PAS components are required for timely mitotic exit

by

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The Best Is Yet To Come

ABSTRACT

PAS Components Are Required For Timely Mitotic Exit

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Master of Science in Molecular Biology and Genetics

Phosphoinositides are evolutionary conserved phospholipid derivatives found in cellular membranes. Each phosphoinositide interacts with specific proteins to perform cellular functions such as membrane trafficking, signal transduction and cytoskeletal organization. Phosphotidylinositol (3,5) Biphosphate (PtdIns(3,5)P_2) is the least abundant phosphoinositide derivative in the cell. PtdIns(3,5)P_2 deficiency is associated with neurological diseases such as ALS and Charcot-Marie-Tooth Disease. Although PtdIns(3,5)P_2 plays roles in membrane trafficking, stress response, vacuole/endolysosome structure/function and transcriptional regulation, no mitotic function has been attributed to PtdIns(3,5)P_2. In this thesis we asked whether PtdIns(3,5)P_2 has a function in exit from mitosis. We first applied genetic approaches to analyze contribution of PtdIns(3,5)P_2 to mitotic exit. We further analysed anaphase duration and actomyosin ring contraction in PtdIns(3,5)P_2 deficient cells. We also investigated the localization of Mitotic Exit Network (MEN) components and its regulators in cells lacking PtdIns(3,5)P_2. Our genetic analysis demonstrated that PtdIns(3,5)P_2 synthesis becomes essential in cells with impaired MEN activity. Consistent with this result, anaphase and actomyosin ring contraction lasted longer in PtdIns(3,5)P_2 deficient cells than in wild type cells. Release of the conserved phosphatase Cdc14 (the most downstream component of the MEN) was also delayed in the absence of PtdIns(3,5)P_2. Taken together, our results suggest that PtdIns(3,5)P_2 synthesis contributes to mitotic exit in budding yeast through yet an unknown mechanism.

Key words: *PtdIns(3,5)P_2, PAS component, Mitotic Exit Pathway*

ÖZETÇE

PAS Bileşenleri Zamanında Mitozdan Çıkış İçin Gereklidir

Barış Bekdaş

Moleküler Biyoloji ve Genetik Yüksek Lisans

Fosfoinositidler, hücre zarında bulunan ve evrimsel olarak korunmuş fosfolipid türevleridir. Her bir fosfoinositid zar trafiği, sinyal iletimi ve hücre iskeleti organizasyonu gibi fonksiyonları yerine getirebilmek için çeşitli proteinlerle etkileşim haline girerler. Fosfotidilinositol (3,5) bifosfat hücre içinde en az bulunan fosfoinositid türevidir. PtdIns(3,5)P_2 eksikliği, ALS ve Charcot-Marie Tooth hastalığı gibi nörolojik rahatsızlıklar ile ilişkilendirilir. PtdIns(3,5)P_2 hücrede zar trafiği, strese karşı yanıt, vakul/endolizozom yapısı ve fonksiyonu ve bunun yanı sıra transkripsiyonel düzenlemede görev alsa da mitotik bir fonksiyonunu olup olmadığına dair bir atıf yapılmamıştır. Bu tezde, PtdIns(3,5)P_2'nin mitozdan çıkışta görev alma ihtimali üzerine durduk. PtdIns(3,5)P_2'ın mitozdan çıkışa katkısını analiz edebilmek adına genetik yaklaşımlar uyguladık. PtdIns(3,5)P_2 eksikliği olan hücrelerde anafaz süresi ve aktomiyozin halkası kasılmasını analiz ettik. Aynı zamanda, bu hücrelerde Mitozdan Çıkış Ağı (MEN) elemanlarının ve düzenleyicilerinin lokalizasyonunu inceledik. Genetik analizler, MEN aktivitesi baltalanmış hücre tiplerinde PtdIns(3,5)P_2 sentezinin hayati önemi olduğunu gösterdi. Bu sonuçlara uyumlu olarak anafaz süresi ve aktomiyozin halka kasılmasının mutant suşlarda yabanıl suşlara göre daha uzun sürdüğü görüldü. Cdc14 fosfataz salınımının PtdIns(3,5)P_2 üretemeyen hücrelerde geciktiği tespit edildi. Toplarsak, sonuçlarımız PtdIns(3,5)P_2'nin mitozdan çıkış aktivitesini bilinmeyen bir mekanizma aracılığı ile tetiklediğini gösteriyor.

Anahtar Kelimeler: *PtdIns(3,5)P_2, PAS komponent, Mitozdan Çıkış Ağı*

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This study is funded by The Scientific and Technological Research Council of Turkey (TÜBİTAK grant no: 117Z232). I would like to give my thanks to TÜBİTAK for its support.

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Dear Hüseyin, you always make whole lab smile "es vermeden". You present professional success and full social life. Those who envy you should be condemned to look you from "below".

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To beauty from İzmir, İdil. You are always calm amongst crazy people of our lab. It gives us chill and provides balance the chaos where we have worked. I inherited you my bench. Please don't let my bench turn into İzmir traffic.

The individual whom I like clothing style and the queen of IP, Cansu. I sincerely gratitude you whenever we need someone to wait and incubate strains of interest. You have better understanding and capable of doing troubleshooting. "Es vermeden" is yours Thank you guys, I love you all…

ABBREVIATIONS

Cdk: Cyclin Dependent Kinase

APC/C: Anaphase-Promoting Complex/Cyclosome

SAC: Spindle Assembly Complex

SPOC: Spindle Position Orientation Checkpoint

FEAR: Fourteen Early Anaphase Release

MEN: Mitotic Exit Network

MTs: Microtubules

SGD: Saccharomyces Genome Database

SPB: Spindle Pole Body

PP1: Protein Phosphatase 1

APS: Ammonium Persulfate

DMSO: Dimethyl Sulfoxide

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Chapter 1. INTRODUCTION

1.1. Budding Yeast

Saccharomyces cerevisiae is a unicellular eukaryotic organism with a round to ovoid shape. Its diameter is 5-10 µm (Feldmann, 2012). Around twelve million base pairs compose the *S. cerevisiae* genome which is organized in 16 chromosomes (Goffeau et al., 1996). *S. cerevisiae* is also known as the budding yeast (Feldmann, 2012). Reproduction of budding yeast occurs by budding which is a type of mitotic division. Budding yeast has two mating types which are a and α (alpha), and each mating type is haploid (Zörgö et al., 2013). "a" type and "α" type cells secrete a-factor and α-factor respectively. These are mating pheromones which initiate a signaling pathway to promote fusion of the haploid yeast cells that are of opposing mating type (Haber, 2012). Mating pheromone response starts with growing a projection known as the shmoo. The act of mating consumes more resource and energy; however, mating increases fitness (Lang et al, 2009). Both haploid and diploid yeast proliferate by budding. Diploid yeast sporulates when facing harsh conditions such as lack of nitrogen (Brauer et al., 2008; Broach, 2012) or lack of glucose (Granot & Snyder, 1991) to produce haploid offspring (Herskowitz, 1988; Mitchell, 1994; Neiman, 2011; Warringer et al., 2011). Ability to exist in the haploid form and high homologous recombination rates results in relatively easy gene editing in budding yeast, which makes budding yeast a famous model system for genetic studies. Owing to the conserved nature of cell biology and all the aforementioned advantages, budding yeast has been widely used to understand fundamental cellular processes such as aging, cell cycle, DNA repair mechanism and autophagy (He, Zhou, & Kennedy, 2018).

1.2. Cell Cycle

Cell cycle is the process by which eukaryotic cells divide to make new cells. Ordered series of phases named G_1, S, G_2, M and cytokinesis constitute the cell cycle. Cells increase their mass and volume in G_1 and under appropriate conditions they commit for entry into the cell cycle by progressing into the S phase where DNA replication takes place (Hartwell, 1974). G_2 is the phase in which cells check whether any DNA damage occurred during the replication process (Weinert, Kiser, Hartwell, 1994). In M phase, chromatin condenses to chromosomes and all genetic material is distributed equally between daughter cells. Later during cytokinesis, daughter cells physically separate (Jansen et al., 1996).

Ordering of cell cycle events is maintained by central regulatory systems in which each event is initiated at predetermined schedules. Once a problem occurs in a way to prevent accurate completion of an event, the following step in the cell cycle can be postponed via many checkpoint mechanisms that monitor the accuracy of specific cell cycle events and delay the cell cycle progression in response. Cyclin dependent kinases (Cdks) are the main components of central regulatory systems that coordinate and order the events of eukaryotic cell division cycle (Morgan, 2007). Cdks are activated by cyclins (**Figure 1.1**). Cyclin levels oscillate during the cell cycle through changes in their expression and their ubiquitin mediated degradation at the proteosome (Morgan, 2007). In response to changes in cyclin association with Cdks, activities of Cdks oscillate during cell cycle. The association of distinct cyclin-Cdk structures with distinct substrates drives particular events of the cell cycle at specific phases such as DNA replication in S phase and chromosome condensation, nuclear envelope breakdown, or mitotic spindle assembly in M phase (Sullivan & Morgan, 2007).

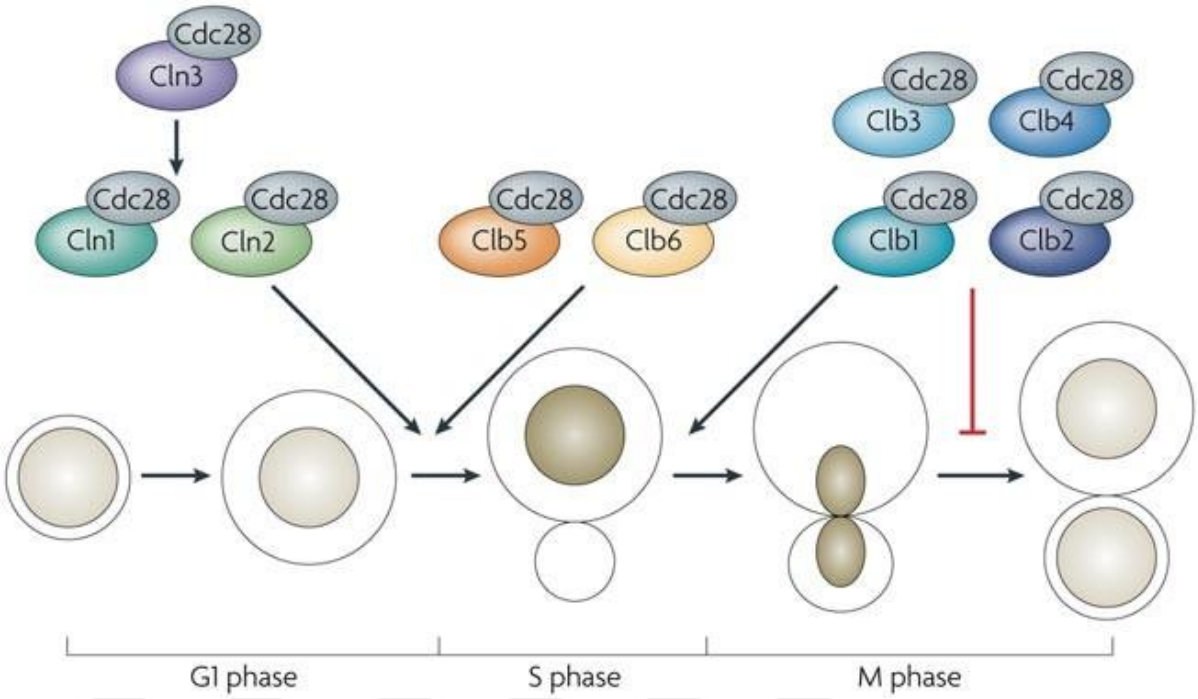

Figure 1.1 **Cyclins and Cdk in a budding yeast** Several cyclin types are present in a budding yeast, which are classified as G_1 , S and M cyclins. Cdc28 is the only cyclin dependent kinase in a budding yeast. Association between cyclin and Cdc28 maintains cell cycle progress. Figure is taken from Cross et al. 2007 (Cross, Frederick R. Bloom, Joanna. 2007).

1.2.1. Cell cycle in budding yeast

When a budding yeast cell reaches an adequate size in G1, it becomes committed for entry into a new cell cycle by committing entry into S phase (Hartwell et al., 1974). This point of decision at G1/S transition point is called as the START. Once the cell passes the START, a daughter cell (bud) starts emerging on the mother cell (**Figure 1.2**) (Charvin, Oikonomou, Siggia, & Cross, 2010). This is immediately followed by DNA replication and spindle pole body (SPB) duplication which occurs in S phase (Hereford & Hartwell, 1974). SPBs are equivalent of centrosomes in budding yeast (See 1.2.2). Microtubules originate from duplicated SPBs to form the mitotic spindle that is essential for segregation of sister chromatids to the mother and daughter cells during M phase (Seybold & Schiebel, 2013). *S. cerevisiae* undergoes closed mitosis. In contrast to open mitosis where nuclear envelope breaks down early in M-phase, nuclear envelope remains intact during closed mitosis (De Souza & Osmani, 2007). Mother and daughter cells stay connected until cytokinesis (**Figure 1.2**). Only after completion of M-phase, cytokinesis takes place to physically separate the mother and the daughter cell (**Figure 1.2**). The daughter cell

and mother cell differ in terms of size, age, and protein content (Yeong, 2005). Thus, budding yeast's mitotic cell division is an asymmetric cell division.

Various specific cyclins drive mitosis in budding yeast, while functions of the different cyclins in budding yeast remarkably overlap during mitosis. However, a single Cdk, Cdc28, plays role in controlling all major events of mitosis in *S. cerevisiae* (Lörincz & Reed, 1984). Various events mediated by Cdc28 occur at different times which means, Cdc28 recognizes different substrates at different times (**Figure 1.3**). This specificity is mediated, in part, by different cyclin partners places (D. O. Morgan, 1995). Cdc28 forms complexes with nine different cyclins that are classified into three groups. Each class of cyclin are named after the phase of mitosis their levels peak. G_1 cyclins are Cln1, Cln2 and Cln3 and S-phase cyclins includes Clb5 and Clb6 whilst Clb1, Clb2, Clb3, Clb4 are members of G_2 cyclins (Futcher, 1996). All phase specific cyclins are expressed at different times by a combination of transcriptional control and protein instability in a budding yeast, which ensures that the different cyclins are available at different times and moreover, interact with Cdc28 at different stages (Mendenhall, Jones, & Reed, 1987; Wittenberg & Reed, 1988).

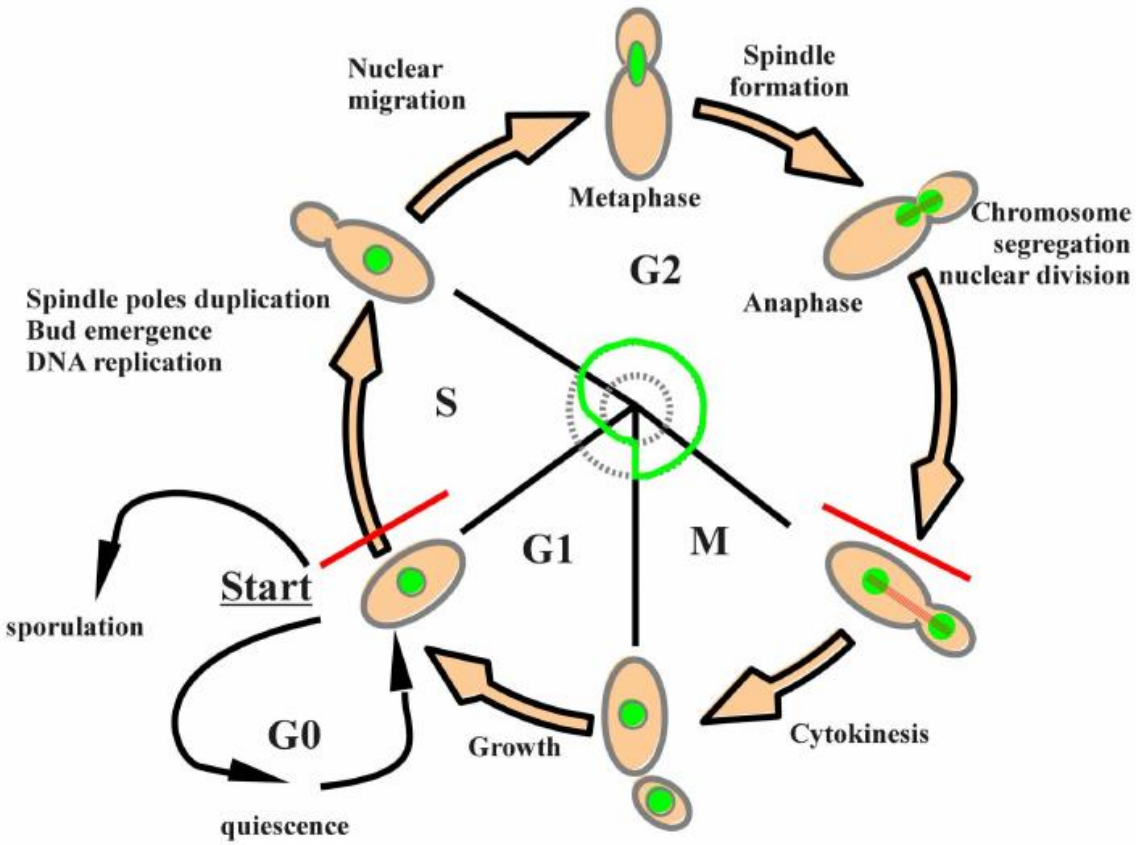

Figure 1.2 **Cell Cycle in Budding Yeast.** After START the bud emerges and DNA replication starts. Spindle pole bodies are equivalent to centrosome in budding yeast. One major difference between mammalian and yeast cell is that nuclear envelope does not disintegrate in *S. cerevisiae* (Tesniere, Delobel, 2014)

To commit a new round of cell cycle, Cln3 associates with Cdc28 and triggers START by activating the SBF and MBF transcription factors (Dirick, Böhm, & Nasmyth, 1995; Dirick, Moll, Auer, & Nasmyth, 1992). START checkpoint constitutes the decision point for cell cycle entry. START monitors cell size, nutrient presence and gene transcription involved in cell cycle progress. Interestingly, budding yeast nearly constitutively expresses Cln3 throughout cell cycle. Cln3 has higher turnover rate and therefore, it is unstable unlike other two G_1 cyclins. Phosphorylation by Cdc28 at C-terminus stabilizes Cln3 (Tyers, 1992). Cln3-Cdc28 phosphorylates Whi5 bound to SBF in nucleus (Nash, Tokiwa, Anand, Erickson, & Futcher, 1988). Whi5 is a transcriptional regulator in a budding yeast and inhibits SBF and MBF which are gene regulator proteins (de Bruin, McDonald, Kalashnikova, Yates, & Wittenberg, 2004; David Owen Morgan, 2007). Phosphorylation of Whi5 by Cln3-Cdc28 causes removal of Whi5 from SBF. Released SBF and MBF induces transcription of the genes that drive DNA replication, SPB

duplication and cyclin production by locating promoter regions of them. Cln1 and Cln2 are products of SBF and MBF activity. Cln1 and Cln2 redundantly induce budding, polarized growth and SPB duplication (Haase, Winey, & Reed, 2001; Lew & Reed, 1993). They also function in repression of the anaphase-promoting complex (APC) till mitotic phase entry and phosphorylation of Cdc28 inhibitors Far1 and Sic1 to promote their destruction (Cross, 1995; Henchoz et al., 1997; Schneider, Yang, & Futcher, 1996; Tyers et al., 1993).

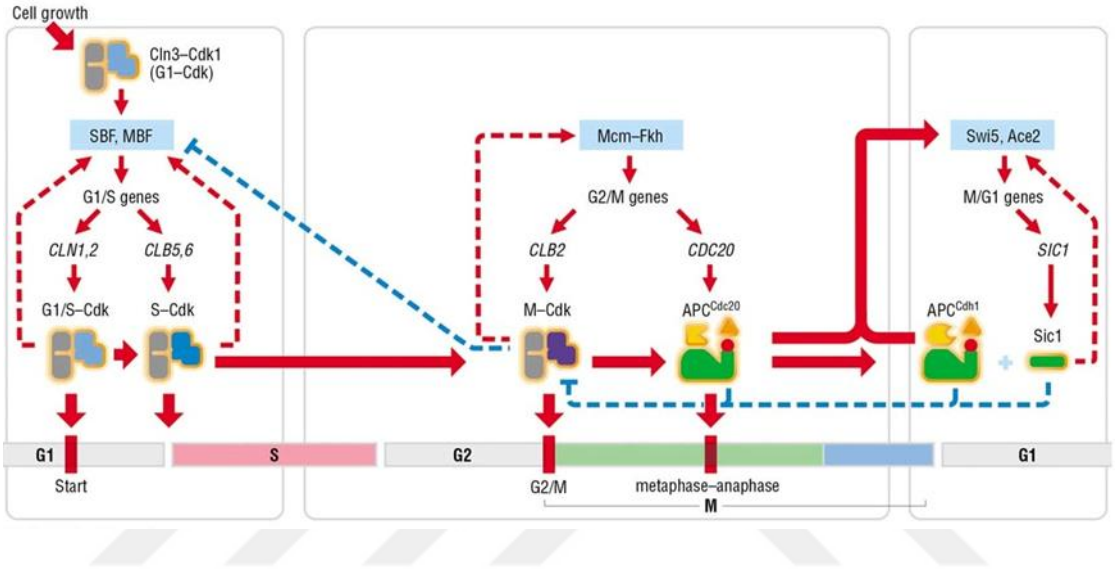

Figure 1.3 **Cdk1 drives cell cycle in budding yeast**: Cdk1 (Cdc28 in *S. cerevisiae*) regulates mitotic events by associating several cyclins. Cdk1 concentration does not change but cyclin level oscillates during mitosis. Discontinuous red arrows demonstrate positive feedback whereas blue ones exhibit negative feedback. Figure is taken from Morgan (Morgan, David O. The Cell Cycle: Principles of Control).

S-phase cyclins are Clb5 and Clb6. They stimulate DNA replication by interacting with Cdc28 and furthermore, induce formation of mitotic spindle along with G_2 cyclin Clb3 and Clb4 (Mendenhall & Hodge, 1998; Schwob & Nasmyth, 1993). The level of Clb5 and Clb6 sharply decreases in mitosis, and they are degraded right before anaphase (Gong et al., 2007; Spellman et al., 1998). All G_2 cyclins (Clb1-4) promote the transition from G_2 to M phase by associating with Cdc28. They also trigger degradation of G_1 cyclins to allow cell cycle progression in budding yeast (Amon, Irniger, & Nasmyth, 1994; Blondel & Mann, 1996). Clb1 and Clb2 accumulates during G_2 to M phase after accumulation of Clb3 and Clb4 between S and G_2 (Ghiara et al., 1991; Richardson, Lew, Henze, Sugimoto, & Reed, 1992). All of the four cyclins take part in bipolar spindle formation and spindle elongation in yeast and induce expression of the genes required mitotic events such as

Cdc20 synthesis (Kuczera, Bayram, Sari, Braus, & Irniger, 2010). Among them, the major mitotic cyclin is Clb2. Mitotic cyclins in association with Cdc28 allow indirectly APC (anaphase-promoting complex) interaction with its regulatory subunit Cdc20. APC in association with Cdc20 ubiquitylates Securin - Pds1 in budding yeast (Visintin, Prinz, & Amon, 1997). Separase - Esp1 in budding yeast - becomes active after degradation of the Securin (Zachariae & Nasmyth, 1999). Active Separase cleaves cohesin which holds sister chromatids each other (Ciosk et al., 1998). Therefore, metaphase-anaphase transition is completed, and the cell enters anaphase. In addition to promoting anaphase entry, APC-Cdc20 partially promotes proteolysis of Clb2 by ubiquitin dependent proteolysis (Queralt & Uhlmann, 2008a). However, complete degradation of Clb2 in budding yeast requires APC association with another specificity factor named Cdh1 (Shirayama, Tóth, Gálová, & Nasmyth, 1999; Wäsch & Cross, 2002). APC-dependent proteolysis of Clb2 is necessary for exit from mitosis. Mitotic Cdk inactivation via Clb2 degradation is not enough for faithful and timely mitotic progression. Phosphatases that counteract Cdk phosphorylation of specific substrates are also important (Barr, Silljé, & Nigg, 2004; Heinrich, Neel, & Rapoport, 2002) (see section 1.2.1).

1.2.2. Spindle Pole Body

Spindle pole body (SPB) is an organelle which is cylindrical shaped and multilayered. It acts as a microtubule organizing center, which is equivalent to centrosomes (Castillo, Meehl, Morgan, Schutz-Geschwender, & Winey, 2002). SPB consists of inner and outer plaque, central plaque and two intermediate layers between outer plaque and central plaque (Bullitt, Rout, Kilmartin, & Akey, 1997; O'Toole, Winey, & McIntosh, 1999) (**Figure 1.4**). SPBs link to cytoplasmic microtubules with its outer plaque (OP) while nuclear envelope surrounds central plaque (CP) of SPB and CP is connected to nuclear envelope. An inner plaque (IP) is a site where nucleation of nuclear microtubules (nMT) starts (Kilmartin, Dyos, Kershaw, & Finch, 1993). Compared to other layers, highly ordered layers are present into CP and IL2. (Adams & Kilmartin, 1999). Budding yeast maintains location of the inner and outer plaques in relation to the nuclear membrane during the entire cell cycle. *MluI* cell cycle boxes whose function is to promote G1 specific gene transcription are found in promoter elements of SPB genes. (Harrington & Andrews, 1996). Each SPB protein can be classified into three essential groups which are

core components, half-bridge components and components required for connection with NE (Jaspersen & Winey, 2004). Nevertheless, no well-known motif or structure building a protein belong to SPB is present, studies demonstrate that known SPB proteins and their genes shares some common features. Proteins with coiled-coil motifs are involved in core of SPB and coiled-coil motifs present opportunities to maintain regular structures by forming dimers either with themselves or with other proteins (Zizlsperger & Keating, 2010). Coiled-coil protein Spc42p is not only the main central plaque component of SPB but also forms a core crystal of SPB (Donaldson & Kilmartin, 1996). Initiation of SPB assembly and its duplication do not occur without Spc42p presence. Two other essential coiled-coil proteins called Spc110p and Spc29p associate with Spc42p (Elliott, Knop, Schlenstedt, & Schiebel, 1999; Knop & Schiebel, 1997). This association is responsible for constructing the nuclear face of the SPB. Spc110 localizes to the central plaque and furthermore, links to Spc29p and calmodulin (Cmd1p) to form inner plaque.

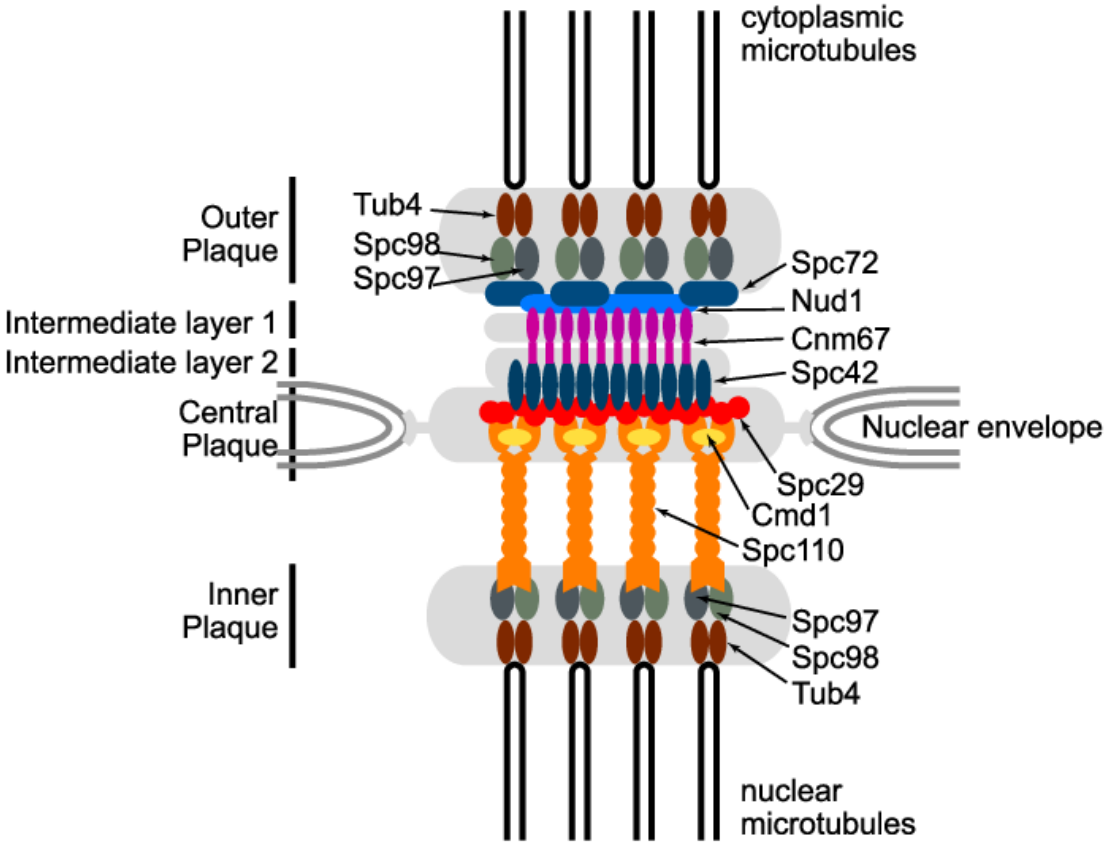

Figure 1.4 **Pole Body Structure:** Spindle Pole Body is a microtubule organizing center and equivalent to centrosome in a budding yeast. It is composed of several layers and acts in mitotic spindle formation and chromosome segregation (Klenchin, Frye, Jones, Winey, & Rayment, 2011).

Spc110p acts as a spacer molecule between the central and inner plaque as well as γ-tubulin complex binding protein while calmodulin plays role in SPB to regulate binding of Spc110p to Spc29p (Geiser, Sundberg, Chang, Muller, & Davis, 1993; Lyon et al., 2016). Spc29 is found in central plaque of SPB with repeating structure and it interacts with Spc42p (Elliott et al., 1999; Wigge et al., 1998). On the other hand, Spc98p and Spc97p found at both outer and inner plaque are two similar yeast γ -tubulin (Tub4p) binding proteins required for microtubule nucleation (Geissler et al., 1996). They are involved in microtubule organization with Tub4p. Spc42 faces the cytoplasm and another interaction of it is binding to coiled coil Cnm67p (chaotic nuclear migration). Cnm67 forms dimers and functions as a spacer between IL2 and IL1. Cnm67 associates with Nud1 which is a part of the outer plaque (Brachat, Kilmartin, Wach, & Philippsen, 1998; Schaerer, Morgan, Winey, & Philippsen, 2001). Nud1 is required for exit from mitosis by acting as a scaffold for Mitotic Exit Network (MEN) proteins (Adams & Kilmartin, 1999; Hotz et al., 2012). Another coiled-coil protein, Spc72p, is also found in the outer plaque. Spc72p associates with Nud1p and to components of the γ-tubulin complex. (Souès & Adams, 1998), For formation of a bipolar mitotic spindle and accurate chromosome segregation, SPB duplication occurs once and only once in each cell cycle. SPB duplication in budding yeast is performed by several steps.

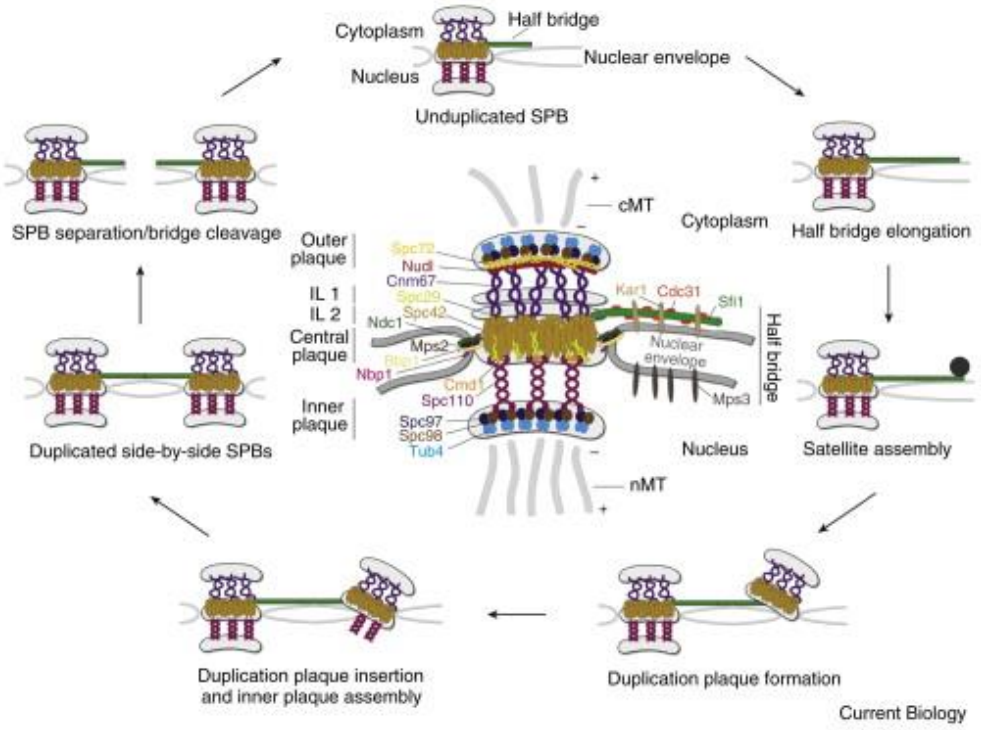

Figure 1.5 **SPB Duplication** SPB duplication starts with half-bridge elongation. It continues inner and outer plaque formation. Each duplicated part is linked by half bridge. Final step is to separation of duplicated complex. See the section 1.2.2 for details. Figure is taken from (Schiebel, Elmar. Seybold, Christian. 2013. Current Biology).

The first step occurs in early G1 when cytoplasmic tip of half-bridge is established by satellite components. Half-bridge of SPB elongates and completes its nuclear and cytoplasmic faces fusion in the second step. In addition to that, a layered structure which is similar to the cytoplasmic half of a mature SPB is formed by satellites to constitute duplication plaque. Insertion of the duplication plaque into the nuclear envelope and assembly of nuclear SPB components is the last step of SPB duplication. *S. cerevisiae* contains two duplicated side-by-side SPBs whose connection is regulated by a complete bridge. Next, SPB nucleates bipolar spindle after bridge separates at the end of G_1 (**Figure 1.5**). SPB continues to grow at the point when mitosis starts, so protein components are able to incorporate into both SPBs throughout the cell cycle.

1.2.3. Exit From Mitosis

Each cell requires complete degradation of the mitotic cyclin Clb2 to achieve mitotic Cdk inactivation. For a successful mitosis, degradation of cyclin is not enough but reversal of mitotic Cdk dependent phosphorylation is also necessary to set the reverse the mitotic events. Cdc14 is the only phosphatase that not only dephosphorylates Cdk substrates but also inhibits mitotic Cdks in budding yeast (Visintin et al., 1998; Wan, Xu, & Grunstein, 1992). Cdc14 is found in nucleolus (D'Amours & Amon, 2004). Its association with Net1/Cfi1 inhibits Cdc14 release from nucleolus till anaphase onset (D'Amours & Amon, 2004; Stegmeier & Amon, 2004). Association with Net1/Cfi1 keeps Cdc14 inactive status (Shou et al., 2001). Dissociation from Net1 releases Cdc14 from nucleolus to nucleus and cytoplasm, where Cdc14 is active (Shou et al., 1999). The Cdc14 activation triggers various processes such as segregation of rDNA, positioning of anaphase nucleus and stabilization of anaphase spindle and mitotic exit (D'Amours, Stegmeier, & Amon, 2004). Activation and release of Cdc14 from nucleolus are based on two regulating pathways: Cdc14 Early Anaphase Release (FEAR) and Mitotic Exit Network (MEN) (Queralt, Lehane, Novak, & Uhlmann, 2006). FEAR and MEN pathways act in sequence to induce the dephosphorylation of distinct Cdk substrate populations and distinct mitotic events.

The FEAR pathway is composed of Separase, its binding protein Slk19, polo-like kinase Cdc5, Spo12, the replication fork block protein Fob1, PP2A phosphatase associated with its regulatory subunit Cdc55, Zds1 and Zds2 - whose function is to perform histone modifications such as H2B ubiquitylation and H3 methylation, and mitotic cyclins Clb1 and Clb2 (Jeremy M. Rock & Angelika Amon, 2009). The role of Fob1 is to stabilize Cdc14-Cfi1/Net1 complex (Stegmeier et al, 2004). APC activation causes degradation of Securin - the Separase inhibitor - at anaphase onset, which triggers activation of the Separase-Slk19 complex ((D'Amours & Amon, 2004; Queralt & Uhlmann, 2008b). This complex along with Zds1 and Zds2 allows Clb1-Cdk and Clb2-Cdk to phosphorylate Cfi1/Net1 to release Cdc14 by counteracting PP2ACdc55 phosphatase activity as shown in **Figure 1.6** (D'Amours & Amon, 2004; Queralt & Uhlmann, 2008b). In addition, Clb1-Cdk and Clb2-Cdk play role in phosphorylation of Spo12 (Tomson et al., 2009). Spo12 acts in regulating Cdc14 release from nucleolus in early anaphase and besides, Spo12 is forced in dephosphorylated state before anaphase by Cdc14 (Shah R, et al. 2001; Tomson et al, 2009).

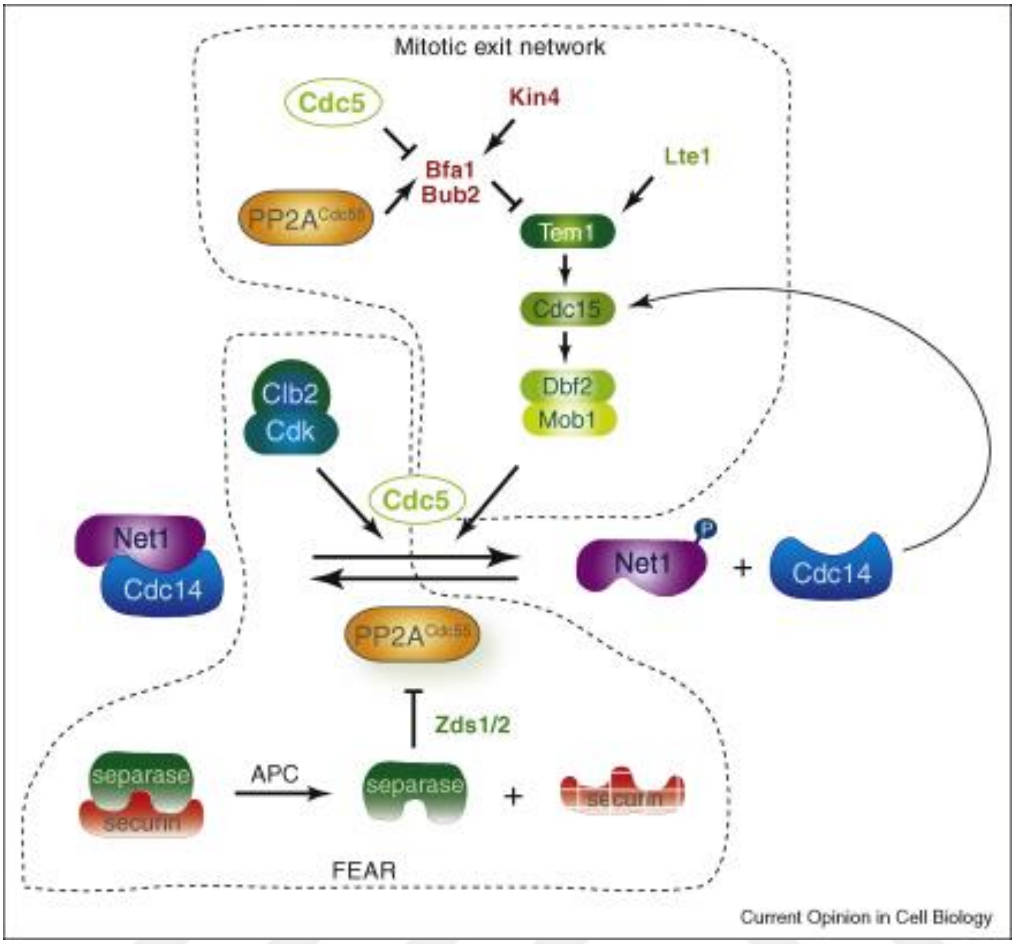

Figure 1.6 **Cdc14 Release:** Exit from mitosis starts with Cdc14 release from nucleolus. Its release is regulated by two pathways, which are FEAR in early anaphase and MEN in late anaphase (Queralt, Uhlmann, 2008). Figure is taken (Queralt, Uhlmann, 2008). . Straight arrow shows activation while vertical line ended arrow means inactivation. Discontinuous line involves indirect effects.

FEAR pathway is activated at the metaphase-anaphase transition concomitant with separase activation (Stegmeier & Amon, 2004). FEAR results in a partial and transient release of Cdc14. This transient release is sufficient to induce anaphase specific events such as spindle stabilization, nuclear positioning and MEN activation (J. M. Rock & A. Amon, 2009). The transiently released Cdc14 also provides MEN pathway a ready-to-start point in late anaphase (Caydasi et al., 2017). However, it is not enough for mitotic exit, mitotic exit requires the fully release of Cdc14 which is triggered by MEN (Caydasi et al., 2017; König, Maekawa, & Schiebel, 2010).

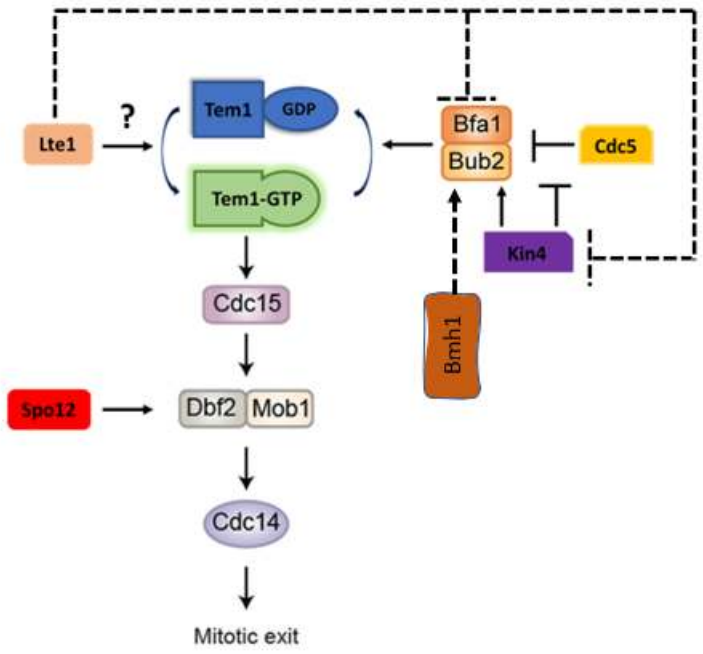

Figure 1.7 **MEN Pathway:** MEN pathway is responsible for exit from mitosis in budding yeast. Cdc15 and Dbf2 are protein kinases whereas Cdc14 is only phosphate to perform dephosphorylating Cdk substrates in yeast. Mob1 is an association factor of Dbf2 while Tem1 is GTPase. When mitotic spindle misalignment occurs, Kin4 inhibits MEN activity by activating Bfa1. Straight arrow shows activation by phosphorylation while vertical blunt-ended arrow means inactivation by phosphorylation. Question mark refers that information given is an estimate not confirmed. Lte1 has a putative GEF domain but not observed related function. Dash lines show that related molecule does not change its catalytically activation but alter localization directly or indirectly.

The MEN is essentially a GTPase triggered signalling cascade. MEN pathway is composed of a Ras like small GTPase named Tem1 and downstream kinases Cdc15 and Dbf2-Mob1 complex that act in a sequence (Queralt & Uhlmann, 2008a) (**Figure 1.7**). This signalling cascade initiates with GTP bound Tem1 which activates Cdc15 kinase (Jeremy M. Rock & Angelika Amon, 2011). It continues with the Cdc15 kinase phosphorylating the protein kinase Dbf2 associated with Mob1 at the SPBs as shown in **Figure 1.7** (Mah, Jang, & Deshaies, 2001). This signalling cascade occurs in the presence of the scaffold protein Nud1 at the SPBs, which provides a template where MEN components interact with each other (Adams & Kilmartin, 1999; Gruneberg, Campbell, Simpson, Grindlay, & Schiebel, 2000). The activation of MEN induces sustained release of Cdc14 into the cytoplasm, disassociating Cfi/Net1-Cdc14 interaction (Manzoni et al., 2010; Tóth, Queralt, Uhlmann, & Novák, 2007). Therein, Cdc14 dephosphorylates

cytoplasmic substrates of mitotic Cdks and furthermore inhibits Cdks. Inhibition of Cdks occurs with Sic1 and Cdh1 dephosphorylation by Cdc14 (Amon, 2008; Visintin et al., 1998). Dephosphorylation of Sic1 -a Cdk inhibitor- by Cdc14 triggers stabilization of Sic1 and furthermore, Swi5 - transcription factor Sic1- dephosphorylation by Cdc14 upregulates Sic1 expression (Visintin et al., 1998; Wäsch & Cross, 2002). In addition to Sic1 and Swi5 activation, Cdc14 dephosphorylation makes Cdh1 active, which allows formation of APC-Cdh1. This complex ubiquitinylates the mitotic cyclin Clb2 (Sullivan & Morgan, 2007; Uhlmann, Lottspeich, & Nasmyth, 1999). Thus, Cdc14 promotes exit from mitosis.

MEN progress is blocked by Bfa1-Bub2 complex in response to microtubule defects, DNA damage and mispositioning of the spindle (Bardin, Visintin, & Amon, 2000; Molk et al., 2004; Pereira, Tanaka, Nasmyth, & Schiebel, 2001). Tem1 inactivation happens since GTP bound to Tem1 is hydrolysed by Bfa1-Bub2 complex in response to microtubule defects, DNA damage or mispositioning of the spindle (Geymonat et al., 2002). On contrary to Bfa1-Bub2 complex, Lte1 acts as a positive regulator of MEN and moreover, it contains a guanosine nucleotide exchange factor (GEF) (Geymonat et al., 2002). Therefore, it has been thought that Lte1 promotes Tem1 activity by exchanging GDP with GTP (Keng et al., 1994). However, studies performed by Geymonat et al in 2009 demonstrated that Lte1 has no GEF activity towards Tem1 and relies on interaction with Ras for localization at the bud cortex rather than providing nucleotide exchange (Geymonat et al., 2009). It has been shown that Lte1 regulates the subcellular localization of Bfa1 and Kin4 by promoting the loss of Bfa1 from the maternal SPB and loss of Kin4 localization from daughter SPB (Bertazzi, Kurtulmus, & Pereira, 2011; M. Geymonat, A. Spanos, G. de Bettignies, & S. G. Sedgwick, 2009).

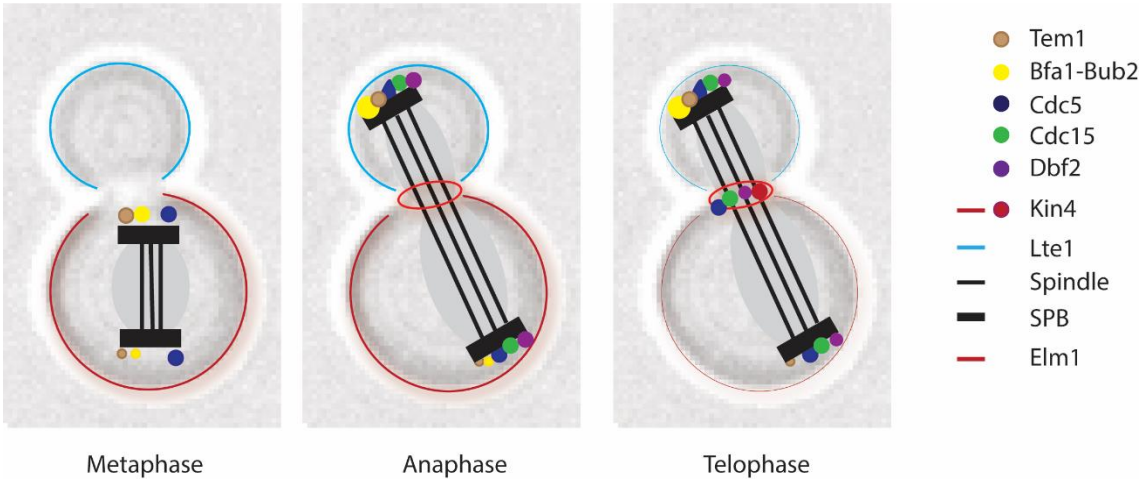

Figure 1.8 **Localization of MEN components at different mitotic stages:** Different proteins and structures are shown with different colors as shown in the figure. In budding yeast, MEN components and its regulators localize different places during cell cycle. Their concentrations at given locations may alter. Most of them localizes at SPB.

All the components of MEN except Lte1 **(Figure 1.8)**, which localizes to the bud cortex, are localized to cytoplasmic face of SPB in a cell cycle dependent manner (Caydasi, Ibrahim, & Pereira, 2010; Molk et al., 2004; Seybold & Schiebel, 2013). Cdc15 and Dbf2 starts to localize both SPBs as cell cycle proceeds. However, localization of Tem1 and Bfa1-Bub2 is mainly observed at daughter SPB (the SPB that migrates to the daughter cell compartment) during M-phase (Pereira, Höfken, Grindlay, Manson, & Schiebel, 2000; Visintin & Amon, 2001; Yoshida & Toh-e, 2001). Interestingly, SPB localized concentration of these MEN components change during mitosis and in response to spindle position and integrity. In addition, the MEN kinases Cdc5 and Dbf2-Mob1 are translocated to the bud neck in telophase (Liang, Jin, Liu, & Wang, 2009; Luca et al., 2001; Song, Grenfell, Garfield, Erikson, & Lee, 2000; Yoshida & Toh-e, 2001). This localization of MEN components is important for cytokinesis (Caydasi et al., 2010).

1.2.4. Spindle Position Checkpoint

Chromosome orientation and segregation are mediated by the mitotic spindle which formed by microtubules and associated proteins. In budding yeast, mitotic spindle must elongate in the mother to daughter cell direction. Mispositioning of mitotic spindle in

budding yeast triggers a surveillance mechanism known as the spindle position checkpoint (SPOC) (Scarfone et al., 2015). In response to spindle orientation defects, SPOC delays mitotic exit by inhibiting the MEN (Caydasi et al., 2010; Caydasi & Pereira, 2012). This way, cells gain time to repair microtubule defects and re-align the position of mitotic spindle. Without SPOC function, misalignment of mitotic spindle causes aneuploidy - defined as a case of abnormal number of chromosomes in a cell (Caydasi et al., 2010; Santaguida & Amon, 2015; Yeh, Skibbens, Cheng, Salmon, & Bloom, 1995).

The key components of the SPOC are Kin4 and Bfa1-Bub2 (Wang, Hu, & Elledge, 2000; Weiss, 2012). Bfa1-Bub2 is a GAP (GTPase activating protein) complex, and the function of this complex is to inactivate Tem1 by hydrolysing GTP (Caydasi et al., 2017). Phosphorylation status of Bfa1 determines arrest or exit from mitosis (J. Kim, Jang, & Song, 2008; Wang et al., 2000). In a healthy condition where spindle is correctly positioned, the polo-like kinase Cdc5 hyperphosphorylates Bfa1 to prevent the GAP complex activation, hence Tem1 is active to trigger MEN (Geymonat, Spanos, Walker, Johnston, & Sedgwick, 2003; Hu et al., 2001; J. M. Rock & A. Amon, 2011). If the spindle is mispositioned, Kin4 kinase phosphorylates Bfa1 to activate GAP complex (Bertazzi, Kurtulmus, & Pereira, 2011; Pereira & Schiebel, 2005). The phosphorylation of Bfa1 by Kin4 prevents the phosphorylation of Bfa1 by Cdc5 (Pereira & Schiebel, 2005). Phosphorylation of Bfa1 by Kin4 triggers to break Bfa1 asymmetry on SPBs yet it is not enough to perform Bfa1 localization change. Kin4 phosphorylation on Bfa1 provides a docking site for Bmh1 to decrease association strength between Bfa1 and centrosome (Caydasi et al, 2014). Consequently, MEN is downregulated by SPOC mechanism and the cell pauses exit from mitosis for a while until spindle position is corrected (Caydasi & Pereira, 2012).

Localization and concentration of MEN and SPOC components change during mitosis. Kin4 localizes at the mother cortex until telophase in an unperturbed mitosis Bfa1-Bub2 GAP complex and Tem1 reside at both SPBs and their concentration in mother SPB decreases from metaphase to telophase (Caydasi et al., 2010). If the elongating mitotic spindle and associated SPBs remain in the mother cell instead of passing through bud neck to bud cortex, Kin4 localizes at both SPBs (Caydasi & Pereira, 2009; Pereira & Schiebel, 2005). Kin4 at both SPBs activates Bfa1 by phosphorylation (D'Aquino et al., 2005). Kin4 phosphorylated Bfa1-Bub2 localizes symmetrically at both SPBs. Active Bfa1-Bub2 inhibits the MEN pathway and blocks mitotic exit (Bertazzi et al., 2011). Cdc5

localizes at both SPBs from metaphase to anaphase in symmetrical way. After anaphase, Cdc5 accumulates at bud neck and its concentration at SPBs decreases (Alexandru, Uhlmann, Mechtler, Poupart, & Nasmyth, 2001; Hu & Elledge, 2002). Localization properties of Cdc5 do not change in misoriented budding yeast (Caydasi, Ibrahim, & Pereira, 2010)..When a yeast cell reaches telophase, Kin4 concentrates in bud neck where cytokinesis occurs (Falk, Chan, & Amon, 2011). On the other hand, Lte1 localizes at bud cortex and disappears in telophase in a healthy division (M. Geymonat et al., 2009). MEN kinases also localize to the bud neck at telophase (Caydasi, 2010).

1.2.5. Cytokinesis in budding yeast

Cytokinesis is a part of cell cycle in which daughter cells are physically separated. Most organisms use contractile ring for cytokinesis. The first step in the process of cytokinesis is to determine a location to place the contractile ring. Secondly, it requires transport of all essential components for assembly of the contractile ring at the determined area. Thirdly, a contractile ring must constrict to generate tension for cleavage. The last step is disassembly of the contractile ring to complete cytokinesis.

In budding yeast, cytokinesis is regulated by two main pathways which are actomyosin dependent pathway and non-actomyosin dependent pathway (Meitinger & Palani, 2016). Generating tension within contractile ring depends on actin filament sliding mediated by myosin on most of organisms (Mendes Pinto, Rubinstein, Kucharavy, Unruh, & Li, 2012; Reichl et al., 2008). However, actin depolymerization between parallel or antiparallel filaments is also capable of generating a constriction without myosin in budding yeast. Cofilin aids to drive constriction in non-actomyosin dependent pathway (Mendes Pinto et al., 2012; Zumdieck, Kruse, Bringmann, Hyman, & Jülicher, 2007). In addition, Septin proteins play major role in maintaining cytokinesis. Septins are a group of GTP-binding and filament forming proteins expressed in all eukaryotic cells except plants (Douglas, Alvarez, McCreary, & Konopka, 2005). Several cell cycle division genes *CDC3, CDC10, CDC11,* and *CDC12* and *SHS1* are classified as members of mitotic septin in budding yeast (Mino et al., 1998; Sanders & Field, 1994). Septin localizes at future bud site at late G_1 and forms a single ring structure. However, this ring structure quickly evolves into an hourglass structure when bud begins to grow (Glomb & Gronemeyer, 2016). Localization of septin at bud site is prerequisite for arrival of Myo1 at bud neck. Septin acts as a

scaffold for Myo1, the two formins Bni1 and Bnr1, and Iqg1 to localize at bud neck (McMurray et al., 2011; Mino et al., 1998; Versele et al., 2004).

Cytokinesis in yeast initiates at late anaphase by Mitotic Exit Network (MEN) activation. In late anaphase, anaphase promoting complex or cyclosome (APC/C) associates with its specificity factor Cdh1 and therefore it triggers exit from mitosis (Visintin et al., 1997). Cytokinesis follows exit from mitosis. Actin filaments forms a ring structure at the bud neck where they are nucleated by formins. Capturing of nucleated filaments occurs by Iqg1 (Epp & Chant, 1997; Lippincott & Li, 1998). Rho GTPase localizes to bud neck to form actomyosin ring complex by activating formins and promoting myosin-II contractility after septin splitting into two rings upon anaphase completion (Meitinger et al., 2013).

1.3. Phosphotidylinositol (3,5) Biphosphate and PAS Complex

Phosphoinositides are an evolutionary conserved phospholipid family found in cellular membranes. Phosphoinositides consists of a glycerol backbone- which is esterified to two fatty acid chains-, a phosphate and a polyol myo-inositol, $(CHOH)_6$ polar head group extending into the cytoplasm (Falkenburger, Jensen, Dickson, Suh, & Hille, 2010). Phosphoinositide consists of free hydroxyl groups at positions D2 through D6. Cytoplasmic lipid kinases readily phosphorylate those at positions D3, D4 and D5 (Falkenburger et al., 2010; Liu & Bankaitis, 2010). Phosphorylation of the inositol ring at different sites gives diversity (see **Figure 1.9**) to phosphoinositide species (Y. J. Kim, Jahan, & Bahk, 2013). Various kinases phosphorylate the inositol ring at the third, fourth and fifth hydroxyl group sites in seven different combinations. Due to steric hindrance, the second and sixth hydroxyl group sites are typically not phosphorylated (Mueller-Roeber & Pical, 2002). Each phosphoinositide interacts with specific proteins to perform their cellular functions, which include membrane trafficking, nuclear events, signal transduction and cytoskeletal organization (Mathews et al., 2005).

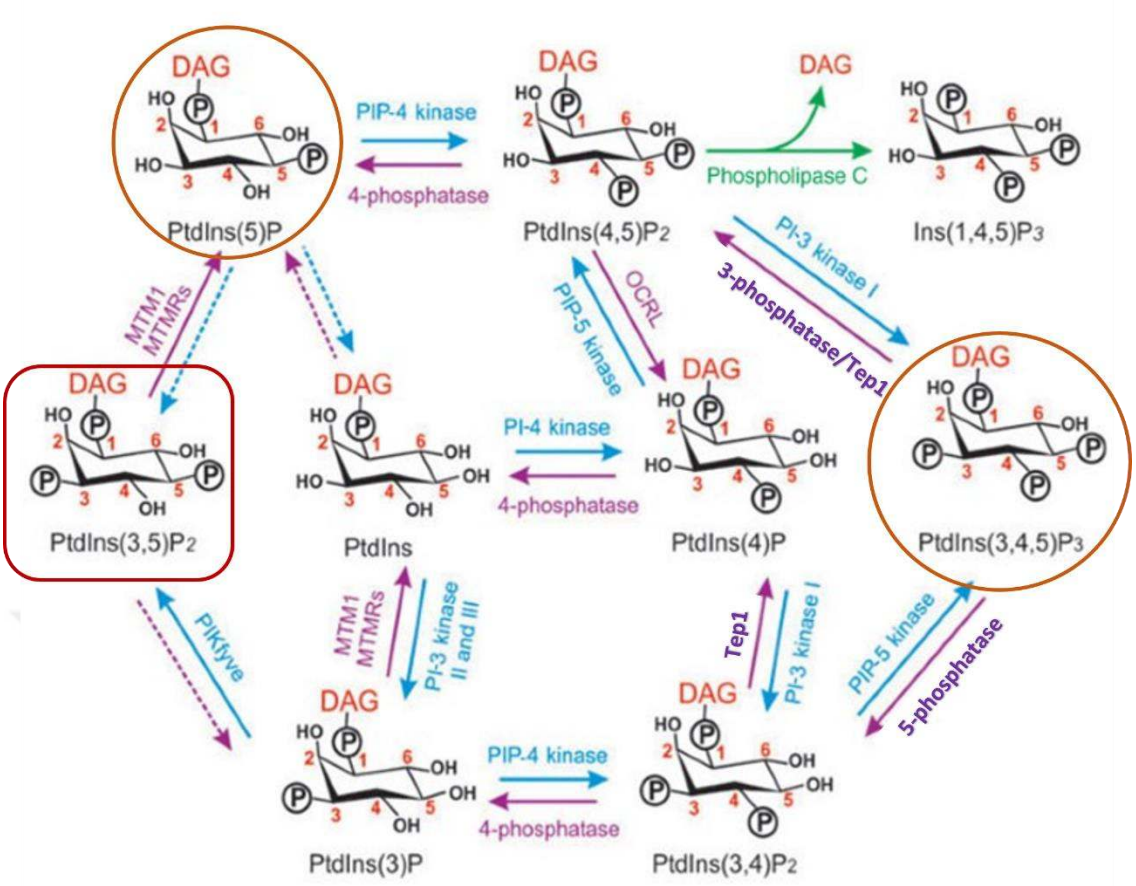

Figure 1.9 **Phosphoinositide Types.** Each phosphoinositide can be converted to other phosphoinositide derivatives by phosphatases (purple arrows) or kinases (blue arrows). The positions on inositol ring are shown in orange numbers. DAG:Diacylglycerol. Tep1: Tepsin like phosphatase. Fig4: Factor-induced Gene aka PtdIns(3,5)P_2 5-phosphatase. MTM1:Myotubularin1 -a lipid phosphatase which dephosphorylates phosphatidylinositol 3-monophosphate and PtdIns(3,5)P_2. MTMR: Myotubularin related proteins. In a budding yeast, phosphoinositide types marked with light brown circle are not found (Strahl & Thorner, 2007; Wera et al.,2001) and PtdIns(3,5)P_2 is pointed with violet rectangular shape The above figure adapted from (Rusten & Stenmark, 2006) .

Phosphotidylinositol (3,5) Biphosphate (PtdIns(3,5)P_2) is a derivative of phosphoinositide phosphorylated at position C3 and C5 (Di Paolo & De Camilli, 2006). PtdIns(3,5)P_2 is less abundant than other phosphoinositide derivatives (Strahl & Thorner, 2007). It is primarily found on late endosomes and lysosomal membranes (Di Paolo & De Camilli, 2006). Change in PtdIns(3,5)P_2 levels depends on extracellular stimuli such as osmotic stress in yeast or stimulation with growth factors and phagocytosis in mammalian cells (Raghu, Joseph, Krishnan, Singh, & Saha, 2019). In response to hyperosmotic stress in yeast, PtdIns(3,5)P_2 level transiently increases (Bonangelino et al., 2002; Duex, Nau, Kauffman, & Weisman, 2006). PtdIns(3,5)P_2 level reaches a peak after

5-10 minutes of the exposure and then it returns to basal levels within 30 minutes (Bonangelino et al., 2002). PtdIns(3,5)P_2 plays roles in membrane trafficking, stress response, vacuole/endolysosome structure and function and transcriptional regulation. Although the underlying molecular mechanism are yet unknown, PtdIns(3,5)P_2 deficiency is associated with some neurological diseases such as Amyotrophic Lateral Sclerosis, Charcot-Marie-Tooth disease (Hasegawa, Strunk, & Weisman, 2017).

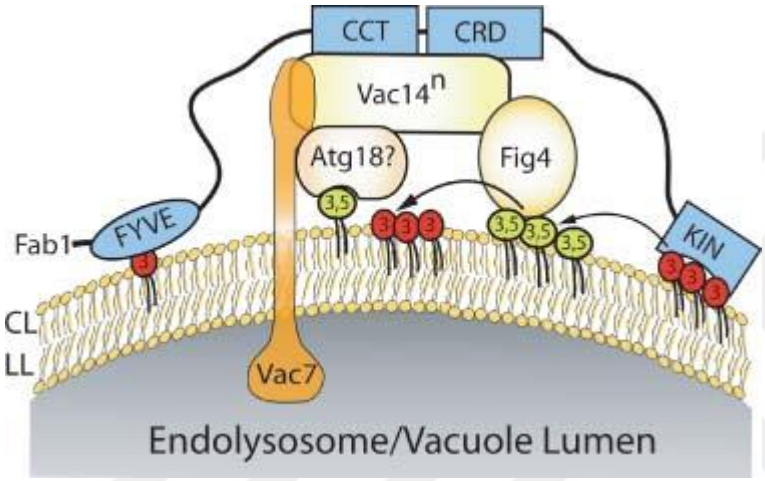

Figure 1.10 **A model of Fab1 complex**: Fab1 associates with a large protein complex composed of several regulatory proteins. Vac7 is a transmembrane protein and induces Fab1 activity similar to Vac14. Atg18 acts as negative regulator. Reduction in PtdIns(3,5)P_2 occurs in presence of Atg18. Fig4 regulates PtdIns(3,5)P2 level at both ways. The figure taken from (Ho, Alghamdi, & Botelho, 2012).

PtdIns(3,5)P_2 synthesis relies on Fab1/PIKfyve association with a large protein complex involving several regulatory proteins which are Fig4, Vac14, Vac7, and Atg18 (Jin, Lang, & Weisman, 2016; McCartney, Zhang, & Weisman, 2014). These proteins form a complex named as the PAS complex to achieve biosynthesis of PtdIns(3,5)P_2 (McCartney, Zhang, & Weisman. 2014). Fab1 (PIKfyve in higher eukaryotes) is the lipid kinase which adds a phosphate group at 5' C in PtdIns(3)P_2 (Sharma et al, 2019). Vac14 (ArPIKfyve in higher eukaryotes) acts as a scaffold protein to form a complex with regulatory proteins and Fab1. Efficient synthesis of PtdIns(3,5)P_2 requires Vac14 binding to all regulatory proteins and Fab1 (Botelho, Efe, Teis, & Emr, 2008; Duex, Tang, & Weisman, 2006; Jin et al., 2008). Similar to Vac14, Vac7 positively regulates Fab1

activity. Vac7 is specific to yeast (Ho, Alghamdi, & Botelho, 2012) and is the only protein containing a transmembrane region in PAS complex (Gary et al., 2002). PtdIns(3,5)P_2 level is negatively regulated by Atg18 (Dove et al., 2004) Atg18 interacts with Vac14 (Efe, Botelho, & Emr, 2007). Atg18 has two PI binding sites for PI3P and PtdIns(3,5)P_2 (Baskaran, Ragusa, Boura, & Hurley, 2012). These binding sites are required for its localization on vacuole membranes (Efe et al., 2007). Fig4 (Sac3 in higher eukaryotes) catalyses PtdIns(3,5)P_2 turnover by dephosphorylating PtdIns(3,5)P_2 at the 5-end to produce PI3P (Rudge, Anderson, & Emr, 2004). Interestingly, both the production and turnover of PtdIns(3,5)P_2 are impaired by specific Fig4 mutations in yeast (Chow et al., 2007; Duex, Nau, et al., 2006; Gary et al., 2002). In mammalian cells, as well, loss of Fig4 function causes a decrease in PtdIns(3,5)P_2 levels. Thereby, Fig4 regulates PtdIns(3,5)P_2 levels in both positively and negatively way (Chow et al., 2007; Hasegawa et al., 2017; Zolov et al., 2012). Fig4 triggers synthesis of PtdIns(3,5)P_2 through its association with Vac14 and Fab1 (Botelho et al., 2008; Hasegawa et al., 2017; Jin et al., 2008).

1.3.1. Molecular mechanisms that depend on PtdIns(3,5)P_2

PtdIns(3,5)P_2 acts in autophagy, endolysosomal structure and function, membrane trafficking, protein sorting and maintenance of intracellular osmolarity and pH in budding yeast (Duex, Nau, et al., 2006; Jin et al., 2014). Autophagy degrades cytosolic content and organelles under limited nutrients and/or stress. PtdIns(3,5)P_2 mediates autophagy by two different ways. First, a decrease in PtdIns(3,5)P_2 levels leads defects in vacuolar degradation of autophagosome since a budding yeast cannot achieve to fully acidify vacuole lumen (Jin et al., 2014). Second, decreased PtdIns(3,5)P_2 levels causes loss of target of rapamycin complex 1 (TORC1) which is a major regulator of cell growth and autophagy in a budding yeast (de Lartigue et al., 2009; Martin et al., 2013).

PtdIns(3,5)P_2 maintains intracellular osmolarity and pH through regulation of the vacuolar proton-translocating ATPase (V-ATPase) and Ca^{2+} channels in response to hyperosmotic shock (Duex, Nau, et al., 2006). In yeast, V-ATPase stability and its reassembly after glucose starvation depends on PtdIns(3,5)P_2 interaction with the V_0 subunit of the V-ATPase (Li et al., 2014). In addition, PtdIns(3,5)P_2 drives flow of

intraluminal Ca^{2+} concentration by regulating Yvc1 - yeast homologue of TRPML1 in mammalian cell - to act as an endolysosomal Ca^{2+} release channel (Dong et al., 2010).

Both retrograde traffic from the yeast vacuole to the Golgi and cargo selection into multivesicular bodies (MVB) requires PtdIns(3,5)P_2 (Dong et al., 2010; Katzmann, Babst, & Emr, 2001). ESCRT machinery is a key point for MVB sorting and PtdIns(3,5)P_2 binds to Vps24 that is a component of ESCRT-III (Katzmann et al., 2001). Furthermore, function of human genes SNX1 and SNX2 -play a role in retrograde traffic- relies on directly binding to PtdIns(3,5)P_2 (Dove et al., 2004). In budding yeast, PtdIns(3,5)P_2 was shown to regulate transcription through interaction with transcriptional regulators Tup1 and Cti6 (Han, & Emr, 2011). These two proteins have high specificity for binding PtdIns(3,5)P_2. Tup1 includes 7 blade ß-propellers in its structure. It shares same structure with Atg18 which binds PtdIns(3,5)P_2 synthesis. These blade ß-propellers provides linkage with PtdIns(3,5)P_2 for Tup1 (Han, & Emr, 2011). PtdIns(3,5)P_2 binding protein Tup1 forms a complex with Cyc8 (Williams, 1991). Cyc8-Tup1 functions as a gene corepressor Tup1 and Cti6 binding to PtdIns(3,5)P_2 assists formation of the Cti6-Cyc8-Tup1 complex which functions as a coactivator (Han, & Emr, 2011) and this is how PtdIns(3,5)P_2 participates in regulation of transcription.

Aim of the thesis

PAS complex components Vac7 and Vac14 were identified in a large-scale genetic screen which was designed to identify putative mitotic exit activators (Caydasi & Pereira, 2012). This data suggested that PtdIns(3,5)P_2 might have a function in mitosis, particularly in exit from mitosis. The purpose of this thesis is to analyze whether and how PAS components influence on mitotic exit parameters. For this, I aimed to analyze (1) genetic interactions between the PAS complex components and known mitotic exit activators (2) the effect of PAS complex on anaphase duration and cytokinesis and (3) the influence of PAS components on localization of proteins that promote and regulate mitotic exit.

Chapter 2. Material and Methods

2.1. Yeast strains and plasmids

All yeast strains used in the thesis study is listed in Table 6.1. All strains were made by PCR cassette or plasmid integration (Janke et al., 2004; Knop et al., 1999). PCR cassette is a nucleotide sequence which constitutes selection markers flanked by homolog sequences (~50bp) matching with desired gene locus at the appropriate position. Homolog sequences in PCR cassette recombines with their counterparts at the chromosome to perform gene replacement or integration. Integration of linearized pRS40x or pRS30x-based plasmids creates homologous sequences in the selection marker or the gene of interest (depending on the site of restriction digestion) through which the plasmid is integrated yeast genome. N-terminally GFP or mCherry tagged *TUB1* was integrated to the chromosome using integration plasmids *pAFS125 (GFP-TUB1*) (Murray, 1996) (Table 7.2).

Gene deletions were confirmed by colony PCR using primers that bind outside of the gene (external) or inside of the gene (internal) (See Table 6.3 for list of primers). C-terminal tagging of genes with GFP or 3xmCherry was confirmed via microscopy.

2.2. Yeast growth conditions

All yeast was grown under aseptic conditions. Yeast was grown on Yeast Extract Peptone Dextrose (YPD) agar or in Yeast Extract Peptone Adenine Dextrose (YPAD) media (Table 3.1: growth media composition) except strains carrying centromeric plasmids. Yeast bearing centromeric plasmids were grown in synthetic complete media (SC) lacking the corresponding amino acid or nucleotide (SC-x) (See Table 3.1) depending on the selection marker of the plasmid (See Table 6.2). For negative selection of the *URA3* based plasmids 5-FOA plates were used. Cells were grown at 30˚C unless otherwise stated. Temperature sensitive mutants were incubated at 23˚C unless the temperature sensitive phenotype was desired to be seen.

All yeast were kept as stocks at -80˚C in 15% glycerol. Yeast was scraped from this glycerol stock on appropriate agar plates and grown at appropriate temperatures. Colonies taken from agar plates were inoculated in appropriate liquid media and grown until

stationary phase in a shaking incubator set at the appropriate temperature. To get logarithmic phase cultures, stationary culture was diluted in fresh medium to an OD600 of 0.15-0.2 and grown until OD600 0.8-1 (~2,5 doubling time).

2.3. *E.coli* transformation

50 µl chemically competent (Inoue method, Cold Spring Harb Protoc, 2006) *DH5α* cells was used for plasmid transformation into *E.coli*. Competent cells taken from -80 ºC freezer were thawed on ice. 1 µl of plasmid of interest was added into thawed competent cells, mixed by tapping the tube and kept on ice for 30 minutes. Then, competent cells were incubated at 42°C for 90 sec. Competent cell mixture was kept on ice for 2-5 min immediately after heat shock. For Ampicillin selection, cells are plated on ampicillin containing agar plates straightaway.

2.4. Plasmid isolation from *E.coli*

Selected plasmid was transformed into *E.coli* $DH_5\alpha$ competent cells. *E.coli* $DH_5\alpha$ was cultured in TY+Amp (See Table 3.1) at 37˚C for about 18 hours. 1-2 ml of sample was pelleted at 6000 rpm for 5 min at room temperature (RT). After removal of the supernatant, 300 µl of P1 (Table 3.1) was added onto the pellet. Pellet was vortexed well. Next, 300 µl P2 (Table 3.1) was added to the suspension of cells and mixed via invertion /rotation. After P2 treatment, 300 µl of cold P3 (Table 3.1) was added and mixed via invertion/rotation. Each sample was incubated on ice for 10 min after addition of the P3 buffer. Then, all samples were centrifuged at 14000 rpm for 10 min and supernatants were transferred into new tubes containing 600 µl of isopropanol. Next, all samples were centrifuged for 20 min at full speed. Supernatants were removed, pellets were treated with 70% cold ethanol and centrifuged at full speed for 5 min. Then, supernatant was discarded, and pellet was air dried. 35 µl of ddH_2O was added to the pellets and plasmids were resuspended by gentle pipetting. Plasmid solutions were kept at -20˚C.

2.5. PCR for cassette preparation

For PCR cassette preparation, S-primers were used (See Table 6.3 for primer list). S1-S2 primers were used for deletion cassettes, whereas S2-S3 primers were used to generate C-terminal tagging cassettes. In addition, S1-S4 primers were used for N-terminal tagging cassettes. Cassettes were generating using aforementioned primers and template plasmid Table 6.2 for plasmid list) from which a selection marker or a selection marker and a tag is amplified. PCR conditions were adjusted like as shown in below Table 2.1. Composition of the PCR reaction was as shown in Table 2.2. After PCR, 3 µl of the product was analyzed in 0.8 % of agarose gel and rest was either directly used for yeast transformation or kept at -20˚C.

Table 2. 1: **Cycle Conditions for PCR cassette (Janke et al., 2004; Taxis & Knop, 2006)**

1	2 min at 95 ºC
2	20 sec. at 95ºC
3	30 sec at 52ºC
4	X min at 68ºC -> jump back to step 2 for 9 times
5	30 sec at 95ºC
6	30 sec at 52ºC
7	X min at 68ºC, increment of 20 sec per step, -> jump back to step 5 for19 times
8	pause, 4ºC

Table 2. 2: **PCR Cassette Reaction Preparation (Janke et al., 2004; Taxis & Knop, 2006)**

Template (20 ng/µl)	5 µl
10 µM Primer 1	3.2 µl
10 µM Primer 2	3.2 µl
2 mM dNTPs	8.75 µl
Buffer 1 (Table 3.1)	5 µl
ddH_2O	24.25 µl
Enzyme mix (Table3.1	0.6 µl
Total	50 µl

2.6. Colony PCR

Small amount of yeast colonies were picked from agar plates and suspended in 50 µl of 0.01% Sarkosyl and 0.02N NaOH mixture. For cell lysis, samples were boiled for 15 min at 95˚C. This lysate was used as a source for chromosomal DNA in Colony PCR reaction.

Colony PCR cycle conditions was as shown in Table 2.3. Reaction was prepared as shown in Table 2.4.

Table 2. 3: **Cycle conditions for Colony PCR (Bergkessel & Guthrie, 2013)**

1	5 min at 95 ºC
2	30 sec. at 95ºC
3	30 sec at 53ºC
4	1 min/1000bp at 68°C back to step 2 for 35x
5	3 min at 68°C
6	Pause at 4ºC

Table 2. 4: **Colony PCR Reaction Preparation (Bergkessel & Guthrie, 2013)**

Yeast Cell /Chromosomal DNA	0.6 / 1 µl
10 µM Primer 1	1.25 µl
10 µM Primer 2	1.25 µl
2 mM dNTPs	2.5 µl
Buffer 1 (Table 3.1)	2.5 µl
ddH_2O	16.75/ 16.35 µl
5U/ µl Taq Polymerase	0.25 µl
Total	25 µl

The same protocol can be used for amplification of DNA using purified yeast chromosomal DNA as a template. If so, 1 µl of chromosomal DNA was added into PCR mixture instead of the cell lysate. After PCR, the product was analyzed in 0.8 % of agarose gel and kept at -20˚C.

2.7. Chromosomal DNA extraction from yeast

Protocol was modified in our lab. Briefly, appropriate yeast strain was cultured in liquid medium overnight at appropriate temperature. 1 ml of the culture was pelleted at full speed for 1 min. Supernatant was discarded. Pellet was resuspended in 300 µl of 6% SET (See Table 3.1) by vortexing and incubated at 65 ˚C for 15 minutes. Then, tubes were transferred onto ice. 150 µl of cold 3M NaOAc -inverting several times to mix - was added into the tubes and kept on ice for 15 min. After incubation on ice, the tubes were centrifuged for 10 min. Supernatant was transferred into a new tube, 500 µl of isopropanol was added and spun for 1 min at full speed. Then, supernatant was discarded and 200 µl

of 70% cold ethanol was added onto the pellet and spun for 1 min at full speed. Supernatant was discarded and pellet was air dried. Next, DNA pellet was resuspended with 35 µl of ddH_2O + 1 µl of 1mg/ml RNase and incubated at 37 ˚C for 15 min.

2.8. Microscopy

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

For still images, light intensity was 20% and exposure time was 50 ms for *BFA1-GFP, MOB1-GFP, LTE1-GFP* and *CDC14-GFP* strains and values for 3mCherry-TUB1 in MEN GFP tagged strains were 50% and 100 ms respectively to investigate localization or intensity of related MEN proteins in wild type and *vac7*Δ strains. For *KIN4*-GFP strains, light intensity was 20% and exposure time was 300 ms and cultured in filter sterilized YPAD. All strains were inoculated into filter sterilized SC Complete or YPAD and kept in log phase at 30˚C to the next day. 1 mL of log phase culture was centrifugated and supernatant was aspirated to the point where 10 µl of medium remained in microcentrifuge tubes. Pellets were dissolved in remaining medium. Then, 1 µl of sample was taken and placed onto microscope glass.

For time-lapse analysis, light intensity was 10% and exposure time was 50 ms for GFP tagged strains and values for 3mCherry tagged strains were 30% and 100 ms respectively. For time-lapse experiments, each sample taken from agar plate was inoculated into filter sterilized SC medium and kept at log phase for 2 days. After incubation, 100 µl Sigma Concanavalin A (6% see Table 3.1) was pipetted onto the glass center of the glass bottom petri dishes (NEST ABOVE 801002) and let sit for 10 min at room temperature. Then, Concanavalin A was pipetted out and the glass bottom petri dishes were washed with ddH_2O four times. After washing, 200 µl of each sample was added onto glass bottom petri dishes and incubated at 30˚C for 30 minutes. When incubation was done, all samples were washed with filter sterilized SC medium four times. Finally, 2-3 mL of SC medium was added on samples and taken into the microscope chamber. All time-lapses were

recorded under 100X objective and samples were photographed with one-minute interval at channel properties mentioned above.

For strain checking, each sample taken from appropriate agar plate was inoculated into filter sterilized appropriate medium - filter sterilized SC-LEU for plasmid carrying strains, SC-URA or SC Complete for integrated plasmid or PCR cassette carrying strains- and kept at log phase for a day. After incubation, 1 mL of sample was taken from each culture and put into microcentrifuge tubes and centrifuged. Supernatants were aspirated to a point where 10 µl of medium remained in microcentrifuge tubes. Pellets were dissolved in remaining medium. Then, 1 µl of sample was taken and placed in between coverslip and slides.

2.9. Analysis of Microscopy data

Cytokinesis kinetics were assessed by analyzing contractile actomyosin ring (CAR) constriction and septin splitting. CAR constriction duration was determined by calculating the time from the time point in which myosin starts to be constricted until the time point in which myosin disappears completely. Timing of septin splitting was analysed by calculating the time from metaphase-anaphase transition until septin splitting - in which the hourglass shaped septin separates into two distinct separated rings. Metaphase-anaphase transition was determined as the starting point of fast spindle elongation.

Anaphase duration was calculated by subtracting the time point of metaphase-anaphase transition from the time point of spindle breakdown.

Bfa1 and Tem1 asymmetry at the SPBs were assessed from log phase cultures through analysis by eye. Images were sum projected before analysis. If signal brightness of the protein of interest is similar at the daughter and mother SPB, it was considered as symmetric localization. If the signal is brighter at one SPB than the other, it was considered as asymmetric. Cells in metaphase and anaphase were quantified separately. Cells with a 1.5-2 µm spindle were considered in metaphase stage whereas cells with a spindle longer than 2.5 µm were considered in anaphase.

To calculate Lte1 intensity, plot profile function of ImageJ was used. For this a line with a thickness of 10 µm was drawn along the bud and mother cell axis of cells. The line started and ended in the regions outside of the cell. Thus, the amount of Lte1 in the bud cortex, cytoplasm and main cell cortex can be relatively assessed.

For Kin4 localization analysis, yeast cells were grown in filter sterilized YPAD and imaged under 100X objective lens. Metaphase and anaphase/telophase stages were selected.

For Cdc14 timelapse analysis, the timepoint where Cdc14 nuclear signal is indistinguishable from its cytoplasmic signal were considered as the time point when Cdc14 is fully released to the cytoplasm. The timing of Cdc14 full release were calculated with respect to the metaphase anaphase transition, simply by subtracting the time point of full release from the timepoint of metaphase anaphase transition. Cdc14 re-appearing in the nucleolus after the full release were also calculated. The time point where dotty signal start to appear in the nucleus were considered as the time of reappearance and this was subtracted from the time point of Cdc14 full release to assess the timing relative to the full release.

2.10. Yeast Competent cell preparation

To make yeast competent cells, 50 mL of logarithmic-phase yeast culture was centrifuged at 3200 rpm for 2 min at room temperature. Supernatant was discarded and pellet was resuspended in 45 mL of sterilized distilled water. Cells were centrifuged as before, and supernatant was discarded. Next, pellet was resuspended in 20 mL of LiSorb (See Table 3.1) and centrifuged as before. Supernatant was discarded. Centrifugation was repeated once again to remove excess LiSorb. Then, cells were resuspended in LiSorb containing denatures salmon sperm DNA (300 µl of LiSorb and 50 µl of denatured salmon sperm DNA for Cell pellet coming from 50 mL 1 OD_{600} culture - Salmon sperm DNA was denatured by heating at 95˚C for 5 min and immediately cooling on ice for 5 min). Competent cells were aliquoted as 50-100µL aliquots and either freshly used or kept at -80 ˚C until usage.

2.11. Yeast Transformation

For yeast transformation, 5 µl of PCR cassette or 1-2 µl of plasmid was pipetted into yeast competent cells, mixed and incubated for 10 minutes at room temperature. Then, 300 µL of cold LiPEG (See Table 3.1) was added into 50 µl competent cells and gently vortexed. Next, 35 µl DMSO was pipetted into the mixture, briefly vortexed and incubated at 42˚C for 15 min. When using temperature sensitive strains, the duration of this incubation was restricted to 12 min. Then, samples were centrifuged at 3200 rpm for 2 minutes. Immediately, samples were resuspended in 200 µl PBS and plated-out onto selective agar medium, if auxotrophy selection is done. For antibiotic selection, cells were incubated in YPAD o/n before plating out.

2.12. Agarose Gel Electrophoresis

0.8% agarose gel was prepared by boiling 0.4 g of agarose SIGMA A9539 in 50 mL TAE. 12 µl of DNA samples are loaded into wells. NEB N3232L DNA ladder was used a DNA marker. After Gels were run at 100 Volt for about 45 min and incubated in EtBr containing water for at least 15 minutes at room temperature. Gels were imaged under UV light using a gel documentation system (Carestream).

2.13. Drop Test

Stationary yeast cultures of yeast grown in appropriate liquid media were diluted to a OD_{600} of 1 using autoclaved PBS (See Table 3.1). From this, 10-fold serial dilutions (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) were performed. 10 µl of each dilution was dropped onto appropriate agar plates and incubated at appropriate temperatures.

2.14. Total Protein Precipitation with TCA

Cells are pelleted at 14000 rpm for 1-2 min at 4˚C. Total proteins were extracted as following: First, pellets were resuspended in 800 µl of ice cold ddH20 by vigorous vortexing. Then 150 µl of 1.85 M NaOH was added and vortexed. Next, 150 µl of ice-

cold 55% TCA was added immediately and vortexed. The mixture was incubated on ice for 5 min and then centrifuged for 15-20 min at 4˚C at 14000 rpm. Supernatant was aspirated. Pellets were centrifuged at 14000 rpm for 2 min at room temperature, and the supernatant was aspirated to get rid of the remaining liquid. The protein pellets were dissolved in HU-DTT (see Table 3.1) by vigorous vortexing and samples were boiled at 65˚C for 15 min.

Chapter 3. Media and Buffer Recipes

Table 3. 1: **Media and Buffer Recipes**

NAME	DESCRIPTION
YPAD	5g Bacto Yeast Extract (Conda pronadisa), 10 g Bacto Peptone (Merck), 10 g D(+)-Glucose (BioFroxx), 0.05 g Adenine (Sigma), ddH2O up to 500 ml
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 ddH2O 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, ddH2O up to 1 L. Ampicillin 100 µg/ml or Kanamycin 50 µg/ml.
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 ddH2O
1X PBS	137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.76 mM KH2PO4. pH 7.2-7.4
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
1M Potassium Phosphate Buffer pH 7.5	83.4 ml 1M K_2HPO_4, 16.6 ml 1M KH_2PO_4
Enzyme Mix	8 µl Taq (5U/µl) + 4 µl Vent (2U/µl). (Keep at -20ºC after mix well)
Concanavalin A	From *Canavalia ensiformis* (Jack Bena) type IV (Sigma C2010-G) %6 w/v

Chapter 4. RESULTS

4.1. *VAC7* and *VAC14* are crucial for timely mitotic exit

Phosphotidylinositol (3,5) Biphosphate (PtdIns(3,5)P_2) is an evolutionary conserved phosphoinositide found in vacuolar/lysosomal membrane (Di Paolo & De Camilli, 2006). It is the least abundant phosphoinositide derivative in cells. PtdIns(3,5)P_2 plays roles in vacuole/endolysosome structure and function, membrane trafficking, stress response, and transcriptional regulation (Ikonomov, Sbrissa, & Shisheva, 2006; Jefferies et al., 2008; Sbrissa et al., 2007). No mitotic function has been attributed to PtdIns(3,5)P_2 so far.

We asked whether PtdIns(3,5)P_2 is involved in mitotic exit regulation. To address this question, first, we took a genetic approach. MEN (Mitotic Exit Network) is responsible for exit from mitosis in budding yeast. It is composed of a GTP-binding protein (Tem1), two downstream kinases (Cdc15 and Dbf2-Mob1), and the most downstream phosphatase Cdc14. We deleted *VAC7* in cells carrying the temperature sensitive mutants of the MEN components and analyzed cell growth at various temperatures upon *VAC7* deletion, which diminishes PtdIns(3,5)P_2 synthesis (Gary et al., 2002) (**Figure 4.1**). *vac7*Δ cells demonstrated synthetic lethality with *dbf2-2*, *mob1-67*, *tem1-3* and *cdc15-1* but not with *cdc14-1* or *cdc14-2* mutants (**Figure 4.1**). *vac14*Δ cells also have greatly reduced PtdIns(3,5)P_2 levels (Rudge et al., 2004). Thus, we also analyzed growth of *vac14*Δ cells in combination with MEN temperature sensitive mutants and got similar results as *vac7*Δ albeit much milder (data not shown).

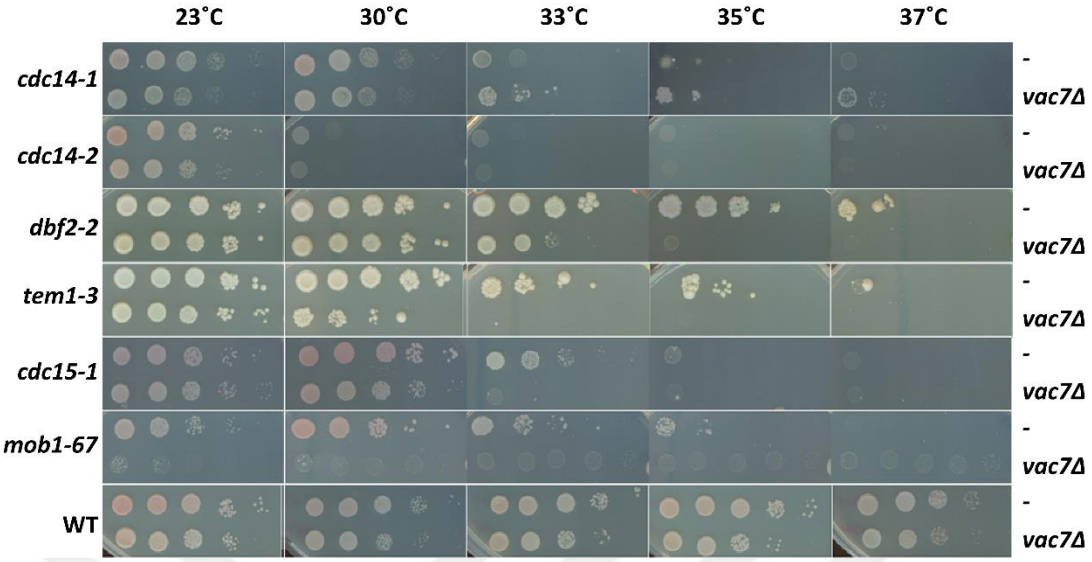

Figure 4.1 ***vac7*Δ shows synthetic lethality with main MEN components:** Serial dilutions of each strain were dropped on SC Complete agar plates and incubated at indicated temperatures (Strains used: YPH499, ESM1249, ESM1276, ESM1278, ESM1309, ESM1361, CVY16, BBY007-1, BBY008-1, BBY014-1, BBY015, BBY016, BBY27-1, BBY032-1)

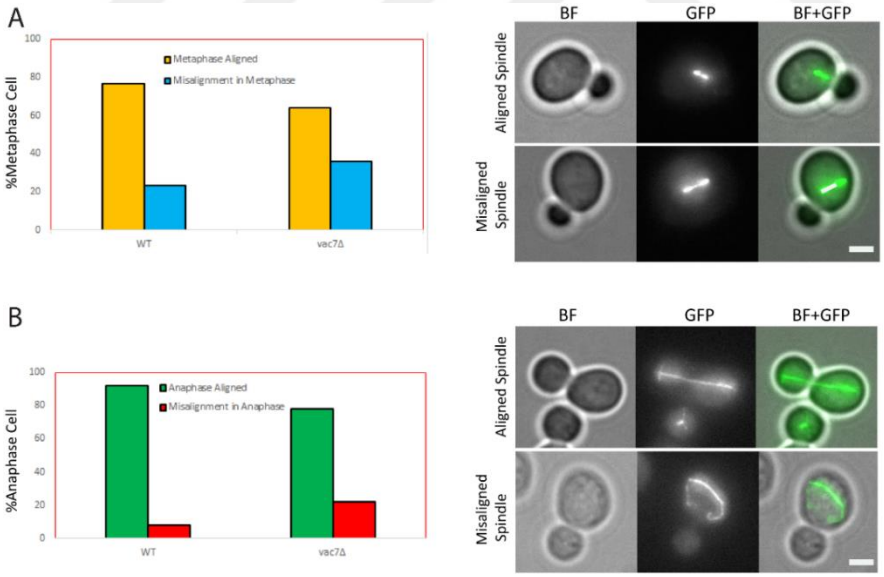

Figure 4.2 ***vac7*Δ increases misalignment of mitotic spindle:** (A) Percentage of cells with misaligned metaphase spindles. Representative images of cells were shown on the right. Sample sizes of WT (SHM295) and *vac7*Δ (BBY024-1) were 138 and 155 in metaphase respectively. (B) Percentage of cells with misaligned anaphase spindles. Representative images of cells were shown on the right. Sample sizes of WT and *vac7*Δ were 99 and 97. Both wild type and mutant strains have GPF tagged *TUB1*. Metaphase and anaphase stages were determined by spindle length. All strains were incubated at 30˚ C and each image was snapped at same temperature. Scale bar is 2 µm. BF = Bright field.

We next took a microscopy-based approach to further understand whether PtdIns(3,5)P_2 plays a role in timely mitotic exit in cells with functional MEN. We employed time-lapse microscopy to analyze the duration of anaphase in cells that fail to produce PtdIns(3,5)P_2. We used GFP-*TUB1* as a microtubule marker and monitored mitotic spindle elongation in cells to calculate anaphase duration in wild type (WT), *vac7*Δ and *vac14*Δ backgrounds (**Figure 4.3**). Anaphase duration was calculated as the time elapsed between metaphase anaphase transition and spindle breakdown (**Figure 4.3a**, below). During our experiments we noticed that some *vac7*Δ cells misposition their spindle (**Figure 4.2**). This is most likely due the enlarged vacuole that physically blocks the bud neck (data not shown) (Rudge, Anderson, & Emr, 2004). Importantly, in our calculation of anaphase durations we only considered cells with correctly aligned spindles. Anaphase lasted 19 min (median) in wild type cells, but 23 (median) and 21 (median) min in *vac7*Δ and *vac14*Δ respectively (**Figure 4.3b**). These data show that mitotic exit is delayed in the absence of *VAC7* or *VAC14* . Similar to our genetic data (**Fig4.1**), *vac7*Δ had more severe phenotype than *vac14*Δ in delaying the mitotic exit. These data indicate that *VAC7* and *VAC14* are crucial for a timely mitotic exit and PtdIns(3,5)P_2 may function in mitotic exit.

4.2. Mitotic exit defects of *vac7*Δ cells can be rescued by hyperactive *FAB1*

We next asked whether the mitotic exit defect of *vac7*Δ and *vac14*Δ cells is dependent on their function in PtdIns(3,5)P_2 biosynthesis. To answer this, a hyperactive form of *FAB1 (FAB1**) was inserted into genome of the *vac7*Δ strain. *FAB1* hyperactive allele produces PtdIns(3,5)P_2 independently of Vac7 (Gary et al., 2002), hence introducing a *FAB1* hyperactive allele into *vac7*Δ strain rescues PtdIns3,5P_2 deficiency. Introducing a *FAB1* hyperactive allele into *vac7*Δ strain decreased anaphase duration in *vac7*Δ cells whereas it did not have an impact on anaphase duration in wild type cells (Fig4.4a). Importantly, anaphase duration of *FAB1** bearing *vac7*Δ cells was similar to the anaphase duration of wild type cells (**Figure 4.4a**). Thus, the mitotic exit delay of *vac7*Δ cells is most likely due to the lack of PtdIns(3,5)P_2.

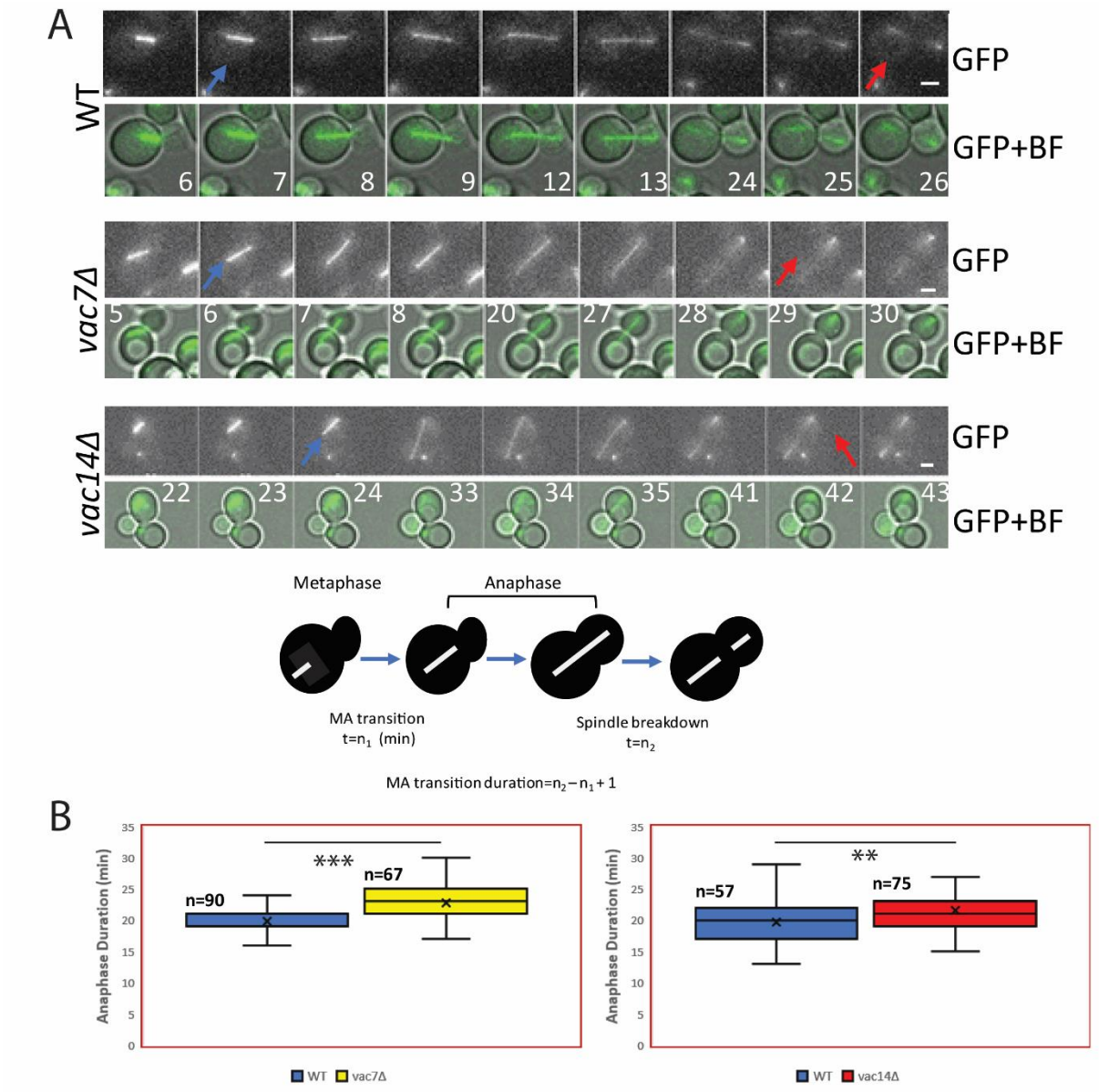

Figure 4.3 **Lack of PAS components cause prolonged anaphase:** (A) Representative still images of time-lapse series. Blue arrows indicate the point when metaphase-anaphase (MA) transition starts, and red arrows demonstrate the point when spindle breakdown happens. Time-lapse images were taken with 1 min time resolution. Anaphase duration was calculated as the time passed from the point of MA transition from timepoint of spindle breakdown as shown in the cartoon below (B) Box and whisker plots showing anaphase duration in indicated strains. "x" is a mean marker and line in whisker-box plot is a median. The bars outside boxes display value interval of anaphase duration for each strain. Median values are 19, 23 and 21 min for WT, *vac7*Δ and *vac14*Δ strains, respectively. Standard deviations for WT and vac7Δ are 2.2 and 2.9. Standard deviations for WT and *vac14*Δ are 3.3 and 4.2. Two tailed students t-test was used for statistics. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ according to t-test and n: sample size. (The strains we used are SHM295-2, BBY023-1 and BBY024-1)

We also analyzed the effect of *FAB1** on synthetic lethality of MEN-ts mutants and *vac7*Δ. *FAB1** rescued the growth of *cdc15-1 vac7*Δ, *dbf2-2 vac7*Δ and *tem1-3 vac7*Δ

(**Figure 4.3b**). These data together show that the mitotic exit defect of *vac7*Δ cells is due to impaired Fab1 activity, hence the lack of PtdIns(3,5)P_2.

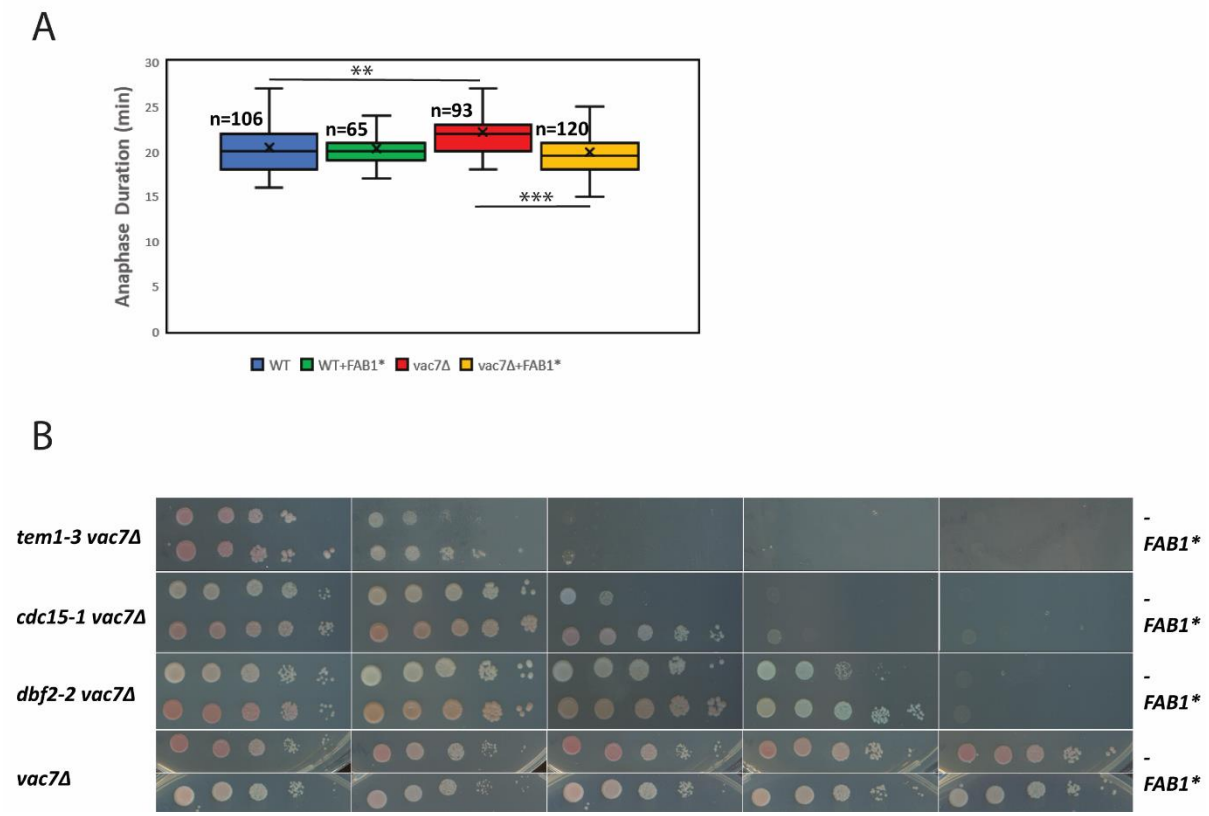

Figure 4.4 **Hyperactive form of *FAB1* (*FAB1**) reverts mitotic exit defects of *vac7*Δ cells**. (A) Box and whisker graph showing the anaphase duration of GFP-Tubulin bearing WT or *vac7*Δ cells with either a *FAB1** containing *LEU2*-based centromeric plasmid (pSE002) or an empty LEU2-based centromeric. N= Sample size. The median values of anaphase duration for wild type, wild type + *FAB1*, vac7*Δ and *vac7*Δ*+FAB1** are 20, 20, 22 and 19.5 respectively. Two tailed students t-test was used for statistics. P<0.05 *, p<0.01 **, p<0.001 *** according to t-test. (B) *FAB1** was integrated in wild type and temperature sensitive *vac7*Δ MEN mutants. Serial dilutions of each strain were dropped on SC- LEU agar plates and incubated at indicated different temperatures. (The strains used were BBY090, BBY091, BBY095, BBY101, BBY096, BBY102, BBY097 and BBY103.)

4.3. Septin and Myo1 behavior in cells lacking PtdIns(3,5)P_2

Septins are cytoskeletal proteins which have a GTP-binding domain and an ability to form filaments as organized rings (Leipe, Wolf, Koonin, & Aravind, 2002) . Budding yeast septins are *CDC3*, *CDC10*, *CDC11*, *CDC12* and *SHS1 (Dolat, Hu, & Spiliotis, 2014)*. In budding yeast, septins form a ring at bud neck in G_1 phase, which turns into an hour-glass shape structure soon after bud emergence (D. Tamborrini, M. A. Juanes, S. Ibanes, G. Rancati, & S. Piatti, 2018). The hour-glass structure splits into two rings (septin splitting) in late anaphase before mitotic spindle breakdown. MEN activity is required for septin splitting (Mostowy & Cossart, 2012). Septin splitting is a prerequisite for contractile actomyosin ring (CAR) constriction for which MEN activity is also required (D. Tamborrini et al., 2018; Tamborrini & Piatti, 2019).

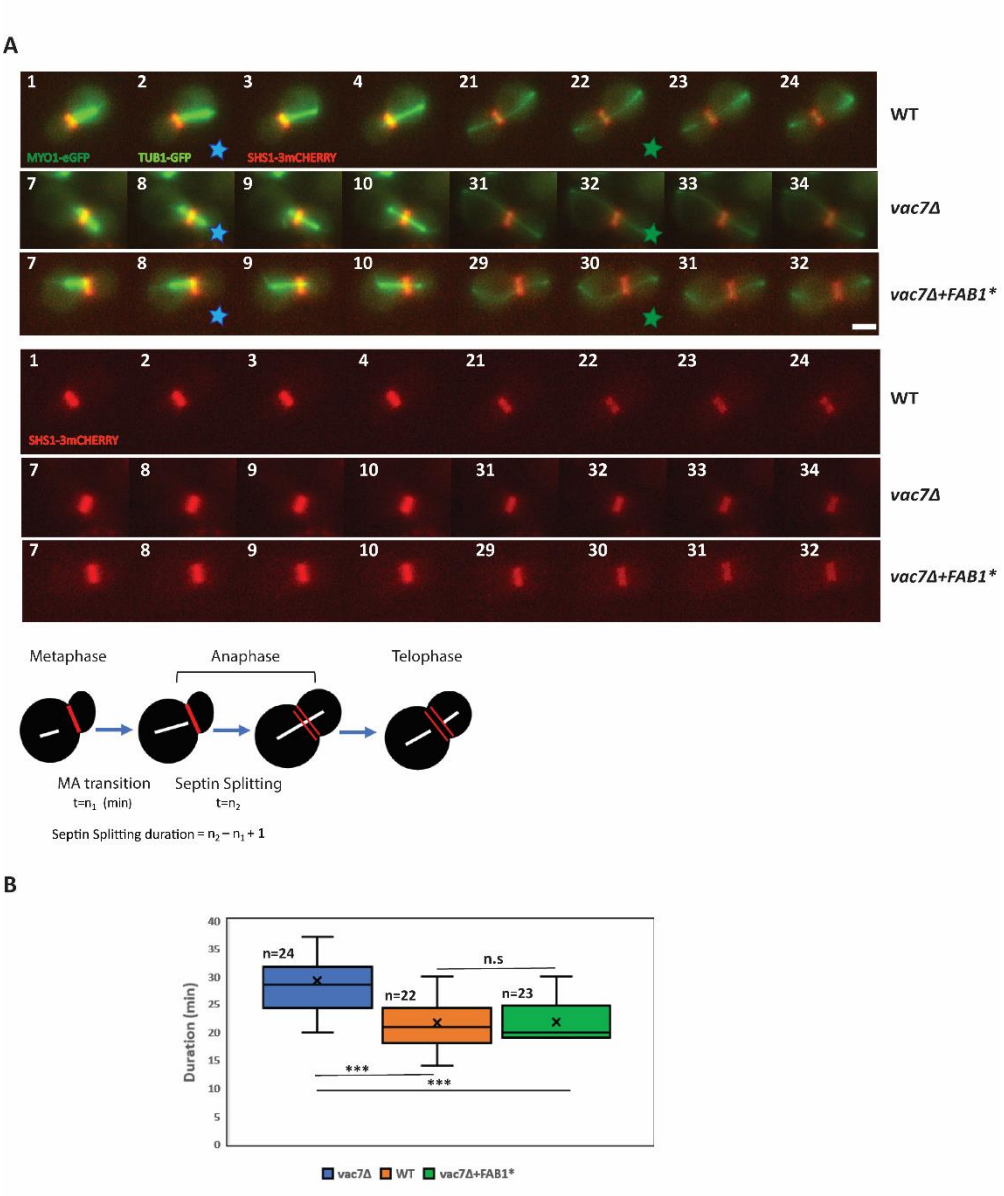

Figure 4.5 **Analysis of septin splitting in *vac7*Δ cells:** (A) Representative still images from time lapse series of *SHS1*-3mCherry *GFP-TUB1* carrying WT (BBY138), *vac7*Δ (BBY137) and FAB1* bearing *vac7*Δ cells (BBY189). Blue asterisks indicate MA transition whilst green asterisks refer the points when septin splitting happens. Scale bar is 2 µm. (B) Box and Whisker plot of duration of septin splitting after MA transition. Mean values of septin splitting after MA transition are 29.3, 21.6 and 22.7 min for v*ac7*Δ*,* WT and v*ac7*Δ *FAB1** respectively. N is sample size. Two tailed students t-test was used for statistics. P<0.05 *, p<0.01 **, p<0.001 *** according to t-test.

We analyzed the effect of *VAC7* deletion on septin splitting and CAR constriction. For this, *MYO1* was tagged with eGFP while *SHS1* was tagged with mCherry3 in wild type and *vac7*Δ.

First, we compared the timing of septin splitting with respect to metaphase-anaphase (MA) transition (**Figure 4.5a**). Septin splitting occurred 29.3 +/-7.4 (min+/- standard deviation) min and 21.6 +/-4.7 min after MA transition in *vac7*Δ and WT cells, respectively. This delay in timing of septin splitting in *vac7*Δ cells was statistically significant, and it was reduced back to the wild type timing by introduction of *FAB1** in *vac7*Δ cells, indicating that the delay was due to impairment of *FAB1* activity (**Figure 4.5b**).

Next, we compared the duration of CAR constriction in *vac7*Δ and WT cells. Myo1 (type II myosin heavy chain) is an essential component of the CAR. Myo1 starts constricting after septin splitting. We monitored the time that passes for a full contraction of Myo1. Myo1 constriction lasted 4 +/-0.67 min and 6 +/-1.67 min in WT and *vac7*Δ cells respectively (**Figure 4.6)**. Adding *FAB1** in *vac7*Δ cells accelerated Myo1 constriction and decreased the duration of constriction back to the wild type kinetics.

Taken together, our results indicate that both septin splitting and CAR constriction is delayed in cells lacking PtdIns(3,5)P_2. Importantly, MEN activity is required for both events (Davide Tamborrini, Maria Angeles Juanes, Sandy Ibanes, Giulia Rancati, & Simonetta Piatti, 2018; Tamborrini & Piatti, 2019). Thus, the delay may be attributed to the impairment of MEN activity in *vac7*Δ cells. This is inline with our previous results that *VAC7* deletion causes synthetic lethality when combined with MEN-ts mutants and causes a prolonged anaphase (**Fig4.1-Fig4.3**).

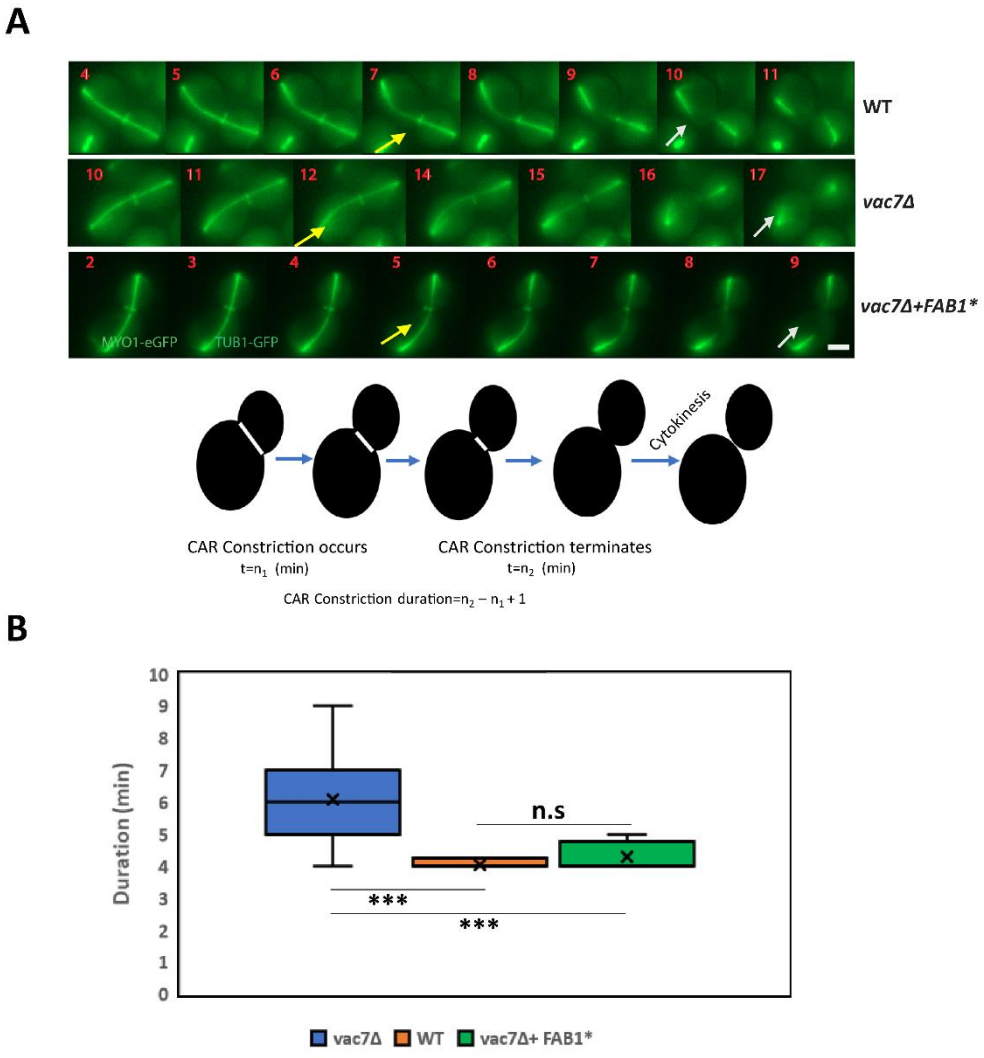

Figure 4.6 **Adding *FAB1** rescues delay in CAR constriction in *vac7*Δ cells:** (A) Representative still images from time lapse series of WT (BBY138), *vac7*Δ *(*BBY137*)* and *vac7*Δ *FAB1** (BBY189) cells carrying Myo1-GFP. Yellow arrows indicate the time point when constriction starts and grey arrows show termination of CAR constriction. Scale bar is 4 µm. (B) Box and Whisker plot of duration of CAR constriction. Duration of CAR constriction is calculated as shown in (a). Sample sizes of WT, *vac7*Δ and *vac7*Δ *FAB1** are 38, 43 and 15, respectively. Mean values of CAR constriction are 6, 4 and 4.26 min for *vac7*Δ, WT and *vac7*Δ *FAB1** respectively. Two tailed students t-test was used for statistics. $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** according to t-test.

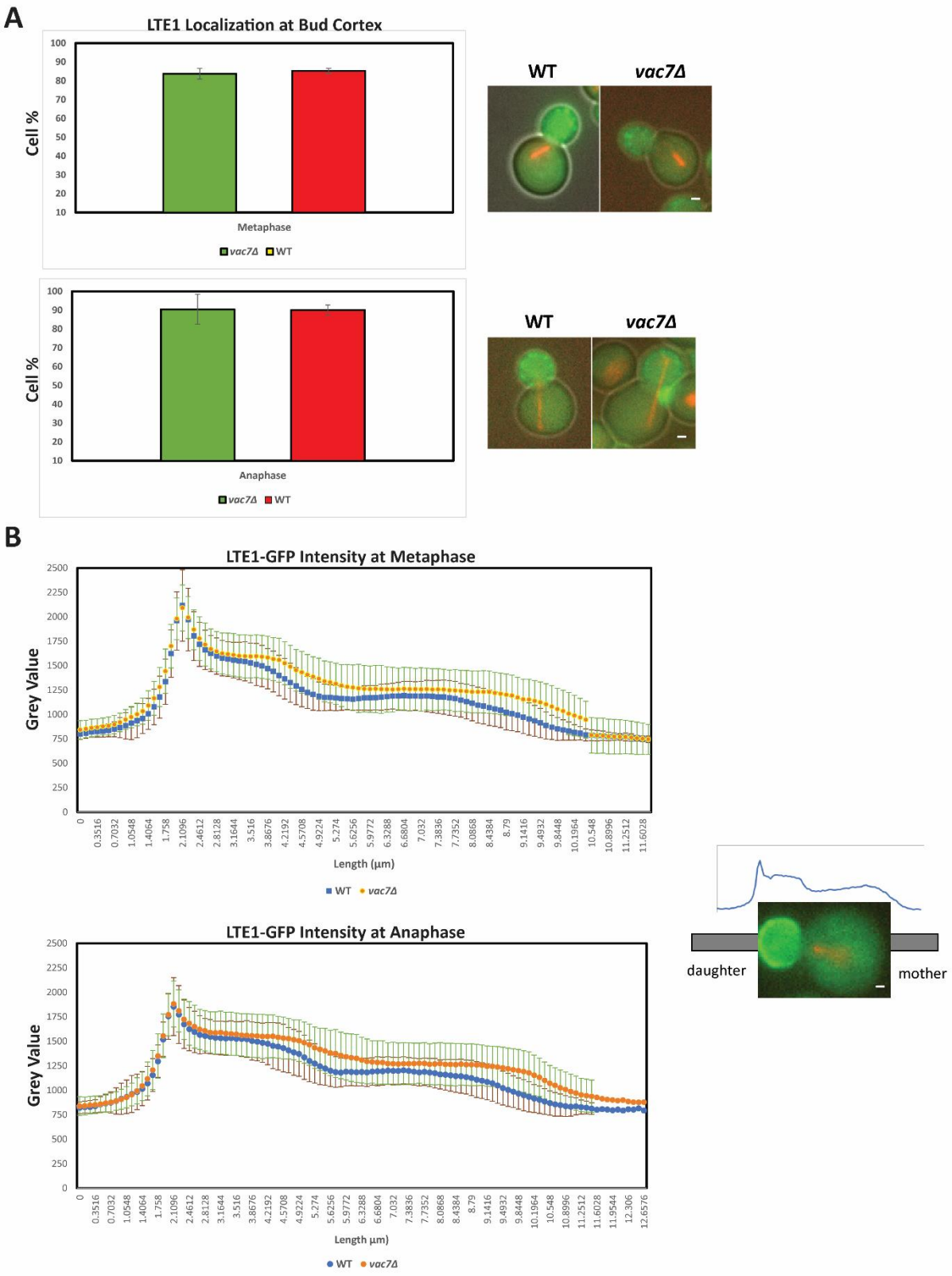
A
LTE1 Localization at Bud Cortex
Cell %
Metaphase
vac7Δ WT
WT
vac7Δ
Anaphase
B
LTE1-GFP Intensity at Metaphase
Grey Value
Length (μm)
WT vac7Δ
LTE1-GFP Intensity at Anaphase
daughter
mother

Figure 4.7 ***vac7*Δ does not impact on Lte1 localization in mitosis:** (A) percentage of metaphase (upper graph) and anaphase cells (lower graph) with Lte1 localization at the bud the cortex. For metaphase, sample size was **258** and **234** for WT (DKY073) and mutant strain (BBY117-1) respectively. For anaphase, sample size was **222** and **190** for WT and mutant strain respectively. For metaphase, the p-value (t-test) yielded 0.56 whilst 0.94 for anaphase. Scale bar is 2 µm (B)Analysis of Lte1-GFP intensity along the cell using Image J plot profile function. On the right representative images of a cell carrying Lte1-GFP is shown. Grey bar in the middle of the cell is the line that is measured by Image J plot profile option. The grey value profile of the representative cell is shown above the cell. For Lte1 intensity at metaphase sample size was **40** and **38** for WT and mutant strain respectively. For anaphase, sample size was **31** and **35** for WT and mutant strain respectively. The graphs shown on the left show the average value and standard deviation. Individual cells' plots were aligned taking the bud neck as a reference point.

4.4. How does PIP(3,5)P_2 influence MEN components and regulators?

So far, we showed that the lack of PtdIns(3,5)P_2 causes synthetic growth defect in MEN-ts mutants (except *CDC14*) and in the absence of PtdIns(3,5)P_2 anaphase is prolonged and cytokinesis is delayed. These data altogether indicate that PtdIns(3,5)P_2 has role in mitotic exit activity. However, we do not know how PtdIns(3,5)P_2 does so. Does PtdIns(3,5)P_2 affect localization and/or activity of MEN components or MEN regulators? Localization of MEN components is crucial for MEN activity. We checked whether MEN components and regulators' localization is changed in *vac7*Δ cells. Reasoning that PtdIns(3,5)P_2 may have a function in membrane protein turnover, we first investigated the localization of the Lte1 protein, which becomes essential fo mitotic exit especially at cold temperatures (Mortimer, Schild, Contopoulou, & Kans, 1989). Lte1 normally localizes at bud cortex (Marco Geymonat, Adonis Spanos, Geoffroy de Bettignies, & Steven G. Sedgwick, 2009; Shirayama, Matsui, Tanaka, & Toh-e, 1994). Under our experimental conditions, we could not detect a significant change in Lte1 cortex localization in the absence of PtdIns(3,5)P_2 (**Figure 4.7**).

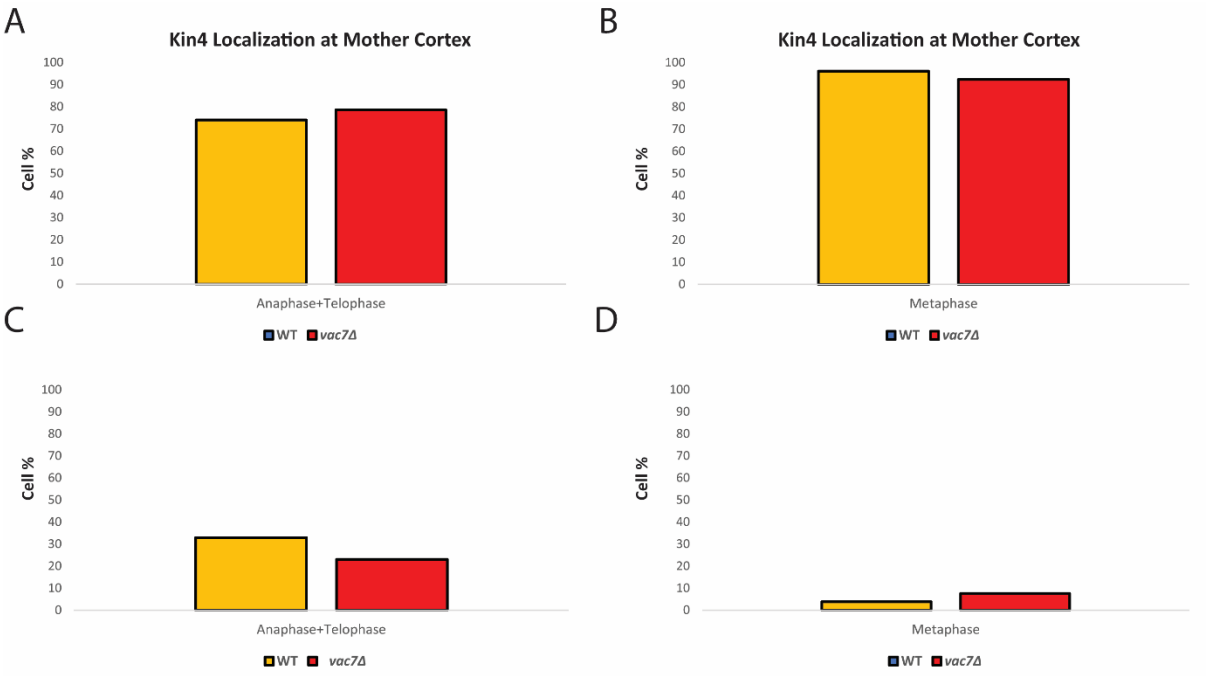

Figure 4.8 **Kin4 localization does not change in *vac7*Δ:** (A and C) Bar graph representing percentage of anaphase and telophase cells with Kin4 localization at mother cell cortex (in a) and bud neck in (c). Sample sizes for wt (BBY168-1) and mutant (BBY172-1) strain were 382 and 369 respectively. (B and D) Bar graph representing percentage of metaphase cells with Kin4 localization at mother cell cortex (in b) and bud neck in (d). Sample sizes for wt (BBY168-1) and mutant (BBY172-1) strain were 104 and 92 respectively. *KIN4* was tagged with GFP and SPB component Spc42 was tagged with eQFP. Mitotic cell phases were determined by the distance between Spc42-eQFP.

Next, we examined Kin4 localization. Kin4 is a protein kinase that inhibits MEN when mitotic spindles are misoriented (Pereira & Schiebel, 2005). In unperturbed mitosis, Kin4 is localized at mother cortex and it moves to bud neck when telophase occurs (D'Aquino et al., 2005). *vac7*Δ cells did have altered Kin4 localization in our experimental set up (**Figure 4.8**).

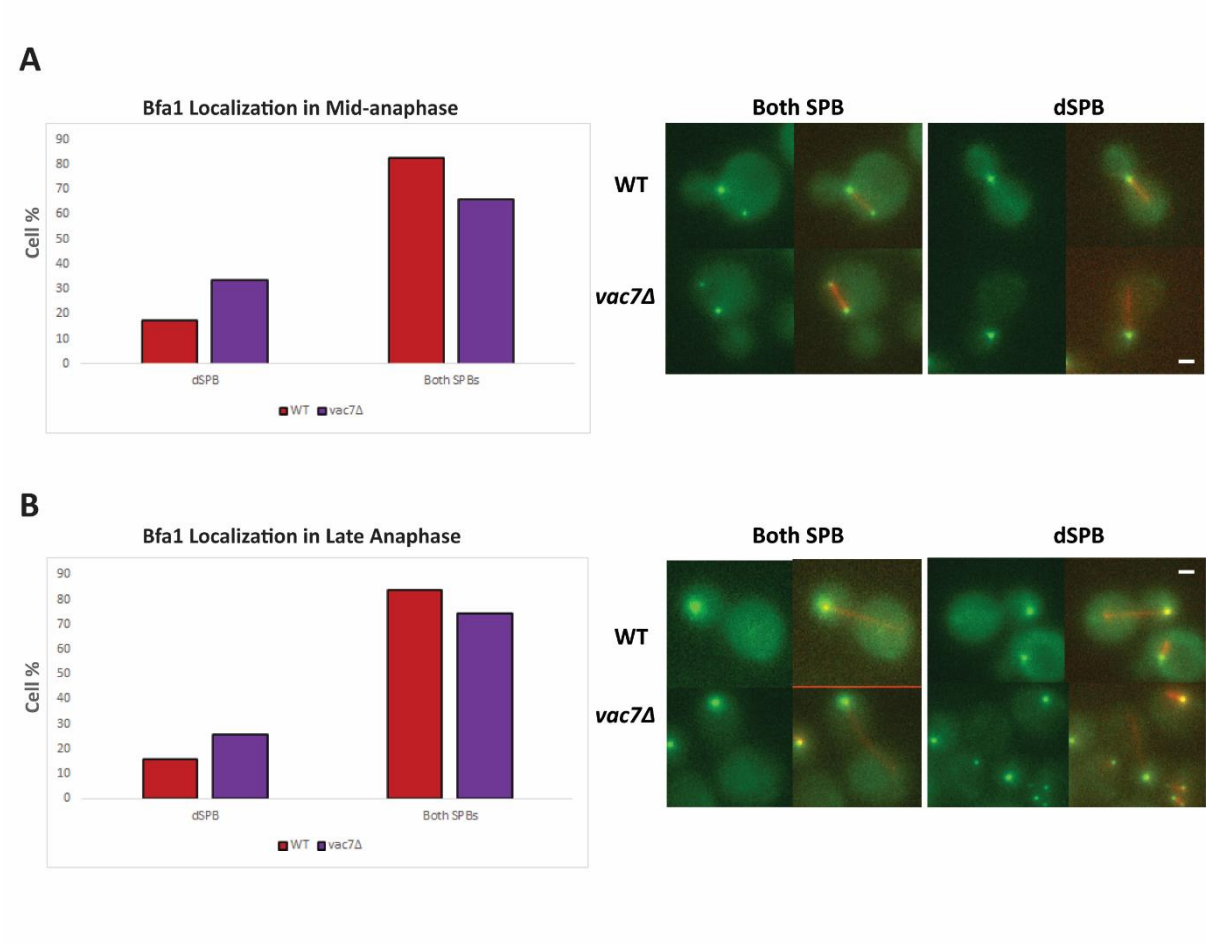

Figure 4.9 **Bfa1 localization is not dependent in *VAC7* activity:** (A) % of mid anaphase cells with Bfa1 at dSPB and both SPBs were calculated (left). Representative still images are shown (right) . (B) % of late anaphase cells with Bfa1 at dSPB and both SPBs were calculated. Scale bar is 2 µm.(WT=BBY164-1 and *vac7*Δ= BBY171-1) *BFA1* was tagged with GFP and cell phases were determined by length of tubulin tagged with mCherry3. Mid-anaphase and anaphase cells were selected and Bfa1 localization was investigated.

Bfa1 is a GTPase-activating protein which inhibits Tem1 by hydrolyzing GTP bound Tem1 in presence of mitotic spindle misorientation (Pereira et al., 2000). Bfa1 mainly localizes to the spindle pole body that moves to the daughter cell (dSPB) (Caydasi & Pereira, 2012). Bfa1 concentration at dSPB increases from metaphase to telophase but its concentration at mSPB decreases as mitotic cycle proceeds. Thus, Bfa1 localization at SPBs is asymmetric throughout mitosis. Bfa1 localization at the SPBs of *vac7*Δ cells was also asymmetric (**Figure 4.9**).

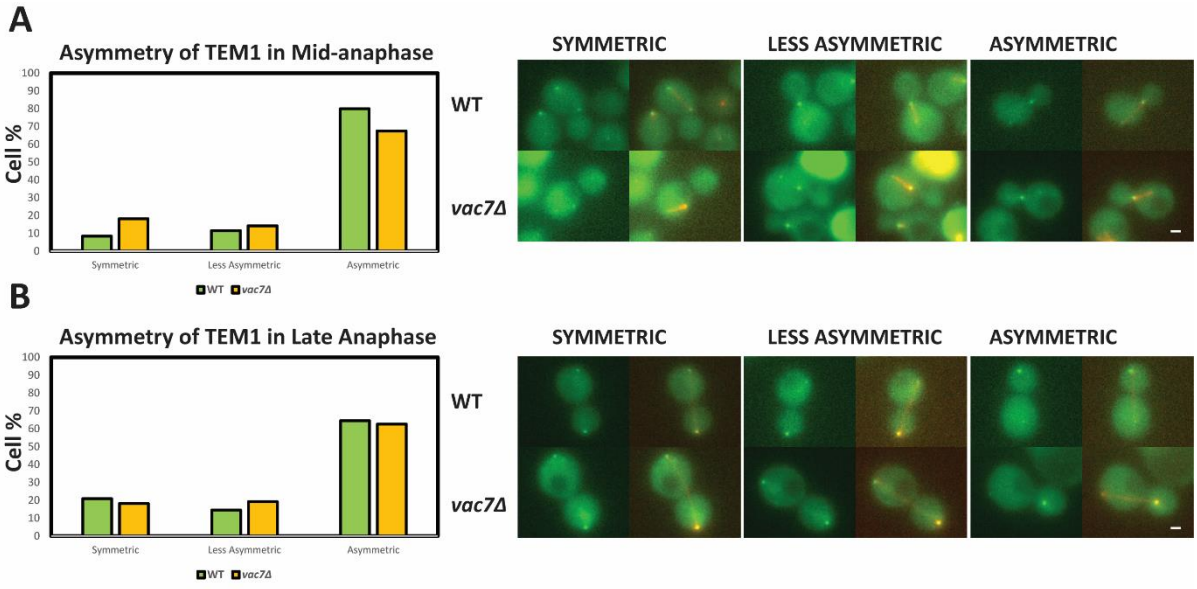

Figure 4.10 **Tem1 asymmetry does not alter in *vac7*Δ cells:** *TEM1* was tagged with GFP and SPB component *Spc42* was tagged with eQFP. In all phases, Tem1 was located at both SPBs and phases were determined by length of mitotic spindles. (A) % of mid-anaphase cells with symmetric, less asymmetric and asymmetric Tem1-GFP localization at SPBs. Less asymmetric cells have slightly but clear Tem1 signal intensity in mother cytoplasm than bud. Asymmetric ones show almost no signal in mother cell. Lte1 sample sizes are 95 and 77 for WT (BBY167-1) and *vac7*Δ (BBY170-1) respectively. On the right, representative still images showing each type of localization is shown. Scale bar is 2 µm. (B) % of late-anaphase cells with symmetric, less asymmetric, and asymmetric Tem1-GFP localization at SPBs. Sample sizes are 110 and 99 for WT and *vac7*Δ respectively. On the right, representative still images showing each type of localization is shown. Scale bar is 2 µm.

Tem1 is a GTP bound protein that initiates the Mitotic Exit Network (Shirayama, Matsui, & Toh, 1994). Tem1 localizes at the SPBs in an asymmetric manner. During cell cycle, Tem1 concentration at dSPB is higher than at mSPB in wild type cells (Caydasi & Pereira, 2012). Tem1 asymmetry at SPBs was not impaired in *vac7*Δ strain (**Figure 4.10**).

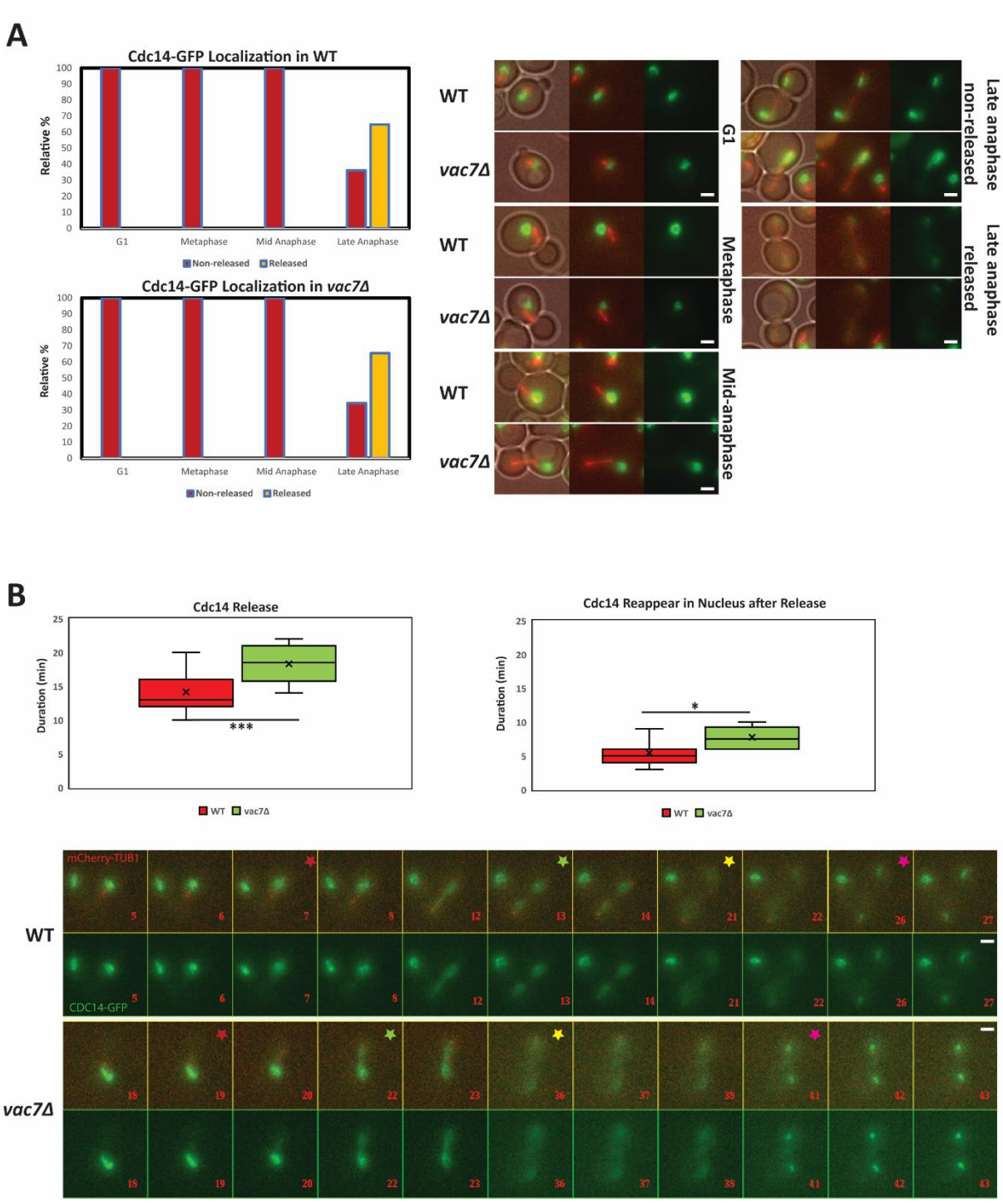

Figure 4.11 **Cdc14 localization is not affected by *VAC7* deficiency but regulated timely :** (A) Analysis of Cdc14 release in WT (DKY062) and *vac7*Δ (BBY174). *CDC14* was tagged with eGFP and phases were determined by length of mitotic spindle tagged with mChery3. Percentage of cells with Cdc14 released and Cdc14 non-released phenotypes were plotted. In wild type, sample size of anaphase cells is 174 whereas related size for *vac7*Δ cells sample size is 122. On the left, representative images of cells from different phases are shown. Scale bar is 2 µm. (B) On the top: box and whisker graphs showing timing of Cdc14 full release (left) and re-appearance back in the nucleus (right). Timing of Cdc14 release after MA transition in WT and mutant type were 14.13 and 18.3 min, in average, respectively. Re-appearance of Cdc14 in the nucleus are 11.3 and 16.2 min for WT and *vac7*Δ cells, in average, respectively. Sample size of WT and *vac7*Δ are 36 and 35, respectively. Scale bar is 2 µm. Two tailed students t-test was used for statistics.

p<0.05 *, p<0.01 **, p<0.001 *** according to t-test. In the bottom: Selected time points of representative WT and *vac7*Δ cells from timelapse analysis shown in A. Red asterisk shows MA transition while green one displays Cdc14 release into nucleus. Yellow asterisk indicates Cdc14 full release into cytoplasm and pink one refers reappear of Cdc14 in daughter and mother cell nucleoli.

Cdc14 is the most downstream component of the MEN pathway and it is a protein phosphatase (Culotti & Hartwell, 1971). Cdc14 normally localizes in the nucleolus and at anaphase onset upon activation of FEAR it is partially released into nucleus. Then after MEN pathway activation, Cdc14 fully release occurs and Cdc14 release into cytoplasm triggers exit from mitosis by inhibiting Cdc28 or dephosphorylating its substrates in budding yeast (Caydasi & Pereira, 2012; Visintin et al., 1998). We analyzed Cdc14 localization through time-lapse microscopy of Cdc14-GFP mCherry-Tub1 bearing WT and *vac7*Δ cells. For this reason, several cell cycle phases which are G_1, metaphase, mid-anaphase and late anaphase were selected. Which step a budding yeast goes through was determined by spindle length. Cdc14 localizes into nucleolus before anaphase. After the onset of anaphase, Cdc14 starts releasing into nucleus (FEAR dependent) and cytoplasm (MEN dependent). *VAC7* deletion had no effect on where Cdc14 releases or its localization at a given time (**Figure 4.11a**). We calculated the time that passes from MA transition until Cdc14 full release into the cytoplasm. We also calculated the time that passes from the time point of Cdc14 full release until Cdc14 reappearance in the nucleolus. *VAC7* deletion caused a delay in timing of both Cdc14 full release and Cdc14 re-appearance in the nucleolus (**Figure 4.11b**).

Chapter 5. Conclusion and Discussion

PtdIns(3,5)P_2 is a derivative of evolutionary conserved phosphoinositide, which is found on late endosomes and lysosomal membranes. It requires to perform essential processes such as membrane trafficking, stress response and vacuole/endolysosome structure. However, no studies attributed mitotic function to PtdIns(3,5)P_2 but Vac7 and Vac14 are positive regulators of PtdIns(3,5)P_2 synthesis. We asked whether PtdIns(3,5)P_2 plays a role in exit from mitosis. We applied genetic approach to detect possible genetic interactions with the MEN. We investigated anaphase and telophase events in WT and PtdIns(3,5)P_2 deficient cells. Also, cytokinesis events were observed.

Our results suggest a novel function for PtdIns(3,5)P_2 in timely mitotic exit and cytokinesis. First, impairment of PtdIns(3,5)P_2 synthesis by deletion of *VAC7* and *VAC14*, caused synthetic lethality in MEN-temperature sensitive mutants. Second, anaphase duration lasted longer in *vac7*Δ and *vac14*Δ cells than in wild type cells (**Figure 4.3**). Third, septin splitting was delayed and actomyosin ring constriction was slowed down in *vac7*Δ cells (**Figure 4.5-4.6**). We also showed that these defects observed in *vac7*Δ and *vac14*Δ cells were due to lack of Fab1 activity and hence PtdIns(3,5)P_2 because introduction of a hyperactive *FAB1* allele which was reported to rescue PtdIns(3,5)P_2 synthesis in these cells also rescued the mitotic exit and cytokinesis defects of *vac7*Δ cells. We thus concluded that PtdIns(3,5)P_2 plays a role in exit from mitosis and cytokinesis in budding yeast. In line with this conclusion, our results showed that Cdc14 release was delayed in *vac7*Δ cells. Thus, we think that the role of PtdIns(3,5)P_2 in mitotic exit is likely upstream of MEN pathway.

This conclusion brings us to another question: How does PtdIns(3,5)P_2 act in exit from mitosis and cytokinesis?. At the moment we failed to understand this. We analyzed localization of some MEN proteins to address this, but we could not detect any phenotype. We do not know whether PtdIns(3,5)P_2 directly regulates MEN or cytokinesis. Observed delay in septin splitting and actomyosin ring constriction is interesting, but it can also be indirect due to defects in MEN, as MEN activity is also important for them. Further experiments are required to address this question. PtdIns(3,5)P_2 may be influencing expression level of key mitotic exit related genes (i.e. MEN genes). PtdIns(3,5)P_2 may be

interacting and regulating the activity of key mitotic exit related proteins (i.e. MEN proteins). In the future, analyzing changes in gene expression and revealing cell cycle dependent interactors of PtdIns(3,5)P_2 may help understand the molecular basis of mitotic exit regulation by PtdIns(3,5)P_2.

Our data demonstrated that PAS complex has a role in exit from mitosis and cytokinesis. We think that this role is through PtdIns(3,5)P_2, as gaining of Fab1 activity in *vac7*Δ cells reverts the *vac7*Δ phenotype and there is no other molecular role defined for Fab1 other than PtdIns(3,5)P_2 synthesis. Nevertheless, more work is required to understand the molecular function of PtdIns(3,5)P_2 in mitotic exit.

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Chapter 7. TABLES

Table 7. 1: **List of strains**

Strain Name	Description	Reference
YPH499	MATa ura3-52 lys2-801amber ade2-101ochre trp1Δ63 his3Δ200 leu2Δ1.	Sikorski and Hieter,1989 "A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae"
ESM356-1	(Spore 2.1) MATa ura3-52 leu2Δ1 his3Δ200 trp1Δ63	Pereira et al., 2001 "Modes of spindle pole body inheritance and segregation of the Bfa1p-Bub2p checkpoint protein complex."
ESM1249-1	YPH499 *tem1-3*.	E. Schiebel. "Funktionelle und molekularbiologische Analyse von Far9, einem neuen Mitoseregulator"
ESM1276-1	YPH499 *cdc14-2*.	E. Schiebel. "Funktionelle und molekularbiologische Analyse von Far9, einem neuen Mitoseregulator"
ESM1278-1	YPH499 *cdc15-1*	E. Schiebel. "Funktionelle und molekularbiologische Analyse von Far9, einem neuen Mitoseregulator"
CVY16-1	YPH499 *cdc14-1*.	E. Schiebel. "Funktionelle und molekularbiologische Analyse von Far9, einem neuen Mitoseregulator"
ESM1309-1	YPH499 *dbf2-2*.	E. Schiebel. "Funktionelle und molekularbiologische Analyse von Far9, einem neuen Mitoseregulator"
ESM1361-1	YPH499 *mob1-67*.	E. Schiebel. "Funktionelle und molekularbiologische Analyse von Far9, einem neuen Mitoseregulator"
SHM295-2	ESM356-1 transform pAFS125-GFP-TUB1 to integrate into URA3 locus.	E. Schiebel. "Funktionelle und molekularbiologische Analyse von Far9, einem neuen Mitoseregulator"
BBY007-1	CVY16-1 (YPH499 *cdc14-1*). *vac7Δ::klTRP3*	Made in this work
BBY008-1	ESM1249-1 (YPH499 *tem1-3*.) *vac7Δ::klTRP3*	Made in this work
BBY059-1	YPH499 *vac14Δ::klTRP3*	Made in this work
BBY010-1	ESM1309-1 (YPH499 *dbf2-2*.) *vac14Δ::klTRP3*	Made in this work

BBY011-1	ESM1276-1 (YPH499 *cdc14-2*) *vac14Δ::klTRP3*	Made in this work
BBY012-1	ESM1249-1 (YPH499 *tem1-3.*) *vac14Δ::klTRP3*	Made in this work
BBY013-1	CVY16-1 (YPH499 *cdc14-1.*) *vac14Δ::klTRP3*	Made in this work
BBY014-1	YPH499 *vac7Δ::klTRP3*	Made in this work
BBY015	ESM1276 (YPH499 *cdc14-2.) vac7Δ::klTRP3*	Made in this work
BBY016	ESM1309 (YPH499 *dbf2-2.*) *vac7Δ::klTRP3*	Made in this work
BBY023-1	SHM295 *vac14Δ::klTRP3*	Made in this work
BBY024-1	SHM295 *vac7Δ::klTRP3*	Made in this work
BBY027-1	ESM1278 *vac7Δ::klTRP3*	Made in this work
BBY028-1	ESM1278 *vac14Δ::klTRP3*	Made in this work
BBY030-1	ESM1361 *vac14Δ::klTRP3*	Made in this work
BBY032-1	ESM1361 *vac7Δ::klTRP3*	Made in this work
DKY073-1	CLY169 *pCL33*	Made in our lab
BBY065-1	*BBY024-1 SHS1-Chery3-kan*	Made in this work
BBY076-1	SHM295 *pSE002-FAB1 hyperactive-LEU*	Made in this work
BBY077-1	SHM295 *pRS315-LEU*	Made in this work
BBY078	BBY24-1 *pSE002-FAB1 hyperactive-LEU*	Made in this work
BBY079	BBY24-1 *pRS315-LEU*	Made in this work
BBY090	BBY014 pSE002-FAB1-LEU	Made in this work
BBY091	BBY014 pRS315-LEU	Made in this work
BBY092-1	YPH499 Mob1-3HA -TRP	Made in this work
BBY093	BBY007-1 pSE002-FAB1	Made in this work
BBY094	BBY015-1 pSE002-FAB1	Made in this work
BBY095	BBY016 pSE002-FAB1	Made in this work
BBY096	BBY008-1 pSE002-FAB1	Made in this work
BBY097	BBY027 pSE002-FAB1	Made in this work
BBY098	BBY032 pSE002-FAB1	Made in this work
BBY099	BBY007-1 pRS315-LEU	Made in this work
BBY100	BBY015-1 pRS315-LEU	Made in this work
BBY101	BBY016 pRS315-LEU	Made in this work
BBY102	BBY008-1 pRS315-LEU	Made in this work
BBY103	BBY027 pRS315-LEU	Made in this work
BBY104	BBY032 pRS315-LEU	Made in this work
BBY117-1	DKY073 *vac7Δ::TRP*	Made in this work
BBY118	ESM1361 *pRS315-LEU*	Made in this work
BBY119	ESM1361 *pSE002-FAB1 hyperactive*	Made in this work
BBY120	CVY16-1 *pRS315-LEU*	Made in this work
BBY121	CVY16-1 *pSE002-FAB1*	Made in this work

	hyperactive	
BBY122	ESM1249 *pRS315-LEU*	Made in this work
BBY123	ESM1249 *pSE002-FAB1 hyperactive*	Made in this work
BBY124	YPH499 *pRS315-LEU*	Made in this work
BBY125	YPH499 *pSE002-FAB1hyperactive*	Made in this work
BBY126	ESM1278 *pRS315-LEU*	Made in this work
BBY127	ESM1278 *pSE002-FAB1hyperactive*	Made in this work
BBY128	ESM1309 *pRS315-LEU*	Made in this work
BBY129	ESM1309 *pSE002-FAB1*	Made in this work
BBY130	ESM1276 *pRS315-LEUhyperactive*	Made in this work
BBY131	ESM1276 *pSE002-FAB1hyperactive*	Made in this work
BBY132	DKY073 *vac14Δ::TRP*	Made in this work
BBY134-1	SHM295 *MYO1-eGFP-HIS.*	Made in this work
BBY137	BBY065 *MYO1-eGFP-HIS.*	Made in this work
BBY138	BBY064 *MYO1-eGFP-HIS*	Made in this work
BBY130	ESM1276 *pRS315-LEU*	Made in this work
BBY131	ESM1276 *pSE002-FAB1 hyperactive*	Made in this work
BBY144-1	BBY087-1 *vac7Δ::TRP*	Made in this work
BBY145-1	BBY089 *vac7Δ::TRP*	Made in this work
BBY146	BBY106 *vac7Δ::TRP*	Made in this work
BBY152-1	BBY107 *vac7Δ::HIS*	Made in this work
BBY159	BBY065 *pRS315-LEU*	Made in this work
BBY160	BBY065 *pSE002-FAB1 hyperactive*	Made in this work
BBY161	BBY064 *pRS315-LEU*	Made in this work
BBY162	BBY064 *pSE002-FAB1 hyperactive*	Made in this work
BBY164-1	GPY1053 *mCherry-TUB1-* URA3.	Made in this work
BBY165-1	GPY1054 *mCherry-TUB1-* URA3.	Made in this work
BBY167-1	GPY1052 *mCherry-TUB1 pAK011*	Made in this work
BBY168-1	GPY1033 *mCherry-TUB1 pAK011*	Made in this work
BBY169-1	BBY154 *mCherry-TUB1 pAK011*	Made in this work
BBY170-1	BBY143 *mCherry-TUB1 pAK011*	Made in this work
BBY171-1	BBY142 *mCherry-TUB1 pAK011*	Made in this work
BBY172-1	BBY141 *mCherry-TUB1*	Made in this work

BBY174	DKY062 *vac7Δ:HIS-eGFP*	Made in this work

Table 7. 2: **Plasmids' description**

Plasmid Name	**Description**	**References**
pRS316	URA3-dependent CEN-based yeast-E.coli shuffle plasmid.	Sikorski and Hieter,(1989) "A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae"
pAFS125-GFP-TUB1	URA;TUB1 promoter-GFP-TUB1	A. Murray (1996) "GFP tagging of budding yeast chromosomes reveals that protein-protein interactions can mediate sister chromatid cohesion
pYM3	6HA-kITRP1 (pFA6a-kanMX4)	M. Knop (1999) "Epitope Tagging of Yeast Genes using a PCR-based Strategy: More Tags and Improved Practical Routines
pYM25	yeGFP-hphNT1	M. Knop (1999) "Epitope Tagging of Yeast Genes using a PCR-based Strategy: More Tags and Improved Practical Routines
Cherry3 with spacer	pFA6a-Spacer-3Cherry-KanMX6	M. Knop (1999) "Epitope Tagging of Yeast Genes using a PCR-based Strategy: More Tags and Improved Practical Routines
pAK011	pRS306-mCherry-TUB1.	Khmelinskii et al., (2007) "Cdc14-regulated midzone assembly controls anaphase B"

pSE002	Prs305- FAB1-T2250A,E1822V,F1833L	Work of Şeyma Nur Erkan from Caydasi Lab.
pYM2	3HA-His3MX6 (pYM1)	M. Knop (1999) "Epitope Tagging of Yeast Genes using a PCR-based Strategy: More Tags and Improved Practical Routines
pKS133	pFA6a-hphNT1	Janke et al. (2004) "A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes
pRS315	LEU2-dependent CEN-based yeast-E.coli shuffle plasmid..	Sikorski and Hieter,(1989) "A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae"

Table 7. 3: **Index of primers**

Bfa1-S1	TGCGTACGCTGCAGGTCGACAGAAAAAAGTTCGAAAACTTTTGTAGCAGTTGAAAGTTTTGTATGCTA
Bfa1-S2	TGTACTCAAGATAACGGTAAAGAAACAGTTATAAGAAGGCTAAAGGGCTAATCGATGAATTCGAGCTCG
Bfa1-S3	AATCCTATATGTATGAAATCAGGAACATGGTAATCAATTCGACAAAAGATCGTACGCTGCAGGTCGAC
BFA1-1	GTGATTCAAATGTTGGGTGAGC
BFA1-2	CGAGTTTTAATCCATTCCACCC
S1-Kin4	TCGCATAATATTCTATCAGGACATTCCGTATACCTGAATATATATACATGCGTACGCTGCAGGTCGAC
S2-Kin4	CACTCTATAATATAATGTAATTGTCGATATAACTATGTACTGAAAACTCAATCGATGAATTCGAGCTCG

S3-Kin4	CAACAGCAAGAAAGGTTCTGAATTTTTTCAAAAGAAGGAGCAT GAGGGTTCGTACGCTGCAGGTCGAC
Kin4-Col1	AATCGCATCGGTGGGAGAG
Kin4-Col2	AACAACTTGCCAGCCAAGAC
MOB1-S2	GCATGGAAGAATACAACCTACAAGCAGACTTATATAAATATAC AATACTAATCGATGAATTCGAGCTCG
MOB1-S3	CGGCTGATTTTGGTCCGCTGTTAGAATTAGTGATGGAGTTGAGG GATAGGCGTACGCTGCAGGTCGAC
DBF2-S2	AAGCTAATTATATCGCGGCGAATGCAAGACAAGAATTCATTTTT ACGCTAATCGATGAATTCGAGCTCG
DBF2-S3	GCATCTTATTCAACGGACTGGAACACTCAGACCCCTTTTCAACC TTTTACCGTACGCTGCAGGTCGAC
CDC14-S2	TAAGTTTTTTTATTATATGATATATATATATATATAAAAATGAAAT AAATTAATCGATGAATTCGAGCTCG
CDC14-S3	CTACAAGCGCCGCCGGTGGTATAAGAAAAATAAGTGGCTCCAT CAAGAAACGTACGCTGCAGGTCGAC
CDC15-S2	GCTGTATTATTCTCTATATATGTATGTATGCACATGCAATTCCTA CATTAATCGATGAATTCGAGCTCG
CDC15-S3	CCAAAGATAAAAGTGACGGCTTTTCCGTCCCCATTACAACATTT CAAACACGTACGCTGCAGGTCGAC
Lte1-10	GAAGCATGAATTAAGATCATCG
LTE1-11	CCGTTGTAGTTATGTACTGAAG
Lte1-s1	TTGCTTTACATAGCCTCTAGCCATTCAAAGATTCGCTACCCTGA ACCATGcgtacgctgcaggtcgac
Ite1-s2	TTTTTTACAAGAGGAATTTGGGAACTGGAGGAAAGTGGCACAA TACCTCAatcgatgaattcgagctcg
VAC14-2	TACGCAGATCCGTCGCGA

VAC14-1	GTCCTATCAGCAGCTAACG
VAC14-S2	CAGGTCCATTTCTTAACCAAAGATGCTTTCAATCAGGTAATGGGTAGTTAATCGATGAATTCGAGCTCG
VAC14-S1	TTGATGCTGCTGTGCTTATCTGCTCAGGCTACAACAGGAACTGGAACATGCGTACGCTGCAGGTCGAC
VAC7-2	ACTATCGCTTCCGCTACTA
VAC7-1	TGTAAGTCTTCCTGGCCAC
VAC7-S2	AAAAAGAAAAATACCCAGCTTTGACGAAAAAGCTACATTCTTAACACTCAATCGATGAATTCGAGCTCG
VAC7-S1	TTATCGTTTCATCTCAGGCAAGTTAAAGCATTTGGGAAACGTGCTAGATGCGTACGCTGCAGGTCGAC
TEM1-S2	TAACGCCAACTCTCACATGGTAGCAGGCGGGTATAGTTGTTTTCAATCGATGAATTCGAGCTCG
TEM1-S3	TCCTCGTCGCCCTCATCTAAGGCACCATCGCCGGGCGTTAATACACGTACGCTGCAGGTCGAC