined as yet; nonselective and selective autophagy. In nonselective autophagy, a bulk of random cytoplasmic components (autophagosome) is delivered to the vacuole for destruction. Light microscopy and EM studies of yeast mutants deficient in vacuolar protease under starvation conditions revealed that autophagic bodies contain lipid granules, glycogen granules, cytoplasmic ribosomes, and mitochondria (Takeshige et al., 1992). Selective autophagy refers to the removal of certain cellular components, such as damaged organelles and aggregated proteins (reviewed in Parzych & Klionsky, 2014). Depending on the organelle that undergoes autophagy, selective autophagy is categorized into different types, such as pexophagy (peroxisome degradation), mitophagy (mitochondria degradation), and reticulophagy (endoplasmic reticulum degradation) (Gatica et al., 2018).

One distinct type of selective autophagy is cytoplasm-to-vacuole targeting (Cvt) which is specific for yeast. The Cvt pathway delivers vacuolar enzymes, such as α-mannosidase (Ams1) and aminopeptidase I (Ape1), from the cytoplasm to the vacuole. Ape1 is synthesized as an inactive proenzyme called precursor Ape1 or prApe1. Atg19, a receptor for Ams1 and prApe1, recognizes prApe1 after its oligomerization. Once Atg19 and preApe1 bound and form a Cvt complex, Atg19 binds to Atg11, which directs the Cvt complex towards a perivacuolar location named phagophore assembly site or pre-autophagosomal structure (PAS), an organizing center to recruit Atg proteins (Baba et al., 1997; Hutchins & Klionsky, 2001). Subsequently, the outer membrane of the Cvt vesicle fuses with the vacuole and releases its cargo in the vacuole lumen. Once prApe1 is delivered to the vacuole, its propeptide is removed by proteinase B to form the active version of the enzyme, which functions in the vacuole lumen (Harding et al., 1996; Sidney V. Scott et al., 1997).

Vesicle formation: In most intracellular trafficking processes, the vesicles are budded from pre-existing organelles. Autophagosomal vesicle formation is a complex process. One view is that autophagy vesicles form de novo in the cytoplasm. Another theory is that the vesicles generate from pre-existing organelles such as the ER, mitochondria outer membrane, nuclear membrane, and plasma membrane. After forming, the autophagosomal vesicle moves to the PAS and forms a sac-like shape termed phagophore. The further extension of the phagophore generates the double membrane autophagosome (reviewed in Li & Zhang, 2019). Based on

current evidence, 16 out of 18 ATG genes are required for autophagosome formation, and it seems Atg17 is the scaffold protein in the PAS organization (K. Suzuki et al., 2007).

Vesicle fusion with vacuole: After autophagosome formation, it must be targeted to the lysosome/vacuole for the fusion process. In yeast, this fusion involves the SNARE proteins, Vam3, Vam7, Vit1, Sec17, Sec18, and Sec19, the Rab small GTPase family, Ypt7, class C Vps/HOPS complex. Later, two more proteins were added to this list, Ccz1, and Mon1 (Fischer Von Mollard & Stevens, 1999; C. W. Wang & Klionsky, 2003). Once the autophagosome fuses to the vacuole, autophagic body is released inside the vacuole lumen.

Vesicle breakdown: The main purpose of autophagy in yeast is to degrade the translocated cargo in the vacuole and recycle the macromolecules to reuse in essential component synthesis. To achieve this goal, the autophagic body must be broken down to expose its cargo to the vacuolar enzymes. This process depends on the acidic pH in the vacuole lumen and proteinase B (Prb1) (Takeshige et al., 1992). Besides, Cvt17/Aut5, a glycosylated integral membrane protein, plays a role in the autophagic body breakdown. Cvt17 contains a lipase active site motif that is essential to disintegrate the lipid membrane of the vesicle (Epple et al., 2001). Two more components are involved in autophagosome breakdown: Atg15 and Atg22. Atg15 induces inner vesicle degradation, whereas Atg22 mediates the efflux of leucine and other amino acids after degradation in the vacuole to the cytoplasm (reviewed in Klionsky, 2005; Song & Kumar, 2012). After the vesicle breaks down, the macromolecules need to release back into the cytosol.

There are pieces of evidence indicating that autophagy regulates centrosome homeostasis. Mouse embryonic fibroblast (MEFs) and hematopoietic cells derived from autophagy-deficient mice accumulate the centrosomal protein, Cep63, increasing the numbers of centrosomes in the cells (Watanabe et al., 2016). Another study has shown the autophagy involvement in centrosome organization and stability by targeting the satellite organizer, PCM1, a process they termed doryphagy (Holdgaard et al., 2019). Conversely, centrosome amplification (CA), a hallmark of cancer cells, has been reported to interfere with autophagosome trafficking and lead to autophagosome accumulation (Denu et al., 2020). In summary, these recent studies highlight the role of autophagy in centrosome homeostasis, but no study has addressed its involvement in SPB regulation.

1.3.3. Proteolysis

The hydrolysis reaction of peptide bonds that leads to protein breakdown into smaller peptides is termed proteolysis and can be performed by either chemicals or enzymes. Enzymatic proteolysis regulates several crucial physiological processes, such as DNA replication and cell cycle progression (Turk, B., 2006). One of the well-defined proteases is separase (Esp1), which cleaves the cohesin complex at the onset of anaphase to separate sister chromatids. The Separase cleavage site has been identified as SxExxR (cleavage after Arg), based on the human cohesin subunit SCC1 (Hauf et al., 2001). Besides sister chromatid separation, separase is involved in centrosome regulation. In Xenopus egg extract, separase plays a key role in centriole disengagement allowing the centrosome to be duplicated in the next cell cycle (Tsou & Stearns, 2006). In addition, Pericentrin (Spc110 in budding yeast) is cleaved by separase at its consensus site, and mutations leading to a non-cleavable pericentrin suppresses centriole disengagement and centrosome duplication (Matsuo et al., 2012). In budding yeast, separase (Esp1) localizes to the SPB and is involved in the anaphase spindle elongation (Baskerville et al., 2008). Esp1 stabilizes spindle elongation by cleaving the kinetochore-associated protein, Slk19 (Sullivan et al., 2004). Although Esp1 colocalizes in SPB, whether SPB components are a substrate of Esp1 remains to be investigated.

2. RESEARCH AIMS

The aim of this thesis was to further our understanding of the fundamental mechanisms that regulate the spindle microtubules and the spindle pole body in budding yeast. To achieve this, the papers presented in this thesis focused on four specific goals:

1. Develop a method to separate old and new SPB subunits for biochemical characterization (Paper I).
2. Identify age-related phosphorylation sites in the SPB protein Spc110 and investigate their potential roles in cell cycle progression (Paper I).
3. Investigate the role of +TIP Bik1 at nuclear MT (Paper II).
4. Study Spc110 dynamics and turnover (Paper III).

3. MATERIALS AND METHODS

This section presents the main methods used in studies I-III. For a comprehensive and detailed description, see each paper's Materials and Methods section in the Appendix.

3.1. *Saccharomyces cerevisiae* as a model organism

The budding yeast *S. cerevisiae* has been one of the most widely used eukaryote model organisms for over a century. In 1986, Mortimer et al. crossed several parental strains to generate the S288 reference strain (Mortimer & Johnston, 1986). The whole genome of the S288 strain was sequenced in 1996 to facilitate genetic and molecular studies (Goffeau et al., 1996). In total, *S. cerevisiae* has 6,275 genes and 23% homologous genes to humans, which makes budding yeast a helpful model (Liti, 2015; Liu et al., 2017). Studies on yeast organelles, including mitochondria, centrosome, nucleus, vacuole, and endoplasmic reticulum, have advanced our understanding of higher eukaryote organelles. For example, discovery of autophagy genes and their underlying mechanisms in budding yeast led to identifying mammalian orthologs (Tsukada, 1993).

Budding yeast has several characteristics that make it a good model organism. First, it has a short generation time, and its growth and maintenance are easy and cheap. Furthermore, its simple genetic manipulation allows the addition or deletion of genes through homologous recombination. Finally, budding yeast can be easily arrested at specific cell cycle phases with different genetic and chemical methods. For example, haploid MATa strains can be reversibly arrested at G1 by adding the peptide pheromone alpha factor (WHWLQLKPGQPMY), which activates the mating pathway.

3.2. Recombination-induced tag exchange (RITE)

The recombination-induced tag exchange (RITE) is a method to label old and new proteins based on a genetic switch of epitope tags by an inducible Cre recombinase (Verziljlbergen et al., 2010). The RITE system is composed of two parts: a RITE DNA cassette integrated at the C-terminal of the gene of interest and a hormone-dependent Cre recombinase that controls the tag exchange. Cre recombinase is a site-specific recombinase that recognizes and cleaves a DNA sequence called the locus of the X-over P1 (loxP) site. Cre recombinase is fused to a derivative from the estrogen-binding domain (EBD) from the human receptor gene, EBD78 (Lindstrom & Gottschling, 2009). Cre-EBD78 is sequestered in the cytoplasm by heat shock

protein Hsp90. Once the β-estradiol is introduced, Cre can translocate to the nucleus, where it recognizes the loxP site and cleaves the DNA.

The RITE cassette encodes two tags, the first tag (old tag) is flanked by two loxP recombination sites, and the second tag (new tag) is integrated downstream of the distal loxP. Adding the hormone β-estradiol to the media activates the Cre recombinase and induces the DNA recombination between the two loxP sites. As a result, the old tag is exchanged for the new tag at the genomic level (epitope-tag switching) (Figure 5). This system allows distinguishing the protein of interest before (old tag) and after switching (new tag). To prevent spontaneous recombination, a selective marker is inserted between the two loxP sites (Verzijlbergen et al., 2010). The RITE system has a number of advantages: i) the gene is tagged with RITE at its endogenous locus; ii) the tag switching is permanent and allows monitoring of the old protein through successive cell divisions; and iii) the old and the new protein can be monitored simultaneously under physiological conditions by microscopy and/or biochemical techniques. RITE has been used in different studies to measure the turnover of histone proteins (Molenaar et al., 2020), to follow the proteasome dynamics in aging cells (van Deventer et al., 2015), and to visualize the inheritance of intracellular components such as the NPC and the SPB during cell division (Menendez-Benito et al., 2013; Lengefeld et al., 2018).

3.3. RITE-based assay to purify old and new Spc110

In paper I, a method was developed based on the RITE system to separate and purify old Spc110 (originated from the previous cell cycle) and newly synthesized Spc110. A plasmid was generated containing a RITE cassette with two short epitope tags amenable for affinity-based purification: 5XFlag (first epitope) and V5 (second epitope) (Figure 5). Next, the SPC110 gene was tagged with the RITE cassette in a yeast strain expressing β-estradiol-inducible Cre-recombinase (Cre-EBD78). In the cell, the SPB proteins can be assembled into the SPBs, into sub-complexes, or unbound. To characterize the phosphosites of the SPB complex, intact SPBs were first purified to eliminate unbound subunits before the affinity purification of old and new Spc110. To isolate intact SPBs, we added a C-terminal TAP-tag to the C-terminus of Spc97, as described by Fong et al. (Fong et al., 2016).

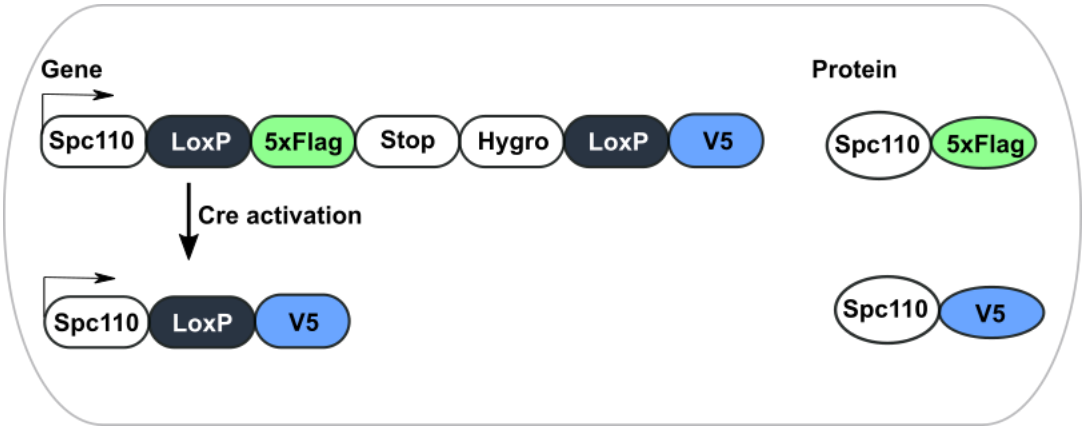

Figure 5. RITE system outline. The RITE cassette contained two epitope tags (Flag and V5) inserted at the Spc110 C-terminus. The first tag is followed with ADH1 terminator sequence (stop), Hygromycin (Hygro) resistance marker, and the second LoxP. Upon Cre activation, the first tag is replaced with the second tag.

To couple the DNA recombination with the cell cycle, the cells were grown until they consumed the glucose and reached the post-diauxic shift (PDS). In this condition, protein synthesis of most proteins (including Spc110) is low, and most of the cells stop dividing but remain viable (Wer -Washburne M et al., 1993). Then β-estradiol was added to activate the Cre recombinase and induce the genetic switch. Once the switching was completed (16 h), the cells were released in fresh media containing glucose. The cells were collected 2.5 h after the release, when most of them had duplicated their DNA and SPBs and lysed by cryogenic grinding with a freezer mill. Afterward, TAP-immunoprecipitation was performed to purify SPB complexes. Menendez-Benito et al. have shown that newly synthesized Spc110 is incorporated in new and old SPBs (Menendez-Benito et al., 2013). Accordingly, the old and the new protein needed to be dissociated in denaturing conditions to prevent co-purification of old- and new-Spc110. To break down the SPB complex, trichloroacetic acid (TCA) was applied as a denaturing component that preserves PTMs. Following, precipitated proteins were dissolved in a mild denaturing buffer. Finally, immunoprecipitation was performed against Flag or V5, to purify old- and new-Spc110, respectively.

In summary, we generated a strain expressing Spc110-RITE (Flag →V5), Spc97-TAP, and an inducible Cre recombinase and followed four steps to purify old and new Spc110: (1) RITE-assay, (2) TAP-enrichment of intact SPBs, (3) Denaturing, and (4) Affinity purification of old and new Spc110.

3.4. CRISPR/Cas9

CRISPR/Cas9 is a genome-editing technology derived from a naturally occurring adaptive immune system of bacteria. CRISPR (clustered regularly interspaced short palindromic repeats) was identified by Dr. Nakata's group (Ishino et al., 1987), which observed an unusual DNA sequence composed of repeats of palindromic sequences of 29 nucleotides separated by non-repetitive DNA sequences, called spacers, in the bacteria Escherichia coli (Mojica et al., 2005; Bolotin et al., 2005). A family of genes is usually in the vicinity of the CRISPR array named CRISPR-associated sequences (Cas), including the gene encoding an endonuclease (Cas9). CRISPR defends bacteria from viruses and plasmids by incorporating their DNA into the CRISPR locus. The defense relies on the cleavage of virus or plasmid DNA into a novel spacer stored upstream of the CRISPR array in the DNA. Once the same virus or plasmid invades, its DNA will be recognized, and the Cas nuclease will cut the DNA of the invading agent apart (Horvath & Barrangou, 2010; Jinek et al., 2012). This mechanism encouraged researchers to adapt the system as a gene editing method named CRISPR/Cas9.

The CRISPR/Cas9 system possesses two parts, a single guide RNA (sgRNA) and a Cas9 endonuclease. The sgRNA consists of a 20-nucleotide-long sequence complementary to the target DNA (spacer) at the 5'-end, followed by a constant part that forms a stem-loop scaffold for Cas9 binding. Cas9 also requires a sequence next to the gRNA-DNA homology region, called the protospacer-adjacent motif (PAM). The PAM sequence depends on the bacterial species of the Cas nuclease. For instance, Cas9 derived from Streptococcus pyogenes (SpCas9) recognizes the PAM sequence 5'-NGG-3'. Once the gRNA spacer binds the complementary DNA region (protospacer) and Cas9 is bound to PAM, Cas9 produces a double-strand break (DSB) in the target DNA. SpCas9, for example, cuts three nucleotides upstream of PAM. The DSB induces DNA repair pathways, non-homologous end joining (NHEJ), or homology-directed repair (HDR), which can result in a sequence insertion or deletion (Heler et al., 2015; Impens et al., 2022). Whereas NHEJ is an error-prone pathway, HDR can be used for precise gene editing, including gene knockout or replacement and point mutation. HDR can be stimulated by adding homologous donor templates (Tang et al., 2019). In my study (Paper I & III), the point mutations were introduced as described by Levi et al. (Levi & Arava, 2020). For each mutant, a 20 nucleotide gRNA was cloned into a single plasmid (bRA66) with a hygromycin selection marker. The donor DNA was designed as an 80-base oligos homolog to the target sequence with the desired point mutation in the middle of the sequence.

CRISPR/Cas9 is easier and more efficient than other gene editing methods. For instance, unlike zinc finger (ZNF) nuclease and transcription activator-like effector nuclease (TALENs), which require the engineering of specific fusion protein pairs for a target site, CRISPR/Cas9 only entails the design of a sgRNA that binds specifically to the target. Furthermore, CRISPR/Cas9 has been developed beyond genome editing for activation or repression of gene expression at specific sites in the genome by fusing a catalytically dead Cas9 to repressors or activator domains (Ma et al., 2014).

3.5. Proximity-dependent biotin identification

Proximity-dependent biotin identification (BioID) was introduced in 2012 by Roux et al. as a new method to screen for protein-protein interactions based on proximity-dependent labeling of proteins (Roux et al., 2012a). In the BioID technique, a mutant of the E. coli biotin protein ligase BirA, a monomeric protein that naturally catalyzes the biotinylation of the biotin carboxyl carrier protein (BCCP), is fused to the protein of interest. Recently, a new version of BirA ligase was introduced, TurboID. The TurboID contains 15 mutations relative to wildtype BirA and is a smaller and more efficient ligase (Branon et al., 2018).

In the presence of biotin, the fusion protein biotinylates those proteins that are in close proximity. Subsequently, biotinylated proteins were purified with streptavidin-coated beads and identified using mass spectrometry or western blot (Sears et al., 2019). BioID has some advantages over more conventional methods to determine protein-protein interactions: it is based on physiological conditions, and since the biotinylation is irreversible and occurs before solubilization, it can detect weak and transient interactions (Roux et al., 2012b).

4. RESULTS AND DISCUSSION

4.1. Paper I – Phosphosites of the yeast centrosome component Spc110 contribute to cell cycle progression and mitotic exit

The SPB is a highly phosphorylated multi-subunits structure. Since many SPB phosphorylation events are cell cycle-dependent, and old (from the preceding cell cycle) and new SPB components appear at different cell-cycle phases, they might acquire different phosphorylations. Indeed, age-dependent phosphosites occur in the OP components Nud1 and Spc72 (Lengefeld et al., 2017; Matellán et al., 2020). However, before the work presented in this thesis, whether the IP subunits acquire diverse age-dependent phosphorylation remained to be addressed. We were interested in Spc110, in which N-terminal phosphorylations activate MT nucleation at the SPB nuclear side. In this study, we developed an assay to detect Spc110 age-dependent phosphorylation residues and explored their potential biological function and impact on cell cycle progression.

4.1.1. Results

Purification of Spc110 from the maternal origin from intact yeast SPBs

Our strategy was to apply the RITE method (sections 3.2 and 3.3) to separate and purify old and new Spc110, followed by mass spectrometry analysis. We generated a yeast strain expressing Spc110-RITE (5xFlag →V5), a β-estradiol inducible Cre recombinase (Cre-EBD78), and Spc97-TAP for purification of intact SPBs. We labeled and purified old (Flag-tagged) and new (V5-tagged) Spc110 following four steps (described in section 3.3): 1) RITE assay, 2) SPB purification, 3) SPB breakdown, and 4) immunoprecipitation.

To label old and new SPBs with RITE, we needed to couple the DNA recombination with the cell cycle. We did so by performing the genetic switching after the post-diauxic shift (PDS), when the cells stopped dividing, and then releasing them in fresh media containing glucose, where the cells re-enter the cell cycle and duplicate the SPB. To assess cell synchronization, we measured the DNA content by flow cytometry. We found a single 1C DNA peak before the release, confirming the efficiency of the arrest using glucose depletion. During the release, cells gradually entered G2/M (2C DNA content), and most (80%) of the cells reached G2/M 2.5 hours post-release. Therefore, we collected the cells 2.5 h post-release to purify old (Flag-tagged) and new (V5-tagged) Spc110. First, we enriched the SPBs by IgG immunoprecipitation of Spc97-TAP, broke down the SPB complex, and affinity-purified old and new Spc110 using

anti-Flag and anti-V5 antibodies. We performed western-blot analyses on the different fractions and confirmed the efficient separation of old and new Spc110 with no apparent cross-contamination.

Identification of phosphosites in Spc110 from maternal origin

Next, we analyzed the purified Spc110-Flag (old, from the previous cell cycle) and Spc110-V5 (new) by mass spectrometry after phosphopeptide enrichment using titanium dioxide (TiO2) and identified two phosphosites on old Spc110, S60, and S11. By contrast, no phosphosites were detected on the new Spc110. With this in mind, we focused on the old Spc110 for further analysis. The mass spectrometry analysis was performed this time without phospho-enrichment, and the outcome was two phosphosites, S36 and S11, on pre-existing Spc110.

Spc110 phosphorylation at S11 and S36 are important for timely cell cycle progression

Prior studies have identified the function of Spc110 phosphosites in the N-terminal region of Spc110 (including S36 and S60) as activators of the MT nucleation (T. C. Lin et al., 2014). The S11 was detected in a high-throughput study (Lanz et al., 2021), yet its function was unknown. Therefore, we generated three yeast strains with non-phosphorylatable mutations (S11A, S36A, and S11AS36A). We measured the growth of the mutated strains using spotting assays at different temperatures (16°C, 30°C, or 37°C), and none showed growth defects.

Next, we asked whether the non-phosphorylatable Spc110 mutants had defects in spindle formation and cell cycle progression. Analysis of the cell cycle showed that single mutated $Spc110^{S36A}$ and double mutated $Spc110^{S11AS36A}$ have a slight delay in SPB separation. Moreover, $Spc110^{S11AS36A}$ had a delay in anaphase progression and a delay to re-enter into the next G1. Another remarkable finding was the $Spc110^{S11A}$ had more robust spindles, mostly notable in anaphase, whereas $Spc110^{S11AS36A}$ has a less intense spindle. Furthermore, we noticed an asymmetric spindle distribution in which spindles nucleate from old SPB has less intensity, in almost half of the $Spc110^{S11A}$ population. This effect was partially decreased in double mutant S11AS36A and single mutant S36A.

Since we observed a slight delay in the cell cycle progression of $Spc110^{S11AS36A}$, next, we investigate the dynamics of the cell cycle regulator Clb2. As explained earlier, Clb2 activates APC/C to trigger metaphase-anaphase transition and anaphase spindle elongation (Rahal & Amon, 2008). In addition, Clb2 is depleted at the end of mitosis, which makes it an indicator

of the mitosis exit (Irniger et al., 1995; Bäumer et al., 2000). We compared the Clb2 expression in wild-type and double-mutated cells. In our experiment, both accumulation and degradation of Clb2 are delayed in double mutant cells, confirming the cell cycle delay in $Spc110^{S11AS36A}$.

Spc110 phosphorylation at S11 and S36 are not required for SPB inheritance

Previous studies have implicated the role of age-dependent phosphorylation in proper SPB inheritance (Hotz et al., 2012; Lengefeld et al., 2017; Matellán et al., 2020). However, measuring the SPB inheritance pattern in wild-type and double mutated $Spc110^{S11AS36A}$, we found that S11 and S36 phosphorylations are not required for SPB inheritance.

Double mutant $Spc110^{S11AS36A}$ cells have a delayed mitotic exit

Our microscopy and western blot analysis indicated a normal SPB inheritance but delayed cell cycle progression in $Spc110^{S11AS36A}$. Next, we looked at the mitotic exit in more detail by following the localization of Cdc14, a key regulator of anaphase and mitotic exit (described in section 1.1.8) after releasing G1-synchronised cells in fresh media and focusing on anaphase cells. Our results showed that $Spc110^{S11AS36A}$ is delayed in fully releasing Cdc14 and re-localizing Cdc14 in the nucleolus. These findings confirm that Spc110 phosphorylation at S11 and S36 is important for timely mitotic exit.

4.1.2. Discussion

The initial objective of the study was to establish a method to purify old and new SPB components to monitor age-dependent phosphorylation within a cell cycle. Several techniques have been developed to label old and new proteins differentially. A common technique used in combination with mass spectrometry is Stable Isotope Labeling with Amino acids in Cell culture (SILAC), a metabolic labeling technique in which essential amino acids, or light label, and isotopic modified amino acids, or heavy label, are added to growing cells to label the proteins. SILAC can be used for several purposes, such as quantitative proteomic analyses that compare treated and untreated cells. Pulse-chase SILAC can label pre-existing and newly synthesized proteins to measure the rate of protein degradation or synthesis (Xixi Wang et al., 2018). Principally, SILAC is one of the most accurate techniques for quantitative proteomics. One limitation of SILAC is the time isotope label required to incorporate into the protein. Previously, it was shown labeling might take a few generations in budding yeast. A recent study has suggested the timing is shorter by testing isotope labeling of nuclear pore

complex components in yeast (Onischenko et al., 2020). The study revealed component labeling differs from 30 min up to 150 minutes depending on protein maturation and exchange dynamics. Since the budding yeast cell cycle is only 90 minutes, SILAC might be unfitting for our purpose to monitor the labeled old and new SPBs within only one cell cycle. To overcome this issue, we applied the RITE system to tag our protein of interest endogenously. The biggest challenge to developing the method was cell cycle synchronization. Although the most common method to arrest budding yeast in G1 is mating pheromones (α-factor), we would need to prolong the arrest via continuous treatment with α-factor for an efficient genetic switch. Hence, we performed cell cycle arrest and genetic switching after reaching the PDS by glucose exhaustion. A caveat of this synchronization method is that about 20% of cells cannot exit the cell cycle arrest.

Previously, a RITE assay was developed to monitor the duplication and inheritance of the SPB by fluorescence microscopy (Menendez-Benito et al., 2013). In this assay, the SPB proteins Spc42 and Spc110 were tagged with a fluorescent RITE (GFP→RFP) cassette, and the distribution of the old (GFP) and new (mRFP) subunit was monitored in the following cell division. Interestingly, these analyses showed that newly synthesized Spc42 and Spc110 were incorporated into both SPBs, while old proteins were primarily localized in the SPB that segregate to the bud. Thus, our upcoming challenge was to purify unmixed old and new components. To solve this limitation, we broke down the SPB complex in an acidic environment combined with high heat. Then, we solubilized the proteins in an environment compatible with Flag and V5 immunoprecipitation to purify old and new Spc110. Eventually, our western blot analysis revealed no visible cross-contamination of old (Flag-labelled) and new (V5-labelled) Spc110. Upon minor adjustment in the protocol, we succeeded to purify the pre-existing and new cytoplasmic SPB component, Nud1. Thus, the method is applicable to enrich other SPB proteins or even other cellular components.

We identified 3 phosphosites on old Spc110: S11, S36, and S60 by mass spectrometry, which, considering the results from flow cytometry, we interpret discovered phosphosites are present at G2/M. In future research, these phosphorylation sites could be verified using phosphosite-specific antibodies, and the method could be refined with additional analysis including internal controls (synthesized phosphopeptides) for quantification.

Several phosphosites have been identified in the N-terminal domain of Spc110, including T18, S36, S60, T64, T68, and S91, both in proteomic studies (Keck et al., 2012; Fong et al., 2018)

and in-depth functional studies (Huisman et al, 2006; T. C. Lin et al., 2014). Our analyses identified S36 and S60 in old Spc110, but low sequence coverage prevented us from detecting the T64, T68, and S91 phosphosites in either old or new Spc110. Furthermore, despite having sufficient coverage in those regions, we could neither find phosphosites at T18 nor T30. Our sample preparation, compared to previous studies (Keck et al., 2012; Fong et al., 2018), includes two additional steps, i.e., denaturing the SPB complex and performing a second immunoprecipitation against Flag/V5, which might cause the phosphosite loss. Another possible explanation would be that the phosphosites were affected during the PDS. This is unlikely since a recent phospho-proteome analysis during PDS identified Spc110 phosphosites at T18, S36, and S60 (Gassaway et al., 2021). Yet, it remains to be proved that the release from the PDS step does not affect the phosphosites.

On the other hand, we identified a phosphosite, S11, which had been reported in a proteome-wide study (Lanz et al., 2021), but was not detected in studies mapping the phosphosites of intact SPBs (Keck et al., 2013; Fong et al., 2018). While the SPB phosphoproteome analyses used cycling cells and cells arrested in G1, G1/S, and mitosis (metaphase/anaphase transition), we used cycling cultures with about 80% cells in G2/M. Hence, if S11 phosphorylation only occurs transiently, for example, in early anaphase, our method would be more likely to detect it.

Exploring the roles of S11 and S36, we found that the non-phosphorylatable mutants Spc110^{S36A} and Spc110^{S11AS36A} (but not Spc110^{S11A}) delayed SPB separation. This finding is in agreement with previous research showing that the Spc110 Cdk sites (S36 and S91) are phosphorylated from S phase (Huisman et al., 2007) to stimulate γ-TuSC nucleation and spindle assembly (T. C. Lin et al., 2014). Furthermore, the mutant Spc110 ^{S11AS36A} showed prolonged anaphase and delayed re-entry in G1, indicating that both S11 and S36 regulate mitotic progression. Interestingly, the spindle in the Spc110^{S11A} mutant was more robust than WT spindles at early anaphase. This phenotype was attenuated in the Spc110^{S11AS36A} mutant, and Spc110^{S36A} spindles had the same intensity as in WT cells. Furthermore, we also found that the spindle was asymmetric (with fewer MTs closer to the old SPB) in Spc110^{S11A} and, to a lesser extent, in the Spc110^{S11AS36A} and the Spc110^{S36A} mutant. The different phenotypes of the mutants indicate that S36 and S11 phosphosites have distinct roles. Whereas S36 has a role in timely SPB separation and spindle elongation, S11 might regulate MT dynamics to maintain the symmetric distribution of MTs in the spindle.

Our analysis of the Cdc14 also showed that Spc110^{S11AS36A} had a delayed exit from mitosis and re-entry to the next G1. The delay of Spc110^{S11AS36A} may be explained by a failure in regulating MT dynamics, resulting in a slow mitotic spindle elongation, a delay in the MEN pathway, and subsequently, a delay in Cdc14 release from the nucleolus. Interestingly, in a recent phosphoproteomic analysis of analog-sensitive alleles of Cdc5, S11 phosphorylation was reported as Cdc5-dependent in anaphase (Zhou et al., 2021, supplementary data). The pole kinase Cdc5 is one of the key regulators in the MEN pathway (section 1.1.8) and Cdc5 might be required to maintain Spc110 phosphorylation on the old SPB, where the MEN pathway is active. Furthermore, we searched the potential kinases for Spc110-S11 using Scansite 4, and the predicated kinase was Cdc15, another effector of the MEN pathway. Hence, S11 phosphorylation might be a potential target for the MEN pathway.

In summary, in this study, we identified three age-dependent phosphorylation sites in Spc110 (S11, S36, and S60) that are important for spindle dynamics and timely cell cycle progression.

4.2. Paper II –The microtubule plus-end tracking protein Bik1 is required for chromosome congression

Bik1 is a +TIP protein that localizes in the cytoplasmic and nuclear MTs. The Bik1 cytoplasmic pool plays a role in mitotic spindle orientation by two pathways, Kar9 and dynein (R. K. Miller et al., 2006), yet its nuclear pool function is not well defined. This study investigated the nuclear Bik1 function during the cell cycle.

4.2.1. Results

Bik1 localizes to the kinetochores in a cell cycle-dependent manner

First, we explored the localization of nuclear Bik1 using a strain expressing Bik1-sfGFP, mTurquoise-Tub1 (MT marker), Spc42-TagRFP-T (SPB marker), and Ndc80-TagRFP-T (kinetochore marker) in the cell cycle. The microscopic observation revealed Bik1 was localized at kinetochores from G1, and further accumulating at this location during metaphase. At anaphase, Bik1 is no longer localized at the kinetochore and gathers in the spindle midzone, then dissociates from the spindle. In addition, we observed Bik1 localization at kinetochore clusters in metaphase-arrested cells. Together, these results demonstrate the Bik1 localized at kinetochore in a cell cycle-dependent manner.

Timely cell cycle progression required nuclear Bik1

Bik1 localization on cytoplasmic MTs is crucial for the Kar9 and Dynein pathway to orient the mitotic spindle properly (Carvalho et al., 2004). To explore the nuclear pool function of Bik1, we used a nuclear export sequence (NES) (Kosugi et al., 2008) to disturb the nuclear pool of Bik1 while maintaining its cytoplasmic pool. First, we confirmed that Bik1-NES was excluded from the nucleus while its localization at the cytoplasm was preserved. Next, we assessed the cell cycle progression in Bik1 (WT) and Bik1-NES cells. We counted the number of cells at G1/S, metaphase, and anaphase, according to the spindle length, based on the mTurquoise-Tub1 marker. Bik1-NES cells were characterized by a broader metaphase peak, indicating a delay in metaphase before spindle elongation.

To confirm our finding, we monitored the cells by time-lapse imaging. In WT cells, Bik1 was localized on the kinetochores from G1 until metaphase, and its colocalization on the kinetochore was not detectable after the spindle elongation at anaphase. Bik1-NES cells had an average delay of 10 min from when two kinetochore clusters appeared to the time of their separation. In addition, 10% of Bik1-NES cells had a characteristic phenotype, where a short spindle is transiently transferred into the daughter cell before spindle elongation.

The absence of nuclear Bik1 induces aberrant kinetochore positioning

We observed an abnormal phenotype with elongated, unfocussed kinetochore stretches in Bik1-NES cells. To measure the frequency of this phenotype, we arrested the cells in metaphase and analyzed the position of kinetochores (Ndc80-sfGFP) relative to SPB (spc42-TagRFP-T) in WT and Bik1-NES. We discovered that 60% of Bik1-NES cells at metaphase have a long stretch of Ndc80 (unclustered kinetochore). In addition, line-scan analyses revealed that the unclustered kinetochores were mispositioning towards the spindle equator.

Bik1 interaction with Cin8 at the spindle

Next, we asked about potential Bik1 interaction partners. We immunoprecipitated Bik1-5XFlag in metaphase-arrested cells and ran mass spectrometry analyses, identifying the kinesins Kip2, Cin8, Kip1, Kip3, and Kar3. Kip2 is a known interaction partner of Bik1 at cytoplasmic MTs (Carvalho et al., 2004). Deleting CIN8, KIP1, or KIP3 results in kinetochore unclustered phenotype (Tytell & Sorger, 2006; Gardner et al., 2008, Wargacki et al., 2010). Furthermore, the unclustered kinetochores appear towards the spindle midzone in Cin8 and Kip1 cells, like the phenotype observed in Bik1-NES. Hence, we focused on Cin8 and Kip1 and investigated their interaction with Bik1 using BioID. We fused Bik1 with TurboID (an

engineered biotin ligase, see section 3.5) in combination with either Cin8-Flag, Kip1-Flag, Kip2-Flag (positive control), or Mre11-Flag (negative control). Cin8, Kip1, and Kip2 were recovered after performing streptavidin affinity purification, indicating close proximity of Bik1 with both Cin8 and Kip1. We utilized bimolecular fluorescence complementation (BiFC) technology, to confirm the observation. Briefly, Bik1 was C-terminally fused to an N-terminal fragment of the fluorescent protein Venus, and Cin8 was C-terminally fused to the complementary C-terminal fragment of Venus. A BiFC signal was observed as one or two foci at the spindle in metaphase cells, whereas only one dot appeared around the spindle midzone at anaphase. In summary, the mass spectrometry and proximity-based assays (BioID and BiFC) prove that Bik1 is localized close to the kinesins Cin8 and Kip1.

Finally, we assessed Bik1 and Cin8 interdependency to localize at the spindle. Cin8 still appeared as two foci in the mitotic spindle in Bik1-NES cells. Intriguingly, Cin8 did not overlap with the unclustered kinetochore. Thus, nuclear Bik1 is not required for Cin8 localization. On the other hand, Cin8 deletion cells showed remarkably reduced Bik1 intensity at the spindle. Therefore, nuclear Bik1 localization on the spindle is partially dependent on Cin8.

4.2.2. Discussion

In the present study, we report that Bik1 localizes near the kinetochore in a cell cycle-dependent manner, starting at G1 and peaking at metaphase. The mitotic spindle must capture the kinetochore before anaphase onset, for efficient chromosome segregation. It has been previously shown that Bik1 is required for kinetochore capture after centromere replication by promoting MT growth that enables lateral attachments of kinetochores (T. U. Tanaka et al., 2005). Furthermore, Bik1 is important in polyploid cells, where it contributes to the pulling forces at the kinetochore-MT interface in the metaphase (H. Lin et al., 2001). However, its corresponding role in haploid cells was not investigated. Therefore, it is possible that Bik1 localization near kinetochores in haploid cells also contributes to the pulling forces at the kinetochore-MT interface and the bi-orientation of sister chromatids. Once all chromosomes are bi-oriented, and SAC is satisfied, Bik1 is no longer required, and change the localization at anaphase. Further work is needed to establish the mechanism regulating the change in Bik1 localization.

In addition, we demonstrated that nuclear Bik1 is required for timely cell cycle progression. In the absence of nuclear Bik1, cells delayed exiting metaphase. Our follow-up microscopy

analyses revealed that cells lacking nuclear Bik1 often have unclustered kinetochores mispositioned towards the spindle equator. Interestingly, the kinesins Cin8, Kip1, and Kip3 also play an essential role in chromosome congression, although deletion of each kinesin leads to a different type of defects in kinetochore clustering (Wargacki et al., 2010; Tytell & Sorger, 2006; Gardner et al., 2008). The kinetochores move toward the spindle equator in cin8Δ and kip1Δ cells while it is positioned closer to the SPBs in the kip3Δ cells (Wargacki et al., 2010). Bik1-NES cells showed the same kinetochore phenotype as cin8Δ and kip1Δ cells. Our findings suggest that Bik1 and Cin8/Kip1 are non-redundant in establishing chromosome congression.

Furthermore, we found out using proximity-binding assays that Bik1 is near Cin8 and Kip1. Cin8, like Bik1, localizes to kinetochore in a cell cycle-dependent manner, reaching the greatest peak at metaphase, and cin8Δ cells have a metaphase delay (Goldstein et al., 2017; A. Suzuki et al., 2018). Cin8 re-localizes to interpolar MT and at the spindle midzone, contributing to the spindle elongation (Gerson-Gurwitz et al., 2011). Based on these observations, we speculate that Bik1 and Cin8 might interact and cooperate to establish chromosome congression at the metaphase to anaphase transition.

Interestingly, we found that in Bik1-NES cells, Cin8 still forms two foci but is not localized to the unclustered kinetochores. This effect is puzzling because Cin8 has been shown to mediate tension at the kinetochore (Suzuki et al., 2016), and bind directly to the kinetochore component Dam1 in vitro (Suzuki et al., 2018). Bik1 could link the kinetochore to the MT plus-ends, thereby facilitating the role of Cin8 in generating tension. cin8Δ cells have longer kMTs during metaphase, and it has been proposed that Cin8 promotes MT plus-end disassembly (Gardner et al., 2008). Bik1 might contribute to this function of Cin8 by increasing the processivity of Cin8, thereby helping Cin8 to stay at long kMTs to depolymerize them for effective chromosome congression.

On the other hand, Cin8 deletion induces a notable decrease in Bik1 levels at the spindle. This decrease could indirectly result from defects in kinetochore-MTs attchment in the absence of Cin8. Indeed, cin8Δ cells have been shown to have severe defects in the mitotic spindle assembly (Hoyt et al., 1992), and loss of tension at the MT-binding domain of the kinetochore component (A. Suzuki et al., 2018), and both effects might contribute to the low Bik1 recruitment to the spindle.

In summary, the current study showed that nuclear Bik1 plays an important role in chromosome congression. Interestingly, CLIP-170 (the human homolog of Bik1) is also involved in chromosome congression and facilitates kinetochore-microtubule attachment (Tanenbaum et al., 2006). Thus, further studies on the mechanisms and regulation of the Bik1 function are also relevant to improve our knowledge about CLIP-170.

4.3. Paper III –Studies on Spc110 turnover

Yoder et al. revealed that Spc110 is dynamic during the cell cycle. Specifically, they showed that 50% of old Spc110 are exchanged with new ones at G1/S (exchange), then Spc110 remains stable until mitosis, where new Spc110 are incorporated (growth) (Yoder et al., 2003). In this study, we compared Spc110 half-life with OP subunits using cycloheximide-chase and tested the role of different degradation pathways in Spc110 turnover.

4.3.1. Results

Spc110 is a short-lived protein

We constructed strains in which Spc110, Spc72, and Nud1 were endogenously tagged with Flag and measured the half-life of these proteins using cycloheximide (CHX)-chase experiments. Briefly, we added cycloheximide, a protein synthesis inhibitor, to the cell culture and harvested samples every 10 minutes. The three proteins had a short half-life, Nud1 is the fastest degraded protein (less than 10 min), Spc110 half-life was around 10 min, and Spc72's was 20 min. Also, the half-life of Spc42 and Cnm67 was tested, which was about 40 minutes, indicating that Spc42 and Cnm67 are stable proteins.

Autophagy is not essential for Spc110 degradation

Holdgaard et al. showed that autophagy deficiency results in centriolar satellites accumulation that leads to centrosomal dysregulation, establishing a role for autophagy in centrosome homeostasis (Holdgaard et al., 2019). Here, we investigated whether autophagy plays a role in Spc110 turnover using ATG1Δ cells expressing Spc110-Flag. The ATG1 gene encodes the Atg1 protein, a serine-threonine kinase essential for autophagosome formation. However, the CHX chase analyses revealed that Spc110 half-life was the same in WT and Atg1 deficient cells, indicating that autophagy is not essential for Spc110 degradation.

Spc110 is a ubiquitin substrate

The UPS system is the main proteolytic pathway responsible for degrading short-lived and regulatory proteins (Finley et al., 2012). Since the Spc110 half-life was short, we sought to investigate if Spc110 is ubiquitylated. To this end, we used cells expressing exogenous 8xHis-Ub and Spc110-Flag and performed Ni-NTA affinity purification in denaturing conditions (to purify His-Ub proteins) followed by a Flag western blot. Spc110 was recovered after His-Ub enrichment, suggesting that Spc110 is a ubiquitin substrate.

Mutating a potential Esp1-target site in Spc110 stabilizes the protein

Apart from UPS and autophagy, proteins can be cleaved by cellular proteases. The human homolog of Spc110, Pericentrin, is cleaved by Separase (Matsuo et al., 2012), a cysteine protease involved in the cleavage of cohesin for sister chromatid separation and in centriole disengagement (Uhlmann et al., 1999) (described in section 1.1.3).

The recognition site of the yeast homolog of Separase, Esp1, has been defined as the sequence "SxExxR" and should be cleaved after the Arginine (R) (Rosen et al., 2019). Spc110 has a potential Separase cleavage site between the amino acids 685 and 690 (SLENDR**)**. We next try to test if Esp1 and Spc110 interact using BioID. Spc110 bound unspecific to streptavidin beads, and we could not perform the experiment. We could neither detect an interaction between Spc110 and Esp1 with co-immunoprecipitation, which might indicate that the interaction could be transient.

To test the Esp1 involvement in Spc110 degradation, we generated a Spc110 mutant by substituting the putative Esp1 cleavage site from SL**E**ND**R** to SL**R**ND**E**. We examined the stability of the mutant Spc110 by CHX chase and observed that disruption of the putative Esp1 site partially stabilized Spc110.

4.3.2. Discussion

Previous studies have proposed that the SPB is dynamic, based on the growth and exchange of the inner plaque γ-TuSC receptor, Spc110 (Yoder et al., 2003). Here, we show that Spc110 and two OP SPB subunits, the γ-TuSC receptor Spc72 and the scaffold protein Nud1, have short half-lives. To the best of our knowledge, there are no published studies on Spc110 and Spc72 degradation pathways in mitotic cells. Nud1 is ubiquitinated by the E3 ligase Dma1/2

at late mitosis to shut off the MEN pathway (Tamborrini et al., 2018). Compared to inner and outer plaque components, the CP subunits, Spc42 and Cnm67, were stable proteins, suggesting the half-life of the SPB proteins might depend on their localization in the complex.

Our pull-down experiments based on ubiquitin overexpression indicate that Spc110 might be a ubiquitin substrate. The SPB components Cmd1, Nud1, and Spc42 were identified as ubiquitin substrates in a proteome-wide mass spectrometry analysis in budding yeast, while Spc110 was not in the list of ubiquitinated proteins (Swaney et al., 2013). This difference suggests that ubiquitinated Spc110 is low abundant and challenging to identify by mass spectrometry. Thus, to determine the Spc110 ubiquitylation residue(s), we propose pulling down Spc110 in denaturing conditions (to remove high abundance proteins that might mask Spc1110 ubiquitylation) followed by mass spectrometry. Furthermore, we should assess if the proteasome has a role in Spc110 degradation using proteasome inhibitors, such as MG132, or the temperature-sensitive proteasome alleles *pre1-1* and *rpt6-1*. If this hypothesis is confirmed, we could screen for the E3 ligase responsible for Spc110 ubiquitylation using yeast two-hybrids.

Autophagy is the main cellular pathway responsible for the clearance of organelles and large protein aggregates and is involved in centrosome homeostasis (Holdgaard et al., 2019). However, our preliminary results indicate that autophagy is not essential for Spc110 degradation. This finding needs to be confirmed with similar experiments inhibiting autophagy genetically by, for example, deleting ATG8, a central player in autophagy that binds to different cargos, or pharmacologically by treating the cells with Bafilomycin A_1 to inhibit autophagosome-vacuole fusion.

The identification of a potential Esp1 consensus site on Spc110 is interesting. It is tempting to speculate that Esp1 cleaves Spc110 at late mitosis, linking Spc110 turnover and the cell cycle. However, the results are still preliminary, and further experimentation is needed. First, we should test whether Spc110 is stabilized using the temperature-sensitive *esp1-1* mutant (Baskerville et al., 2008) at the restrictive temperature. To rigorously demonstrate that Esp1 can cleave Spc110, we could perform an *in vitro* cleave assay using immunoprecipitated Scp110 and Esp1 purified from overexpressing yeast (Uhlmann et al., 1999). Finally, the Esp1 cleavage should produce two fragments with different molecular weights: 81KDa (N-terminal fragment) and 9.8KDa (C-terminal fragment). The first amino acid in the N-terminal fragment is Leucine, which is destabilizing according to the N-end rule (Bachmair et al., 1986), explaining

why we do not detect this fragment in our experiments by western blot. To explore the role of the putative Esp1-site of Spc110, we could treat WT and mutant Spc110 with proteasome inhibitors, followed by western blot analysis to test whether a 9.8 KDa band, corresponding to the putative C-terminal fragment accumulates in the WT strain. Alternatively, tagging Spc110 at the N-terminus would allow us to test for the appearance of the N-terminal 81KDa fragment.

Our data show a short half-life of Spc110. Nevertheless, we found Spc110 degradation is not dependent on autophagy and is partially stabilized by mutating the Esp1 cleavage site. Yet, the mechanism of Spc110 turnover remains unclear and additional studies will be needed to uncover all potential pathways.

5. CONCLUDING REMARKS

This thesis aimed to advance our understanding of the general principles regulating spindle MTs and SPB in budding yeast. Our studies contributed to the field with the results and findings summarized below.

In **Paper I**, we introduced a new method to identify age-associated PTMs in Spc110. Minor adjustments in the protocol helped us to purify the old and new Nud1 as well. Follow-up mass spectrometry analyses would be valuable for verifying (or identifying new) age-dependent phosphosites in Nud1 with a role in SPB inheritance, expanding previous studies by the Barral group (Lengefeld et al., 2017). Also, our assay could be a valuable tool for identifying other age-associated PTMs such as ubiquitylation and sumoylation.

We have found three phosphosites (S11, S36, and S60) on old (from the previous cell cycle) Spc110. While S36 and S60 had been previously characterized, the function of S11 was unknown. Our studies with Spc110 phosphomutant revealed that S11 phosphorylation is important for proper MT distribution in early anaphase. These results warrant further studies exploring the effect of S11 on the arrangement and dynamics of the spindle MTs, and on genetic stability. Furthermore, we showed the involvement of two of these phosphosites (S11 and S36) in cell cycle progression and mitotic exit, highlighting the relevance of S11 phosphorylation. Further research should focus on determining when in the cell cycle S11 is phosphorylated and by which kinase.

In **Paper II,** we discovered that nuclear Bik1 localizes to the kinetochores in a cell-cycle dependent manner and participates in chromosome congression with the kinesins Cin8 and Kip1. There are still open questions on how Bik1 mediates its function. For instance, the molecular mechanisms that regulate the cell-cycle localization of Bik1 at KT-MTs and how Bik1 is evicted after metaphase/transition remain unclear. Further studies should also define how Bik1 mediates chromosome congression. Different, but not exclusive, mechanisms could be by regulating MT dynamics, facilitating kinetochore capture (Tanaka et al., 2005), or controlling the kinetochore-MT interphase.

In **Paper III**, we showed that the SPB components Spc110, Spc72, and Nud1 have a short half-life, while the CP component Spc42 was relatively stable. Although preliminary, this finding suggests that SPB proteins' turnover depends on their localization. This finding is intriguing and should be followed by more detailed experiments, such as the CHX-chase of synchronized

cultures and microscopy-based measurements of SPB components' dynamics. Although preliminary data suggested involvement of Esp1 in Spc110 degradation, the mechanisms of Spc110 turnover remains elusive. Additional investigations should be carried out to explore the role of the UPS and the potential involvement of Spearase (Esp1) in controlling Spc110 turnover.

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