Characterization of the Role of a Novel Mitotic Exit Inhibitor in *S. cerevisiae*

by

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To My Brother and Lab Mates

ABSTRACT

Characterization of the Role of a Novel Mitotic Exit Inhibitor in *S. cerevisiae*
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Master of Science in Molecular Biology and Genetics

Saccharomyces cerevisiae, also known as the budding yeast, is an ideal model for investigation of how spindle orientation impinges on asymmetric cell division. Every cell division in *S. cerevisiae* is asymmetric, giving rise to a young daughter and an old mother cell with distinct fates. The budding yeast, orients and elongates its mitotic spindle along its polarity axis in order to segregate one copy of its genomic DNA to the daughter cell. If accurate positioning of the mitotic spindle fails, a surveillance mechanism, named the Spindle POsition Checkpoint (SPOC), prevents cells from exiting mitosis unless the spindle orientation is corrected. Such mitotic arrest provides cells time to align their spindle correctly before cell division is completed. Mutants with a defective SPOC loss their genomic integrity, become multiploid and aneuploid. Thus, SPOC is a crucial checkpoint for the budding yeast. Yet, a comprehensive understanding of how SPOC mechanism works is lacking. In this study, we identified Bud14 as a novel checkpoint protein. We showed that Bud14 in association with the type 1 protein phosphatase, Glc7, has a mitotic exit inhibitory function. *bud14*Δ cells accumulated multinucleated phenotypes when spindle positioning was impaired in these cells. In addition, deletion of *BUD14* promoted growth of mutants defective in mitotic exit. The function of Bud14 in SPOC dependent on its ability to bind Glc7. Cells bearing versions of Bud14 that cannot interact with Glc7 was SPOC deficient. Furthermore, they were unable to rescue growth of mitotic exit mutants. Mitotic exit inhibitory role of Bud14 was not through its role in formin regulation as a Bud14 mutant that cannot bind to the formin Bnr1 was functional in terms of mitotic exit inhibition. Our data indicate that Bud14-Glc7 inhibits mitotic exit by promoting dephosphorylation of Bfa1 during anaphase. Our results support a model in which Bud14-Glc7 works parallel to the SPOC kinase Kin4 in regulating Bfa1, the most downstream effector of SPOC that inhibits mitotic exit.

ÖZETÇE

Yeni Bir Mitozdan Çıkış İnhibitörünün Moleküler Rolünün Ekmek Mayasında Karakterizasyonu

Dilara Kocakaplan
Moleküler Biyoloji ve Genetik, Yüksek Lisans Tezi

Saccharomyces cerevisiae adıyla bilinen ekmek mayası, iğ ipliği oryantasyonun asimetrik hücre bölünmesi üzerine etkisini incelemek açısından ideal bir modeldir. *S. cerevisiae'*daki her hücre bölünmesi asimetriktir ve farklı özelliklere sahip genç bir yavru hücre ile yaşlı bir anne hücre meydana getirir. Anne ve yavru hücrenin herbirine eşit birer DNA kopyası aktarabilmek için ekmek mayası iğ ipliğini polarite ekseni boyunca yönlendirir ve uzatır. Eğer iğ ipliği düzgün yönlendirilmezse, iğ ipliği yönü kontrol noktası (SPOC) adı verilen bir gözetleme mekanizması hücrelerin mitozdan çıkışını, hücreler iğ ipliği yönünü düzeltene kadar, engeller. Eğer SPOC mekanizması düzgün işlemezse, hücreler genom kararlılığını kaybeder, poliploidi ve anöploidi görülür. Kısaca, SPOC ekmek mayası için elzem bir kontrol noktasıdır. Buna rağmen, SPOC mekanizmasının nasıl çalıştığı tam olarak anlaşılmamıştır. Bu çalışmada, SPOC mekanizmasında görevli yeni bir protein keşfettik. Bud14 adlı bu proteinin, protein fosfataz 1 (PP1) Glc7 ile birlikte çalışarak, mitozdan çıkışı baskıladığını gösterdik. Bud14'in genomdan silinmesi iğ ipliğinin yanlış yönlendiği durumlarda çok çekirdeklilik fenotipine neden oldu ve mitozdan çıkış mutantlarının mitozdan çıkışına destek oldu. Bud14 ve Glc7 ile etkileşiminin, Bud14'ün SPOC fonksiyonu için gerekli olduğunu gösterdik. Glc7'e bağlanamayan Bud14 mutantlarında SPOC mekanizmasının çalışmadığını gözlemledik. Aynı zamanda, bu mutantlar mitozdan çıkış mutantlarının yaşarlılığını da geri getirmedi. Bud14-Glc7'nin mitozdan çıkışı baskılayıcı rolünün, Bud14'ün formin regülasyonundaki rolü üzerinden olmadığını gösterdik, formin Bnr1'a bağlanamayan Bud14 mutantı mitozdan çıkışı inhibe etme rolünü gerçekleştirebiliyordu. Bud14-Glc7'nin mitozdan çıkışı baskılayıcı görevinin Bfa1'in anafazda defosforilasyonunu sağlayarak gerçekleştirebileceği gösteren sonuçlar elde ettik. Elde ettiğimiz veriler, Bud14-Glc7'nin SPOC kinazı Kin4'a paralel bir şekilde SPOC'un en aşağıdaki efektörü Bfa1'i regüle ederek mitozdan çıkışı inhibe ettiğine işaret ediyor.

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I would not be able to decide getting into a scientific career to understand how the cells function if my dear brother, Tolga was not born when I was 15. He is my inspiration and his humour remind me to enjoy every aspect of life. Thank you, my beloved family, for providing me proper set of chromosomes and peaceful home. Thank you, my thesis committee members, Asst. Prof. Elif Nur Fırat Karalar and Prof. Zeynep Petek Çakar.

This thesis would have a special place for me, not only because it is my first publication. The lockdown days were very difficult for me like everyone experienced. I am thankful for myself that I channelled my pain into writing the chapters of this thesis.

ABBREVIATIONS

Cdk: Cyclin Dependent Kinase

APC/C: Anaphase-Promoting Complex/Cyclosome

SAC: Spindle Assembly Complex

SPOC: Spindle Position Orientation Complex

FEAR: Fourteen Early Anaphase Release

MEN: Mitotic Exit Network

MTs: Microtubules

SPB: Spindle Pole Body

PP1: Protein Phosphatase 1

APS: Ammonium Persulfate

DMSO: Dimethyl Sulfoxide

SGA: Synthetic Genome Array

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Chapter 1 **INTRODUCTION**

1.1 Budding Yeast as a Model Organism

Saccharomyces. cerevisiae, also known as budding yeast, is a single-celled eukaryotic organism that is enclosed by a cell wall and has a size of 3–5 mm in diameter (Botstein & Fink, 1988). The budding yeast *S. cerevisiae* is a choice of organism in experimental studies due to its physiological and genetical features. These advantages are ease in culture maintenance, having haploid and diploid life cycles, availability of the efficient gene manipulation techniques, fully sequenced genome and conserved cell biology to that of higher eukaryotes.

Budding yeast can be stored in 15% glycerol for several years at -80 ^{o}C and maintained in growth media easily made from dry ingredients (Kaiser et al., 1994). In addition, it can be cultured in a relatively short period of time because of its fast-grow rate (1.5-5 hr doubling time) (Herskowitz, 1988).

The life cycle of the *S. cerevisiae* consists of cycling of two stages, namely haploid and diploid stages (Botstein et al., 1997). The haploid stage having single copies of each chromosomes has two mating types as MAT-a and MAT-α (Herskowitz, 1988) (Figure 1.1). When the mating types secrete the mating factors, G1 arrest is triggered and opposing mating types fuse to generate the diploid with two copies of chromosomes. In starvation conditions, diploid cells sporulate undergoing meiosis to form haploid cells (Haber, 1998). Yeast cells used in most experimental studies are genetically modified to restrict their proliferation only in the haploid stage (Duina et al., 2014; Morgan, 2012). In haploid stage gene manipulations can easily be done, as there is only one copy of the gene to edit. Furthermore, mating haploid yeasts allows for genetic complementation and backcross experiments. Thus, having haploid- diploid life cycle is advantageous for genetic studies.

Another reason that makes yeast a powerful organism for genetic studies is availability of highly standardized and efficient methods for gene manipulations such as gene deletions and epitope tagging of genes at their N and C termini (Duina et al., 2014). These methods are based on homologous recombination of DNA fragments with the yeast chromosomal DNA and high homologous recombination rate of budding yeast eases such genetic manipulations.

The discovery of genes that governs the biological processes in yeast is largely based on isolation of conditional mutants, such as temperature-sensitive mutants (Mortimer & Johnston, 1986). Temperature sensitive mutants are conditional mutants whose gene products are inactive at high temperatures. Such mutants can be obtained through screening of randomly mutagenized haploid yeast subjected to mutagenic agents. Presence of only single gene copies in the haploid state allows for such experiments. To allow propagation of cells, cells are maintained at permissive condition in which the gene product is functional, such as lower temperatures. The effect of the mutation is observed upon shifting cells to restrictive condition where the gene product is unfunctional, such as high temperatures. For example, genes that govern the cell cycle were discovered by isolation of the temperature sensitive cell division cycle (cdc) mutants which arrest at the cell cycle phase where they have a function (Leland H. Hartwell et al., 1970, 1974).

In 1996, the budding yeast genome has been fully sequenced as the first fully sequenced eukaryotic organism, and revealed 12 million base pairs arranged in 16 chromosomes and approximately 6000 genes in the haploid cell (Botstein et al., 1997; Goffeau et al., 1996; Liu et al., 2017). Genetic screens using conditional mutants revealed the molecular mechanisms underlying eukaryotic cell biology (Leland H. Hartwell et al., 1970). Cell biology of budding yeast highly resembles to that of higher eukaryotic organisms. Indeed, about one third of the genes in budding yeast have homologous genes in human (Botstein et al., 1997). Such conserved cell biology between yeast and human cells in addition to the genetic power of yeast place the budding yeast at a unique place among model organisms. Thus, budding yeast has been a choice of model organism in many studies that aim to solve molecular mechanisms of complex cellular processes and human diseases.

Studying cell cycle using the model organism *S. cerevisiae* is especially advantageous compared to human cell lines. There are couple of reasons behind this. First, as explained above, to investigate the phenotypic effect of a gene, one can easily mutate the gene in the haploid state, which would be much more difficult in other organisms due to the presence of at least two gene copies. Secondly, most human cell lines carry mutations to enable them to grow indefinitely in petri dishes which interrupts the regular cell cycle control mechanisms. For example, transformed human cells do not stop dividing in serum-free culture or when they loss petri dish contact. Lastly, budding yeast has an inherited polarization and divides asymmetrically where the proper orientation of the

spindle must be checked. Therefore, *S. cerevisiae* is very suitable organism for especially asymmetric cell division studies and hence it is the model organism of choice for our investigations on communication of spindle orientation and exit from mitosis.

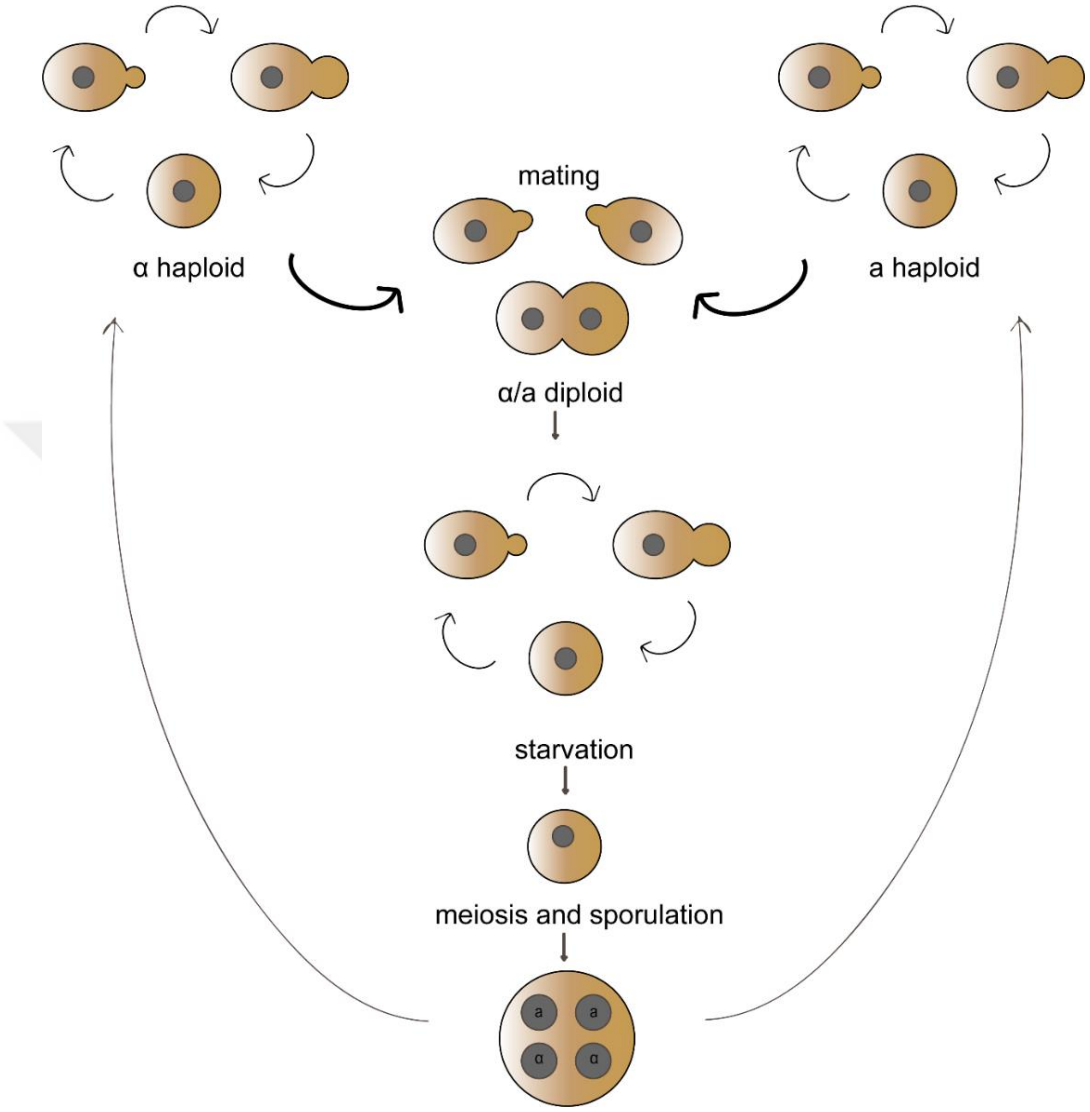


Figure 1.1: **The life cycle of *S. cerevisiae*.** Budding yeast has two haploid mating types named MATa and MATα which undergoes mitosis by budding (Haber, 1998). Two opposite mating types secrete pheromones mate with each other in shmoo form and generate diploid cell which can undergo meiosis upon starvation (Herskowitz, 1988). In laboratory, the yeast strains are usually kept stable in haploid stage by the absence of a functional HO endonuclease (Haber & George, 1979). The figure is adapted from (Morgan, 2012).

1.2 Cell Cycle in Budding Yeast

The cell cycle is the orderly continuation of the G1, S, G2 and M phases ('The Cell Cycle', 1994). *S. cerevisiae* cells divide by budding and enter to a new cycle when they reach a certain size threshold in G1 phase, where the cell growth takes place. The budding takes place concomitantly with the transition from G1 to S phase (Leland H. Hartwell, 1974). During this transition, the bud starts to arise at the bud emergence site which is defined

by cortical landmarks from the previous division (Casamayor & Snyder, 2002). The bud is also referred as the daughter cell (Figure 1.2). The daughter cell stays connected to the mother cell as daughter cell expands. The bud emergence site, also called as the bud neck, defines the future cytokinesis site. The budding process is based on polarization of the yeast cell in a process that involves delivery of vesicles to the bud along actin cables (Moseley & Goode, 2006). Polarization cues located at the bud emerge site constructs actin cables along the mother-to-bud polarity axis (Leland H. Hartwell, 1974; Slaughter et al., 2009). Actin cables provide polarized growth, localization of the actin patches and secretory vesicles and determination of the spindle axis (Pruyne et al., 1998; Yin et al., 2000).

At the S-phase, chromosomes and the microtubule organizing centre called Spindle Pole Body (SPB) is duplicated (Adams & Kilmartin, 1999). The G2 gap is the period between end of the replication and the start of the mitosis. It is very short or undetectable in budding yeast (‘The Cell Cycle’, 1994).

During mitosis sister chromatids, the two copies of chromosomes are folded up into condensed chromosomes which are held together by the Cohesin ring until the anaphase stage of mitosis. Kinetochores, built around the centromere region of chromosomes, provides sites for microtubule attachment. In anaphase, when Cohesin is cleaved, sister chromatids are separated and each chromatid is pulled away towards to opposite the spindle poles (Mitchison & Salmon, 2001). Yeast undergoes “closed mitosis”, in which nuclear envelope does not break down (Boettcher & Barral, 2013). Therefore, opposite to vertebrates, nuclear envelope is not broken down after chromosome condensation and not reformed around decondensing yeast chromosomes during telophase. Rather, elongated anaphase nucleus is partitioned between the mother and daughter cells. At the end of mitosis, cells undergo cytokinesis by actomyosin ring contraction and septum deposition at the bud neck (Bhavsar-Jog & Bi, 2017; McCollum & Gould, 2001). This way, the mother and the daughter cells containing identical genomic material are physically separated.

1.2.1 Cell Cycle Regulation

What is the molecular machinery that triggers the cell cycle events in an orderly fashion? The cyclin-dependent kinases (Cdk) form a robust switch-like system to maintain the order of cycle events that are conserved from yeast to humans. To modulate the timing of these events at the correct order, Cdk activity is regulated by several means. The major

regulators of Cdk activity are proteins called cyclins. Cdks are activated by their interactions with specific cyclins. Cyclins also provide substrate specificity of Cdks. Changes in rates of cyclin gene expression and cyclin degradation are key regulators of Cdk activity. Protein levels of cyclins oscillate, and their peak concentrations initiate the specific phases of the cell cycle (Nasmyth, 1993).

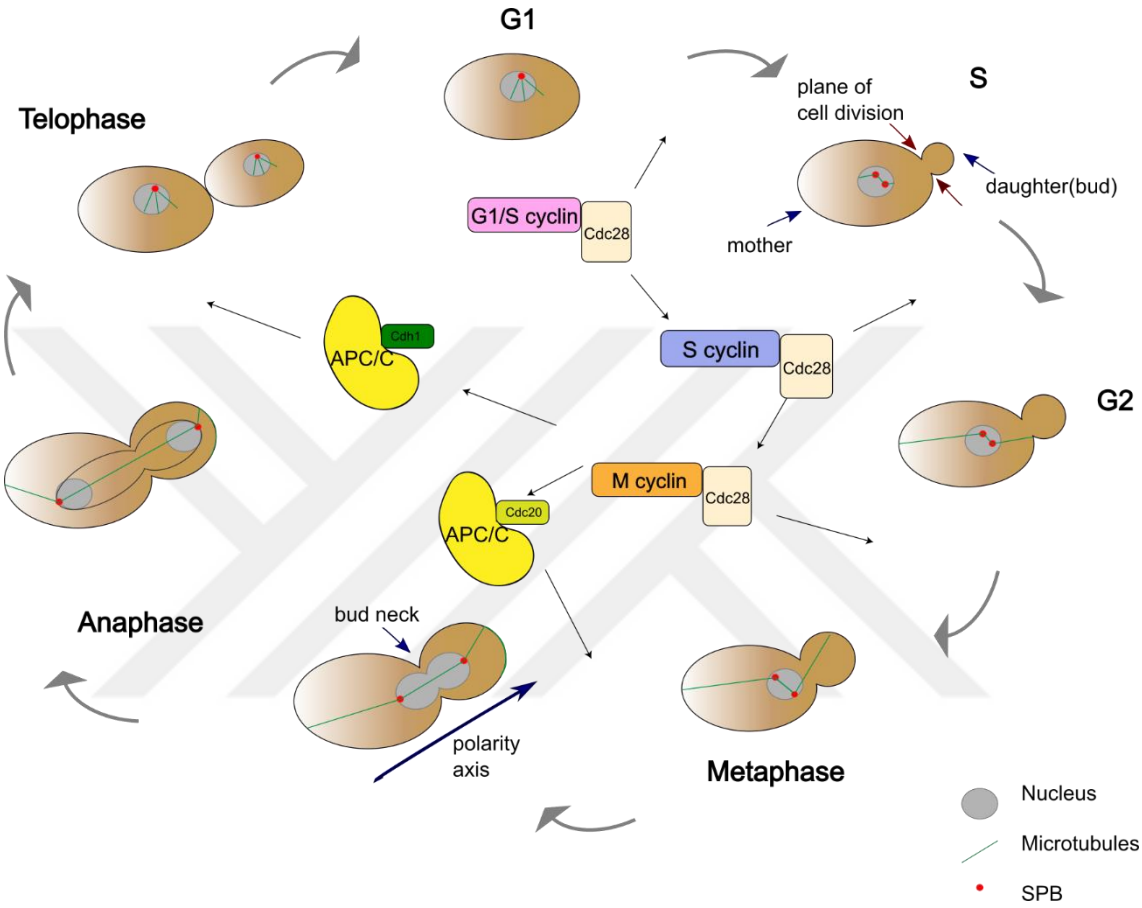


Figure 1.2: **Cell cycle with Cyclins-Cdks complexes operate for continuity of the cell cycle in a timely manner.** G1/S- and S-phase cyclin–Cdk activation results in DNA and centrosome replication. G1/S- and S-phase Cdks activates M-phase cyclin–Cdk which provides spindle assembly and other mitotic events. M-phase cyclin–Cdk stimulates APC/C which triggers destruction of cyclins, sister-chromatid separation and spindle disassembly (Mendenhall & Hodge, 1998; M. Shirayama et al., 1999). Blue arrows show location of daughter cell (bud), mother cell, bud neck and polarity axis. Red arrow shows the plane of cell division.

There is a single cyclin-dependent kinase in budding yeast called Cdc28 (Mendenhall & Hodge, 1998). The cyclins that are associated with this Cdk are (Cln1-3) for G1, (Clb5-6) for S phase, and (Clb1-4) cyclins for mitosis (Fitch et al., 1992; Hadwiger et al., 1989; Schwob & Nasmyth, 1993) (Figure 1.2).

Entry into a new cell cycle is committed by formation of G1/S cyclin–Cdk complexes in the late G1 phase (Dunphy, 1994). G1/S–, S– and M–Cdks are kept inactive in G1 by inhibitory mechanisms to prevent improper commitment to the next cell cycle events (Costanzo et al., 2004). These inhibitory mechanisms include suppression of cyclin genes,

degradation of mitotic cyclin by APC and activation of Cdk inhibitors in G1 (Irniger & Nasmyth, 1997).

How G1/S transition is activated? G1/S–Cdk complexes stimulate the activation of the S–Cdk complexes for DNA replication at the S phase (Costanzo et al., 2004). Then, S–Cdk levels drop and M-Cdk activation allows the mitotic spindle to be assembled. Completion of the mitosis and cytokinesis is initiated by the inactivation of the S and M cyclins by Anaphase Promoting Complex-Cyclosome (APC/C) activity (M. Shirayama et al., 1999; Wäsch & Cross, 2002). APC/C is a ubiquitin protein ligase (E3) which attaches ubiquitin to specific proteins to lead their degradation by proteasome. APC/C in association with its regulatory subunit Cdh1 or Cdc20 targets S and M cyclins, resulting in inactivation of Cdks in late mitosis and, triggering mitotic exit (Peters, 2006). Another target of APC/C is a protein called Securin which is important for metaphase-to-anaphase transition (Thornton & Toczyski, 2003). It is specifically the APC/C that in association with its regulatory subunit Cdc20 that ubiquitinylates Securin. Securin is inhibitor of a protease called Seperase. Upon degradation of Securin, Seperase is liberated. Seperase cleaves the single subunit of cohesin complex, leading to sister chromatid separation (Uhlmann et al., 1999).

1.2.2 Cell Cycle Checkpoints

Checkpoints exist throughout the cell cycle to monitor accurate completion of events such as growth to a certain size, proper chromosome duplication, correct assembly of the mitotic spindle and accurate segregation of the chromosomes (Figure 1.3). In the presence of mistakes in such events, checkpoints inhibit progression of the cell cycle and thus give cell time to correct those mistakes.

The first checkpoint is G1/S or Start where the size of the cell, nutrient availability and DNA damage are controlled to commit to enter the cell cycle. Internal and external conditions affect the decision of whether the cell enters the quiescent state called G_o or proceed to the entry. If conditions are suitable, signals activate the G1/S and S-phase cyclin-Cdk (Clb5,6–Cdk1) complexes to trigger DNA replication initiation and SPB duplication (Labib, 2010; Tanaka & Araki, 2010). During G1, transcription of G1/S genes is required to progress through to the next phases of cell cycle (Iyer et al., 2001). These transcriptions are regulated by SBF (E2F) and MBF that can activate G1/S transcription if Whi5 is dissociated by phosphorylation by Cln3-Cdk1 complex (Bruin et al., 2004).

Dilution of Whi5 concentration contributes to the determination of adequate cell size (Schmoller et al., 2015).

ATR and ATM are conserved protein kinases that act in DNA damage checkpoint. ATR and ATM are activated upon damaged or unreplicated DNA. Their activation leads to the DNA damage response which results in cell cycle arrest and activation of DNA repair genes (Foiani et al., 1998). In budding yeast, Mec1 and Tel1 kinases (ATR and ATM in mammals, respectively), phosphorylate the adaptor proteins Rad9 and Mrc1 in response to checkpoint activation. This activates Chk1 and Rad53 kinases that functions in two separate pathways to block metaphase-to-anaphase transition (Branzei & Foiani, 2006; Melo et al., 2001; Sanchez et al., 1999; Sweeney et al., 2005; Tercero & Diffley, 2001). Chk1 phosphorylates Pds1 (Securin) to prevent APC/C-Cdc20 dependent degradation (H. Wang et al., 2001). As Pds1 is Esp1 (Separase) inhibitor, this way the sister chromatids are kept together, and sister chromatid separation is inhibited. Rad53 acts by providing Pds1 stability, preventing Pds1 and Cdc20 interaction that results in metaphase-to-anaphase transition arrest (Agarwal et al., 2003). DNA damage or replication fork defects in S phase also arrest cells in S phase by inhibiting the replication origin firing. Rad53 phosphorylates and inhibits Dbf4 and Sld3 which are replication initiation factors (Lopez-Mosqueda et al., 2010; Zegerman & Diffley, 2010).

Spindle Assembly Checkpoint (SAC) arrest the metaphase-to-anaphase transition if the bipolar attachment of sister chromatids to spindles is incorrect. A signal from improperly attached or unattached kinetochores inhibits APC activity that results in prevention of early sister chromatid separation and block anaphase (Y. Wang et al., 2014). Mechanistically, Mad1 binds to unattached kinetochores, and recruits Mad2 (Luo et al., 2000, p. 2). Mad2 conformation is changed to the “closed” form where Mad2 sequesters APC/C activator Cdc20 to block its activation (Yu, 2006). Mad3/BubR1 and Bub3 bind to Cdc20 and form the mitotic checkpoint complex (MCC) (Fang et al., 1998; Hardwick et al., 2000; Sczaniecka et al., 2008). Therefore, upon SAC activation, Pds1 (Securin) cannot be degraded by APC/C and the anaphase onset is inhibited (Cohen-Fix et al., 1996).

Spindle POsition Checkpoint (SPOC) is a mitotic surveillance mechanism that monitors alignment of the mitotic spindle along the mother-daughter axis. SPOC prevents exit from mitosis when spindle is mispositioned. I will describe SPOC in detail in the following sections, as it is the main focus of the thesis.

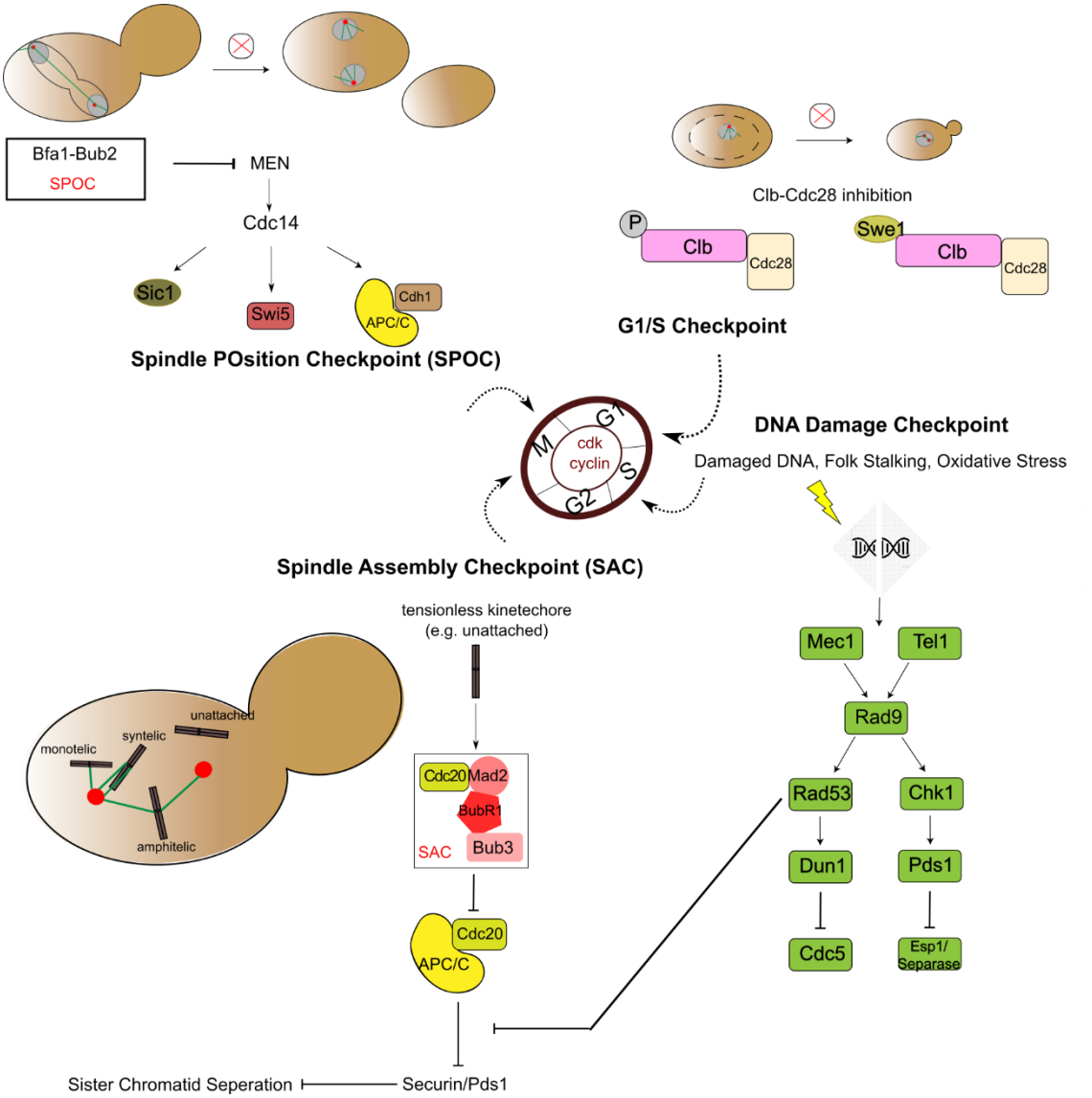


Figure 1.3: **Checkpoint mechanisms in budding yeast**. G1/S checkpoint, DNA Damage checkpoint, Spindle Assembly Checkpoint (SAC) and Spindle POsition Checkpoint (SPOC). The circumstances that activate the checkpoints and the molecular mechanisms to respond as checkpoints are shown. Upon activation the checkpoint, the progression of the cell cycle is inhibited by corresponding cyclin-Cdk inhibition (Branzei & Foiani, 2006; Caydasi & Pereira, 2012; Sanchez et al., 1999; Y. Wang et al., 2014). Point arrows represent activation, while block arrows represent inhibition. Dashed arrows represent checkpoint activation.

1.3 Yeast MTOC: Spindle Pole Body Structure and Function

Spindle Pole Bodies (SPB) are centrosome equivalent in fungal cells. Locating at the nuclear envelope, SPB controls the astral mictotubules, which is the cytoplasmic microtubule equivalent in yeast and the intranuclear spindle (Byers et al., 1978). Structurally, SPB has a cylindrical multi-layered shape, consisting of the outer, central, and inner plaques (Jaspersen & Winey, 2004). Outer plaque faces the cytoplasm and nucleate cytoplasmic microtubules which function in spindle positioning. Inner plaques

face the nucleoplasm and nucleate nuclear microtubules (intranuclear spindle) which mediate chromosome segregation. The central plaque of the SPB crosses the nuclear membrane, connecting the outer and the inner plaques. (Bullitt et al., 1997; Byers & Goetsch, 1974, 1975; Moens & Rapport, 1971; Robinow & Marak, 1966).

One of the core SPB component is Spc42 at the central core of the SPB (Donaldson & Kilmartin, 1996). It is a coiled-coil protein and assembles as trimers of Spc42 dimers at the central core (Bullitt et al., 1997). The C terminus of Spc42 binds to the C terminus of Cnm67 (Adams & Kilmartin, 1999). The N terminus of Cnm67 interacts with the to the outer plaque protein Nud1, an SPB protein required for exit from mitosis (Adams & Kilmartin, 1999; Elliott et al., 1999). Spc72 places in the outer plaque and binds to γ -tubulin complex via its N terminus and binds to Nud1 via its C terminus, thus constating a γ-tubulin receptor at the cytoplasmic face of SPB (Chen et al., 1998; Gruneberg et al., 2000; Knop & Schiebel, 1998; Wigge et al., 1998). Another γ-tubulin receptor named Spc110 resides at the SPB inner plaque to nucleate nuclear microtubules (M. Knop & Schiebel, 1997).

Proper SPB duplication is crucial for genomic stability. When the chromosome is segregated with the monopolar spindle, cells can result with having multinucleate or anucleate DNA content (Chial & Winey, 1999).

How is SPB duplicated? The new SPB arises from the half-bridge region of the SPB. Half-bridge is asymmetrically attached to a side of the SPB and has membrane-associated attachment to the SPB at the central plaque on the nuclear envelope at the early G1 (Byers & Goetsch, 1975). For SPB duplication, first, the half-bridge extends, and satellite material is accumulated. Then, the satellite is expanded into a duplication plaque and the half-bridge is retracted. Next, the duplication plaque is implanted into the nuclear envelope and inner plaque is built. After the SPB duplication, SPBs are moved on the opposing sides of the nuclear envelope. To separate the duplicated SPBs, the action of the microtubules and the kinesin-like motor proteins Cin8 and Kip1 are involved (Jacobs et al., 1988; Roof et al., 1992).

In budding yeast, the spindle assembly starts in S phase after separation of the duplicated SPBs (Byers & Goetsch, 1974, 1975). The phosphorylation of Spc110 by Mps1 promotes binding of the γ -tubulin complex for nucleation of microtubules. This phosphorylation also activates Cdc28-Clb complexes to form the spindle assembly (Friedman et al., 2001). Two main components involved at the spindle positioning, named Kar9 and Dynein

localize at SPB in a microtubule-dependent manner (Rita K. Miller & Rose, 1998; Yeh et al., 1995). Interestingly, the old SPB preferentially moves to the daughter cell (Pereira et al., 2001).

SPB is the location where microtubule-dependent functions take place such as chromosome segregation, nuclear positioning, and spindle orientation. In addition to these microtubule dependent functions, SPB serves as a scaffold for Mitotic Exit Network (MEN) and the SPOC. In this regard, SPB works as a signaling hub. For example, MEN components are not properly localized at the SPBs in *nud1* mutants and thus *nud1* mutants cannot exit mitosis (Gruneberg et al., 2000). Localization of MEN and SPOC components at SPBs is essential for their function, which is described in detail 1.5 and 1.6 sections (Bardin & Amon, 2001; McCollum & Gould, 2001).

1.4 Spindle Positioning in Budding Yeast

In budding yeast, the mother-bud axis determines the division site is determined before the spindle formation by the mother-to-bud polarity axis. Hence, positioning of the spindle along mother-bud axis is important for proper segregation of the nuclei in mother and bud derived cells. The positioning of the spindle is governed by two redundant pathways, namely the "early" and "late" pathways, in budding yeast (Siller & Doe, 2009). Both pathways require polarity cues and the yeast cytoskeleton to orient the spindle.

Budding yeast establishes a polarity axis during bud emergence. This occurs mainly through local activation of the Rho-family GTPase Cdc42 at the incipient bud site. The site of bud emergence and Cdc42 activation is determined by polarity landmarks inherited from the previous cell division cycle. Several effectors of Cdc42 establishes and maintains yeast mother-to-daughter polarity. Local activation of the formin Bni1 at the bud tip leads to actin cable formation in the bud to mother direction (Beach et al., 2000; Huisman et al., 2004; R. K. Miller et al., 1999).

The early pathway acts before anaphase and aligns the spindle along the polarity axis using the actin cable architecture that decorates the cells in the bud to mother direction. The early pathway is also referred as the Kar9-dependent pathway. Kar9 protein is the homolog of mammalian Adenomatous Polyposis Coli (APC). Kar9 is localized at the SPBs, preferentially at the old SPB and transported to the plus ends of the cytoplasmic microtubules by the plus end tracking protein Bim1 (EB1 in human). Bim1-bound Kar9 interacts with Myo2 myosin motor and directs the MT plus-ends to the bud along the actin cables. MT plus ends that are directed to the bud are captured at the bud cortex by a

process that involves the Bud6 protein, which is an actin and formin binding protein localized to the bud tip and to the bud neck (Huisman et al., 2004). This is how one of the SPBs is positioned towards the bud direction by the early pathway. Of importance, it is mostly the Kar9 localized old SPB that moves towards the bud (Paoletti & Bornens, 2003; G. Pereira et al., 2001).

The late pathway acts in anaphase and generates the pulling force that inserts the mitotic spindle into the bud. This process requires cortical dynein (Adames & Cooper, 2000; Carminati & Stearns, 1997; Li et al., 1993). The kinesin motor Kip2 transports Pac1-dynein-dynactin complex from SPB to the microtubule ends (Rita K. Miller et al., 2006). The cortical anchor protein Num1 activates off-loaded dynein-dynactin complex at cortex where Num1-dynein-dynactin complex now generates pulling force on MT to translocate the SPB to the bud (Farkasovsky & Küntzel, 2001; Lee et al., 2003).

The cyclin control on the spindle positioning is established by phosphorylation of Kar9 by Clb3–Cdc28 and Clb4–Cdc28 to prevent Kar9 association with mother-bound SPB. The asymmetrical localization of Kar9 to bud-bound SPB and astral MTs emanating from it have a functional importance. Bud-bound SPB that accommodates Kar9, undergoes fast reorientation, while MTs from the mother-bound SPB pole do not display that reorientation. In, *clb4Δ* or cells with mutated Cdc28 phosphorylation sites of Kar9 exhibits defective spindle orientation (Paoletti & Bornens, 2003).

Also, Dyn1 is phosphorylated by Clb1–Cdc28 and Clb2–Cdc28 to prevent its association with the mother SPB until anaphase where Clb1 and Clb2 are in their most abundant concentration (Bloom & Cross, 2007). During anaphase, Clb1 and Clb2 levels decrease and dynein start to localize to both SPBs. The symmetrical localization of dynein is physiologically important for pulling two SPBs away from each other to facilitate spindle elongation (Barral, 2004; Grava et al., 2006).

Simultaneous impairment of both early and the late pathway of spindle positioning machinery causes lethality (Rita K. Miller & Rose, 1998). But elimination of either pathway results in viable cells that frequently misposition their mitotic spindle. Cells defective in one of the pathways of spindle positioning can still segregate their nuclei correctly because, they can still re-align their spindle due to the presence of the other pathway (Yeh et al., 1995). Such cells with mispositioned spindle arrest their cell cycle in late anaphase due to SPOC until the spindle re-aligns. Thus, such cells rely on SPOC for a faithful mitosis.

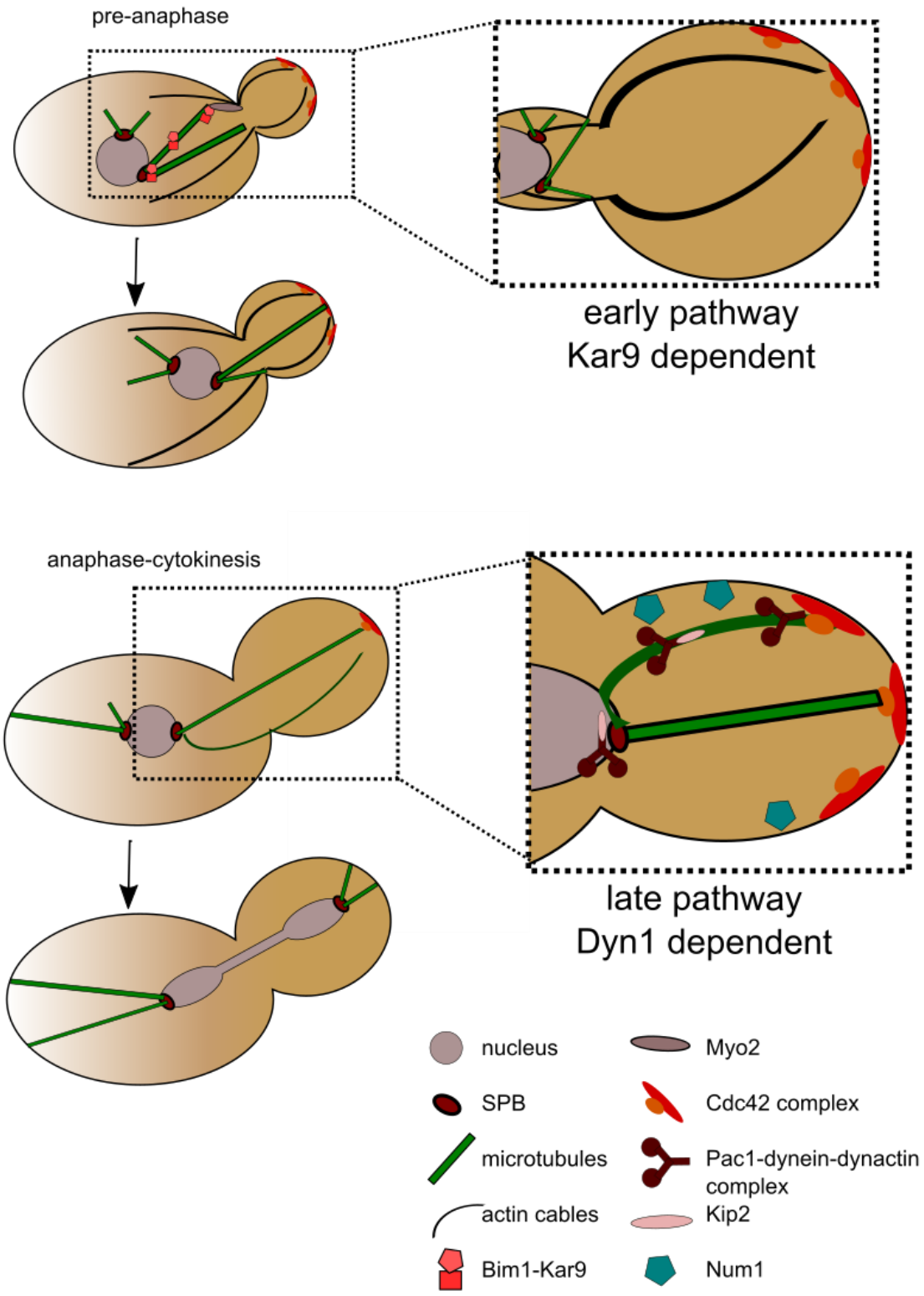


Figure 1.4**: Kar9 and Dyn1 dependent redundant pathways controlling the spindle orientation.** Adapted from (Siller & Doe, 2009). See the text for the molecular mechanisms.

1.5 Mitotic Exit in Budding Yeast

After the metaphase-anaphase transition the late mitotic events are triggered by inactivation of Clb-Cdks. The inactivation of Clb-Cdks is mainly performed by Cdc14 phosphatase through dephosphorylation of three main substrates. First, Cdc14 dephosphorylates APC/C specificity factor Cdh1, which in turn promotes Clb cyclins degradation. Second, Cdc14 dephosphorylates the Clb-Cdk inhibitor Sic1. This dephosphorylation causes stabilization of Sic1 (Verma et al., 1997). Swi5 which is a Sic1 transcription factor is also dephosphorylated by Cdc14 (Toyn et al., 1997). In addition, Cdc14 dephosphorylates other CDK substrates such as Ask1 in chromosome segregation, Fin1 in spindle stabilization and Cdh1 for spindle disassembly (Bouchoux & Uhlmann, 2011; Higuchi & Uhlmann, 2005; Jaspersen et al., 1999; Gislene Pereira & Schiebel, 2003; Stegmeier & Amon, 2004; Visintin et al., 1998; Woodbury & Morgan, 2007).

Cdc14 is kept inactive in the nucleolus until anaphase through its association with Net1 (Shou et al., 1999; Visintin et al., 1998). In early anaphase, Cdc14 is transiently and partially released from nucleolus into the nucleoplasm by the FEAR network (Cdc Fourteen Early Anaphase Release network) (D'Amours & Amon, 2004). This transiently released Cdc14 regulates multiple mitotic events such as rDNA segregation, sharpening metaphase to anaphase transition and spindle stabilization (Rock & Amon, 2009). FEAR network is not essential for mitotic exit; however, the MEN (Mitotic Exit Network) is (Rock & Amon, 2009). In late anaphase, the Cdc14 is fully released from the nucleolus into the cytoplasm by the MEN mechanism. It is this fully released Cdc14 that promotes mitotic exit (Jaspersen et al., 1998; Visintin et al., 1998)

The MEN is a GTPase driven kinase cascade (Figure 1.5). It is an essential pathway that cells arrest in late anaphase if MEN is defective (Geymonat et al., 2002). A Ras-like GTPase Tem1 protein is located at the top of MEN (Shou et al., 1999). Tem1 binds to SPB outer plaque. When Tem1 is bound to GTP, it promotes binding of Cdc15 kinase to the SPBs (Rock & Amon, 2011). Cdc15 phosphorylates Dbf2 and recruits Dbf2-Mob1 to the SPB and activates Dbf2-Mob1 (Rock & Amon, 2011). Mob1 is the regulatory subunit of Dbf2 which is required for Dbf2 activity (Komarnitsky et al., 1998). Phosphorylated Dbf2-Mob1 complex facilitates Cdc14 release from the nucleus (Mohl et al., 2009). Also, Nud1 is a MEN component that function as a scaffold on spindle pole bodies (SPBs) that other MEN components reside (Valerio-Santiago & Monje-Casas, 2011).

MEN components asymmetrically localize to SPBs (Scarfone & Piatti, 2017) (Figure 1.5). Also, SPOC component Kin4 localization creates a MEN inhibitory zone in the mother cortex and Lte1 creates a MEN activating zone in the bud cortex (Bertazzi et al., 2011; J. E. Falk et al., 2011). This way, MEN activation take place when MEN GTPase-bearing SPB moves to the bud during anaphase and making MEN to be responsive to the spindle position (Leon Y. Chan & Amon, 2010; Jill Elaine Falk et al., 2016). But this "zone model" does not explain the metaphase arrested cells with nucleus moving into the bud still exit from mitosis (Palmer et al., 1992). The FEAR network that is activated only after anaphase onset contributes to mitotic exit through dephosphorylation of CDK phosphorylated SPOC component Bfa1 and the MEN kinase Cdc15 (Caydasi et al., 2017), which may explain why cells in metaphase do not exit mitosis. Recent work also showed that MEN activation in metaphase is inhibited by high mitotic CDK activity even when a MEN GTPase-bearing SPB translocate into the bud (Campbell et al., 2019)

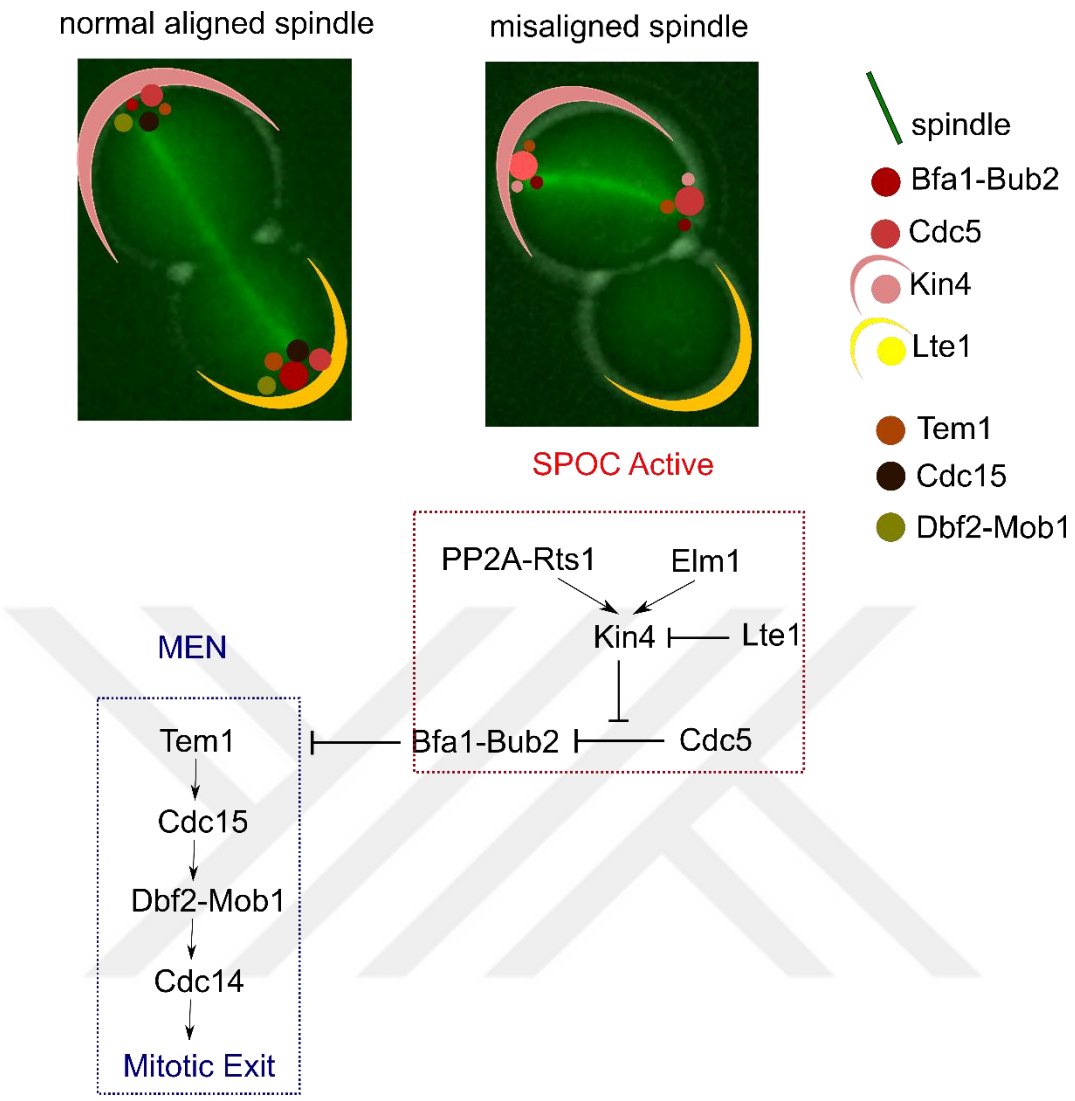


Figure 1.5 **MEN signaling pathways and SPOC mechanisms are shown**. Cartoons shows localization of MEN and SPOC components to the SPBs and mother and daughter cell cortexes. Upper left is a cell with normally aligned spindle, whereas upper right is a cell with misaligned anaphase spindle. The spindle is shown as green threads inside cells. Different proteins are marked with different colors. The mother cell faces up and the daughter cell faces downside of the cartoon. MEN and SPOC pathways are shown inside blue and red boxes, respectively. As the GTPase bearing SPB enters to the bud mitotic exit is permitted by the MEN signal transduction pathway. If the spindle is misaligned, SPOC becomes active and mitotic exit is inhibited. Note that the localization of components changes when spindle is misaligned. Point arrows represent activation, while block arrows represent inhibition.

1.6 Spindle Position Checkpoint in Budding Yeast

To partition the genetic material that is segregated during the mitotic events, cells must exit from the mitosis with a following cytokinesis. There are two crucial events for faithful chromosome segregation in budding yeast. First, all microtubules should attach to the kinetochores in a bipolar manner before anaphase onset. This process is assured by the mitotic checkpoint SAC which is a conserved checkpoint from yeast to mammals. Second, the mitotic spindle should align and elongate in the mother to daughter direction, delivering one set of chromosomes to the daughter cell compartment.

Another mitotic checkpoint named the Spindle POsition Checkpoint (SPOC) guaranties that correct orientation of the mitotic spindle is achieved before cells exit from mitosis. SPOC prevents cell cycle progression of cells with misaligned spindles by inhibiting the Mitotic Exit Network (MEN) (Figure 1.5). Yeast cells with defective spindle positioning pathways (see Figure 1.4 the early and the late pathways) or cells with impaired microtubules relies on SPOC for faithful chromosome segregation. Absence of SPOC causes multinucleation, enucleation and aneuploidy in budding yeast. Although a SPOC like checkpoint also exists in Drosophila (Cheng et al., 2011), whether SPOC like mechanisms exist in mammals yet remains to be investigated.

How does SPOC inhibit the MEN? The regulation of mitotic exit regarding spindle orientation is exerted by the SPOC component Bfa1-Bub2 complex. Bfa1-Bub2, composed of two proteins namely Bfa1 and Bub2, is a two-component GAP (GTPase activating protein) that inhibits Tem1 activity by promoting its GTP hydrolysis (Geymonat et al., 2002). GDP bound Tem1 cannot execute the MEN signaling cascade, therefore mitotic exit is prevented. To favor mitotic exit when the spindle is correctly aligned, the polo like kinase Cdc5 phosphorylates Bfa1, in a way to prevent Bfa1-Bub2 GAP activity towards Tem1 (Hu et al., 2001a). In the presence of spindle misalignment, Kin4 kinase phosphorylates Bfa1 (Maekawa et al., 2007). This phosphorylation prevents inhibitory phosphorylation of Bfa1 by Cdc5 kinase (Gislene Pereira & Schiebel, 2005). Consequently, Bfa1-Bub2 GAP complex activity is promoted, Tem1 activity is prevented, and exit from mitosis is inhibited (Figure 1.5).

Kin4 is the main activating kinase in SPOC. Kin4 localization and activity is controlled by the protein phosphatase 2A (PP2A) in association with its regulatory unit Rts1 and by the bud neck kinase Elm1. PP2A-Rts1 dephosphorylates Kin4 at entry into cell cycle and keeps Kin4 dephosphorylated at mitosis. Rts1 is required for Kin4 localize at the mother bound SPB in an unperturbed cell cycle and for SPOC activation response. Also, restricted mother cortex localization of Kin4 is significantly lost in *rts1*Δ mutants (L. Y. Chan & Amon, 2009). Elm1 is a kinase that catalytically activates Kin4 by phosphorylating a conserved residue within its activation loop of Kin4 (Caydasi et al., 2010a).

Specific localization of SPOC and MEN components are crucial for coordination of the spindle position and the timing of mitotic exit. When the spindle is properly oriented, most of the SPOC and MEN components (Bfa1, Bub2, Tem1) reside in a preferential,

asymmetrical manner at the SPB that moves to the bud (Gislene Pereira et al., 2000). On contrary, Kin4 localizes exclusively to the mother SPB during an unperturbed anaphase. Thus, Kin4 SPB localization is also asymmetric albeit in a reverse manner. Unlike aforementioned components of the MEN and SPOC, Cdc5 kinase is symmetrically localized to both SPBs (Maekawa et al., 2007). In addition, Kin4 localizes to the mother cell cortex throughout the cell cycle. Whereas, localization of the kinase Kin4 is restricted to the mother cell cortex, the mitotic activator Lte1 is localized to the daughter cell cortex. Daughter cortex localized Lte1, inhibits Kin4 from interacting with the dSPB, thereby activating Tem1 (Bertazzi et al., 2011). Lte1 also promotes hyper-phosphorylation of Kin4 and inactivation of Kin4. Strict localization of Kin4 in the mother compartment is important for a timely mitotic exit. It has been shown that daughter localized Kin4 causes mitotic exit delay (Leon Y. Chan & Amon, 2010). Strict localization of Lte1 at the daughter cell compartment is crucial for SPOC as leakage of Lte1 to the mother cell or forced localization of Lte1 to the mother cell cortex causes SPOC deficiency (Bardin et al., 2000; Bertazzi et al., 2011; Castillon et al., 2003; Geymonat et al., 2009).

Spindle misalignment alters the SPB localization of SPOC components (Caydasi & Pereira, 2009) (Figure 1.5). Kin4 localize to both SPBs if the spindle is misaligned (Gislene Pereira & Schiebel, 2005). The Bfa1-Bub2 localization is also changed from dSPB to both SPBs by phosphorylation of Bfa1 by Kin4 (Bertazzi et al., 2011). This phosphorylation causes formation of a docking site on Bfa1 for the 14-3-3 family protein, Bmh1. (Caydasi et al., 2014). Bmh1 binding to Bfa1 weakens Bfa1 interaction with SPBs and causes an increase in the turn-over rate of Bfa1 at the SPBs. Hence, the amount of Bfa1-Bub2 at both SPBs decreases, while the cytoplasmic amount of them is increased (Caydasi & Pereira, 2009). The release of Bfa1-Bub2 from SPBs keeps Bfa1-Bub2 away from the inhibitory kinase Cdc5 and thus prevents inactivation of Bfa1-Bub2 by Cdc5.

Taken together, the compartmentalization of the SPOC and MEN components to defined mother or daughter cortex or SPB locations controls mitotic exit in a spatial and temporal manner.

1.7 Open Questions in the Field of SPOC

Although the most downstream molecular events that lead to inhibition of mitotic exit upon spindle misorientation is relatively well understood, the most upstream events such as how the spindle position is sensed and how this information is translated to the SPOC machinery still remains unresolved. The main view is that compartmentalization of a

dividing budding yeast cell into two distinct parts (mother and daughter compartments) and the dependency of the MEN cascade on SPBs as a scaffold are the key for regulation of mitotic exit with respect to the spindle position. In cells with a normally aligned anaphase spindle one SPB enters the daughter cell compartment whereas in cells with a misaligned spindle both SPBs reside in the mother cell compartment. Consequently, restricting localization of the SPOC activating kinase Kin4 explicitly in the mother cell and localization of mitotic exit promoting proteins such as Lte1 and Ste20 in the daughter cell compartment allows cells to activate MEN only when one SPB enters into the bud during anaphase. However, entry of one SPB into the bud in metaphase is not sufficient for cells to exit mitosis. This is partly due to the function of the FEAR network released Cdc14 in Bfa1 deactivation and in MEN priming (Baro et al., 2013). Intriguingly, activation of the SPOC machinery can still occur in cells arrested in metaphase when the cells are treated with the microtubule depolymerizing drug nocodazole. Microtubule depolymerization causes Kin4 recruitment to the SPBs and consequent phosphorylation of Bfa1 by Kin4 (Caydasi & Pereira, 2009; Maekawa et al., 2007). Laser ablation of the daughter cortex associated cytoplasmic microtubules also results in activation of the SPOC as evident from the increased Bfa1-SPB binding dynamics at the daughter SPB (Monje-Casas & Amon, 2009). Such responses of Kin4 and Bfa1 to microtubule defects suggests that there is a microtubule dependent sensor and the absence of microtubule-cortex interactions may trigger this sensor independently of the mother-daughter compartmentalization. Yet, the molecular mechanisms underlying such a sensory mechanism remains elusive.

So far, the most upstream component of SPOC is the kinase Kin4. Yet how Kin4 localization is restricted to the mother cell cortex and to the mother SPB is not well understood. Kin4 at the daughter SPB is inhibited by Lte1 through a process that involves phosphoregulation of Kin4 (Bertazzi et al., 2011; J. E. Falk et al., 2011). However how Lte1 is able to do so and how indeed Kin4 SPB and cortex localization is regulated remains largely unknown. It has been shown that the PP2A phosphatase in association with its regulatory subunit Rts1 is involved in localizing Kin4 to the mother cortex and SPBs (L. Y. Chan & Amon, 2009). Kin4 appears hyperphosphorylated and it does not localize to the cortex or SPBs in cells lacking Rts1, indicating a requirement for PP2A-Rts1 dependent Kin4 dephosphorylation for Kin4 localization. Nevertheless, how PP2A-Rts1 regulates Kin4 at molecular level is currently not known. As mentioned before, upon

spindle misalignment Kin4 localizes to both SPBs to phosphorylate Bfa1, but the upstream mechanism that directs such a change in Kin4 localization in response to defective spindle position and defective microtubules remains to be revealed.

Kin4 phosphorylation on Bfa1 creates a docking site on Bfa1 for Bmh1 to regulate Bfa1 turn-over at SPBs, which is suggested to prevent phosphorylation of Bfa1 by Cdc5 (Caydasi et al., 2014). Interestingly cells lacking Bmh1 does not have Cdc5 phosphorylated Bfa1 during the metaphase arrest imposed by nocodazole treatment. This suggest the presence of additional mechanisms that prevent Cdc5 phosphorylation of Bfa1, which remains unknown.

Asymmetric localization of Bfa1-Bub2 at the daughter directed SPB is one of the most intriguing aspects of MEN regulation. How this asymmetry is established and maintained is not fully understood. It is not the age of the SPB but the location of SPB what promotes Bfa1 asymmetric localization at SPBs (G. Pereira et al., 2001). Cell polarity determinants such as Cdc42 are involved in establishment of symmetrical Bfa1 localization pattern in cells with correctly aligned spindles (Monje-Casas & Amon, 2009). Lte1 as a daughter localized protein is shown to be important for clearance of Bfa1 from the mother directed SPB (Geymonat et al., 2009). Yet, underlying molecular mechanisms of this regulation is not known. Another daughter localized protein important for mitotic exit is the PAK kinase Ste20 (Hofken, 2002).

The molecular function of Ste20 in the control of mitotic exit appears to be independent of its function in MAPK signaling and remains to be investigated (Caydasi et al., 2017).

To be able to tackle these open questions, it is crucial to discover interacting protein partners that play a role in the regulation of SPOC and MEN pathways. Therefore, we approached to address these questions by conducting a genetic screen to reveal novel mitotic exit inhibitors that could play a role in MEN and SPOC regulation.

1.8 Aims of The Thesis

In order to discover novel proteins that may potentially elucidate the unresolved mechanisms in mitotic exit, we conducted a genetic screen using a Synthetic-Genetic Array (SGA) based screening platform. For this we made use of *lte1*Δ *spo12*Δ cells that are unable to exit mitosis. We screened every non-essential single gene deletion in *S. cerevisiae* in combination with the double mutant *lte1*Δ *spo12*Δ and identified the gene deletions that enable these cells to divide. Such genes are expected to code for mitotic exit inhibitors. As expected, the known genes such as *KIN4*, *BFA1* and *BUB2* were among

the hits of the screen. In addition to those, we identified 26 genes that rescued the synthetic lethality of *lte1*Δ *spo12*Δ cells. We further screened those genes to find out whether the gene deletion causes SPOC deficiency. The absence of one of the genes; named *BUD14*, caused SPOC deficiency when spindle misalignment was induced through deletion of *KAR9*. So far, Bud14 has not been shown to play a role in cell cycle checkpoints and has never been investigated with respect to mitotic exit. The aim of this thesis is understanding the role of Bud14 in SPOC.

1.9 Known Roles of Bud14 protein

Bud14 (Bud Site Selection protein 14) is a non-essential gene whose phenotype was first characterized by the null mutant that exhibits a random budding pattern (Ni & Snyder, 2001). It localizes to sites of the polarized growth at the bud tips, bud cortex and the bud neck during the cell cycle. Bud14 concentration does not change during cell cycle (Knaus et al., 2005). Bud14 is composed of domains named SH3 domain, Bnr1 inhibitory domain and Glc7 binding domain (Figure 1.6). Overexpression of the Bud14 protein is toxic to the cells (Cullen & Sprague, 2002; Knaus et al., 2005). On contrary, the null mutants of Bud14 does not cause lethality, but shows elongated bud shapes, random bud site selection and fewer, longer and more stable actin cable formation (Chesarone et al., 2009; Gould et al., 2014). One of the known functions of Bud14 is being a regulatory protein of Glc7 in stabilizing the microtubule interactions. The other established role of Bud14 is contributing to the actin cable architecture by inhibiting FH2 domain of Bnr1 to displace it from growing barbed ends (Chesarone et al., 2009). Also, Bud14 was shown to be involved in filamentous growth and pheromone response pathways (Cullen & Sprague, 2002).

Bud14 is one of the regulators of an essential protein named Protein Phosphatase I (PP1), Glc7. It binds Glc7 through conserved motif (R/K-X-V/I-X-F) at 375–379 amino acid sequence. In *bud14*Δ cells, Glc7p is not localized at the bud cortex while it can be observed at SPBs, therefore Bud14 recruits Glc7 to the daughter cortex. Bud14 SH3 domain is important for its cortex localization and it also contributes to the Bud14 binding to Glc7 (Knaus et al., 2005). Cells lacking Bud14 showed unstable pre-anaphase spindle positioning near the mother-bud neck. On contrary, cells overexpressing Bud14 shows arrest with large buds and 2N-DNA content. In addition, the mitotic spindle was mispositioned and tend to be pulled at the bud at G2/M in Bud14 overexpressed cells, suggesting that MT–cortex interactions increases in bud cortex. Bud14 overexpression

also caused excessive cortical sliding of MTs is depending on Dyn1 and Num1. Together, these experiments suggested that Bud14–Glc7 complex increases activity of dynein/dynactin complex by stabilizing the microtubule interactions (Cullen & Sprague, 2002; Knaus et al., 2005). Bud14/Glc7 presumably regulates the unloading and/or activation of the dynein steps, rather than the loading of dynein on microtubule event. Therefore, the specific target(s) of Bud14/Glc7 in these processes remains to be elucidated. In addition, the temperature sensitive mutant of Glc7 cells (*glc7-10*) arrest at metaphase-anaphase transition due to the fault in microtubule-kinetochore attachment by SAC activation. Given that the overexpressed Bud14 caused arrest is not by this checkpoint, the substrates of the possible dephosphorylation event by Bud14/Glc7 complex and whether the defects in the processes this complex cause another checkpoint activation remains to be discovered (Knaus et al., 2005; Pinsky et al., 2009).

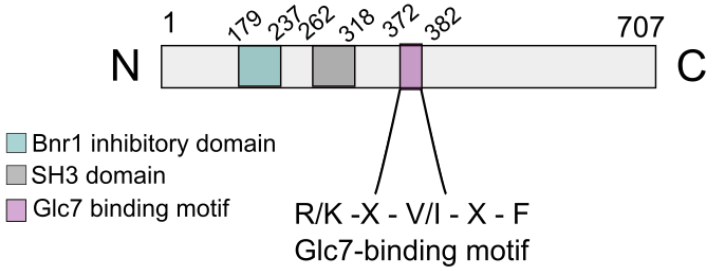


Figure 1.6: **Bud14 full length structure** (Chesarone et al., 2009; Knaus et al., 2005)

Bud14 is also involved in actin cable construction where Bud14 binds to the formin Bnr1. The formin proteins are actin-nucleation promoting proteins that function in regulating the actin and microtubule cytoskeleton to participate the cellular events such as cell polarity, cytokinesis, and migration (Goode & Eck, 2007). In budding yeast, Bni1 and Bnr1 proteins were shown to be two formins that are specialized by their localization to bud cortex and bud neck, respectively. They assemble actin filaments from these sites to build the actin cables, thereby provide polarized paths for transportation of secretory vesicles by myosin V-dependent manner (Moseley & Goode, 2005). Studies showed that Bud14 also plays a role in controlling the actin cable architecture by inhibiting FH2 domain of Bnr1 to displace it from growing barbed ends (Chesarone et al., 2009; Gould et al., 2014). Following research has identified Kel1 and Kel2 proteins, forming a stable complex with Bud14 in regulating actin cable architecture at polarized growth sites by inhibiting Bnr1 (Chesarone et al., 2009; Eskin et al., 2016; Gould et al., 2014). Kel1 and Kel2 are the member of Kelch protein family which localizes to the bud tips and bud neck where they physically interact with Bud14. Also, the deletion of these proteins causes the

actin cable defect morphology, similar to Bud14 (Gould et al., 2014). Interestingly, Kelch proteins form complexes with Lte1 and Tem1, and negatively regulate the mitotic exit (Bertazzi et al., 2011; Hofken, 2002; Seshan et al., 2002).

Moreover, Bud14 has a role in filamentous growth and pheromone response pathways. Bud14 mutants show elongated morphology and hyperfilamentous growth. These two pathways have another shared protein named p21-activated kinase Ste20. When *BUD14* is overexpressed, long thin budded cells are observed. That phenotype is even higher in *ste20* mutants when *BUD14* is overexpressed. In addition, the septin mislocalization phenotype observed in cells with overexpression of BUD14 has increased in *ste20* mutants. These data suggested that Bud14 negatively regulates the filamentous growth pathway by inhibiting Ste20 (Cullen & Sprague, 2002).

Schizosaccharomyces pombe, also known as fission yeast, is another single-celled yeast model organism and have diverged from the *S. cerevisiae* from several hundred million years ago. Tea4 is a SH3 domain protein in fission yeast *S. pombe* and is a homolog of Bud14 in budding yeast (Martin & Arkowitz, 2014). Tea4 has been shown to be a critical determinant of polarized growth by marking the cell poles with the support of microtubule cytoskeleton proteins (Huisman & Brunner, 2011; Kokkoris et al., 2014; Martin et al., 2005). In addition, Tea4 is involved in polarization of the actin cables at the cell poles by Tea4 recruiting a formin, reminiscing Bud14 interaction with Bnr1 formin to promote short actin filament formation (Chesarone et al., 2009; Valinluck et al., 2014). Tea1 and Tea3 are the interacting proteins with Tea4 that are crucial for its localization, same as the budding yeast Bud14 makes a complex with Kel1 and Kel2 proteins (Martin & Arkowitz, 2014; Tatebe et al., 2005). Similar to Bud14, Tea4 also associates with the catalytic subunit of PP1 (Dis2 in fission yeast) targets it to the cell tips to localize DYRK-family protein kinase Pom1 (Hachet et al., 2011). On contrary, there has not been any kinase affected by Bud14/Glc7 in budding yeast so far. In summary, Bud14 and Tea4 homologs, interacts with the same proteins to exert similar functions in providing the cytoskeletal roles in the cell polarity.

Bud14 has no homolog in human genome. But, in animal cells there are Kelch proteins called KLEIP and human Keap1 that have similar functions to that of Kelch proteins in yeast. They play a role in actin-rich cell-cell junctions where formins is required for the assembly of the complex (Velichkova & Hasson, 2003). Also, the mouse Kelch protein called Nd1 is involved in actin cytoskeleton arrangement which is dependent on Rho

protein (Sasagawa et al., 2002; Spence et al., 2000). The rat Kelch protein Krp1 localizes at cell tips with F-actins where they perform pseudopod elongation (Spence et al., 2000, p. 1).

In summary, Bud14 has been shown to function in two identified roles in budding yeast. Bud14 interacts with Glc7 at bud cortex and Bud14-Kel1-Kel2 complex interact with Bnr1 at bud neck. In this thesis I identified a novel role of Bud14/Glc7 in regulation of mitotic exit. It is a possibility that the data in this thesis connects these previously identified processes related to the spindle positioning and the actin cable regulation as being factors that contribute the mitotic exit inhibition.

Chapter 2 **MATERIALS AND METHODS**

2.1 S. cerevisiae Methods

The name and description of all media, buffer and reagent compositions are listed in Table in 6.4 section.

2.1.1 Strains and Growth Conditions

All yeast strains used are isogenic with S288C and are listed in Table 6-1. The auxotroph yeast strains were grown in SC-X medium supplemented with D(+)-Glucose (BioFroxx) as a carbon source, unless otherwise stated. YPAD medium was used for general use. For Raffinose and/or Galactose as carbon source(s), 2% D-Raffinose (AFG Bioscience #379827), 3% D(+)-Galactose (AFG Bioscience #307688), was used instead of 2% D(+)-Glucose. For the agar versions of these mediums, 20 g Bacto Agar (Carl Roth) was added for 1 L agar medium.

Yeast strains were grown at 30 °C unless otherwise stated. The temperature sensitive mutants were maintained at 23 °C and phenotypes were observed at 18 °C, 23 °C, 30 °C, 33 °C, 35 °C and 37 °C. *kar9*Δ cells were grown at 23 °C and shifted to 30 °C for phenotypic investigation.

In order to obtain the stationary phase of yeast cultures, yeast cells taken from agar plates were inoculated in appropriate growth medium and grown at an appropriate temperature until a culture of OD_{600} is greater than 5 was obtained. This usually takes 12-14 hr at 30 °C. This stationary culture was diluted in a fresh media to give an initial OD_{600} of 0.15-0.2 OD_{600}. Measurements were performed in a spectrophotometer (PG Instruments, T60

Visible Spectrophotometer). From this point on, the culture was further grown until OD_{600} 0.8-1.0. This takes about 2.5 cell divisions.

2.2 PCR Methods

Cassette PCR-based gene editing method was used for chromosomal gene deletion and C-terminal or N-terminal (Janke et al., 2004). Yeast colony PCR was performed to confirm gene deletions. Epitope tagging was confirmed by Western blotting or by microscopy. Promoter exchange was confirmed by growth in the lack of the promoter.
The plasmids and primers used in this experiment are listed in Table 6-2 and Table 6-3 respectively.

2.2.1 Cassette PCR

Cassette PCR was performed as described in (Taxis & Knop, 2006) using Biometra T-Advanced Analytikjena PCR machine. For 50 µl of cassette PCR reaction, 5 µl of the plasmid template (20 ng/µl), 3.2 µl of the forward -S1 or -S3 primers (10 µM), 3.2 µl of the reverse -S2 or -S4 primers (10 µM), 0,6 µl of the polymerase, enzyme mix (8 µl Taq DNA Polymerase (5U/µl, NEB #M0273L) + 4 µl Vent (2U/µl, NEB #M0254S), 8.75 µl of 2mM dNTPs mix (NEB #N0446S), 5 µl of 10X Buffer 1 and 24.25 µl ddH_2O was mixed. Primer couples were gene-S1/-S2 for gene deletion, gene-S2/-S3 for C-terminal tagging, and gene-S1/S4 for N-terminal tagging.
Cassette PCR cycle conditions were set as following: Step1: 2 min at 94 ºC, Step 2: 20 sec. at 94ºC, Step3: 30 sec at 52ºC, Step 4: X min at 68ºC , (X: the amount of time corresponds to 1 minute per 1000bp of the expected product size). Steps 2 to 4 were repeated for 9 times. Step 5: 30 sec at 94ºC, Step 6. 30 sec at 52ºC, Step 7: X+ increment min at 68ºC (increment: 20 sec per step). Steps 5 to 7 were repeated 19 times and the reaction was finally kept at 4ºC. The size and amount of the PCR product was analyzed at 0.8% agarose gel prepared in TAE buffer.

2.2.2 Yeast Colony PCR

Yeast colony PCR mix for 25 µl reaction was prepared with the stocks of 10 µM Primer1 1.25 µl, 10 µM Primer2 1.25 µl, 10 x Taq buffer w/ $Mg2+$ 2.5 µl, 2 mM dNTPs 2.5 µl, 5 U/µl Taq-pol, 0.2 µl, ddH_2O 16.8 µl and Yeast cells 0.5 µl. The yeast cells were extracted by suspending small pick of yeast colonies with 50 µl of 0.01% Sarkosyl and 0.02N NaOH and incubating this suspension at 95°C with vigorous shaking for 10 minutes. PCR conditions were: Step 1. 5 min at 94°C, Step 2. 30 sec. at 94°C, Step 3. 30

sec. at 53°C, Step 4. 1 min/1000bp (calculated by the expected size of the product) at 68°C, back to step 2 for 35x, followed by 3 min at 68°C, 4°C. The half of the reaction was used to analyze the size of the product at 0.8% agarose (Sigma) gel with DNA ladder (NEB # N3232L) (Bergkessel & Guthrie, 2013).

2.2.3 Yeast Transformation

50 ml log phase cultures of 1 OD_{600} were centrifuged at 3200 rpm for 2 min at room temperature. Supernatant was discarded, and pellet was resuspended in 45 ml ddH_2O. The resuspension was centrifuged at 3200 rpm, 2 min room temperature, supernatant was discarded, and pellet was resuspended in 25 ml LiSorb. The resuspension was centrifuged at 3200 rpm, 2 min room temperature, supernatant was discarded. Pellet was centrifuged once again to remove all liquid and then resuspended in 300 µl LiSorb and 50 µl denatured salmon sperm DNA. Salmon sperm DNA (ThermoFisher Scientific #1563250) was denatured by boiling at 95°C for 5 min and followed by incubation on ice for 5 min). The amount of reagents given is for 50 ml of 1 OD_{600} culture and is scaled proportionally to the volume and optical density of the culture used. Competent cells were aliquoted and stored at -80 ^oC or used right away for transformation.

Cassette PCR products or plasmids were added to 50 µl competent cells and incubated for 10-15 minutes at room temperature. For transformation with a cassette 5 µl of PCR product, for integration of integration plasmids 2-5 µl of 3-5 µg plasmid digested with the appropriate enzyme, for plasmid transformation 1-3 µl of circular plasmid DNA was used. Then, 300 µl of LiPEG was added to the mix and, vortexed. After, incubation of the mixture for 15-20 min at room temperature. 35 µl of DMSO (Fisher Scientific #BP231-1) was added, vortexed and incubated straightaway in the 42^oC water bath for 15 min. If transforming temperature sensitive mutants, incubation time was reduced at 42°C to 12 min. After the incubation, the mix was centrifuged at 3200 rpm for 2 minutes, supernatant was removed by aspiration. The pellet was resuspended in 200 µl of sterile PBS. If auxotrophy markers were used, cells were plated out onto appropriate auxotrophy selective plates right away. If antibiotic resistance markers were used, cells were recovered in nonselective medium for 6-12 hours before pelleting, resuspending in PBS and plating out on appropriate agar plates.

2.2.4 Drop test

Yeast cultures were inoculated in appropriate growth conditions for at least 24-48 hours. The OD_{600} values of the stationary cultures were measured by spectrophotometer. The

OD_{600} of the cultures were adjusted to obtain 1 OD in 1 ml using sterile 1x PBS. 10 fold serial dilutions were made using 1x PBS. 7.5-10 µl of dilutions were spotted on the plates and grown at appropriate temperatures for 1-3 days.

2.3 Molecular Biology Methods

2.3.1 Manual E. coli Plasmid DNA isolation

A colony of E. coli was inoculated in 3 ml TY with appropriate antibiotics and grown at 37 °C overnight at shaker. The culture was pelleted at 6000 rpm 5 min at RT. The supernatant was removed, and the pellet was resuspended with 300 µl P1. 300 µl P2 was added to the suspension of cells and inverted the tubes to mix. 300 µl cold P3 was added to the mix and the tube was inverted. The mix was centrifuged at 14000 rpm for 10 min at RT. The supernatant was taken and mixed with 600 µL isopropanol (ACS #0312.2500) by inversion. The mix was centrifuged at 14000 rpm for 1 min at RT. The pellet washed with 70 % EtOH. The pellet was air dried and resuspended in 30 µl of dH2O.

2.3.2 Yeast DNA Isolation

Yeast cultures were inoculated at appropriate growth medium overnight. The stationary cultures were centrifuged at 14000 rpm for 20 seconds. The supernatant was poured off and resuspended with 300 µl %6 SET. The mix was inoculated in 65 degree for 15 min and transferred to ice. 150 µl of cold 3M NaOAc was added and left on ice for 15 minutes. Centrifuged 14000 rpm for 10 min the supernatant was transferred to a new tube.500 µl of isopropanol was added, inverted vigorously and centrifuged at 14000 rpm for 1min. The pellet was rinsed with 200 µl cold %70 ethanol (Merck #1.00983.2500). DNA was resuspended in 30 µl ddH_2O and 1 µl 1mg/ml RNAase (Neofroxx #1263) and incubated at 37 ^oC for 15 minutes.

2.4 Cell cycle synchronizations

2.4.1 G1 arrest by Alpha factor release

The cells that are in log-phase were synchronised at G1 phase by addition of 1:100 of the culture volume of 1 mg/1ml α-factor (Sigma #T6901) dissolved in DMSO. α-factor added cultures were grown for the 1.5 multiple time of their doubling time at appropriate incubator with shaker. After the incubation time, the percent of cells that are arrested were analysed by investigating the shmoo appearance. If at least 95% of cells had shmoo phenotype, the cells were transferred from the flask to falcon tubes and centrifuged at 3200 rpm for 2 minutes, washed with the appropriate medium and the centrifuge-wash

cycle was repeated for 2 times. In the last wash, the pellet was resuspended with the medium and the OD_{600} was measured. The cell density was adjusted to 0.3 OD_{600} and the cells were transferred to flask to release the cells from G1.

2.4.2 Metaphase arrest by Nocodazole

The cells that are in log-phase were synchronised at metaphase phase using 1:100 culture volume of 1.5 mg/1ml nocodazole (Sigma #M1404) dissolved in DMSO. Culture was grown in YPAD for 1.5 multiple time of their doubling time at appropriate incubator with shaker. The arrest was confirmed by microscopy after ethanol fixation of the cells and staining the nucleus with DAPI. The arrested cell phenotype should be 1 DAPI with large-budded cells. For nocodazole arrest from G1 synchronized cells, cells were first treated with α-factor and released in nocodazole containing YPAD.

2.4.3 Late anaphase arrest by Tem1 depletion

For Tem1 depletion, Gal1-UPL-Tem1 bearing cells were grown to log-phase in Raffinose-Galactose containing medium. Then the culture was centrifuged at 3200 rpm for 2 min and the supernatant was aspirated. The pellet was resuspended in YPAD. Cells were allowed to grow until the arrest phenotype was observed. The arrest was confirmed by microscopy after ethanol fixation of the cells and staining the nucleus with DAPI. The arrested cell phenotype should be 2 DAPI with large-budded cells. In experiments where cells were synchronized with α-factor, α-factor was added to the log-phase Gal1-UPL-Tem1 cells in Raffinose-Galactose medium. 30 min before G1 synchronization was completed, glucose was added to the culture to give 2% final concentration. Then cells were released from α-factor in YPAD.

2.5 Biochemical Analysis

2.5.1 Total cell protein extraction

The 2 ml of culture of known OD_{600} yeast cells were centrifuged at 14000 rpm for 2 minutes. The pellets were resuspended in in 800 µl cold H_2O and 150 µl of 1.85M NaOH, vortexed and kept on ice for 5 minutes. 150 µl of TCA (Merck #100807) 55% (w/w) was added, vortexed and kept on ice for 10 min. Centrifuged at 14,000 rpm for 15-20 min at 4°C. Supernatant was aspirated and pellet was resuspended in HU-DTT. 150 (or 75 µl) HU-DTT was used for 1 OD_{600}, 1 mL culture pellet of abundant (or less abundant) proteins. The amount of HU-DTT added was scaled according to the OD_{600} value x volume of the culture pelleted. For SDS-PAGE loading, the samples were heated up at

65°C for 15 min, centrifuged at 14000 rpm for 2 min at room temperature and loaded 15 µl per lane.

2.5.2 SDS-PAGE

Protein electrophoresis was performed by using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels and the SDS-PAGE gels were prepared by using Mini-Protean II gel apparatus. The separating gel was prepared by the stock concentrations of the 1.5M Tris-HCl pH 8.8, 10 (w/v)% SDS, 6-15% polyacrylamide mixture (30%: 0.8% w/v acrylamide:bisacrylamide) and polymerised by addition of 10%(w/v) ammonium persulfate (APS) (Sigma #A3678) and TEMED (Sigma). Stacking gels contained 0.5 M Tris-HCl pH 6.8, 10% SDS, 4% polyacrylamide mixture, and were polymerised with 10% APS and TEMED.

Electrophoresis was performed at 100V in 1X Laemmli buffer and at ice.

2.5.3 Western Blot

After the proteins were resolved by SDS-PAGE and transferred onto nitrocellulose membrane (Merck, GE10600002) in blotting buffer for 1 hr and 30 min at 200mA using Bio-Rad Mini-Trans-Blot cell apparatus. Membrane were stained with Ponceau. S, lanes were marked, and the Ponceau S was destained using 1X PBS-T.

The membrane was blocked in PBS-T buffer supplemented with 5% nonfat dried milk (PanReac AppliChem) for 1 hr at RT or overnight at 4°C with gentle shaking. The membrane was incubated for 1 hr at RT or overnight at 4°C in primary antibody diluted in PBS-T supplemented with 3% non-fat dried milk with gentle shaking. Primary antibodies were Mouse-anti-HA (1:50 in 3% milk with PBS-T), Rabbit-anti-clb2 (1:3000 in 3% milk with PST-T; Gift from Gislene Pereira), Rabbit-anti-tubulin (1:50000 in 3% milk with PBS-T; Abcam EPR13799). Membrane was washed 3 times with PBS-T for 5 min each and then incubated with secondary antibodies in PBS-T containing 3% non-fat dried milk for 1 hr at RT. Secondary antibodies were Goat-anti-rabbit (Advansta #R-05062-500) or Goat-anti-mouse (Advansta #R-05071-500) horse radish peroxidase (HRP) conjugated secondary antibody diluted 1:10000. The membrane was washed 3 times with PBS-T for 5 min each. The protein bands were detected after incubation with home-made enhanced chemiluminescence ECL and exposing on X-ray films (Carestream Medical X-ray Green/MXG Film).

2.6 Microscopy Methods

All fluorescence microscopy was performed using Carl ZEISS Axio Observer 7 motorized inverted epifluorescence microscope equipped with Colibri 7 LED light source, Axiocam 702 Monochrome camera, 100X and 63X Plan Apochromat immersion oil objective lenses, Filter sets 95 (GFP/Cherry), 20 (Rhodamin), FITC and DAPI, and an XL incubation and climate chamber.

2.6.1 Live-cell imaging

For time-lapse experiments, cells were prepared on glass bottom petri dishes (WVR 10810-054 Matsunami) as following: 150 µl of 6% Concanavalin A (*Canavalia ensiformis* Jack Bean, type IV Sigma C2010-G) was pipetted on the glass center of the dish and waited for 10 minutes. The Concanavalin A was taken back, and the dish was washed with dH_2O for 6 times. 150 µl of culture was pipetted on the dish and incubated at 30 °C for 30 min. After incubation, cells were aspirated gently and washed with pre-warmed fresh media four times. After the final wash, the dish was filled with enough media. The dish was left on the microscope stage in preheated chamber for at least 1 hour before the start of the time-lapse.

2.6.2 Fixed-cell imaging

For the Ethanol fixation, 300 µl culture was mixed with 700 µl 100% ethanol and inverted rotated. Before the staining, cell suspension was centrifuged at 3200 rpm for 2 minutes and the ethanol was aspirated. The pellet was resuspended by 15 µl 1 mg/ml DAPI in PBS (1:10.000).

For Formaldehyde fixation, 900µL of culture was mixed with 100µL of 37% formaldehyde by inversion and incubate the tube at RT for 10-15 minutes. The mix was centrifuged at 6000 rpm for 2 minutes and the supernatant was aspirated. Pellet was resuspended 1000µl 0.1M Potassium Phosphate Buffer pH 7.5 (83.4 ml 1M K_2HPO_4, 16.6 ml 1M KH_2PO_4 for 1M Potassium Phosphate Buffer pH 7.5) and centrifuged at 6000rpm for 3 min, then resuspended in 100µl 0.1M Potassium Phosphate Buffer pH 7.5.

2.6.3 Image Analysis

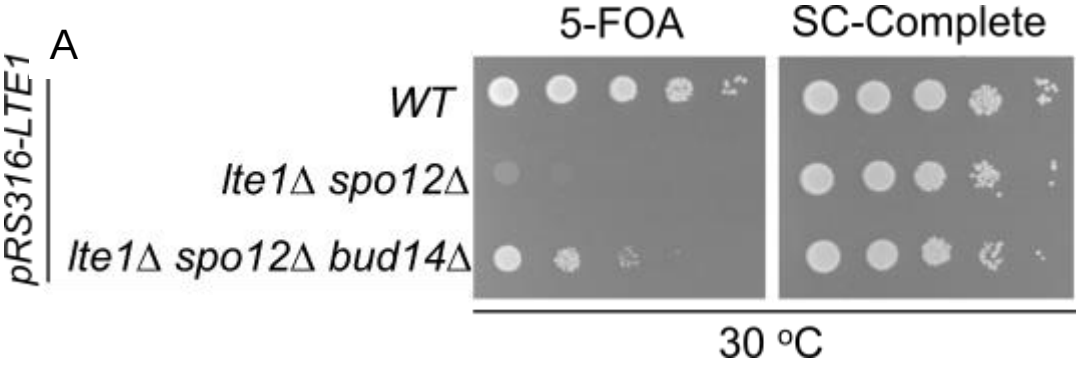
Image analysis was done in Image J. Statistical results, average and standard deviation values were computed and plotted by using Prism (GraphPad, La Jolla, CA). Two-tailed t-test were applied to compare the statistical significance of the measurements. Following

key is followed for asterisk placeholders for p-values in the figures: *P < 0.05, **P < 0.01, ***P < 0.001, **** P < 0.0001

Chapter 3 **BUD14 IS A NOVEL SPOC COMPONENT**

3.1 BUD14 is a Novel Mitotic Exit Inhibitor

Lte1 is a mitotic exit activator that becomes essential for mitotic exit at cold temperatures (<16 ºC) (Masaki Shirayama et al., 1994; Wickner et al., 1987). Although the exact function of Lte1 in mitotic exit is not fully understood, one of the ways that Lte1 promotes mitotic exit is by preventing binding of the SPOC kinase Kin4 to the SPB that has migrated into the bud and by inhibiting its activity therein (Bertazzi et al., 2011; J. E. Falk et al., 2011). At physiological temperatures (i.e. 30 ºC) *lte1*Δ cells rely on the presence of another mitotic exit activating pathway, namely the FEAR network (Stegmeier et al., 2002). Deletion of FEAR network components such as *SPO12* or *SLK19* in *lte1*Δ cells causes lethality due to failure of mitotic exit (Heil-Chapdelaine et al., 2000). Lethality of the *lte1Δ spo12Δ* (or *lte1Δ slk19Δ*) double mutants can be rescued by deletion of the known mitotic exit inhibitors *BFA1*, *BUB2* and *KIN4* (Caydasi et al., 2017). In order to identify novel mitotic exit inhibitors, we designed a genetic screen that looked for single gene deletions rescuing *lte1Δ spo12Δ* lethality. In addition to the known mitotic exit inhibitors *BFA1*, *BUB2* and *KIN4*, we identified *BUD14* as a novel gene that contributes to the mitotic arrest of *lte1Δ spo12Δ* cells (Caydasi AK, unpublished data). Deletion of *BUD14* rescued lethality of *lte1Δ spo12Δ* cells (Figure 3.1A). Deletion of *BUD14* also rescued the cold sensitivity of *lte1Δ* cells at 18 ºC (Figure 3.1B). This data indicate that Bud14 is a novel inhibitor of mitotic exit.



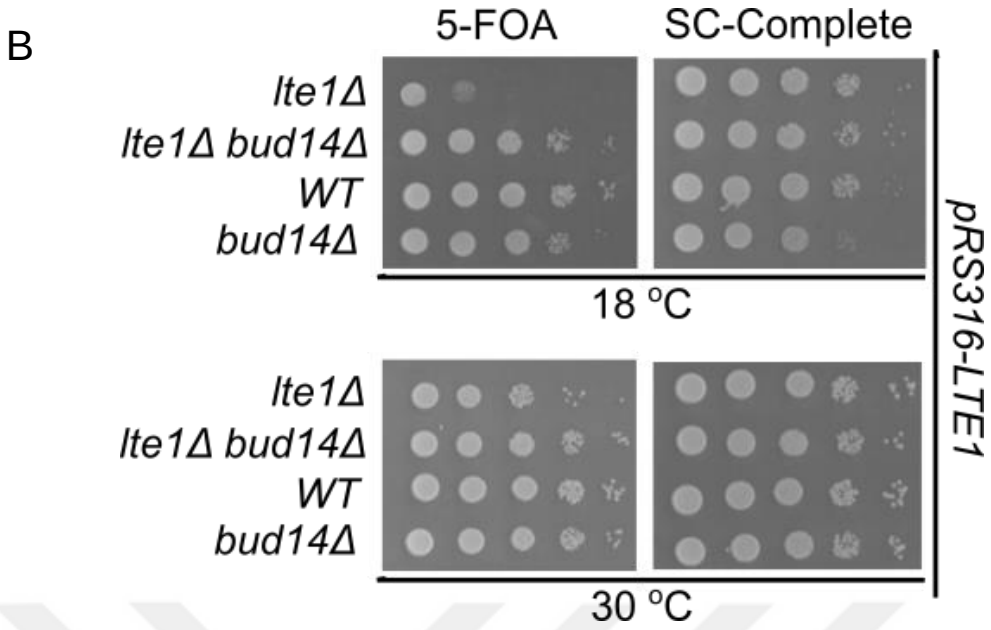


Figure 3.1 **Bud14 deletion rescues growth of cells with impaired mitotic exit.** A. *bud14*Δ cells rescue the synthetic lethality of *lte1*Δ *spo12*Δ cells. B. *bud14*Δ cells rescue the lethality of *lte1*Δ cells at 18 ºC. Serial dilutions of indicated strains were spotted on indicated plates and grown at given temperatures. 5-FOA plates negatively selects for the URA3 based plasmids (pRS316-LTE1), thus only the cells that have lost this plasmid can grow on 5-FOA plates where genetic interactions can be observed.

Deletion of mitotic exit inhibitors such as *BFA1*, *BUB2* and *KIN4* also rescues growth of mitotic exit network (MEN) mutants (Scarfone et al., 2015). Thus, we next asked whether deletion of *BUD14* also promotes the growth of MEN mutants. For this, we made use of the temperature sensitive (*ts*) alleles of MEN components (Grandin et al., 1998). We deleted *BUD14* in several MEN-*ts* mutants and compared their growth at different temperatures. In line with its mitotic exit inhibitory role, deletion of *BUD14* promoted growth of *dbf2-2, cdc15-1* and *mob1-67* mutants at 37 ºC, 35 ºC and 33 ºC respectively (Figure 3.2)

Notably, *bud14Δ* did not rescue the growth of the *cdc14-2* mutant (Figure 3.2). Cdc14 is the essential phosphatase that is activated at the very downstream of the MEN pathway. The fact that *bud14*Δ rescues the MEN-*ts* mutants but not *cdc14-ts* suggest that Bud14 may function upstream of the MEN pathway.

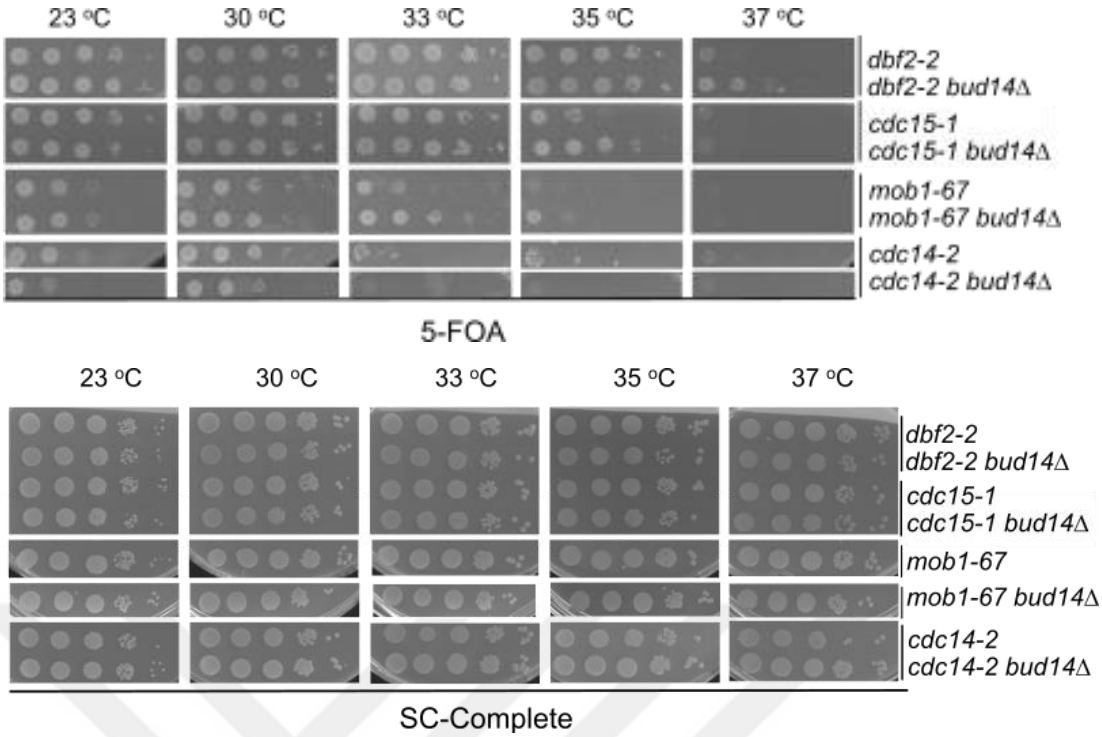


Figure 3.2 ***bud14*Δ rescues lethality of MEN-ts mutants**. All MEN temperature sensitive mutants are initially complemented with plasmids carrying the wild type counterpart of the corresponding ts allele on a *URA3* based plasmid (not shown). Serial dilutions of indicated strains were spotted on indicated plates and grown at given temperatures. 5-FOA plates negatively selects for the uracil-based plasmids, thus only the cells that have lost this plasmid can grow on 5-FOA plates where genetic interactions can be observed. SC-complete plates serve as a control for the cell density of serial dilutions.

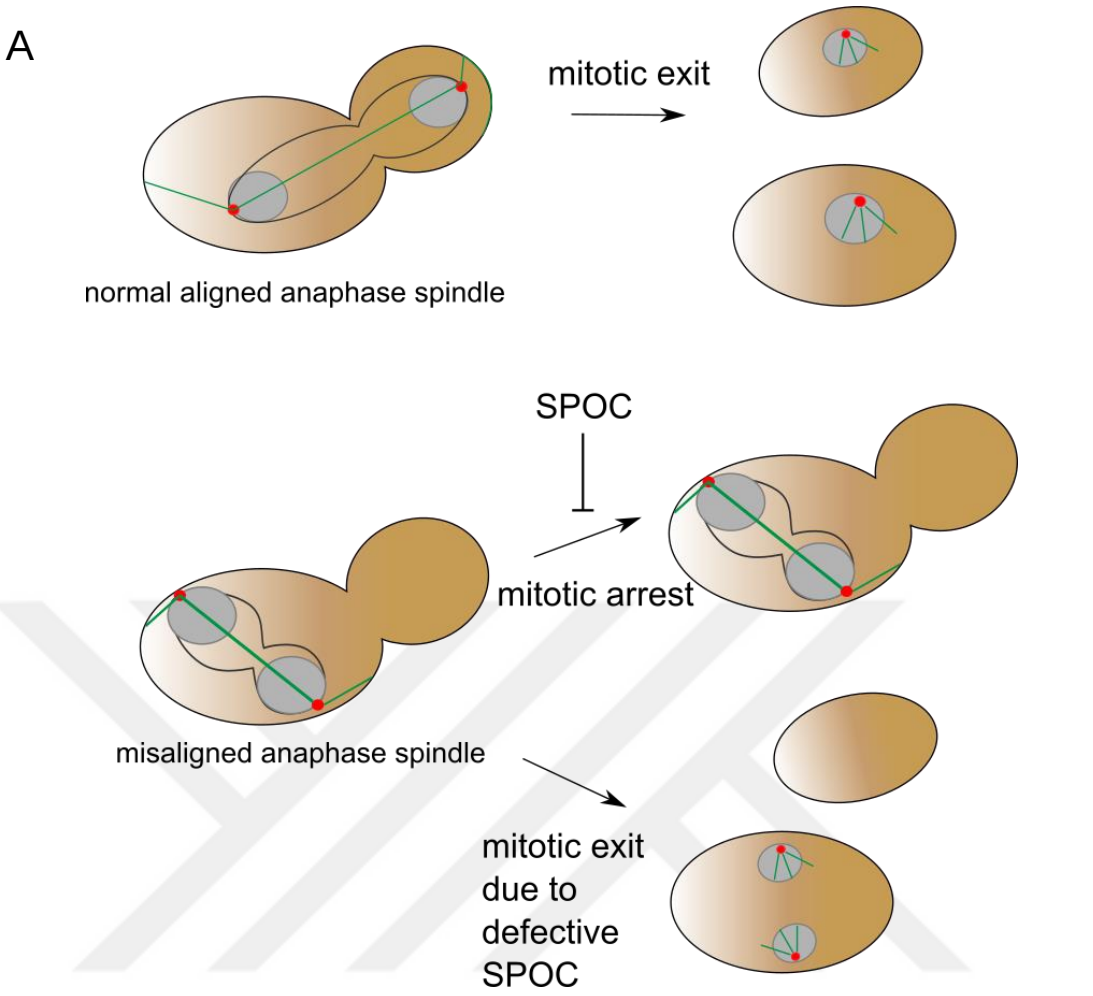
*3.2 bud14*Δ *cells are SPOC deficient*

The known mitotic exit inhibitors Bfa1, Bub2 and Kin4 are key components of the Spindle Position Checkpoint (SPOC), which is a surveillance mechanism that inhibits the MEN upon mispositioning of the anaphase spindle (D'Aquino et al., 2005; Hu et al., 2001a). We next, investigated whether Bud14 has a role in SPOC. To assess SPOC activity in *bud14*Δ cells, we first used a *kar9*Δ background. Kar9 (homolog of the mammalian tumor suppressor Adenomatous polyposis coli, APC) is a microtubule and actin associated protein, that links the astral microtubules with the actin cables to allow for spindle positioning along the mother-to-daughter polarity axis. Deletion of *KAR9* causes frequent spindle mispositioning at 30 °C and above. These cells, however, can correct their mispositioned spindle due to the presence of another spindle positioning pathway that is dependent on the minus-end directed microtubule motor protein Dynein. Upon spindle mispositioning, cells with a functional SPOC arrest in anaphase until the spindle position is corrected; whereas SPOC deficient cells continue cell cycle progression resulting in formation of multinucleated and enucleated cells (Figure 3.3A). As expected *kin4*Δ *kar9*Δ or *bfa1*Δ *kar9*Δ cultures accumulated multinucleated cells while

*kar9*Δ cells did not (Figure 3.3B). *bud14*Δ *kar9*Δ cells also accumulated such multinucleated phenotypes, indicating that Bud14 is crucial for maintenance of ploidy in cells lacking *KAR9* (Figure 3.3B)

Bud14 was shown to have a role in promoting dynein/dynactin activity at the bud cortex (Knaus et al., 2005). Considering the function of Dynein in spindle positioning, we asked whether the reason of multinucleation in *bud14*Δ *kar9*Δ cells was the lack of Dynein activity which in turn would prevent re-aligning of the anaphase spindle. If this was the case, we would expect to see no significant multinucleation phenotype when the SPOC activity was assayed in a background that already lacked Dynein activity. For this we used *dyn1*Δ cells that are deleted for the dynein heavy chain. *bud14*Δ *dyn1*Δ cells exhibited remarkable multinucleation, similar to SPOC deficient *kin4*Δ *dyn1*Δ cells (Figure 3.3C). This data indicates that the role of Bud14 in Dynein activation does not account for the observed multinucleated phenotypes of *bud14*Δ *kar9*Δ or *bud14*Δ *dyn1*Δ and that Bud14 is a novel SPOC component.

These data altogether suggest that Bud14 is needed as a part of the SPOC mechanism to arrest the cells in mitosis upon spindle mispositioning.


A
mitotic exit
normal aligned anaphase spindle
SPOC
mitotic arrest
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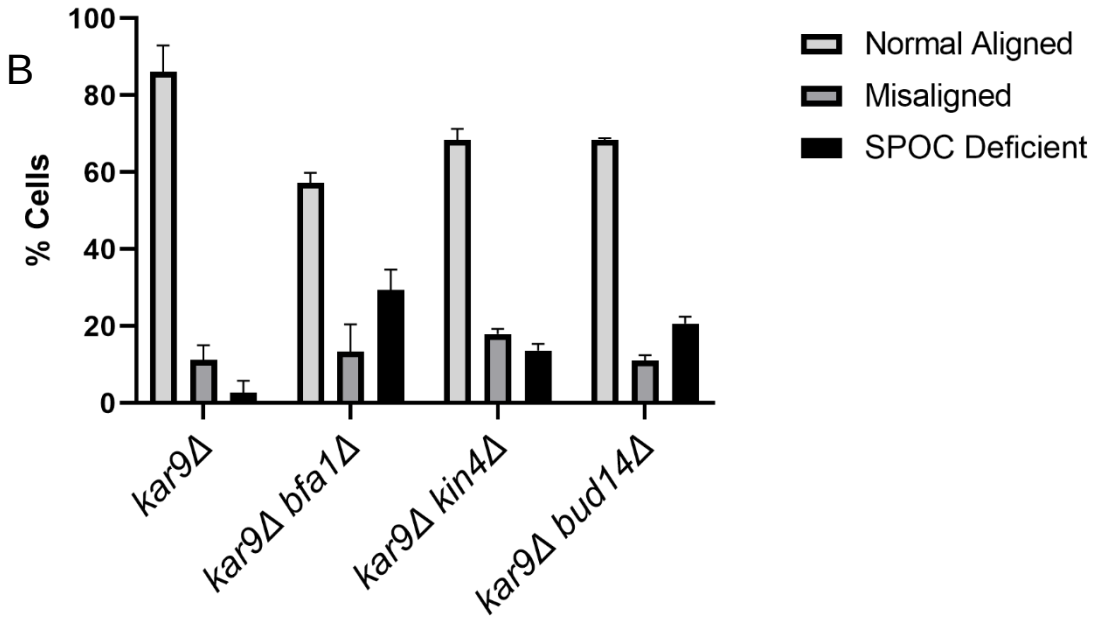
defective
SPOC


B
% Cells
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kar9Δ bfa1Δ
kar9Δ kin4Δ
kar9Δ bud14Δ

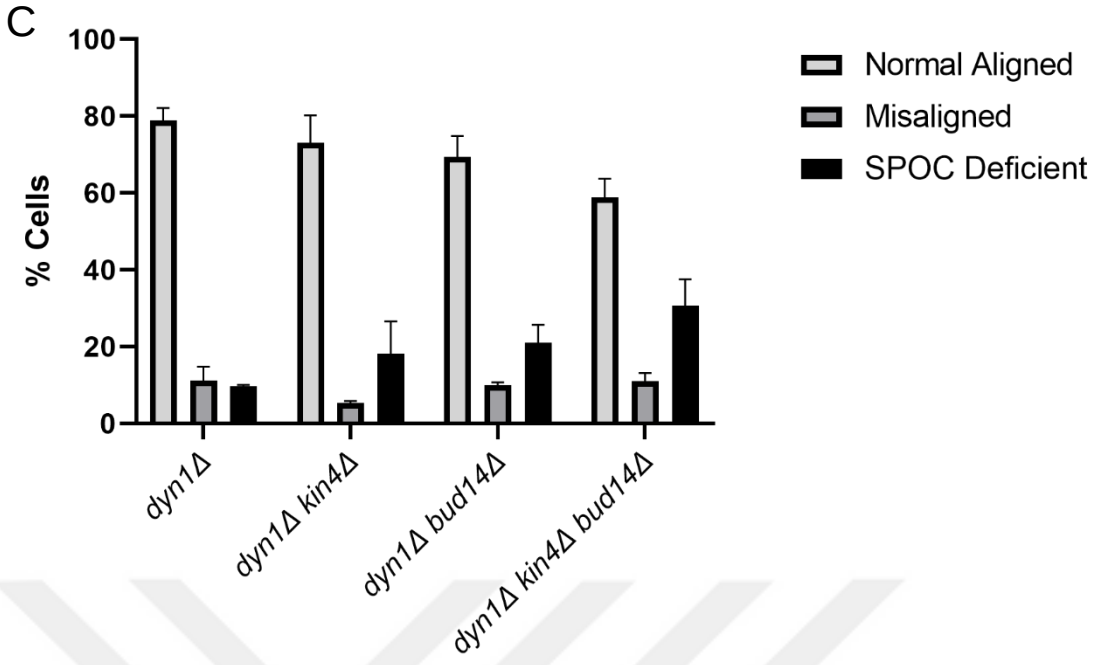


Figure 3.3 ***bud14*Δ cells are SPOC deficient A.** Cartoon showing the fates of SPOC proficient and SPOC deficient cells. When the spindle is misaligned, and SPOC works properly mitotic exit and cytokinesis is prevented. When spindle is misaligned and SPOC does not work (i.e. SPOC component is defective), mitotic exit proceeds resulting in multinucleated cells and enucleated cells, which we call as SPOC deficient phenotypes. **B.** *kar9*Δ cells with indicated gene deletions were grown at 23 ºC until logarithmic growth phase and shifted to 30 ºC for about 2 doubling times. Then the cells were fixed with ethanol fixation and stained with DAPI. Indicated phenotypes were analyzed by microscopy. **C.** *dyn1*Δ cells with indicated gene deletions were grown at 30 ºC until log phase and then cultures were grown at 18 ºC overnight. The cells were fixed with ethanol fixation and stained with DAPI. The percentage of cells with normal positioned nuclei, mispositioned nuclei and SPOC deficient phenotypes were plotted. The experiments have repeated 3 times and the average values are shown. Error bars show standard deviation. A minimum of 100 cells were count for each strain in each experiment.

These data altogether suggest that Bud14 is needed as a part of the SPOC mechanism to arrest the cells in mitosis upon spindle mispositioning.

3.3 Bud14 inhibits mitotic exit though its interaction with Glc7 and SH3 domain but not through its role in formin regulation

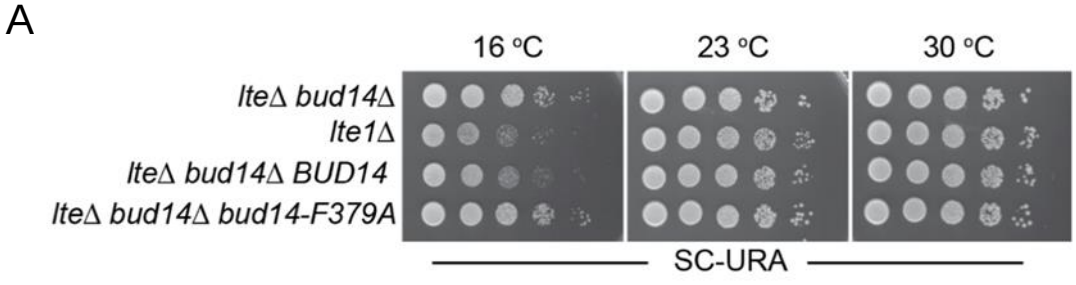
Bud14 is a regulatory subunit of the type I Protein phosphatase (PP1) Glc7 (Cullen & Sprague, 2002). Therefore, we asked whether Bud14 exerts its mitotic exit inhibitory role through Glc7. To address this question, we impaired Glc7-Bud14 function using several mutants. First, we used a mutant version of Bud14 that cannot bind Gcl7 (*bud14-F379A*) (Knaus et al., 2005) and analysed its ability to rescue *lte1*Δ cold sensitivity. Deletion of *BUD14* rescued the growth of *lte1*Δ cells at 16 ºC as shown earlier (Figure 3.1B). Introducing wild type *BUD14* but not the *bud14-F379A* mutant into *lte1*Δ *bud14*Δ cells restored cold sensitivity of *lte1*Δ cells (Figure 3.4A). This data indicates that Glc7-Bud14 interaction is essential for mitotic exit inhibitory function of Bud14. In line with this notion, a temperature sensitive *GLC7* mutant *glc7-12* (Andrews & Stark, 2000), rescued

the lethality of *lte1Δ spo12Δ* cells at its semi-permissive temperature (33 ^{o}C), but not at its permissive temperature (18 ^{o}C)(Figure 3.4B). These data altogether suggest that Bud14-Glc7 complex has a mitotic exit inhibitory function.

Bud14 contains an SH3 domain at its amino terminal (amino acids 262-318). Deletion of this domain diminishes Bud14 bud cortex localization, as well as Bud14-Glc7 interaction (Knaus et al., 2005). A truncated version of *BUD14* that lacks the SH3 domain (*bud14*$^{262\text{-}318\Delta}$) also rescued *lte1*Δ cold sensitivity. We thus conclude that the SH3 domain of Bud14 is important for Bud14 function in mitotic exit, most likely due to impairment of Glc7-Bud14 interaction (Figure 3.4C).

Bud14 is an inhibitor of the formin Bnr1 (Chesarone et al., 2009). It regulates actin cable formation and secretory trafficking through its formin-regulatory motif (amino acids 135-150) (Chesarone et al., 2009; Eskin et al., 2016). *bud14-5A* mutant with five alanine amino acid substitutions at the formin-regulatory motif lacks actin regulation by Bud14. Using this mutant, we addressed the contribution of actin regulatory role of Bud14 to its mitotic exit inhibitory function. Unlike *bud14-F379A* or *bud14-sh3*Δ*, bud14-5A* complemented the deletion of *BUD14* in *lte1*Δ cells to the same level as wild type *BUD14* (Figure 3.4C). Thus, Bud14 function in inhibition of mitotic exit is independent of its role in actin cable regulation but dependent on its association with Glc7.

Next, we investigated functional importance of Bud14-Glc7 interaction in SPOC. *kar9Δ bud14Δ* cells exhibit SPOC deficiency phenotype as we showed earlier (Figure 3.3B). When *kar9Δ bud14Δ* cells were supplemented with a wild type copy of *BUD14* SPOC deficient phenotypes were diminished, indicating restoration of SPOC activity. However, SPOC activity was not restored when *bud14-F379A* was introduced into *kar9Δ bud14Δ* cells (Figure 3.4D). This data suggests that Bud14-Glc7 complex has crucial role in SPOC functionality.



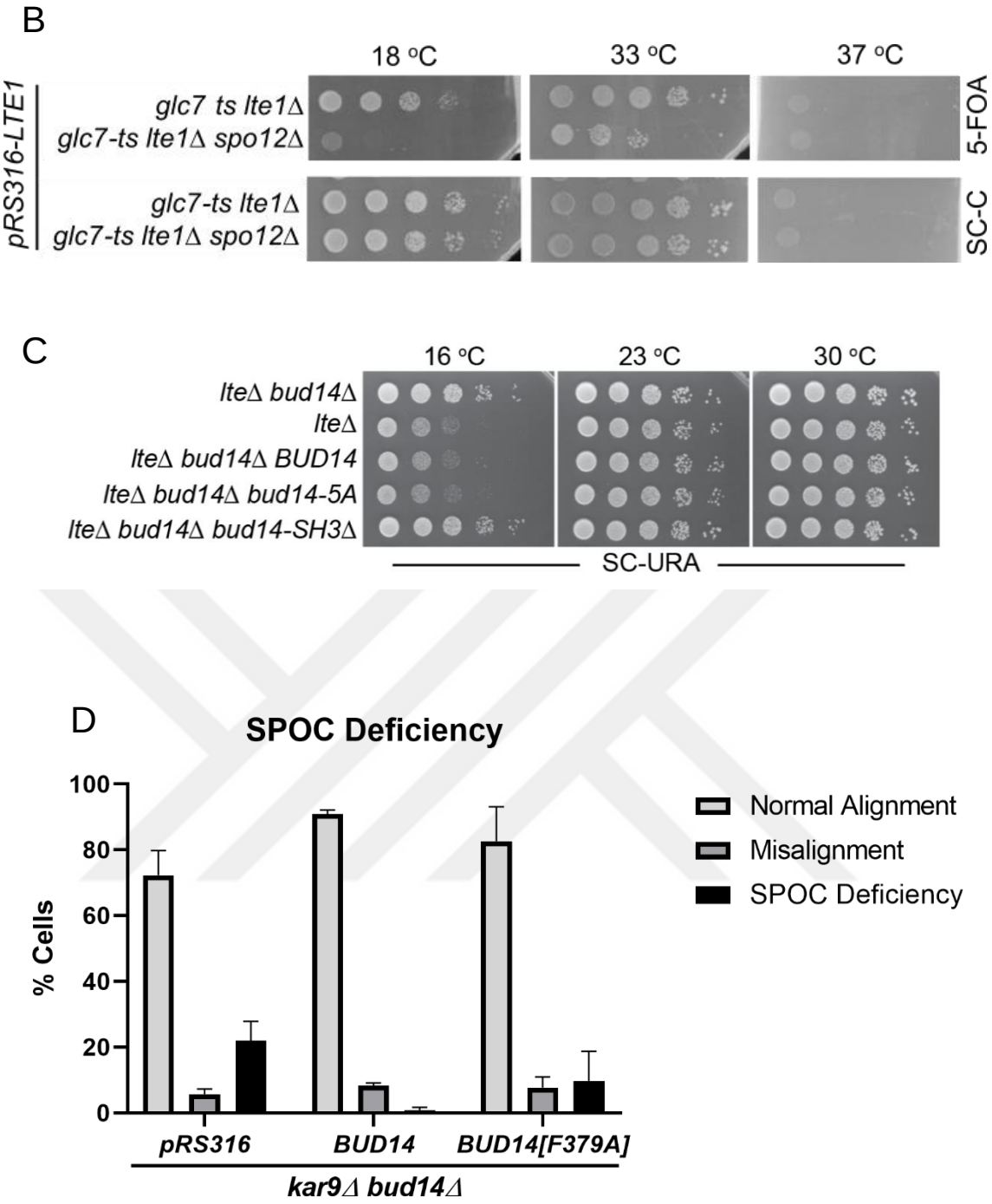


Figure 3.4 **Bud14-Glc7 interaction and SH3 domain plays a role in mitotic exit inhibitory role of Bud14. A** Serial dilutions of indicated strains bearing *URA3* based empty plasmid (not indicated on figure) or BUD14- containing *URA3* based plasmids (*BUD14, bud14-F379A*) were spotted on indicated plates and grown at indicated temperatures **B. S**erial dilutions of indicated strains bearing the *LTE1* on URA3 based pRS316 plasmid were spotted on 5-FOA and SC-Complete (SC-C) plates and grown at indicated temperatures. 5-FOA negatively selects for *URA3* containing plasmids, thus cells lose their pRS316-*LTE1* plasmids on 5-FOA plates and genetic interactions can be observed on this plate. **C.** Serial dilutions of indicated strains bearing *URA3* based empty plasmid (not indicated on figure) or BUD14- containing *URA3* based plasmids (*BUD14, bud14-SH3 or bud14-4A*) were spotted on SC-Ura and grown at indicated temperatures **D.** *kar9*Δ *bud14*Δ strains were transformed with the empty (pRS316) plasmid or *BUD14* containing plasmids. *kar9*Δ*bud14*Δ cells bearing indicated plasmids were grown at 23 °C until logarithmic growth phase and shifted to 30 °C for about 2 doubling times. Cells were fixed with ethanol fixation and stained with DAPI. The percentage of cells with normal positioned nuclei, mispositioned nuclei and SPOC deficient phenotypes were plotted. The experiment was repeated 3 times and the average values are shown

in the graph. Error bars are standard deviation. A minimum of 100 cells were count for each strain in each experiment.

3.4 Localization of Bud14 and Glc7 during spindle misalignment

Bud14 was shown to localize to bud tips, bud cortex and bud neck in a cell cycle dependent manner (Knaus et al., 2005). We investigated the Bud14 localization in cells with misaligned spindles (Figure 3.5).

In consistence with previous reports, *BUD14-GFP* localized to the future bud sites at the start of the cell cycle and enlarged there like a crest shape at the bud cortex as the bud grew. As the spindle elongated correctly in anaphase (Figure 3.5, upper cell, where start of anaphase is marked with a yellow pointed arrow) the bud cortex localization reduced gradually and finally disappeared as it started to accumulate at the bud neck. Bud neck accumulation of Bud14-GFP coincided with spindle breakdown (indicated with squared arrow) which marks the end of mitosis. When the anaphase spindle was misaligned, Bud14 localized to the bud tip similar to cells with a normally aligned spindle (Figure 3.5, lower cell, where start of the anaphase is marked with a yellow pointed arrow). Bud14 localization at the bud cortex gradually disappeared as the cells stayed arrested in anaphase, in a way similar to cells with correctly aligned spindles. However, Bud14 did not localize to the bud neck. More experiments must be done to understand the nature of Bud14 localization in cells with misaligned spindles.

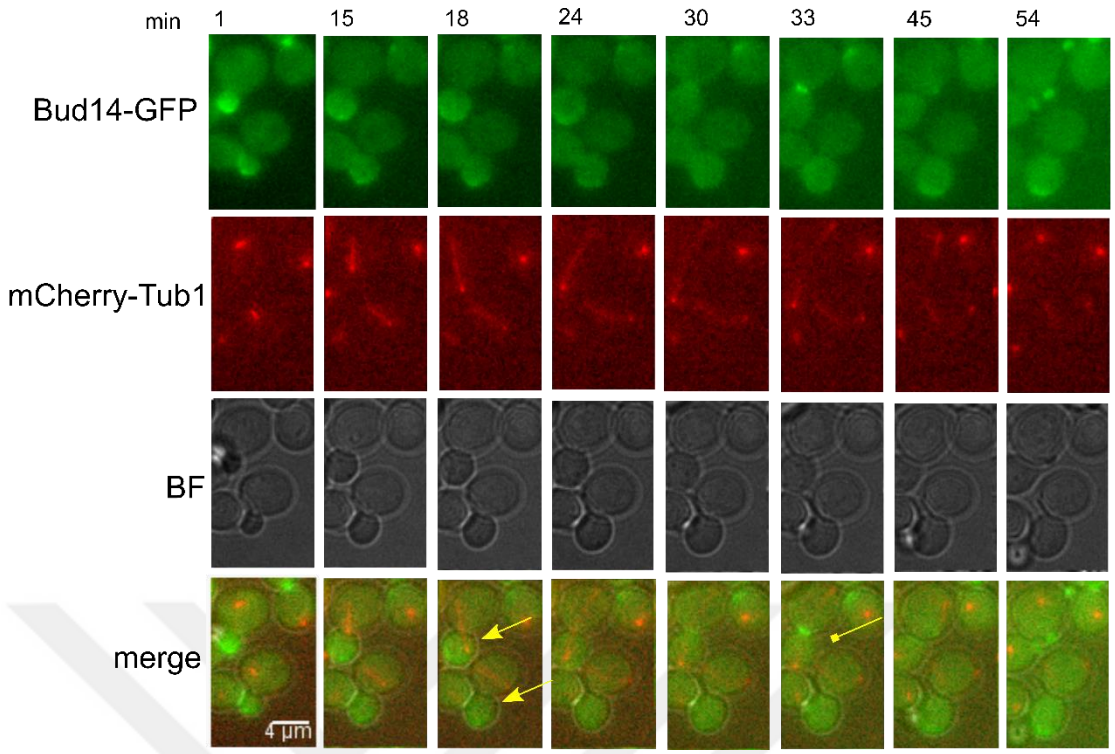


Figure 3.5 **Localization of Bud14 in normal and misaligned cells by live-imaging.** Selected frames from time-lapse series of *BUD14-GFP mCherry-TUB1 cells*. 2 cells, one of which has a normally aligned spindle and the other has a misaligned anaphase spindle were indicated yellow arrow. Pointed arrows indicate the start of the anaphase, squared arrow indicates end of the anaphase with spindle breakage. Scale bar shows 4 µm.

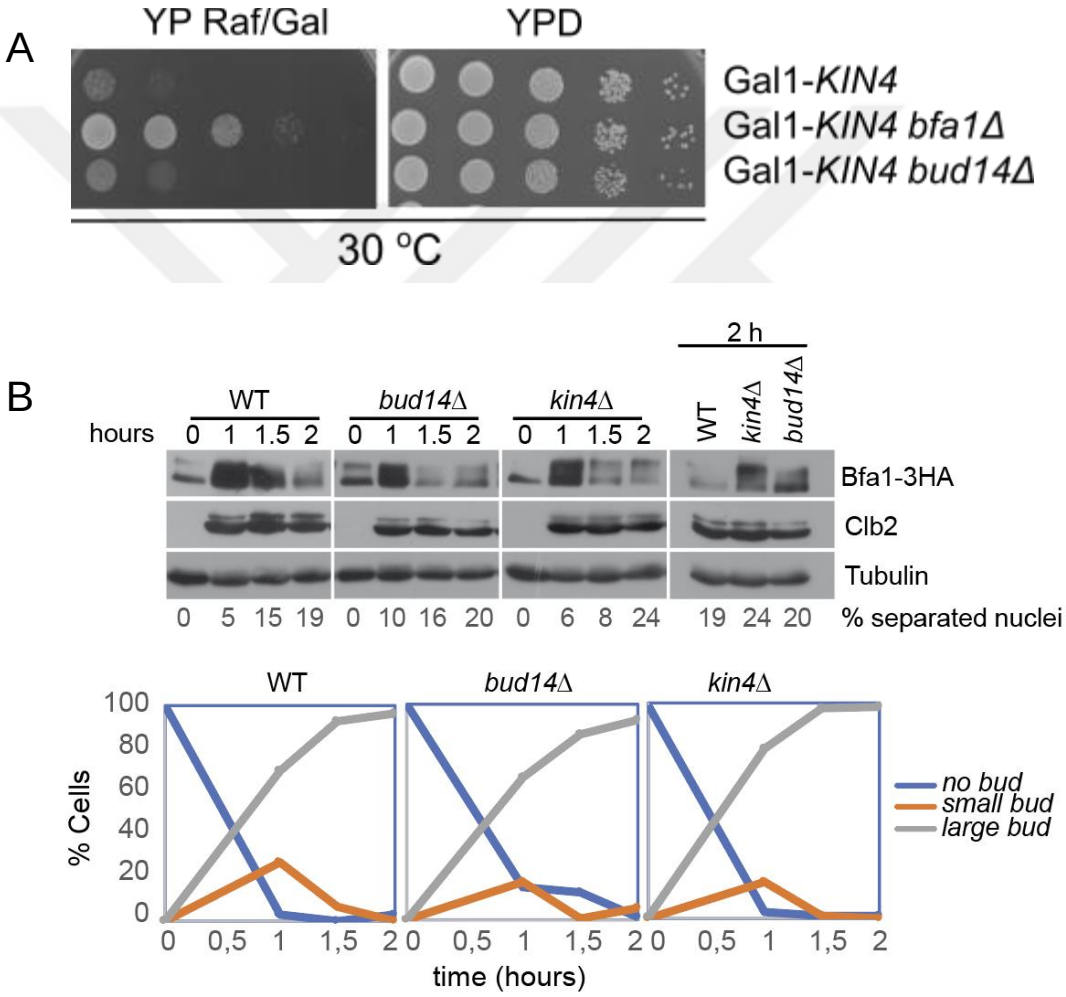
3.5.Bud14 does not work in Kin4 branch of SPOC

Kin4 is the key SPOC kinase that phosphorylates Bfa1 upon spindle misalignment to promote Bfa1-Bub2 GAP activity. Kin4 overexpression blocks exit from mitosis through constitutive Bfa1-Bub2 activation and thus results in lethality (D'Aquino et al., 2005). This lethality can be rescued by deletion of *BFA1* or *BUB2*, which are the most downstream SPOC components. Lethality of Kin4 overexpression can as well be rescued by deletion of *RTS1* or *ELM1*, which are Kin4 regulators and deletion of *BMH1*, which works downstream of *KIN4* (Caydasi et al., 2010a). Unlike aforementioned gene deletions, deletion of *BUD14* did not rescue the lethality of Kin4 overexpression (Figure 3.6A), indicating that Kin4 regulation of Bfa1 is still functional in the absence of Bud14. This data suggests that *BUD14* most likely acts in a pathway parallel to Kin4 to prevent mitotic exit.

To further investigate the effect of Bud14 on Kin4 phosphorylation of Bfa1, we assayed Kin4 activity in cells treated with the microtubule depolymerizing drug nocodazole. Nocodazole treatment promotes Kin4 phosphorylation of Bfa1 that in turn prevents Cdc5 phosphorylation of Bfa1. Consequently, Bfa1 becomes vastly hyperphosphorylated by

Cdc5 in the absence of Kin4, which is not the case in *KIN4* bearing wild type cells. Such hyperphosphorylated forms of Bfa1 can be observed as slow migrating forms of Bfa1 on SDS-PAGE which is absent in wild type cells (Figure 3.6B). Like in wild type cells, Bfa1 in *bud14*Δ cells lacked the hyperphosphorylated forms of Bfa1 (Figure 3.6B). This data indicates that Kin4 is still able to act upon Bfa1 in the absence of Bud14. In line with this statement, Bud14 did not affect Kin4 localization or mobility shift on SDS-PAGE during the nocodazole arrest (Figure 3.6C,D).

These data altogether suggest that Bud14 works independently of Kin4 branch of Bfa1.



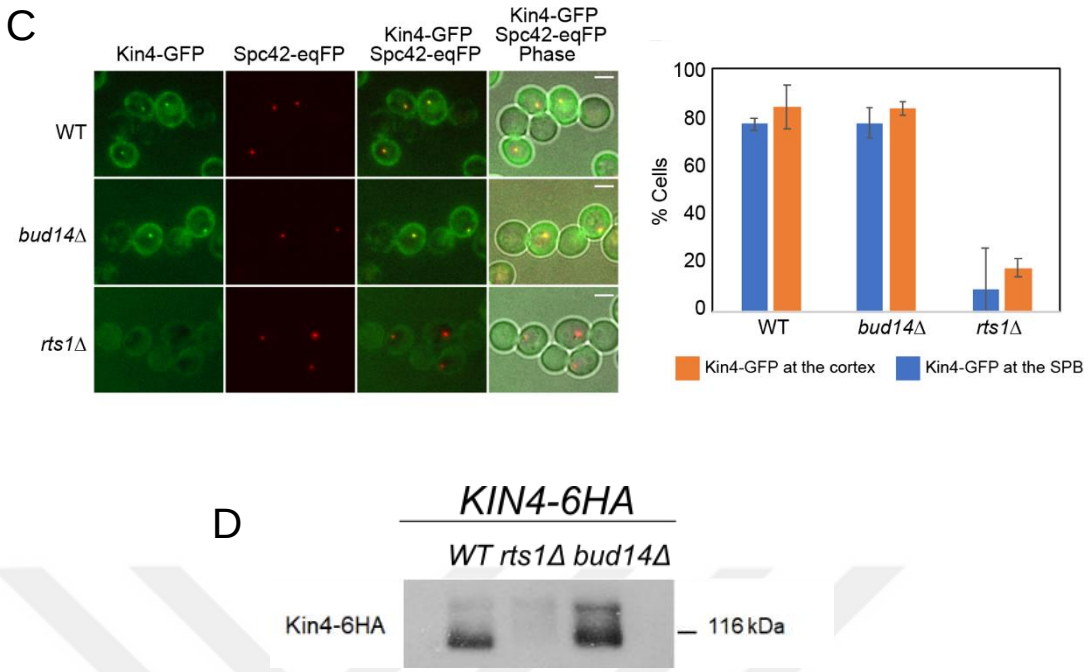


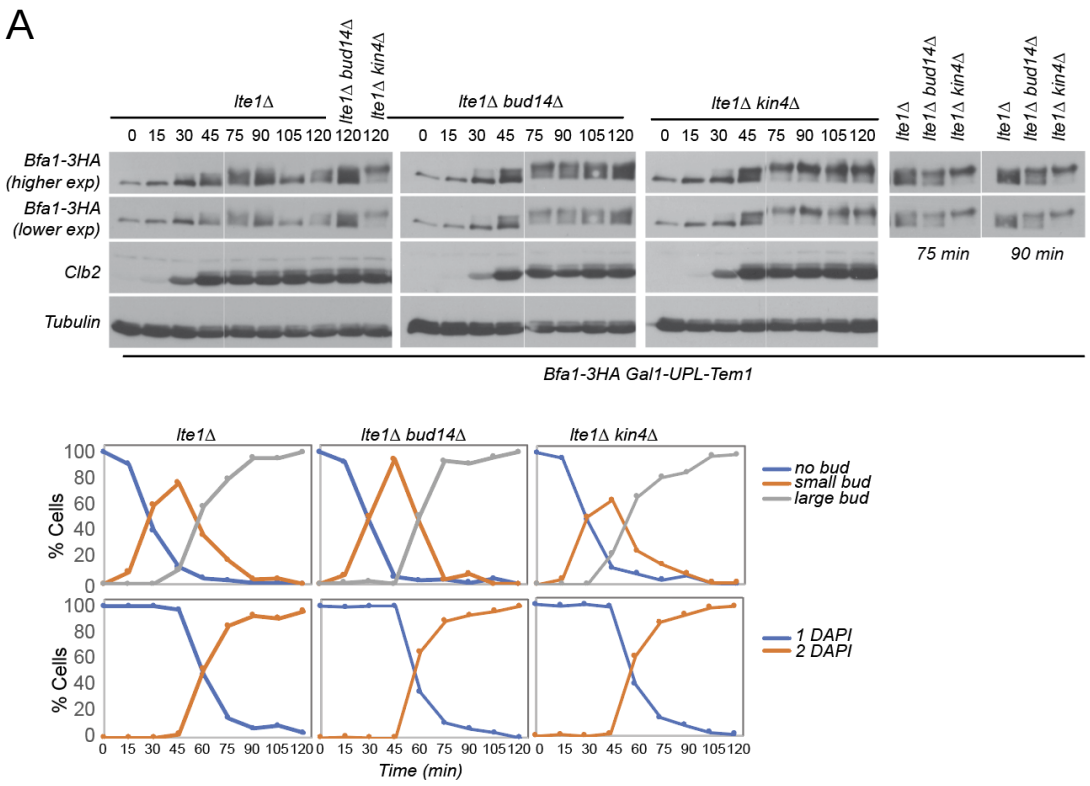
Figure 3.6 **Bud14 does not affect Kin4 in SPOC function. A.** Serial dilutions of indicated strains were spotted on Glucose containing (YPD) and Galactose containing (YP Raf/Gal) agar plates. Gal1-Kin4 overexpression is induced on Galactose containing plates. **B.** Cells arrested in G1 by alpha factor treatment (t=0) were released in alpha factor free nocodazole containing media. Samples were collected at indicated time points. Bfa1-3HA mobility shift was analyzed via western blotting using anti-HA antibodies. Clb2 served as a marker for mitotic progression and Tubulin served as loading control. Percentage of separated nuclei and the budding index was assessed by microscopy after DAPI staining and serve as a control for the metaphase arrest impinged by nocodazole. **D.** Logarithmic phase strains were treated by nocodazole and samples for total cell protein extraction and ethanol fixation were collected after 2 h. Kin4-6HA mobility shift was analyzed by western blotting. *rts1*Δ served as a control for hyperphosphorylated Kin4.

3.5 Bud14 affects Bfa1 phospho-regulation during anaphase

In order to understand how Bud14 contributes to inhibition of mitotic exit, we made use of cells bearing Gal1-UPL-Tem1, which allows Tem1 depletion in the presence of glucose. Thus, these cells can be arrested in telophase. Wild type cells arrest in telophase with hyperphosphorylated Bfa1 (Hu et al., 2001a) (Figure 3.7A). This form of Bfa1 is established to be inactive through phosphorylation by Cdc5 (Hu et al., 2001b). *lte1*Δ cells fail to accumulate such hyperphosphorylated Bfa1 due to Kin4 localization at dSPB of *lte1*Δ cells (Bertazzi et al., 2011) (Figure 3.7A). Consequently, Bfa1 hyperphosphorylation is restored in *lte1*Δ *kin4*Δ cells (Bertazzi et al., 2011) (Figure 3.7A). We asked whether *BUD14* deletion affects Bfa1 phosphorylation in *lte1*Δ cells. Remarkably, deletion of *BUD14* in *lte1*Δ cells resulted in appearance of slow migrating Bfa1 forms during anaphase (t ≥ 75min, cells with separated nucleus ≥ %80). This form of Bfa1 was less abundant in *lte1*Δ *bud14*Δ than in *lte1*Δ *kin4*Δ cells and ceased away

upon prolonged arrest (120min). Our data indicate that Bud14 may promote dephosphorylation of Bfa1 in a transient manner during anaphase.

We next asked whether deletion of *BUD14* affected Kin4 localization in *lte1*Δ cells, which may account for the observed Bfa1 hypershift in *lte1*Δ *bud14*Δ. In *lte1*Δ cells, Kin4 localizes to both SPBs during anaphase (Bertazzi et al., 2011)(Figure 3.7B). This localization of Kin4 causes phosphorylation of Bfa1 by Kin4, which prevents Cdc5 phosphorylation of Bfa1 that appears as hyperphosphorylated forms of Bfa1 (Gislene Pereira & Schiebel, 2005). Similar to *lte1*Δ cells, Kin4 localized on both SPBs of *lte1*Δ *bud14*Δ cells during anaphase. Our data thus show that Bud14 affects Bfa1 phosphorylation in *lte1*Δ cells independently of Kin4 localization.



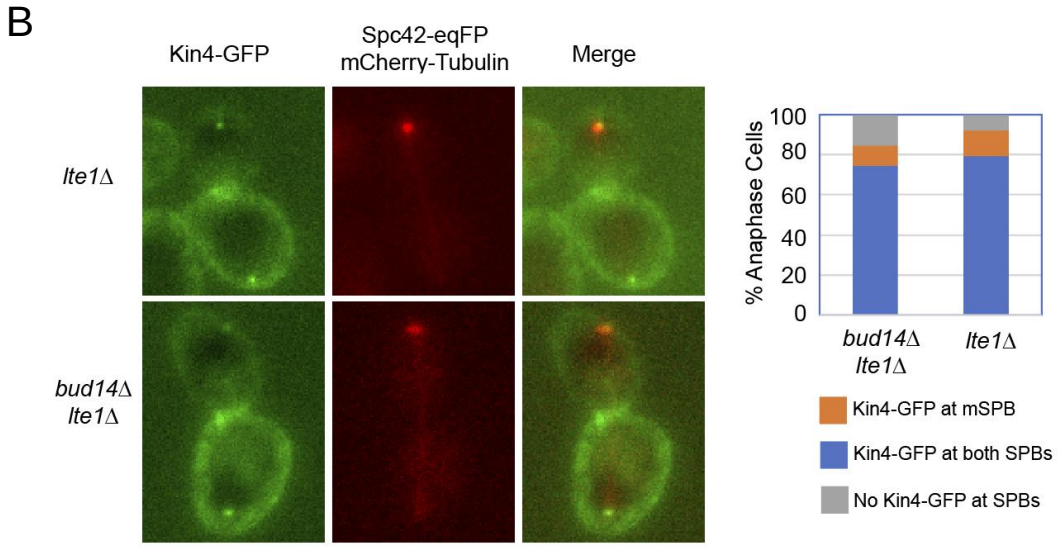


Figure 3.7 **Bud14 affects Bfa1 phospho-regulation independent of Kin4. A.** Bfa1-3HA Gal1-UPL-Tem1 containing strains were released from G1 arrest (t=0) imposed by alpha factor treatment and arrested in alpha factor free medium supplemented with glucose to achieve Tem1 depletion. Cell cycle progression and the telophase arrest was monitored by budding index and DAPI plots by microscopy as well as Clb2 levels by western blotting. Bfa1-3HA mobility shift is shown in a low exposed as well as a higher exposed film for better visualization of ratios of up and down shifted protein bands. **B.** Logarithmic growing cultures of Kin4-GFP Spc42-eqFP mCherry-*TUB1* bearing cells were imaged live. Kin4 localization at the cortex and SPBs were analyzed in anaphase cells (spindle length >5). Images of representative cells and one representative graph out of 3 experiments was shown. A minimum of 100 cells were counted from each sample.

3.6 Bud14 overexpression lethality is not rescued by deletion of SPOC components

The overexpression of Bud14 is toxic to cells by unknown reasons (Cullen & Sprague, 2002) (Figure 3.8). We asked whether this lethality is related with the role of Bud14 in inhibition of mitotic exit. For this we constructed Gal1-Bud14-GFP bearing strains. This strain grew very poor on Galactose containing agar plates. We reasoned that if toxicity of *BUD14* overexpression is through hyperactivation of the SPOC, deletion SPOC components *BFA1* and *KIN4* should rescue this lethality. However, lethality of Bud14 overexpression persisted in *bfa1Δ* and *kin4Δ* cells at various temperatures tested. Thus, we conclude that over expression Bud14 is lethal independently of MEN inhibition. This is inline with the observation that *Gal1-BUD14* cells arrest with pre-anaphase like spindle that moves into the bud (Knaus et al., 2005).

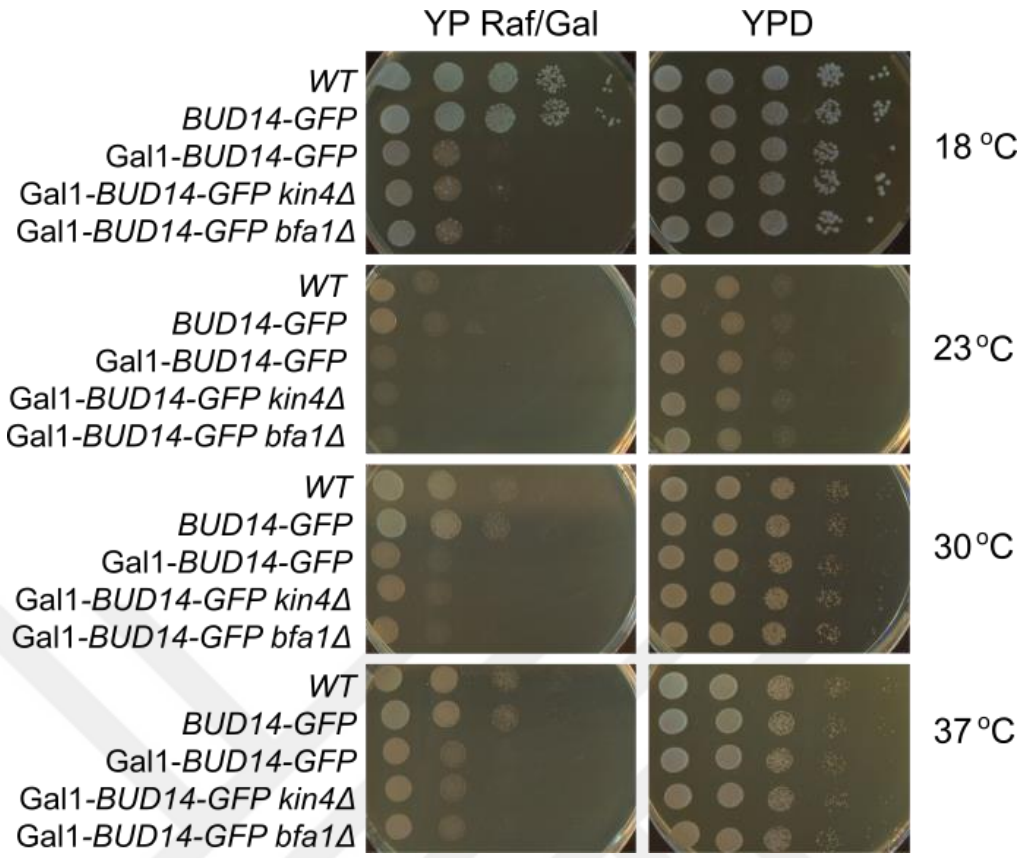


Figure 3.8 **Bud14 overexpression lethality is not rescued by deletion of SPOC components.** Stationary phase cultures were diluted to 1.0 OD_{600}. Serial dilutions were performed by 10-fold dilutions with PBS. 2% Raffinose, 3% Galactose was used in YP Raf/Gal agar plates.

Chapter 4 **DISCUSSION, CONCLUSION and OUTLOOK**

Our data showed that Bud14 is a novel mitotic exit inhibitor. Several lines of evidence led us to this conclusion. *BUD14* deletion rescued the lethality of *lte1Δ spo12Δ*, similar to known mitotic exit inhibitors *BFA1*, *BUB2* and *KIN4* (Figure 3.1A). In addition, deletion of *BUD14* rescued cold sensitivity of *lte1Δ* cells (Figure 3.1B) and the temperature sensitivity of MEN mutants *cdc15-1*, *dbf2-2* and *mob1-67*. Our data suggests that Bud14 functions upstream of the MEN pathway, as deletion of *BUD14* did not rescue growth of *cdc14-2*, the temperature sensitive allele of Cdc14, the very downstream component of MEN (Figure 3.2).

When spindle misalignment was induced in cells by deletion of *KAR9* or *DYN1*, *bud14*Δ cells showed significant multinucleation phenotype similar to *kin4*Δ cells, implying that *bud14*Δ cells are deficient in mitotic arrest upon mispositioning of the anaphase spindle (Figure 3.3). Importantly, Bud14 was implicated in the Dynein pathway of spindle positioning (Knaus et al., 2005). However, deletion of *BUD14* caused accumulation of multinucleated phenotypes in both *kar9*Δ and *dyn1*Δ backgrounds indicating that the

function of Bud14 in Dynein regulation is not contributing to the observed multinucleation phenotype. These data suggest that Bud14 is required for inhibition of mitotic exit in cells with misaligned spindles and thus for SPOC functionality.

Bud14 is a regulatory subunit of the protein phosphatase 1 (PP1) Glc7. Bud14 recruits Glc7 to the bud cortex and regulates microtubule dynamics and polarization therein (Knaus et al., 2005). Bud14-Glc7 complex also has a transcription regulatory role through posttranslational modification of Msn2, a transcriptional activator (Lenssen et al., 2005). Our data that a Bud14 mutant that cannot bind Glc7 (*bud14-F379A*) (Knaus et al., 2005) was deficient in SPOC show that the role of Bud14 in SPOC is through its interaction with Glc7 (Figure 3.4 A,B,D). Interestingly a truncated version of Bud14 that lacks the SH3 domain is also unable to inhibit mitotic exit (Figure 3.4C). This truncated Bud14 is unable to bind to bud cortex and also have reduced binding to Glc7 (Knaus et al., 2005). We think that *Bud14-SH3*Δ may fail in mitotic exit inhibition due to its reduced association with Glc7. Bud cortex localization of Bud14 may as well be important for this function but our observations that Bud14 bud cortex localization remains low during spindle misalignment may argue against this notion (Figure 3.5),

The SPOC component Bfa1 is a highly phosphorylated protein in a cell cycle dependent manner. So far Bfa1 has been shown to be the target of kinases Cdk1, Cdc5 and Kin4 (Geymonat et al., 2003; Gislene Pereira & Schiebel, 2005). It is possible that other kinases may also phosphorylate Bfa1. There has been an ongoing curiosity for phosphatases that act on Bfa1. So far, Cdc14 was shown to counteract Cdk1 phosphorylation (Visintin et al., 1998) and PP2A-Cdc55 was shown to counteract Cdc5 dependent dephosphorylation of Bfa1 (Baro et al., 2013). Our data that Bfa1 appears hyperphosphorylated in *lte1*Δ *bud14*Δ cells in anaphase indicate that Glc7-Bud14 promotes dephosphorylation of Bfa1 during anaphase (Figure 3.7A). It should yet be investigated whether this is due to direct dephosphorylation of Bfa1 by Glc7-Bud14. Glc7-Bud14 may directly dephosphorylate Bfa1. We were able to observe interaction of Bfa1 and Bud14 in yeast two hybrid assays, however we were unable to observe co-immunoprecipitation of Bfa1 and Bud14 (unpublished data). This might be due to a transient interaction of Bud14 with Bfa1. Alternatively, Bud14 may exert its effect on Bfa1 through regulation of Kin4 or other proteins that regulate Bfa1 phosphorylation. Our data showed that Bud14 does not affect Kin4 localization and phosphorylation. In line with this, Kin4 overexpression still causes toxicity in *BUD14* deleted cells indicating that Kin4 is still active and prevents Cdc5

phosphorylation of Bfa1 in *bud14*Δ cells. Furthermore, our preliminary time-lapse analysis of Bfa1 localization during spindle misalignment suggests that Bfa1 is being removed from SPBs of cells with misaligned spindles, in a way similar to *KIN4* cells rather than *kin4*Δ cells (data not shown). These data altogether exclude the possibility that Bud14 affects Bfa1 via Kin4.

The hyperphosphorylated Bfa1 observed in *lte1*Δ *bud14*Δ cells resemble those seen in *lte1*Δ *kin4*Δ. Kin4 prevents hyperphosphorylation of Bfa1 by inhibiting phosphorylation of Bfa1 by Cdc5 (Figure 3.6B). This function of Kin4 is active both in anaphase and metaphase (D'Aquino et al., 2005). However, unlike Kin4, the effect of Glc7-Bud14 on Bfa1 phosphorylation can only been seen during anaphase, and even then, in a transient manner. This discrepancy between Kin4 and Glc7-Bud14 may indicate alternative scenarios: First, Glc7-Bud14 may promote dephosphorylation of sites other than those phosphorylated by Cdc5. Cdc5 dependency of the slower migrating Bfa1 forms observed in *lte1*Δ *bud14*Δ cells can be analyzed by taking advantage of the *cdc5-10* temperature sensitive mutant that is unable to phosphorylate Bfa1 at its restrictive temperature. Second, there may be redundant mechanisms of Bfa1 dephosphorylation. For example, PP2A-Cdc55 was shown to counteract Bfa1 phosphorylation, both in metaphase and anaphase. Therefore, it could be one possibility that PP2A-Cdc55 and Glc7-Bud14 may work redundantly to dephosphorylate Cdc5 phosphorylated Bfa1. This hypothesis is yet to be tested. If it is true, we expect to see a stable and additive increase Bfa1 hyperphosphorylation in a *cdc55*Δ *bud14*Δ double mutants.

Interestingly Glc7 was shown to localize to spindle poles but not Bud14 (Bloecher & Tatchell, 2000; Knaus et al., 2005). At the moment, we are unable to comment on importance of this localization for the function of Bud14-Glc7 in SPOC. Although Glc7 localizes to the poles of cells with normally aligned anaphase spindles (Bloecher & Tatchell, 2000) as well as cells with misaligned anaphase spindles (data not shown), we were unable to detect Bud14 at the poles in either condition (data not shown). Furthermore, we forced Bud14-GFP to the SPBs in a strain bearing GFP-binding-protein (GBP) tagged Spc42 but the resultant mutant had mitotic exit kinetics and Bfa1 protein mobility similar to cells lacking Spc42-GBP (data not shown), indicating that presence of Bud14 at the SPB is not sufficient or necessary for its mitotic exit inhibitory function. Bud14 accumulates at the bud tip but is also present diffusely in the cytoplasm especially of the daughter cell. Bud14 may dephosphorylate Bfa1 at these locations. The fact that

Bfa1 stays hyperphosphorylated in *kin4*Δ cells in which Bfa1 stays at the SPBs also supports this notion.

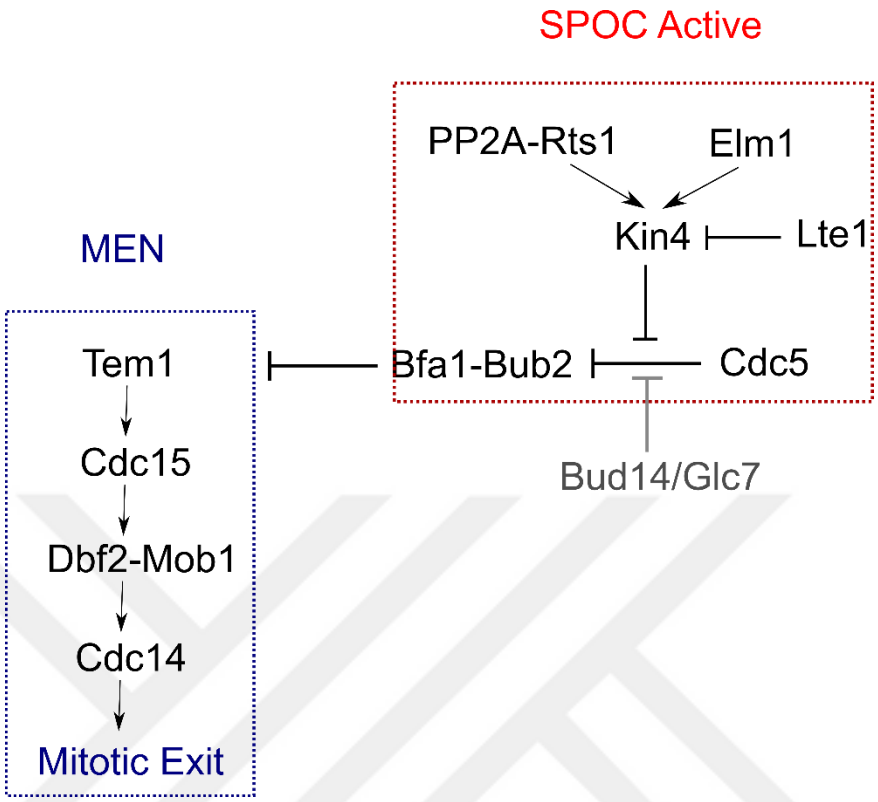


Figure 4.1 Mechanism of action of Bud14/Glc7 role in mitotic exit. Our model depicts that Bud14/Glc7 function in SPOC through promoting Bfa1 dephosphorylation, parallel to Kin4. Point arrows represent activation, while block arrows represent inhibition.

Taken together, our data is consistent with a model in which Glc7-Bud14 acts in Bfa1 activation in a way parallel to Kin4. Kin4 prevents access of Cdc5 to Bfa1 by removing Bfa1 from spindle poles, and Glc7-Bud14 may dephosphorylate this released Bfa1 in the cytoplasm (Figure 4.1).

How spindle alignment is sensed, and this information activates SPOC still remain unknown. Data based on Kin4 activity on Bfa1 indicates that SPOC mechanism can be activated by treatment with microtubule depolymerizing drug nocodazole (Caydasi & Pereira, 2009; Maekawa et al., 2007; Monje-Casas & Amon, 2009; Gislene Pereira & Schiebel, 2005). Therefore, microtubule-cortex interactions may be sensed. Due to their known function in regulating microtubule-cortex interactions, Bud14-Glc7 constitutes a great candidate for sensing of such interactions (Knaus et al., 2005). Considering that Glc7 (PP1) is a conserved phosphatase, similar mechanisms may be employed by higher eukaryotes. Future work will show whether Bud14-Glc7 may act as a sensor that translates the unattached microtubule-cortex interactions to the downstream SPOC machinery.

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Chapter 6 **APPENDIX**

6.1 Table of strains

Table 6-1 Table of strains

Strain Name	**Description**	**Associated Figure**	**Reference**
ESM356-1	*MATa ura3-52 leu2Δ1 his3Δ200 trp1Δ63*	Figure *3.8*	G. Pereira et al., 2001
GPY491-1	*YPH499 cdc14-2 pRS316- CDC14*	Figure *3.2*	(Caydasi et al., 2017)
ESM2285	*YPH499 mob1-67 pRS316-MOB1*	Figure *3.2*	(Caydasi et al., 2017)
JOY79-1	*YPH499 cdc5-10 pRS316-CDC5*	Figure *3.2*	(Caydasi et al., 2017)
ESM2283	*YPH499 dbf2-2 pRS316-DBF2*	Figure *3.2*	(Caydasi et al., 2017)
ESM2282	*YPH499 cdc15-1 pRS316-CDC15*	Figure *3.2*	(Caydasi et al., 2017)
FAY145-1	*MATa ura3-52 his3Δ200 leu2Δ1 lte1Δ::kanMX6 pRS316-LTE1*	Figure *3.1*	(Bertazzi et al., 2011)
AKY2526-1	*MATa ura3-52 his3Δ200 leu2Δ1 lte1Δ::kanMX6 pRS316-LTE1 spo12Δ::natNT2*	Figure *3.1*	(Caydasi et al., 2017)
GPY1033	*ESM356-1 KIN4-GFP-his3MX6 Spc42-eqFP-natNT2*	Figure *3.7*	(Gislene Pereira & Schiebel, 2005)
AKY260	*ESM356 kar9Δ::his3MX6 pRS316-KAR9*	Figure 3.3	(Caydasi et al., 2017)
AKY315-1	*ESM356 kar9Δ::his3MX6 pRS316-KAR9 bfa1Δ::klTRP*	Figure 3.3	(Caydasi et al., 2017)
AKY321-1	*ESM356 kar9Δ::his3MX6 pRS316-KAR9 kin4Δ::klTRP*	Figure 3.3	(Caydasi et al., 2010b)
YDA101-1	*ESM356 kar9Δ::his3MX6 bud14Δ::klTRP*	Figure 3.3	This study
AKY2916-1	*MATa ura3-52 his3Δ200 leu2Δ1 lte1Δ::kanMX6 pRS316-LTE1 spo12Δ::natNT2 bud14Δ::his3MX6*	Figure *3.1*	This study

AKY2917-1	*YPH499 ura3::URA3-GFP-Tub1 dyn1Δ::klTRP1 bud14Δ::his3MX6*	Figure 3.3	This study
AKY2918-1	*YPH499 ura3::URA3-GFP-Tub1 dyn1Δ::klTRP1 kin4Δ::hphNT1 bud14Δ::his3MX6*	Figure 3.3	This study
PAY704-1	*W303 MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 can1-100 ssd1-d2 glc7::LEU2 trp1-1::GLC7::TRP1*	Figure *3.4*	(Andrews & Stark, 2000)
PAY701-2	*W303 MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 can1-100 ssd1-d2 glc7::LEU2 trp1-1::glc7-12::TRP1*	Figure *3.4*	(Andrews & Stark, 2000)
AKY887-1	*ESM356-1 BFA1-3HA-hphNT1 kin4Δ::his3MX6*	Figure *3.7* Figure *3.6*	(Gislene Pereira & Schiebel, 2005)
BKY091-1	*ESM356-1 BFA1-3HA-hphNT1*	Figure *3.7* Figure *3.6*	(Gislene Pereira & Schiebel, 2005)
ESM2326-1	*ESM356-1 KIN4-6HA-hphNT1*	Figure *3.6*	(Caydasi et al., 2010, p. 1)
AKY415	*ESM356-1 KIN4-6HA-hphNT1 rts1Δ::klTRP*	Figure *3.6*	(Caydasi et al., 2010a, p. 1)
DGY001-1	*ESM356-1 KIN4-6HA-hphNT1 bud14Δ::KlTrp*	Figure *3.6*	This study
DGY002-1	*ESM356-1 BFA1-3HA-hph kin4Δ::his3MX6 bud14Δ::klTRP*	Figure *3.7* Figure *3.6*	This study
DGY003-1	*ESM356-1 BFA1-3HA-hphNT1 bud14Δ::KlTrp*	Figure *3.7* Figure *3.6*	This study
DGY004-1	*ESM356-1 KIN4-GFP-his3MX6 Spc42-eqFP-natNT2 bud14Δ::klTRP*	Figure *3.6*	This study
SHM562-1	*YPH499 GFP-Tub1-URA3 dyn1Δ::klTRP1*	Figure 3.3	(Gislene Pereira & Schiebel, 2005)
ESM2156-2	*YPH499 GFP-Tub1-URA3 dyn1Δ::klTRP1 kin4Δ::hphNT1*	Figure 3.3	(Gislene Pereira & Schiebel, 2005)
DKY057-1	*YPH499 cdc14-2 pRS316-CDC14 bud14Δ::klTRP3*	Figure *3.2*	This study
DKY058-1	*YPH499 mob1-67 pRS316-MOB1 bud14Δ::klTRP3*	Figure *3.2*	This study
DKY060-1	*YPH499 dbf2-2 pRS316-DBF2 bud14Δ::klTRP3*	Figure *3.2*	This study

DKY061-1	*YPH499 cdc15-1 pRS316-CDC15 bud14Δ::klTRP3*	Figure *3.2*	This study
DKY063-1	*KIN4-GFP-his3MX6 Spc42-eqFP611-natNT2 bud14Δ::klTRP*	Figure *3.6*	This study
DKY069-1	*MATa ura3-52 his3Δ200 leu2Δ1 lte1Δ::kanMX6 pRS316-LTE1 bud14Δ::his3MX6*	Figure *3.1*	This study
DKY074-1	*W303 MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 can1-100 ssd1-d2 glc7::LEU2 trp1-1::glc7-12::TRP1 lte1Δ::his3MX6*	Figure *3.4*	This study
DKY075-1	*W303 MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 can1-100 ssd1-d2 glc7::LEU2 trp1-1::GLC7::TRP1 lte1Δ::his3MX6*	Figure *3.4*	This study
DKY078-1	*W303 MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 can1-100 ssd1-d2 glc7::LEU2 trp1-1::GLC7::TRP1 lte1Δ::his3MX6 pRS316-LTE1*	Figure *3.4*	This study
DKY079-1	*W303 MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 can1-100 ssd1-d2 glc7::LEU2 trp1-1::glc7-12::TRP1 lte1Δ::his3MX6 pRS316-LTE1*	Figure *3.4*	This study
DKY080-1	*W303 MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 can1-100 ssd1-d2 glc7::LEU2 trp1-1::glc7-12::TRP1 lte1Δ::his3MX6 pRS316-LTE1 spo12Δ::hpn*	Figure *3.4*	This study
DKY118	*ESM356 GAL1-UPL-TEM1::kITRP BFA1-3HA-hphNT1 lte1Δ::natNT2*	Figure *3.7*	This study
DKY125-1	*ESM356 GAL1-UPL-TEM1::kITRP BFA1-3HA-hphNT1 kin4Δ::natNT2 bud14Δ::his3MX6*	Figure *3.6*	This study
DKY126-1	*ESM356 (GAL1-UPL-TEM1::kITRP) BFA1-3HA-hphNT1 lte1Δ::natNT2 bud14Δ::his3MX6*	Figure *3.7*	This study
DKY131-1	*lte1Δ::kanMX6 pRS316-LTE1 bud14Δ::his3MX6 GFP BUD14 CEN URA*	Figure *3.4*	This study
DKY132	*ESM356 bud14Δ::klTrp GFP BUD14 CEN URA)*	Figure *3.4*	This study
DKY135-1	*lte1Δ::kanMX6 pRS316-LTE1 bud14Δ::his3MX6 pRS416-ADH-BUD14*	Figure *3.4*	This study
DKY137-1	*lte1Δ::kanMX6 pRS316-LTE1 bud14Δ::his3MX6 pRS416-ADH-BUD14 [F379A]*	Figure *3.4*	This study
DKY145-1	*ESM356 kar9Δ::his3MX6 bud14Δ::klTRP pRS316*	Figure *3.4*	This study

DKY146-1	*ESM356 kar9Δ::his3MX6 bud14Δ::klTRP GFP BUD14 CEN URA*	Figure *3.4*	This study
DKY147-1	*ESM356 kar9Δ::his3MX6 bud14Δ::klTRP pRS416-ADH-BUD14*	Figure *3.4*	This study
DKY149-1	*ESM356 kar9Δ::his3MX6 bud14Δ::klTRP pRS416-ADH-BUD14 [F379A]*	Figure *3.4*	This study
DKY168-1	*ESM356-1 BFA1-3HA-hphNT1 kin4Δ::his3MX6 BUD14-GFP-klTRP Gal1-BUD14-natNT2*	Figure *3.8*	This study
DKY178-1	*ESM356-1 Spc42-eqFP611-kanMX6 BUD14-GFP-hisMX6 Gal1-BUD14-natNT2 kin4Δ::klTRP*	Figure *3.8*	This study
DKY189	*ESM356-1 Spc42-eqFP611-kanMX6 BUD14-GFP-hisMX6 Gal1-BUD14-natNT2 bfa1Δ::klTRP*	Figure *3.8*	This study
AKY4070-1	*ESM356-1 Spc42-eqFP611-kanMX6 BUD14-GFP-hisMX6 mCherry-TUB1-URA3 kar9Δ::klTRP1*	Figure *3.5*	This study

6.2 Table of plasmids

Table 6-2 Table of plasmids

Plasmid Name	**Description**	**Reference**
pRS316	*URA3-dependent CEN-based yeast-E.coli shuffle plasmid. AmpR.*	(Sikorski & Hieter, 1989)
pWS103-1	*pRS304-Gal1-UPL-TEM1*	(Shou et al., 1999)
pMK60	*pRS416-endogenous BUD14-promoter-GFP-BUD14*	(Knaus et al., 2005)
pMK125	*pRS416 ADH-BUD14*	(Knaus et al., 2005)
pMK131	*pRS416 ADH-BUD14^{F379A}*	(Knaus et al., 2005)
pSM903-4	*pRS316-LTE1*	(Hofken, 2002)
pGW399-1	*pRS316-KAR9*	(Hofken, 2002)
pDKY001	*pRS416-endogenous BUD14-promoter-GFP-BUD14$^{5A\ (135A\ 137A\ 138A\ 139A\ 140A)}$*	This Study
pDKY003	*pRS416-endogenous BUD14-promoter-GFP-BUD14$^{\Delta SH3(\ \Delta\ aa\ 262–318)}$*	This Study

6.3 Table of primers

Table 6-3 Table of primers

Primer Name	**Description**
S3-DYN1	GCTAAAGAGGTGGCTAGCTCAACTGAACAACTTCTTCAAGAAATGCGTACGCTGCAGGTCGAC
S2-DYN1	TGAGAAAACCGCGGACAAGCAAGACACGCGTACCTGAAAAGGCTAATCGATGAATTCGAGCTCG
S1-DYN1	CAGAGCTTAAATTGGAAAGTACGTCAAAACGTTTTTTAGGCAATGCGTACGCTGCAGGTCGAC
KAR9-S2	AGGATATATAAAAATGTATAAGTATACAGTTTTAGGTTAGTATCAATCGATGAATTCGAGCTCG
KAR9-S1	ATCTTTGTCTGTAACAGCCTTAAAGATTTCAGTAGCACTGCCATGCGTACGCTGCAGGTCGAC
Bud14-T-rv	CTGACTCGAGCTTGCAAGGAAGAGGTCATC
Bud14-P-fw	GATCAGATCTCCAACCGTCTTACCTCAAAG
Bud14-col2-fow A	CACGACTAAGTAGTTGTCG
Bud14-col1 rev	GCAAGAGTCAGACTGACTCG
Bud14-S1	GTGAAATACACGTAGTTTTTAGATAACATTCTCTGCTTGGTAAGAGAATGCGTACGCTGCAGGTCGAC
Bud14-S2	AAGTTCGTTGGATGAGAAAAAGACCAGGCTTTATTGTAAGGACAATATCAATCGATGAATTCGAGCTCG
Bud14-S3	TTGACAGAATGGATGTGTTGATGAAACAATTGGATGAAATTATTCGTAAACGTACGCTGCAGGTCGAC
Bud14-S4	TCCTTGACACCACTTGCGGAAGTCTCATCAACATGCTCTTCCTTATTACTCATCGATGAATTCTCTGTCG

Bfa1-S1	TGCGTACGCTGCAGGTCGACAGAAAAAAGTTCGAAAACTTTTGTAGCAGTTGAAAGTTTTGTATGCTA
Bfa1-S2	TGTACTCAAGATAACGGTAAAGAAACAGTTATAAGAAGGCTAAAGGGCTAATCGATGAATTCGAGCTCG
Bfa1-S3	AATCCTATATGTATGAAATCAGGAACATGGTAATCAATTCGACAAAAGATCGTACGCTGCAGGTCGAC
BFA1-1	GTGATTCAAATGTTGGGTGAGC
BFA1-2	CGAGTTTTAATCCATTCCACCC
BUB2-1	AGAGGAGTCGCTGCAGCTGGTGTC
BUB2-2	CGCCTTTGGCTGTCATGAAGGTGG
BUB2-S1	AGGTAAAAGAAACAACAGACTTTTAAACTTGTTAACTTTTGCATGCGTACGCTGCAGGTCGAC
BUB2-S2	TTGTAGAATTAAACGATAAAATATAATATTTCTTCACATAGTTTAATCGATGAATTCGAGCTCG
BUB2-S3	GACCTATTGGTAGACCACTTGACCGACCCAGACATATATATACCGCGTACGCTGCAGGTCGAC
S1-Kin4	TCGCATAATATTCTATCAGGACATTCCGTATACCTGAATATATATACATGCGTACGCTGCAGGTCGAC
S2-Kin4	CACTCTATAATATAATGTAATTGTCGATATAACTATGTACTGAAAACTCAATCGATGAATTCGAGCTCG
S3-Kin4	CAACAGCAAGAAAGGTTCTGAATTTTTTCAAAAGAAGGAGCATGAGGGTTCGTACGCTGCAGGTCGAC
Kin4-Col1	AATCGCATCGGTGGGAGAG
Kin4-Col2	AACAACTTGCCAGCCAAGAC
S1-SPO12	AACAAAATAACATATACAGTAAGAACAATAGAAAACGTATTTATGCGTACGCTGCAGGTCGA
S2-SPO12	TAGTGTAGCATTTGGCTATTTTTGGATGACTAGAAAGGCAGATTTTTTTAATCGATGAATTCGAGCTCG
SPO12-1	CCCATTGTATTGCCTCTTCCC

SPO12-2	CGGGTGCCCGCTACTTTATTACAGAGG
MOB1-S2	GCATGGAAGAATACAACCTACAAGCAGACTTATATAAATATACAATACTAATCGATGAATTCGAGCTCG
MOB1-S3	CGGCTGATTTTGGTCCGCTGTTAGAATTAGTGATGGAGTTGAGGGATAGGCGTACGCTGCAGGTCGAC
DBF2-S2	AAGCTAATTATATCGCGGCGAATGCAAGACAAGAATTCATTTTTACGCTAATCGATGAATTCGAGCTCG
DBF2-S3	GCATCTTATTCAACGGACTGGAACACTCAGACCCCTTTTCAACCTTTTACCGTACGCTGCAGGTCGAC
CDC14-S2	TAAGTTTTTTTATTATATGATATATATATATATATAAAAATGAAATAAATTAATCGATGAATTCGAGCTCG
CDC14-S3	CTACAAGCGCCGCCGGTGGTATAAGAAAAATAAGTGGCTCCATCAAGAAACGTACGCTGCAGGTCGAC
CDC15-S2	GCTGTATTATTCTCTATATATGTATGTATGCACATGCAATTCCTACATTAATCGATGAATTCGAGCTCG
CDC15-S3	CCAAAGATAAAAGTGACGGCTTTTCCGTCCCCATTACAACATTTCAAACACGTACGCTGCAGGTCGAC
S2-CDC5	TGGACTGGTAATTTCGTATTCGTATTTCTTTCTACTTTAATATTGGTTTAATCGATGAATTCGAGCTCG
S3-CDC5	TTTGATAAAGGAAGGTTTGAAGCAGAAGTCCACAATTGTTACCGTAGATCGTACGCTGCAGGTCGAC
Lte1-10	GAAGCATGAATTAAGATCATCG
LTE1-11	CCGTTGTAGTTATGTACTGAAG
Lte1-seq	TACAGGATTCACGATTCGAG
Lte1-s1	TTGCTTTACATAGCCTCTAGCCATTCAAAGATTCGCTACCCTGAACCATGcgtacgctgcaggtcgac
Ite1-s2	TTTTTTACAAGAGGAATTTGGGAACTGGAGGAAAGTGGCACAATACCTCAatcgatgaattcgagctcg
GLC7-S2	TAATAAGTATTTTCCTTTTTAAACTTTGATTTAGGACGTGAATCTATTTAatcgatgaattcgagctcg

GLC7-S3	AGCCAGCCCAAAAAAGTCTACCAAGGCAAGCTGGGGGTAGAAAGAAAAAAcgtacgctgcaggtcgac
GLC7-S1	TGGGGAGAGGAAAGCAGATTACCACAATATACATTCAAATTAAAGAAATGcgtacgctgcaggtcgac
GLC7-S4	ACTTCCAATAATCTATCGATGATATTATCAACGTCAACTGGTTGTGAGTCcatcgatgaattctctgtcg

6.4 Media, Buffer and Reagent Compositions

Table 6.4 **Media, Buffer and Reagent Compositions**

Name	**Description**
SC-X (X= URA or LEU or HIS or TRP or Complete)	3.4 g Bacto yeast nitrogen base without amino acids without ammonium sulfate (Conda pronadisa), 1 g SC-X drop out mix, 10 g D(+)-Glucose (BioFroxx), Adenine (Sigma # A8626), 0.05 g ddH_2O up to 500 ml
YPAD	5g Bacto Yeast Extract (Conda pronadisa), 10 g Bacto Peptone (Merck), 10 g D(+)-Glucose (BioFroxx), 0.05 g Adenine (Sigma), ddH_2O up to 500 ml
YPD+G418	20 g/L Bacto agar, 1.7 g/L Bacto yeast nitrogen base without amino acids without ammonium sulfate (Conda pronadisa), 1 g/L monosodium glutamic acid, 2 g drop-out mix, 20 g D(+)-Glucose (BioFroxx), 200 mg/L G418
YPD+Nat	Nourseothricin (Werner BioAgents, Jena-Cospeda) for 100 mg/l of Nat final concentration, 0.5 ml of 200 mg/ml stock in 1 L of autoclaved YPD-Agar
YPD+hph	Hygromycin B (from Cayla, Toulouse) for 300 mg/l hygromycin B final concentration, 3 ml of 100 mg/ml stock in1 l of autoclaved YPD-Agar.
TY-Medium	5 g NaCl, 5 g Yeast Extract, 10 g Bacto Tryptone, ddH_2O up to 1 L. Ampicillin 100 µg/ml or Kanamycin 50 µg/ml.
5-FOA	6.7 g Bacto yeast nitrogen base without amino acids without ammonium sulfate (Conda pronadisa), 2 g SC-URA drop-out mix, 20 g Glucose, 0.1 g Uracil, 1 g 5-FOA (USbiological), ddH_2O up to 500

	ml were stirred and filter-sterilized, then mixed with autoclaved 20 g Bacto-agar in 500 ml ddH_2O
LiSorb	100 mM Lithium acetate (LiOAc), 10 mM Tris pH 8, 1 mM EDTA pH 8, 1 M Sorbitol (Merck), filter sterilized
LiPEG	100 mM LiOAc, 10 mM Tris pH 8, 1 mM EDTA pH 8, 40% PEG3350; filter sterilized
%6 SET	3ml %20 SDS + 0.2ml 0,5 M EDTA PH 8.0 + 0.3 ml 1M Tris PH 8.0 + 6.5 ml ddH_2O
1X PBS	137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4. pH 7.2-7.4
1X PBS-T	137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4 . pH 7.2-7.4, 0.05% Tween-20 (AppliChem)
P1 buffer	50mM Tris HCl pH:8, 10mM EDTA + 0.1 mg/ml RNAse A
P2 buffer	200mM NaOH, 1%SDS
P3 buffer	3M KAc pH: 5.5
2 mM dNTPs mix	20 µl dATP (100 mM), 20 µl dCTP (100 mM), 20 µl dTTP (100 mM) and 20 µl dGTP (100 mM) + 920 µl ddH_2O
10X Buffer 1	500 mM Tris-HCl (pH 9.2), 160 mM $(NH_4)_2SO_4$, 17.5 mM $MgCl_2$
HU-DTT	8 M urea, 5% SDS, 200 mM Tris-Cl pH 6.8, 0.1 mM EDTA, bromophenol blue, 100 mM 15 mg/ml DTT
1X Laemmli buffer	25 mM Tris base, 192 mM Glycine, 0.1% SDS, pH 8.3
Ponceau	0,2 % (w/w) Ponceau S (Sigma #P3504) 3% (w/w) TCA in ddH_2O
1X Blotting buffer	25 mM Tris-base, 190 mM glycine, 0.025% SDS, 20% methanol
Home-made ECL	Solution 1: 20 ml 1 M Tris pH 8.5, 2 ml 250 mM luminol (in DMSO, Sigma # A-8511), 889 µl 90 mM p-coumaric acid (in DMSO, Sigma # C-9008), 178 ml H_2O Solution 2: 20 ml 1 M Tris, pH 8.5, 180 ml H_2O, 123 µl 30% H_2O_2 (Sigma # H-1009)
1M Potassium Phosphate Buffer pH 7.5	83.4 ml 1M K_2HPO_4, 16.6 ml 1M KH_2PO_4