The cytokinetic inhibitors Boi1 and Boi2 are required for activation of the exocyst complex by Rho GTPases

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Abstract

Cell growth and division requires delivery of new membrane and remodelling factors to the cell surface. In budding yeast, this involves actin-dependent transport of secretory vesicles to sites of growth, followed by vesicle fusion to the plasma membrane. Rho-type GTPases regulate actin polarization and vesicle fusion, but how they signal to diverse effectors controlling these separate processes is not well understood. Here, we show that the cortical proteins Boi1 and Boi2 work together with Rho GTPase signalling to regulate the exocyst, a complex that mediates the tethering of secretory vesicles to the plasma membrane. Cells lacking both Boi proteins show normal actin polarity but are defective in bud emergence, bud growth, and fusion of Bgl2-containing vesicles to the plasma membrane. A gain-of-function version of Exo70, a component of the exocyst and effector of Cdc42 and Rho3, restores growth of *boi1 boi2* mutant cells. Furthermore, hyperactivation of Rho-GTPase signalling rescues *boi* defects, suggesting that the essential function of Boi proteins is to mediate Rho-dependent activation of the exocyst during cell growth. Finally, we find that Boi1 and Boi2-dependent inhibition of abscission in cells with chromatin bridges, via their function in the NoCut checkpoint, is bypassed by expression of gain-of-function *EXO70*, suggesting the NoCut pathway also involves regulation of the exocyst.

Resum

Tant el creixement com la divisió cel·lular requereix el transport de membrana i altres factors a la superfície cel·lular. En cèl·lules de *S. cerevisiae*, aquests processos necessiten el transport de vesícules de secreció a través dels cables d'actina fins a les zones de creixement actiu, on es fusionen. Les Rho GTPases regulen la polarització de l'actina i la fusió de vesícules, però es desconeix com les GTPases senyalitzen els diversos efectors i com regulen els dos tipus de funcions.

En aquest estudi, demostrem que les proteines Boi1 i Boi2 treballen conjuntament amb la senyalització de les Rho GTPases, per tal de regular la funció del complexe "exocyst" que media els contactes inicials entre vesícules de secreció i la membrana plasmàtica. Cèl·lules sense Boi1/2 tenen el citoesquelet d'actina polaritzat, però la cèl·lula filla no pot emergir ni créixer. Un al·lel d'Exo70, una subunitat de l'"exocyst", que és efector de les GTPases Rho3 i Cdc42, restaura el creixement de cel·lules defectuoses en la funció de Boi1/2. A més a més, l'hiperactivació de GTPases rescata defectes dels mutants de Boi1/2, suggerint que la funció essencial de Boi1 i Boi2 és promoure l'activació de l'"exocyst", depenent de Rho, durant el creixement cel·lular.

Finalment, hem demostrat que la inibició de la divisió cel·lular que controlen via NoCut els efectors Boi1/2 en cèl·lules amb defectes en la segregació de cromosomes, es rescata amb l'al·lel d'Exo70 descrit anteriorment. Aquestes observacions suggereixen que NoCut podria funcionar també a través de la regulació de l'"exocyst".

Preface

In order to achieve faithful division, cells must make sure chromosomes are fully segregated before mother and daughter physically divide. Premature cytokinesis before clearance of chromosome arms from the cleavage plane could lead to DNA damage. Indeed, delays in chromosome segregation lead to corresponding delays in the completion of cytokinesis. In budding yeast, these delays depend on Aurora-B kinase and the cortical proteins Boi1 and Boi2. Inactivation of these proteins results in DNA damage after cytokinesis. These observations led to the proposal that a cytokinesis checkpoint, known as NoCut, coordinates cytokinesis with chromosome segregation to protect genome integrity (Norden et al. 2006; Mendoza et al. 2009).

How the NoCut effectors Boi1 and Boi2 regulate cytokinesis is not known. However, these proteins are also determinants of cell polarity (Bender, Lo, H. Lee, Kokojan, J. Peterson, et al. 1996a; Matsui et al. 1996). Because delivery of new material and specific cargoes to the plasma membrane and the extracellular space during budding and during cytokinesis are essential for cell viability, one possibility would be that Boi1 and Boi2 could link those two processes: polarized growth and cytokinesis. In this study I investigated what is the molecular function of Boi1 and Boi2 proteins in the context of bud growth and cytokinesis.

Contents

1. Introduction

In this introduction, I will summarize the current knowledge about the mechanisms of cell growth and cytokinesis and how these processes are coordinated with the cell cycle. Because the model organism used for this study is the budding yeast *Saccharomyces cerevisiae*, the particularities and differences to the rest of eukaryotic cells will be commented as well.

1.1. The cell cycle in eukaryotes

Cell reproduction is a fundamental feature of all living organisms. It occurs by an elaborate series of events called the cell cycle, governed

by a complex network of proteins. All cells arise by division of existing cells, and every cell living today is thought to be descended from a single ancestral cell that lived billions of years ago. Throughout all this time, evolution of cells and organisms has depended on the transmission of genetic information by cell division (Morgan 2007).

1.1.1. Events of the eukaryotic cell cycle

The division cycle of most cells consists of four coordinated processes; cell growth, DNA replication, distribution of the duplicated chromosomes to daughter cells, and cell division. However, the timing of those processes differs in different cell types. In bacteria, cell growth and DNA replication take place throughout most of the cell cycle, and duplicated chromosomes are distributed to daughter cells in association with the plasma membrane. Eukaryotic cells undergo a more complex cell cycle that is divided in four discrete steps defined by chromosomal events: G1 (growth or gap phase 1), S (synthesis phase), G2 (gap phase 2) and M (Mitosis) (Fig. 1.1) (Morgan 2007). There is another kind of cell division, meiosis, used by diploid organisms in order to generate haploid progeny. In that case, there is one S-phase followed by two rounds of division.

During G1 phase, high rates of protein synthesis increase cell size and mass. This phase is followed by duplication of the DNA content and the MTOCs (centrosomes or SPBs) during S phase. Proteins known as helicases open the DNA double helix at replication origins, exposing it

to DNA polymerases that will faithfully duplicate the chromosomes generating sister chromatids. The duration of the second growth phase G2 is very variable and in some species, like *S. cerevisiae*, almost completely absent. Gap phases also serve as important regulatory transitions, in which progression to the next cell-cycle stage can be controlled by different signals. Finally, during M phase there are two major events; nuclear division (mitosis) and cell division (cytokinesis). The period from the end of one M phase to the beginning of the next is called interphase (Figure 1.1).

During mitosis, sister chromatids are pulled apart from each other by the mitotic spindle to form two independent nuclei. In most plants and animal cells, in which chromosomes are easily visualized, this process can be subdivided in four steps depending on the chromosomal state: prophase, metaphase, anaphase and telophase. At the onset of prophase, sister chromatids start condensing and the spindles start to form as the centrioles move to opposite poles and microtubules begin to polymerize from the centrosomes. Next, spindle microtubules coming from opposite poles attach to the kinetocores of the sister chromatids forming a bipolar structure. At this point, cells reach metaphase. During anaphase, sister chromatid cohesion is lost by cleavage of the protein complex that hold them together, cohesin, allowing the spindle to pull them apart. At the same time, the mitotic spindle elongates resulting in the complete segregation of the chromatids to opposite poles of the cell. Mitosis ends in telophase, when chromatin is packed into two identical

daughter nuclei and the spindle disassembles. (Reviewed in (Morgan 2007)).

Following mitosis, cells physically separate by a process called cytokinesis (see (Glotzer 2005) for review). New plasma membrane, and cell wall in some cases, is deposited at a position that bisects the long axis of the mitotic spindle, ensuring that the daughter nuclei are distributed into the two cells. In most cell types, there is an acto-myosin ring (AMR) that contracts at the site of division guiding the deposition of new membrane material (Bi et al. 1998; Guertin et al. 2002).

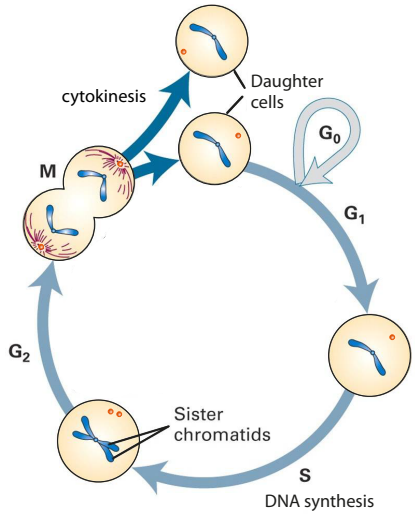

Figure 1.1. The events of the eukaryotic cell cycle. The central events of cell reproduction are chromosome duplication during S-phase, followed by chromosome segregation and division during M phase. G1 is the gap phase between S and M and G2 is the gap between M and S. (Lodish 2008)

The cell cycle machinery is well conserved among all eukaryotes. Budding and fission yeast have been powerful tools for the analysis of cell-cycle and other fundamental biological processes. In particular, the isolation of yeast mutants with perturbed cell cycle control phenotypes helped identify genes involved in the sequential execution of cell cycle phase transitions (Hartwell et al. 1974; Nurse et al. 1976). As a model organism, yeasts have some advantages such as their simplicity, powerful genetics, the existence of highly optimized cell synchronization protocols and the fact that they have high rate of homologous recombination, which allows fast and easy introduction of genetic modifications. One important difference between *S. cerevisiae* and *S. pombe* compared to animal and plant cells is that they undergo a closed mitosis: the nuclear envelope does not break while mitosis takes place. Instead, the nuclear envelope is stretched as chromosome segregation takes place and it is divided as the actomyosin ring contracts (Botstein et al. 1997).

1.1.2. The cell cycle control system

The cell cycle control system is a complex assembly of oscillating protein kinase activities that determines the order and timing of the cell cycle events. Early studies in yeasts and *X. laevis* oocytes lead to the identification of the genes responsible for cell cycle progression (Hartwell et al. 1974; Nurse 1975; Gautier et al. 1988; Nurse et al. 1976).

The main components of the cell-cycle control system are the cyclin-dependent kinases (Cdk) which phosphorylate and modulate the activity of several proteins that govern cell-cycle processes. For example, an increase of Cdk activity at the beginning of S-phase causes phosphorylation-dependent activation of proteins that will replicate the DNA such as Sld2 and Sld3 (Tanaka, Umemori, et al. 2007b; Tanaka, Tak, et al. 2007a). Expression levels of Cdk are constant through the cycle, however, its enzymatic activity oscillates depending on cyclin proteins, that are expressed and degraded at particular cell cycle stages (Evans et al. 1983; Morgan 1997). There are three main types of cyclins; G1/S-, S-, and M-cyclin. Different cdk-cyclin complexes are formed during the cycle, and those complexes have different targets that will drive consecutive cell cycle events (Figure 1.2).

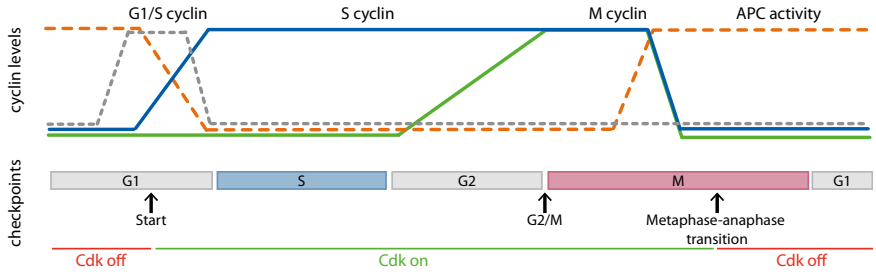

Figure 1.2. Schematic view of the cell cycle regulation. Oscillation of cyclin levels during the cell cycle progression is represented at the top. At the bottom checkpoint-controlled transitions are represented as arrows. Adapted from (Morgan 2007).

In order to ensure faithful cell division, transitions through the cell cycle are tightly regulated as mistakes that occur during the cell cycle can have severe consequences. For instance, errors in the chromosomes attachment to the mitotic spindle can result in aneuploidies, which are linked to the onset of cancer in higher eukaryotes (Musacchio & Salmon 2007). There are signaling pathways called "cell cycle checkpoints" that guarantee that cell cycle events occur in the right order (Hartwell & Weinert 1989). Thus, if an error is detected, a checkpoint will become active preventing progression to the next phase and activating mechanisms in order to repair the errors. There are three main checkpoints that monitor accurate completion of each cell cycle phase: the G1 checkpoint or Start, the G2/M transition and the metaphase to anaphase transition (Figure 1.2).

1.1.3. Chromosome segregation in Budding yeast

The transition from metaphase to anaphase leads to sister-chromatid segregation, mitotic exit and cytokinesis. First, the spindle assembly checkpoint (SAC) ensures that all the chromosome kinetochores are properly attached to the metaphase spindle. The SAC components, like Mad2, bind to kinetochores that are not attached to microtubules (Eytan et al. 2008), and inhibit the anaphase-promoting complex (APC) (Schwab et al. 2001; Passmore et al. 2003; Vodermaier et al. 2003). The APC is a complex that poliubuquitinates proteins to send them to degradation by the proteasome. It has two major substrates; on one hand, Securin (Yamamoto et al. 1996), the inhibitor of Separase that will

cleave the subunit Scc1 of the cohesin complex, allowing chromosome segregation (Uhlmann et al. 1999; Hornig et al. 2002) (Figure 1.3). On the other hand, the APC polyubiquitinates M-cyclins, resulting in the inactivation of Cdk activity. Thus, APC activation results into two outcomes: chromosome segragation by degrading securin and cytokinesis by degrading M-cyclins and allowing exit from mitosis. If those two processes are not coordinated, the cytokinetic machinery would create chromosome cuts with severe consequences for the cell. For that reason, the NoCut checkpoint delays cytokinesis in cells with of chromatin bridges or midzone defects (see section 1.2.5.)

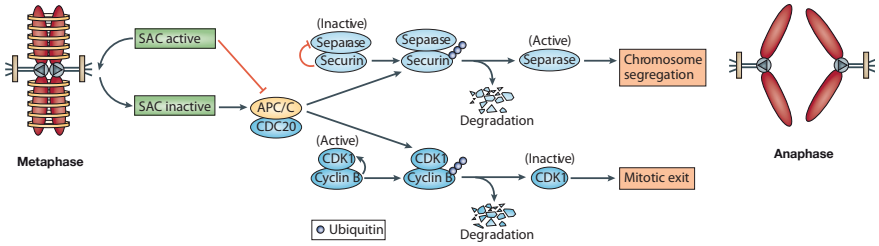

Figure 1.3. Main events during the metaphase to anaphase transition. The Anaphase promoting complex (APC) has two main substrates; securin, that is repressing Separase, that once active will eliminate sister-chromatid cohesion, and S- and M-cyclins that will inactivate Cdk activity allowing completion of mitosis and cytokinesis. Adapted from (Yanagida 2009; Marston & Amon 2004).

After chromosome segregation occurs, in order for the cells to exit mitosis, Cdk-Clb2 phosphorylations are reversed by the Cdc14 phosphatase (Visintin et al. 1998). Cdc14 is sequestered at the

nucleolus by Net1 until the onset of anaphase and it is released in two waves. In early anaphase it is released for a short period of time into the nucleus and the cytosol. This transient change of localization is induced by the Cdc14 early anaphase release (FEAR) pathway. Complete activation and relocation of Cdc14 to the cytosol results in exit of mitosis and requires the Mitotic Exit Network (MEN) pathway. It is important that Cdc14 releases on time because some of its targets are components of the actomyosin ring and the cytokinetic machinery (like the chitin synthase Chs2 or the formins Bni1 and Bnr1) (Chin et al. 2012; Bloom et al. 2011). Therefore, Cdc14 activity is essential to promote cytokinesis.

1.2. Budding yeast Cytokinesis

Efficient cell proliferation requires accurate splitting of the dividing cell into two independent individuals. When mitosis is complete, the cell cycle ends with the partition of the cytoplasm by cytokinesis (Guertin et al. 2002; Glotzer 2005). Despite from a microscopic perspective cytokinesis appears distinct in different organisms, the molecular mechanisms are conserved in fundamental organization through all cell types. In this section I will describe central events of cell separation, as well as the mechanisms that drive this process.

1.2.1. Mechanisms and regulation

In most eukaryotes, cytokinesis depends on two basic mechanisms; the contraction of an actomyosin ring (AMR) that is attached to the inner leaflet of the plasma membrane at the site of division, and the addition of new membranes that will increase the surface area (Finger & White 2002). In addition, yeast cells need to form a septum between mother and daughter.

In budding yeast, after ring contraction, there is targeted vesicle fusion at the site of division that allows deposition of the a specialized chitin structure, the primary septum (PS) (Cid et al. 1995) and presumably membrane expansion too. Following primary septum deposition, a secondary septum (SS) made of glucans and mannans, is assembled at both sides of the primary septum (Fig. 1.9). After cytokinesis, the primary septum and part of the secondary septum are degraded by enzymes from the daughter side (like Eng1), leading to cell separation (Kuranda & Robbins 1991; Baladrón et al. 2002). As remnant of this process, a bud scar is left on the mother cell wall.

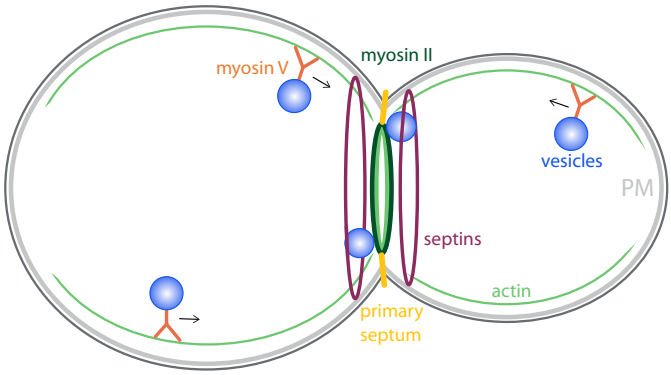

Figure 1.9. Schematic view of cytokinesis in *S.cerevisiae*. Actomyosin ring contraction is coupled to primary septum formation through vesicle fusion. Adapted from (Bi & Park 2012).

1.2.1.1. Actomyosin ring assembly and disassembly

The main components of the ring are the filamentous protein actin and the motor myosin II, which form contractile bundles that will invaginate the plasma membrane forming the cleavage furrow. In budding yeast, there are other six types of protein required for assembly of a functional actomyosin ring; septins, myosin II, IQGAP, Rho1, formins and tropomyosin.

Septins are a family of small GTPase proteins that form filaments, and are essential for cytokinesis (Hartwell 1971). Septins form a ring at the future bud site in late G1 and act as a scaffold to recruit all the other ring components (Bi et al. 1998; Lippincott & Li 1998). The septin ring broadens forming an hourglass structure at the bud neck until telophase when it splits into two cortical rings flanking the AMR (Cid

et al. 2001; Dobbelaere & Barral 2004). Septins are thought to play a second role at the bud neck, the split rings may provide a diffusion barrier to retain diffusible factors (Dobbelaere & Barral 2004). There is some data suggesting that this barrier is not essential for cytokinesis. As seen in *cdc10*Δ and *bud4*Δ, AMR assembly and constriction, the localization of membrane-trafficking, cytokinesis, and cell-wall-septum formation all occur efficiently, yet septin split rings do not form (Wloka et al. 2011).

Myo1, the budding yeast myosin II heavy chain, forms a two-headed structure and is arranged into higher-order filaments, similar to myosin II filaments in animal cells. Myo1 is targeted to the contractile ring through interaction with Bni5 (P. R. Lee et al. 2002), a septin-binding protein, and later on with its essential light chain Mlc1 and the IQGAP protein Iqg1 (Epp & Chant 1997; Lippincott & Li 1998). Mlc1 is also a light chain for Myo1 and it is essential for Iqg1 localization to the division site. In turn, Iqg1 is required for actin ring assembly (Epp & Chant 1997; Shannon & Li 2000; Boyne et al. 2000). Several studies reported that unlike fission yeast and animal cells, the actomyosin ring is not essential for cell viability of *S. cerevisiae* cells (Watts et al. 1987; Rodriguez & Paterson 1990; Bi et al. 1998). However, others have found that the actomyosin ring is crucial for successful cytokinesis, but, that in the absence of myosin II function, new cytokinetic mechanisms can evolve through genetic changes that can bypass Myo1 function. Actually, it has been demonstrated that in *myo1*Δ cells there are whole chromosome aneuploidies that change

gene expression patterns and result in formation of a remedial septum (Watts et al. 1987; Tolliday et al. 2003; Rancati et al. 2008).

Rho1, formins and tropomyosin are also involved in actin ring assembly. The formins Bni1 and Bnr1 are essential for nucleation of actin filaments both during polarized bud growth and cytokinesis (Moseley & Goode 2006; Park & Bi 2007). Their localization is partially dependent on the mitotic exit phosphatase Cdc14 that drives Bnr1 disappearance and localization of Bni1 at the neck (Bloom et al. 2011). The RhoA homolog in yeast, Rho1 promotes actomyosin ring formation through regulation of Bni1 (Tolliday et al. 2002). Rho1 activity during this process is, in turn, controlled mainly by the Polo-like kinase Cdc5 that regulate its GEFs Tus1 and Rom2 (Yoshida et al. 2006). In animal cells, RhoA acts in cytokinesis by regulating AMR assembly (Van Aelst & D'Souza-Schorey 1997).

The behavior of the actomyosin ring in budding yeast is very similar to the one of animal cells. However, there are some important differences like the timing of assembly. In budding yeast the ring assembly starts at late G1 by the localization of type II myosin, Myo1, at the future bud site, and it remains there until late anaphase, when filamentous actin is recruited to the ring to form a functional contractile structure (Pringle et al. 1995; Drubin & W. J. Nelson 1996; Bi et al. 1998; Lippincott & Li 1998). In contrast, F-actin and myosin II in animal cells localize at the cleavage site determined by the position of the mitotic spindle (Satterwhite & Pollard 1992).

How the contractile ring constricts is not fully understood, but contraction seems to be coupled to its disassembly. It seems not to function as the sarcomere in skeletal muscle, with actin and myosin II filaments sliding past each other (Schroeder 1968; Balasubramanian et al. 2004; Eggert et al. 2006). To date, some observations suggest that actin filament depolymerization and the motor domain of Myo1 play a major role in AMR contraction and disassembly. By using actin stabilizing drugs and cofilin mutants, it was observed that AMR disassembly slowed down (Mendes Pinto et al. 2012). An allele of MYO1 lacking the entire head domain is able to assemble an AMR however, is clearly defective in ring disassembly (Lord et al. 2005; Fang et al. 2010; Straight et al. 2003). Also, there is a cell cycle-regulated mechanism of ring disassembly that works through the APC-dependent degradation of Iqg1 (Ko et al. 2007; Tully et al. 2009).

1.2.1.2. Membrane trafficking and cytokinesis

In order to complete cell division, insertion of new membrane is required at the division site in all kind of organisms; animals, fungi and specially plants. It has long been known that targeted secretion plays an essential role in division of plant cells (SHARP 1943; WHALEY & MOLLENHAUER 1963; Jürgens 2005). These cells form a specialized structure derived from the anaphase spindle, the phragmoplast, that serves as a docking platform for vesicle delivery (D. Zhang et al. 1993; Lambert 1993). The vesicles, then, fuse at the center of the two daughter cells building the cell plate that grows

outwards reaching the plasma membrane and separating them (Samuels et al. 1995; Strompen et al. 2002).

In animal cells, there is evidence suggesting that vesicle fusion of Golgi-derived secretory vesicles and recycling endosomes mediate several aspects of cytokinesis. The plasma membrane Soluble N-ethylmaleimide attachment protein receptor (t-SNARE) Syntaxin1, which mediate the actual fusion step of vesicles to the plasma emembrane, is required for cell division in *D. melanogaster*, sea urchin, and *C. elegans embryos*. Syntaxin mutants have defective cytokinesis and as a result cells become multinucleated (Burgess et al. 1997; Jantsch-Plunger & Glotzer 1999; Conner & Wessel 1999; Lecuit & Wieschaus 2000). Also, injection of fruit fly's embryos with either the microtubule depolimerizing drug Colcemid, or the Golgi inhibitor Brefeldin A (BFA), blocks the export of newly synthesized proteins from the Golgi to the furrow, and membrane invagination (Lecuit & Wieschaus 2000; Sisson et al. 2000). These data suggest that the membrane addition for furrowing in animal cells is mediated by vesicle fusion events.

From experiments with *C. elegans* embryos, it seems that membrane fusion is also required to seal off the plasma membrane at the division site. BFA-treated *C. elegans* embryos were blocked in cytokinesis at a late stage, after furrowing (Skop et al. 2001). Also, mammalian cells show an accumulation of exocytic mid-zone membrane accumulations at both sides of the midbody, that then becomes asymmetric and upon

fusion, the connection between the two daughter cells disappears (Gromley et al. 2005; Goss & Toomre 2008).

In budding yeast, the growth machinery is repolarized towards the division site at the onset of telophase. Exocytic vesicles are transported to the actomyosin ring through actin cables. This opens the possibility that in yeast, as in animal cells, it is important to add new membrane in order to expand the surface and to seal the connection between the two daughter cells. Moreover, cargoes are delivered to do specific functions and promote abscission. One of the relevant cargoes delivered to the bud neck that are important for cytokinesis is Chs2, the chitin synthase II. This enzyme is essential for primary septum formation (Sburlati & Cabib 1986; Shaw et al. 1991; VerPlank & Li 2005) and requires the exocyst complex for its normal localization. The exocyst is a multiprotein complex required for vesicle tethering at the plasma membrane. Most of its subunits localize to the surface of secretory vesicles (Boyd et al. 2004) (see section 1.3.4.). Once vesicles arrive at the division site, they are likely captured by the AMR. This mechanism would ensure that surface expansion at the division site is coupled with AMR constriction and septum deposition in time and space. At the plasma membrane, Sso1 and Sso2 t-SNARE proteins act subsequently mediating fusion. Cells lacking Sso1/2 function, accumulate secretory vesicles around the division site as observed by electron microscopy.(Jäntti et al. 2002).

In addition, membrane trafficking is important for the insertion of specific lipids at the division site that may be required for localization of the cytokinetic machinery or to alter membrane curvature. In animal cells, disturbing the Phosphoinositides (PIs) metabolism cause defects in cytokinesis (Forer & Sillers 1987; Becchetti & Whitaker 1997; Sagona et al. 2010; Jianhua Zhang et al. 2010; Montagnac & Chavrier 2010; Echard 2012). In yeast, Rho1 localization during abscission has been shown to be dependent on PI 4,5-bisphosphate (PI(4,5)P2) (Yoshida et al. 2009). This suggests that the lipid composition at the plasma membrane is essential for efficient cytokinesis.

In *myo1*Δ mutants, which do not form a proper AMR, secretory vesicles are delivered to the bud neck where they fuse with the plasma membrane between the split septin rings, as they form a remedial septum. The bud neck of yeast haploid cells is about 1μm in diameter (Bi et al. 1998; Lippincott & Li 1998), whereas animal cells are usually over 15-20μm (Schroeder 1972; Carvalho et al. 2009). Since animal and fungal cytokinesis have similar duration (<20 min) and their post-Golgi vesicles are similar in size (80-100nm in diameter) (Novick et al. 1980; Lehman et al. 1988; Rambourg et al. 1989), a large difference in gap dimension at the division site suggests that different degrees of vesicle fusions might be required to seal the division site (Fang et al. 2010). In budding yeast, only around 50 post-Golgi vesicles would be enough to fill both sides of the neck, and perhaps this explains why

AMR assembly defects can be presumably bypassed by multiple vesicle fusion events, resulting in a remedial septum. This hypothesis is consistent with electron microscopy images showing thick and abnormal multiple setpa in *myo1*Δ yeast cells (Rodriguez & Paterson 1990; Schmidt et al. 2002; Tolliday et al. 2003). In contrast, in animal cells, AMR constriction is presumably required to reduce the membrane demand during cytokinesis, which explains why cytokinesis does not take place if there is no AMR contraction (Straight et al. 2003).

1.2.2. Septum formation

1.2.2.1. Primary septum deposition

During budding yeast cytokinesis, the primary septum separates mother and bud at the same time that the AMR contracts. The PS is mainly made of chitin, a polymer N-acetylglucosamine that is synthesized in situ by the chitin synthase II (Chs2) (Sburlati & Cabib 1986; Shaw et al. 1991).

Chs2 is expressed in early mitosis (Chuang & Schekman 1996), and its activity is cell cycle-regulated. In order to ensure that the chitin synthase is not active until the cell exits mitosis, Chs2 is retained at the endoplasmic reticulum (ER) through a Cdk1 phosphorylation (Teh et al. 2009), and it is not until it is dephosphorylated by the phosphatase Cdc14 in late anaphase, that Chs2 enters the secretory pathway and is

delivered at the bud neck (Chuang & Schekman 1996; VerPlank & Li 2005; G. Zhang et al. 2006; Chin et al. 2012). Interestingly, there is evidence suggesting another level of regulation of Chs2 activity; it reaches the division site in its inactive form, and is activated in situ by interacting with Inn1 and Cyk3 (Nishihama et al. 2009; Devrekanli et al. 2012). Inn1 is essential for Chs2 function but not for its localization (Nishihama et al. 2009), and a dominant CHS2 allele suppresses cytokinetic defects of Inn1 and Cyk3 mutants, suggesting that it acts upstream of Inn1 and Cyk3 (Devrekanli et al. 2012).

Although Chs2 was found to be essential for viability, specifically during PS formation (Sburlati & Cabib 1986; Shaw et al. 1991), as for *myo1*Δ mutants, there is some controversy concerning its essentiality. Viable *chs2*Δ strains have been isolated, suggesting that these cells accumulated suppressor mutations accounting for their viability (Bulawa & Osmond 1990). In *chs2*Δ cells, which show abnormal septa, the AMR is assembled but contracts asymmetrically (Bi 2001; Schmidt et al. 2002; VerPlank & Li 2005). This results support the idea of mutual dependency between the PS and the AMR function.

Deletion of CHS2 and the chitin synthase III (CHS3) together causes cell lethality with cytokinesis arrest. It is thought that in the absence of Chs2, Chs3 promotes the assembly of a misshaped "remedial" septum that allows cell viability (Cabib & Schmidt 2003). Thus, Chs2 function is thightly regulated, and intimately connected with actomyosin ring

contraction and polarization of membrane trafficking to the division site (Bi 2001; Schmidt et al. 2002; Oh et al. 2012).

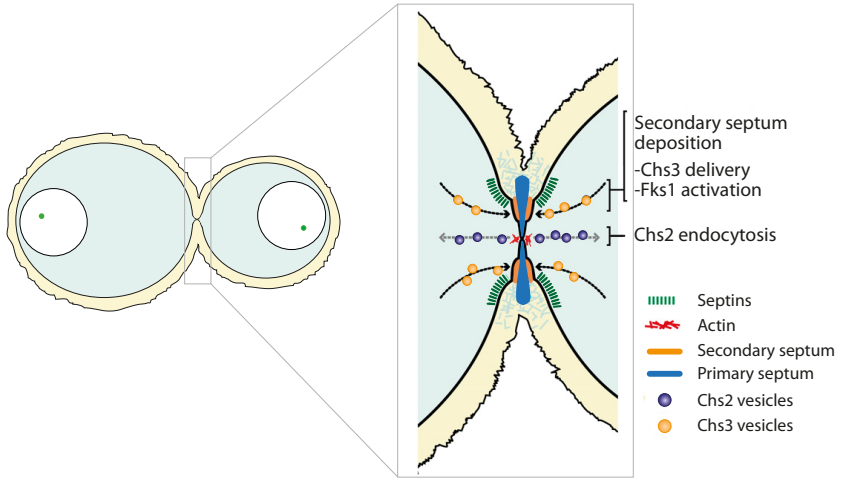

Figure 1.10. Septum deposition at the mother-daughter bud neck. At the same time that the AMR is disassembled (red), the primary septum (blue) is completed and Chs2 is internalized by endocytic vesicles (purple circles). Deposition of the secondary septum (orange) by Fks1 and Chs3 (orange circles), which is delivered by exocytic vesicles along with other material, occurs on both sides of the primary septum. Adapted from (Weiss 2012).

1.2.2.2. Secondary septum deposition

Following deposition of the primary septum, new material is added on either side forming a secondary septum, so that subsequent digestion of the primary septum leads to cell separation. The secondary septum is mainly made of β1–3 glucan, and it is synthesized by the Fks1 glucan synthase (Cabib et al. 2001; Lesage et al. 2004; Lesage & Bussey 2006). The chitin synthase Chs3 also localizes to the site of secondary

septum formation, providing additional structural reinforcement with chitin (Ziman et al. 1998; Cabib et al. 2001; Schmidt et al. 2002; Cabib & Schmidt 2003). Delivery of other wall components by secretion is probably important, as there is a strong concentration of exocytic machinery in this site (Dobbelaere & Barral 2004; G. Zhang et al. 2006). For example, the exocyst component Sec3 acts in septation parallel to actomyosin ring contraction, suggesting that delivery of material through the secretory system promotes secondary septum formation (Dobbelaere & Barral 2004). Also, cells defective in the exocyst subunit Sec10 show abnormal Chs2 localization and defective cytokinesis (VerPlank & Li 2005).

Secondary septum deposition is a very robust process and it takes place even in the absence of either primary septum synthesis or actomyosin ring contraction. This occurs via synthesis of the "remedial septum" mentioned before, which is essentially a secondary septum deposited at the bud neck in a way that is disorganized yet sufficient to separate mother and daughter cells (Cabib & Schmidt 2003). In fact, cells that lack chitin synthase activity, rapidly acquire suppressor mutations that compensate by deposition of other polymers in this remedial structure (Schmidt 2004).

In addition to AMR constriction and targeted membrane deposition, remodeling of the extracellular matrix (ECM) which might be analogous to septation in yeast is also a hallmark of animal cytokinesis(Bi & Park 2012). Defective glycosaminglycan synthesis

cause embryonic lethality and cytokinetic arrest in *C. elegans* and mice (Mizuguchi et al. 2003; Izumikawa et al. 2010).

1.2.3. Cell separation

In order to complete cell separation, the PS and part of the SS are degraded by an endochitinase (Cts1) (Kuranda & Robbins 1991) and glucanases (Eng1 and Egt2) (Kovacech et al. 1996; Baladrón et al. 2002) from the daughter side (Lesage & Bussey 2006). The asymmetric localization and activation of Ace2, which is the daughter cell specific factor that control the expression of the Cts1 and Eng1 (King & Butler 1998; O'Conallain et al. 1999; Colman-Lerner et al. 2001) depends on the RAM pathway, a conserved signaling network involved in polarized cell growth and cell separation (Colman-Lerner et al. 2001; B. Nelson et al. 2003; Mazanka et al. 2008). The main regulator of the RAM pathway is the kinase Cbk1, which activation depends on the mitotic exit network (MEN) (Brace et al. 2011). Direct Cbk1 phosphorylation activates Ace2, which initiates and maintains the asymmetric localization of Ace2 in the daughter nucleus (Mazanka et al. 2008). After cell separation, Ace2 is translocated to the cytoplasm by CDK phosphorylation and remains there until the next division (Mazanka & Weiss 2010).

1.2.4. GTPases in yeast cytokinesis

Rho family GTPases are critical for eukaryotic cytokinesis, and concentration of active Rho at the cell division site with proper timing is important for normal progression (Y. Nishimura et al. 1998; Bement et al. 2005; Piekny et al. 2005).

Rho1

The budding yeast homolog of the RhoA GTPase, Rho1, plays is essential for spatial organization of cell growth and membrane trafficking. During cytokinesis, Rho1 localizes at the division site and is required for actomyosin ring assembly. Specifically, it triggers formin-mediated actin cable assembly (Qadota et al. 1996; Kohno et al. 1996; Yoshida et al. 2009). In addition, it has been recently shown that Rho1 is inhibited by Cyk3 during primary septum formation, and it is reactivated afterwards to promote secondary septum deposition (Onishi et al. 2013). This suggests that Rho1 is essential for secondary septum formation. In animal cells, the active form of RhoA binds to the Rho-associated protein kinase (ROCK) through which it regulates myosin II contractility.

Cdc42

Cdc42 has several functions in actin organization, membrane trafficking and it is considered the master regulator of cell polarity in

budding yeast (Johnson & Pringle 1990; Adams et al. 1990). In G1, Cdc42 is essential for actin polarization and septin assembly (Johnson & Pringle 1990; Gladfelter et al. 2002; Caviston et al. 2003). Later in the cycle, during secondary septum deposition and abscission, Cdc42 is inactive, predominantly bound to GDP (Onishi et al. 2013; Atkins et al. 2013). Recent reports show that Cdc42 must be inactivated during cytokinesis. Overexpression of the Cdc42 GAPs RGA1 and BEM3 suppressed *cyk3*Δ *hof1*Δ mutants, suggesting that inactivation of Cdc42 is sufficient to suppress deffects in PS formation by inducing SS formation (Onishi et al. 2013). Also, the polo-like protein kinase, Cdc5, regulates Cdc42 inactivation by phosphorylating Bem2 and Bem3, which are GTPase-activating proteins for Cdc42. Failure to down-regulate Cdc42 (in *bem2-84* mutants) during mitotic exit impairs the normal localization of key cytokinesis regulators, Iqg1 and Inn1, at the division site, and results in an abnormal septum (Atkins et al. 2013). In conclusion, both genetic data and the phenotype of bem2-ts mutants suggests that inactivation of Cdc42 is essential for proper localization of proteins important for actomyosin ring constriction and secondary septum assembly to the division site.

Rho3

In *S. pombe*, lack of Rho3 causes accumulation of vesicle-like structures. Also, at higher temperatures, results in a cell arrest with two or more nuclei separated by uncleaved septa (H. Wang et al. 2003). As this phenotype is very similar to an exocyst loss of function, it has

been proposed that Rho3 modulates exocyst function during cytokinesis (H. Wang et al. 2002). In budding yeast, it is not clear whether Rho3 has a function during cytokinesis. Overexpression of RGD1, which encodes a GAP for Rho3 and Rho4 (Prouzet-Mauleon et al. 2008) suppresses lethality of *chs2*Δ, *iqg1*Δ and *hof*Δ *cyk3*Δ mutants, suggesting that these proteins may also have a role in cytokinesis (Onishi et al. 2013). In addition, Tos2, an anchor protein that localizes at the bud tip and bud neck, regulates Rho3 activity through, Rgd1 (Claret et al. 2011). However it is not known what is the specific function at the neck. Tos2 mutants are viable and grow with normal morphology, but overexpression, in contrast, cause aberrant Cdc3 septin localization, defective Hof1 recruitment and, as a result, cytokinetic defects.

Rho4

Rho4 has redundant function with Rho3 during polarized cell growth, as RHO4 overexpression can rescue viability of rho3Δ cells (Matsui & Toh-E 1992). It localizes at the division site in late anaphase, where might regulate Bnr1 interaction with Hof1, as by yeast two hybrid it was observed that Bnr1 and Hof1 interaction depends on Rho4-GTP (Kamei et al. 1998). Also, an interesting observation is that expression of GTP-locked Rho4 results in an increase of large and round cells (30%) with a very broad bud neck indicating possible defects in cytokinesis (Fernandes et al. 2006).

1.2.5. The NoCut checkpoint

In budding yeast and HeLa cells, a pathway called NoCut delays abscission when chromosomal segregation is defective (Norden et al. 2006; Steigemann & Gerlich 2009). After anaphase onset, sister chromatids are pulled apart from each other to opposite poles of the cells, and it is not until the cleavage site is clear of chromatin that the membranes are resolved. In animal cells, spindle-midzone defects cause cytokinetic furrow regression, which results in the formation of binucleated cells (Glotzer 2005; Guertin et al. 2002; McCollum 2004). In yeast, spindle midzone-defective cells like *ase1*Δ, which break the spindle prematurely, show an increased time of abscission measured as the time between contraction and resolution of the membranes at the bud neck. Cells with contracted membranes were not able to separate even in the presence on the enzyme zymolyase that degrades the cell wall, suggesting that the defect in cell separation was in membrane resolution and not related to cell wall defects (Norden et al. 2006). Inactivation of kinetochore proteins with functions at the anaphase spindle midzone (Ndc10, Ndc80) showed defects in abscission too. Because cytokinesis defects were not observed in other kinetochore mutants, the abscission phenotypes of *ndc10* and *ndc80* mutants have been attributed to their function in stabilizing the spindle midzone.

Interestingly, cells with intact spindle midzone but that were unable to separate their chromosomes, by expressing an uncleavable version of the cohesin subunit Scc1 (scc1RRDD) showed delays in cytokinesis

dependent on the NoCut effectors Boi1 and Boi2. A similar example are topoisomerase II mutants, which have chromosomal bridges in late anaphase caused by catenations that are unable to resolve, but have an intact midzone, and show increased percentage of large budded cells with contracted membranes. These observations showed that chromosome segregation defects cause a delay in membrane resolution at the bud neck (Mendoza et al. 2009). Because the cytokinesis defects in cells with midzone and chromosome segregation defects were similar in nature, and were suppressed by mutations in the same genes (see below) it was proposed that the primary defect in both types of mutants was the same: namely, a failure in the removal of chromosome arms away from the mid-spindle region.

The mechanism(s) that inhibits abscission in cells with midzone and/or chromosome segregation defects is not fully understood. However, a number of proteins have been identified that are required for this inhibition, which together define the NoCut pathway. By inactivating the Aurora kinase (Ipl1 in yeast) or by preventing its localization to the spindle midzone, abscsission is restored in mutants with midzone or chromosome segregation defects. This suggests that Aurora regulates this response (Norden et al. 2006; Mendoza et al. 2009). Ipl1 is part of the chromosome passenger complex (CPC), and together with its regulator INCENP/Sli15, it localizes on the spindle midzone during anaphase. The fact that the histone acetyltransferase component of the ADA complex Ahc1, is also required for *ase1*Δ abscission defect, raised the possibility that acetylated chromatin is

directly involved in NoCut signaling, perhaps by activating Ipl1. Moreover, forcing Ipl1-chromatin interactions triggered a delay in cytokinesis, suggesting that Ipl1 would be the sensor of the pathway activating a signaling cascade (Mendoza et al. 2009).

In animal cells, Aurora B also mediate abscission delays caused by chromosome segregation defetcs (Steigemann et al. 2009). This mechanism depends on Aurora-B-mediated phosphorylation of CHMP4C, a component of the endosomal sorting complex required for transport (ESCRT) that mediates abscission (Carlton et al. 2012). In the presence of chromatin bridges, ANCHR (Abscission/NoCut Checkpoint Regulator; ZFYVE19) together with CHMP4C, associates with VPS4, the most downstream component of the ESCRT machinery, at the midbody. After inactivation of Aurora B signaling, VPS4-ANCHR complex dissociates allowing VPS4 to relocate to the abscission site (Thoresen et al. 2014). Thus, NoCut delays are controlled through retention of VPS4 at the midbody ring by ANCHR and CHMP4C.

In addition to Aurora-B and Ahc1, the Boi1 and Boi2 proteins are also required for abscission inhibition in cells with midzone and chromosome segregation defects. Boi1 and Boi2 are functionally redundant scaffold proteins with very similar structure, the main features being a pleckstrin-homology (PH) and SRC homology 3 (SH3) domain (Bender, Lo, H. Lee, Kokojan, J. Peterson, et al. 1996a; Matsui et al. 1996). Boi1 and Boi2 localize at the bud cortex during

interphase and transfer to the bud neck during cytokinesis. Localization of Boi1-GFP and Boi2-GFP was found to be dependent on Ipl1 activity (Norden et al. 2006). Abscission delays caused by midzone or chromosome segregation defects also depend on Boi1/2 function as deletion of both Boi1 and Boi2 rescues abscission defects observed in *ase1*Δ, *top2-4 and ndc10-1* cells. Their function is important for the maintenance of genetic stability. Triple mutants *ase1 boi1 boi2* show an increase in the number of DNA damage foci after cytokinesis. The number of foci is reduced in septin mutants (*cdc12-6*), which do not complete division, suggesting that the DNA breaks are caused by the cytokinetic machinery. However, it is important to mention that *boi1*Δ *boi2*Δ mutants have already a higher percentage of cells showing DNA damage foci than wild-type, suggesting that boi mutants are genetically unstable but the reason is not clear.

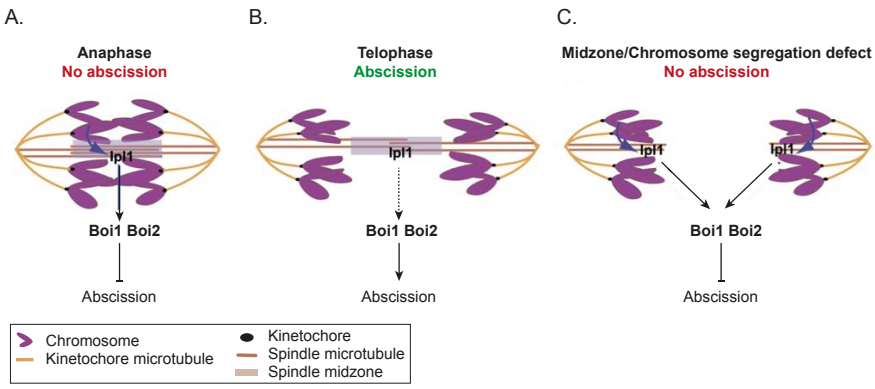

Figure 1.4. The NoCut pathway. (A) During anaphase, Ipl1 kinase is at the midzone and senses the presence of segregating chromosomes. (B) Once the midzone is clear of chromosomes, Ipl1 does not signal Boi1/2 anymore and abscission proceeds. (C) In the presence of midzone or chromosome segregation

defects, Ipl1 is in contact with the chromatin signaling Boi1/2 which inhibit abscission.. Adapted from (Norden et al. 2006)

All together, the evidence described above suggested that the Boi1/2 proteins are effectors of the NoCut pathway (Norden et al. 2006; Mendoza et al. 2009). A model was proposed whereby during chromosome segregation, Ipl1 sits on the spindle midzone and sends a signal to Boi1/2 proteins that during cytokinesis localize at the bud neck and inhibit abscission. Once the midzone is clear of chromatin, the checkpoint signal is off and abscission proceeds. In midzone or chromosome segregation defective cells, the chromatin is in contact with the spindle midzone and Ipl1 for longer, thus prolonging the signaling of Aurora to Boi proteins that delay abscission in order to prevent DNA cuts.

Several questions remain however open. Among those, the mechanism by which Boi1 and Boi2 inhibit abscission was not clear. My work is focused on the Boi1 and Boi2 mechanism of function during cytokinesis and during interphase. From our results on Boi1/2 function during interphase, we know that Boi1 and Boi2 are essential for cell growth (see section 1.3.6). For this reason I will give an introduction to this topic in the next chapter.

1.3. Polarized cell growth

Maintenance of cell size requires doubling of cell mass in each cycle by synthesis de novo of new proteins, membranes and organelles. As it has been seen for cell division, cell growth is controlled by a combination of intrinsic and extrinsic signals, like external nutrients in yeast or growth factors produced by other cells in animals. In order to coordinate growth and division, the signalling pathways governing cell growth are generally linked in some way to those controlling cell division. At the same time, cell polarity is crucial for asymmetric cell divisions, a developmental mechanism that is required for cell fate determination and *S.cerevisiae* normal division.

1.3.1. Polarized cell growth in eukaryotic cells

Cell polarity is a common feature of eukaryotic cells and it refers to spatial differences in shape, structure or function (Figure 1.4). One example is epithelial tissue initiation, which occurs during development and involves congression of mesenchymal cells into aggregates that differentiate to form polarized apical–basal cell monolayers (W. J. Nelson 2009). Perturbations in cell polarity contribute to a number of tissue pathologies like lissencephaly, a brain formation pathology (Valiente & Marín 2010).

The budding yeast *Saccharomyces cerevisiae* undergoes asymmetric cell division and provides an excellent model system to study cell polarity.

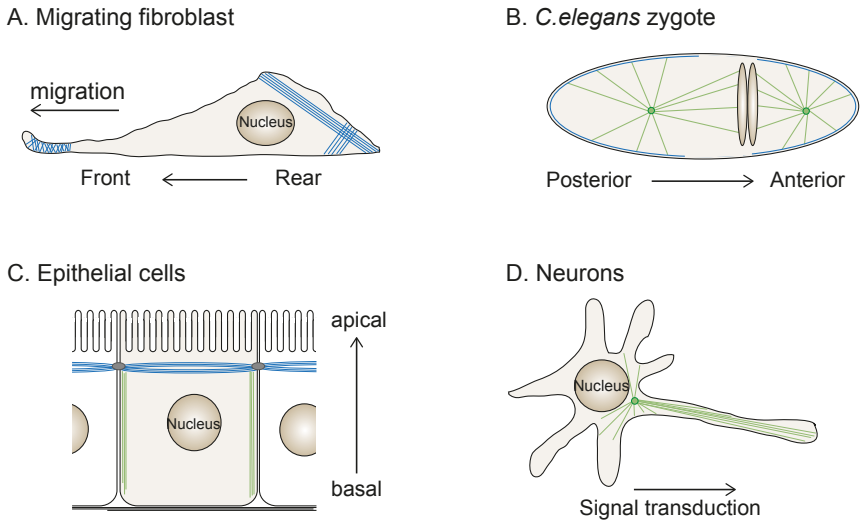

Figure 1.4. Different examples of polarized cell types and functions. (A) In migrating fibroblasts, the actin cytoskeleton promotes extension of the leading edge and retraction of the rear of the cell. (B) In the *C. elegans* zygote, the first division is asymmetric. The microtubule system is polarized, leading to an asymmetric positioning of the mitotic spindle. (C) In differentiated epithelial cells, the entire cell organization is polarized and allows the segregation of apical and baso-lateral proteins and membrane domains separated by tight junctions. (D) Polarity is essential in neurons for long range communication. Adapted from (Etienne-Manneville 2004)

The main mechanism by which polarized cell growth takes place is by exocytosis. Directed exocytosis of vesicles allows the secretion of intracellular proteins such as hormones, neurotransmitters or enzymes

outside of the cell as well as the incorporation of membrane, proteins and lipids to specific domains of the plasma membrane.

1.3.2. Budding yeast actin cytoskeleton polarizes growth

The yeast actin cytoskeleton guides growth by directing the delivery of secretory vesicles and other factors towards the plasma membrane (Bretscher et al. 1994; Finger et al. 1998). Actin is found in two states; monomeric (G-actin) and filamentous (F-actin). The transition between these two states is regulated by different factors like nucleotide hydrolysis, ions, and a large number of actin binding proteins. F-actin forms long polarized chains called microfilaments, which are arranged in a two-stranded helix.

In budding yeast, the actin cytoskeleton undergoes periodic changes in polarization during the cell cycle, and as a consequence the areas of exocytic vesicle deposition change depending on the cell cycle stage (Adams & Pringle 1984; Lew & Reed 1995b; Amberg 1998). These changes in the actin cytoskeleton are mainly controlled by Rho GTPase function. Localization of the Rho GTPase Cdc42, is essential for the overall polarity state of these cells. The septin ring, which is assembled in G1, also undergoes periodic changes over the cycle (Figure 1.5).

In G1, actin cables are randomly distributed all around the cytoplasm, resulting in uniform vesicle delivery, isotropic growth, and round cell morphology. When cells enter into S phase, the actin cytoskeleton

becomes polarized, and exocytic vesicle fusion takes place exclusively at the future bud site. After bud emergence, growth becomes apical and is restricted to the daughter cell. Then, in order to form a spherical bud, growth becomes isotropic. In mitosis, the actin cytoskeleton reorients toward the mother-bud neck to allow cell division (Pruyne et al. 2004; Moseley & Goode 2006; Park & Bi 2007).

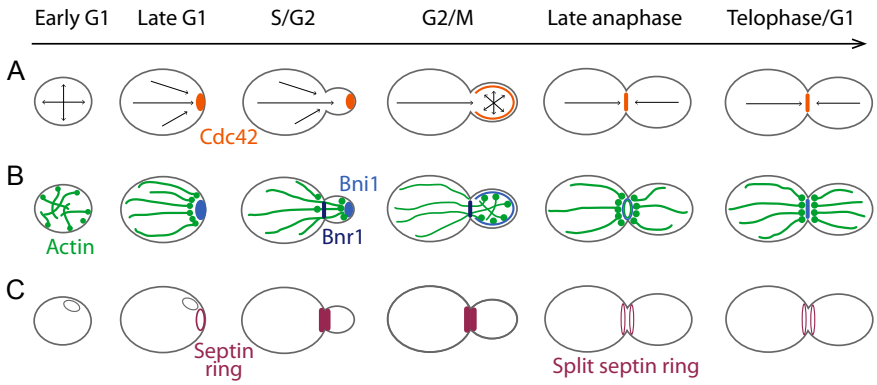

Figure 1.5. Polarized cell growth during the cell cycle. (A) Directionality of polarized growth (arrows) during the cell cycle and localization of Cdc42 (orange). (B) The actin cytoskeleton organization is depicted in green; actin patches (spots), cables (lines) and contractile ring (circle). During isotropic bud growth, actin cables nucleated by formins Bni1 (blue) and Bnr1 (dark blue), extend from the mother cell into a network in the bud guiding polarized exocytosis. (C) The septin ring forms a polarized patch that will direct apical growth. Upon bud emergence, the discrete septin ring is broadened acquiring the shape of an hourglass that later on splits in two by the activity of MEN. Adapted from (Park & Bi 2007).

Depolymerization of F actin by the monomer-sequestering drug latrunculin A or depolarization of the cytoskeleton by disrupting

certain protein functions, like *cdc24-4*, blocks polarized growth: mother cells arrest bud growth and grow isotropically into large round cells (Sloat et al. 1981; Ayscough et al. 1997). In contrast, mutations that hyperpolarize the actin cytoskeleton, like *cla4*Δ, generate highly elongated buds (Cvrcková et al. 1995).

Unlike budding yeast, animals and many fungi use microtubules to polarize growth. In animals, microtubules mediate long-range transport of membranous organelles to the cell periphery, whereas actin mediates short-range transport and anchorage (Brown 1999). In contrast, budding yeast microtubules regulate only nuclear and chromosomal movements. The reduced size of budding yeast cells may have made short-range actin-dependent transport sufficient for the purpose.

1.3.3. Establishment and maintenance of polarized cell growth in *S. cerevisiae*

Regulation of the overall polarity state in *S. cerevisiae* is under the coordinated control of cyclin-dependent protein kinases (Cdk) and Rho GTPases (Pruyne & Bretscher 2000). Feedback signals between these pathways allow yeast to switch between alternative stable patterns of growth.

The essential Rho GTPase Cdc42 is the master regulator of cell polarity. A key polarizing event is the recruitment of Cdc42 to growth sites on the plasma membrane, where the GTPase activates effectors that control the actin cytoskeleton and promotes vesicle fusion with the plasma membrane (Ziman et al. 1993; Adamo et al. 2001) (Fig. 1.5). Cdc42 is initially polarized to the presumptive bud site independently of the actin cytoskeleton. However, for bud emergence it is important that endocytosis occurs surrounding the exocytic sites to maintain polarization at one restricted area. Thus, F-actin affects Cdc42 distribution through its effects on exocytosis and endocytosis (Irazoqui et al. 2005; Jose et al. 2013).

Once Cdc42 is polarized, it interacts with all its downstream effectors: PAKs Ste20, Cla4, the formin Bni1, the Gic proteins Gic1 and Gic2, and the exocyst subunit Sec3 (Figure 1.6). These proteins organize septin assembly, the actin cytoskeleton, and vesicle secretion that lead to polarized cell growth .

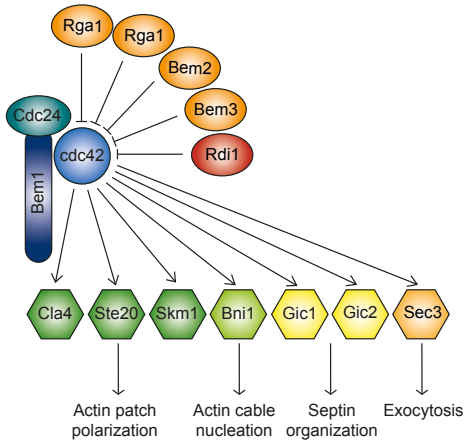

Figure 1.6. Cdc42 regulators and effectors. Schematic representation of GAPs, GEF, and targets of the master regulator of cell polarity in S. cerevisiae. Adapted from (Perez & Rincón 2010).

Like all Rho GTPases, Cdc42 signals to effectors only in the active GTP-bound state. The GTPase activity of cdc42 is stimulated by the GTPase activating proteins (GAPs) Bem2, Bem3, Rga1 and Rga2 (Zheng et al. 1993; Stevenson et al. 1995; Smith et al. 2002). These GAPs are not essential for viability, suggesting their functions are, at least, partially overlapping. Once GTP is hydrolyzed, the guanine-nucleotide-exchange factor (GEF) Cdc24 promotes the exchange from GDP to GTP (Figure 1.7). Mutations in Cdc24 (*cdc24-1*, *cdc24-4*) depolarize actin and growth (Hartwell et al. 1974; Sloat et al. 1981; J. Peterson et al. 1994).

CDK function is essential for bud emergence and maintenance of cell polarity (McCusker et al. 2007). CDK acts in this process in at least three different levels. First, before Start, Cdc24 is sequestered by Far1 at the nucleus. Phosphorylation of Far1 by CDK-G1 cyclin, triggers its degradation and Cdc24 is transported to the cytoplasm (O'Shea & Herskowitz 2000). Mutations that disturb the interaction between Cdc24 and Far1 allow Cdc24 to escape from the nucleus in the absence of G1 cyclins, however, Cdc24 does not localize to the bud cortex and Cdc42 is not polarized (Gulli et al. 2000). Second, Cdc24 GEF interacts with Bem1, Boi1, Boi2, Rga2 and other polarized proteins during bud emergence. Some of those proteins are substrates of CDK-G1 cyclin. In particular, phosphorylation of Boi1/2 is essential to maintain cell polarity under certain conditions (37ºC), but not in others (30ºC) (McCusker et al. 2007). Finally, the Cdc42 GAPs Rga2, Bem2 and Bem3, are inhibited by Cdk-Cln2 phosphorylation (Sopko et al. 2007; Knaus et al. 2007).

In summary, G1 cyclin-Cdk-dependent phosphorylation promotes the activation of Cdc42 by both activating its GEF Cdc24 and inhibiting the GAPs.

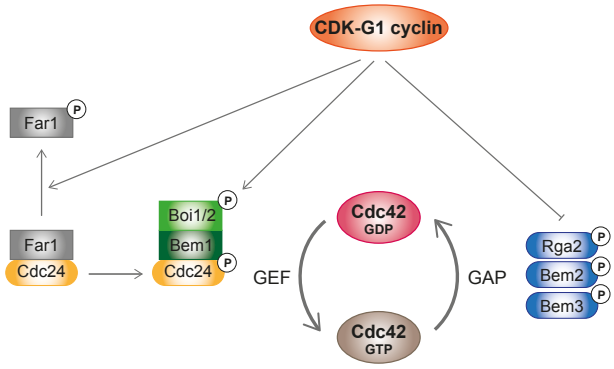

Figure 1.7. G1 cyclin–CDK activates Cdc42 by multiple pathways. CDK, cyclin-dependent kinase; GAP, GTPase-activating protein; GEF, guanine-nucleotide exchange factor; P, phosphorylation. Adapted from (Yoshida & Pellman 2008)

1.3.4. Polarized exocytosis

In order to grow in size, cells must fuse new lipid vesicles with the cell membrane. For most eukaryotic cells exocytosis is polarized, and especially in budding yeast, which divides asymmetrically, this is a tightly regulated process.

Polarized secretion at the cell surface can be described in three steps. First, Golgi-derived secretory vesicles are targeted to the vicinity of designated plasma membrane domains via myosin function and actin cables. This rapid transport of vesicles leads to a local accumulation (Govindan et al. 1995; Chuang & Schekman 1996; Santos & Snyder 1997). Second, after the vesicles arrive at the plasma membrane, they

are tethered to specific domains where the evolutionarily conserved exocyst complex localizes, and mediates the initial contact of vesicles with the plasma membrane (TerBush et al. 1996; Guo et al. 2000; Pfeffer 1999). Finally, interactions between the integral membrane proteins v-SNAREs and t-SNAREs (soluble N-ethylmaleimide-sensitive fusion attachment protein receptors), catalyse the final step of fusion of the exocytic vesicle with the plasma membrane. This fusion event allows on one hand the delivery of the cargoes to the extracellular space and on the other the incorporation of membrane proteins at the plasma membrane (Söllner et al. 1993).

In order to grow a bud, exocytic vesicles deliver proteins and lipids to the daughter membrane. In addition, as yeast cells are surrounded by a rigid cell wall, they also need to secrete enzymes that allow surface expansion. Two major classes of exocytic vesicles have been identified in budding yeast, which carry different cargoes that are delivered at sites of growth. One class carries plasma membrane proteins and cell wall modification enzymes such as Bgl2, whereas the other class contains proteins such as the periplasmic enzyme invertase (Harsay & Bretscher 1995). Most of the exocytic machinery is common for both types of vesicles, however there are some specificities. The exo70-38 allele for example, only accumulates Bgl2 but not invertase vesicles in the cytoplasm (He, Xi, Jian Zhang, et al. 2007a).

The exocyst complex

The exocyst complex, is required for efficient polarized exocytosis from yeast to mammals. In *S. pombe* however, this complex although essential for viability, is apparently not required for cell growth or cytokinesis, but only for cell separation (H. Wang et al. 2002). The exocyst is formed by eight components: Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84. The sec core of proteins was identified in a genetic screen using *S. cerevisiae* in the late 70s (Novick & Schekman 1979; Novick et al. 1980). Sec mutants accumulated 80-100nm post-golgi vesicles at the non-permissive temperature. The ones that belong to the exocyst, together with SEC1, SEC2, SEC4 and SEC9, were classified as 'late acting' secretory genes, as their function was essential only after the cargo was packed into exocytic vesicles at the Golgi, but before vesicle fusion with the plasma membrane (Figure 1.8). While genetic screens identified individual exocyst subunits, biochemical purification allowed the characterization of those proteins as components of the exocyst complex as well as of additional exocyst subunits missed by the genetic methods (Bowser & Novick 1991). It was in the 90s when Exo70 was discovered by performing immuno-precipitations of Sec8 from yeast extracts (TerBush et al. 1996) and EXO84 was identified later on, and after its mammalian homolog (Guo et al. 1999).

The exocyst complex is concentrated on sites of active vesicle fusion at the plasma membrane, which as we have seen, change during the cell cycle (TerBush & Novick 1995; Mondésert et al. 1997; Finger et al.

1998). Fluorescence recovery after photobleaching (FRAP) experiments in the presence of LatA, showed that while most subunits of the exocyst are delivered to exocytic sites on secretory vesicles, Sec3-GFP and Exo70-GFP localize at growing sites independently of actin cables (Finger et al. 1998; Boyd et al. 2004). Localization of Sec3p and Exo70 rely on its interaction with phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2] (He, Xi, Jian Zhang, et al. 2007a; X. Zhang et al. 2008) and small GTPases Rho1, Rho3p and Cdc42p (Wu et al. 2008; He & Guo 2009), RalA in metazoans (Moskalenko et al. 2002).

Thus, a subcomplex of the exocyst containing seven subunits (Sec5, Sec6, Sec8, Sec10, Sec15, Exo84 and Exo70) is recruited to secretory vesicles by the Rab protein Sec4 and transported to the plasma membrane. The remaining exocyst subunits, Sec3 and Exo70, localize at sites of secretion in an actin-independent manner. The interaction between both exocyst subcomplexes results in the tethering of the secretory vesicles, that will then be fused to the plasma membrane through SNARE function (Figure 1.8).

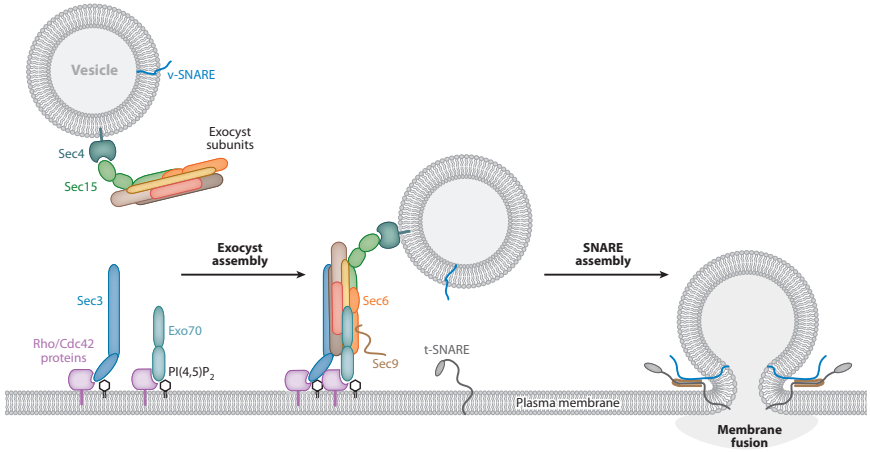

Figure 1.8. Model of secretory vesicle tethering, docking and fusion. Exocyst assembly mediates vesicle tethering. Sec3 and Exo70 are on the plasma membrane interacting to PI(4,5)P and Rho GTPases. The other subset of components of the exocyst are delivered to the plasma membrane through secretory vesicles. V-SNARE, interacts with the t-SNARE on the plasma membrane and together with Sec9 mediates the last step of fusion. Adapted from (Yu & Hughson 2010).

Exocyst function is cell cycle regulated. The exo84 subunit of the complex is phosphorylated by Cdk1-Clb2. This regulatory event prevents binding to other exocyst subunits like Sec15 and Exo70 and results in the inhibition of Bgl2-vesicles secretion during mitosis, more specifically during metaphase (Luo et al. 2013). This result suggests that during metaphase, growth is prevented by disassembly of the exocyst complex.

Cells defective in exocyst function like *exo70-38*, *sec3-2*, or GAL-Exo84 accumulate secretory vesicles at the vicinity of the plasma membrane

as seen by EM (Guo et al. 1999; He, Xi, Jian Zhang, et al. 2007a). Analysis of rapidly secreted cargoes can also assess defects in vesicle fusion. *Sec3-2, sec6-4, sec10-2 or exo84-121* show accumulation in the cytoplasm of the two main cargoes, Invertase and Bgl2. Other mutants like *exo70-38* cells have normal invertase secretion but interestingly, they accumulate internal Bgl2 (He, Xi, Jian Zhang, et al. 2007a). Some exocyst mutants are defective in cell division like *sec10-1* cells which show abnormal Chs2 delivery at the bud neck (VerPlank & Li 2005) or *sec3-4* mutants that are unable to finish cell separation (Dobbelaere & Barral 2004).

In *S. pombe*, the exocyst function is essential for cell separation but not for cell growth (H. Wang et al. 2002). In higher eukaryotes, exocyst mutants or knock-downs are associated with cell growth, cytokinesis and developmental defects. Exocyst function focuses a number of different signaling pathways, and regulates very diverse biological processes like cell migration, phagocytosis or formation of cilia (reviewed in (Heider & Munson 2012)).

1.3.5. Regulation of cell polarity and growth by RhoGTPases

As we have seen in the previous chapter, there are six members of Rho GTPases in yeast; Rho1, Rho2, Rho3, Rho4, Rho5 and Cdc42. Localization of the exocyst complex is critical for its function, as

exocytosis has to take place at the right sites in order to maintain cell polarity.

Polarization of the exocyst is lost in some *rho1*, *rho3* and *cdc42* mutant alleles, suggesting that the exocyst is an effector of those Rho GTPases (Guo et al. 2001; X. Zhang et al. 2001). More specifically, Rho1 and Cdc42 interact physically with Sec3, whereas Rho3 in its GTP-bound form, directly interacts with Exo70 (Adamo et al. 1999; Robinson et al. 1999). It could be that the changes in exocyst localization in Rho GTPase mutants are caused by defects in actin cytoskeleton. Nevertheless, it has been shown that Rho3 and Cdc42 have direct functions in exocytosis which are independent of their role in regulating actin polarity (Adamo et al. 2001; Adamo et al. 1999). Specific alles defective in exocytosis of Rho3 and Cdc42 have been isolated. While GFP-tagged exocyst components are depolarized in *cdc42-13* or *cdc42-201* cells (X. Zhang et al. 2001), a Cdc42 allele that is defective only in exocytosis, *cdc42-6,* shows normal exocyst localization and accumulates secretory vesicles (Adamo et al. 2001). The same is true for the Rho3 allele *rho3-v51* (Adamo et al. 1999).

Interaction of exocyst subunits with lipids and Rho GTPases restrict exocyst function to specific plasma membrane domains but also, these interactions might determine different regulation of specific types of vesicles (Grosshans et al. 2006). It has been proposed that the purpose of some GTPase-exocyst interactions, like Exo70-Rho3, involve exocyst function regulation a part from localization (Wu et al. 2008).

1.3.6. Boi1 and Boi2 as regulators of cell polarity

Boi1 and Boi2 are highly redundant proteins involved in cell polarity. They contain an SRC homology 3 (SH3) domain, which mediate protein-protein interactions and is essential for bud neck localization (Hallett et al. 2002), a sterile-alpha motif (SAM), a prolin-rich region, that usually interact wiyh SH3 domains, and a Pleckstrin Homology (PH) domain at the C-terminal part which is essential for the interaction with the plasma membrane (Bender, Lo, H. Lee, Kokojan, J. Peterson, et al. 1996a; Matsui et al. 1996). Boi proteins localize at the bud cortex during interphase and later on, during mitosis, they relocate to the bud neck. This transition is thought to be regulated by Cdk activity. Both Boi1 and Boi2 are phosphorylated by Cln2-Cdk and this phosphorylation is important of initiating and maintaining cell polarity at high temperatures (McCusker et al. 2007).

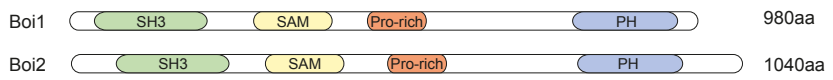

Figure 1.9. Boi1 and Boi2 structure. Both proteins have a similar structure. At the N-terminal region they have an SH3 domain. At the central part there is a SAM motif and a prolin-rich region, and at the C-terminal part there is the PH domain.

Deletion of either Boi1 or Boi2 does not affect cell viability or growth rates. In (Matsui et al. 1996) study, by tetrad dissection, only one out of ~200 *boil*Δ *boi2*Δ spores isolated from boilΔ/+ boi2Δ/+

heterozygous diploid cells formed a visible colony. The single *boi1*Δ *boi2*Δ spore that formed a colony, grew very poorly. Cells lacking Boi1 and Boi2 had an abnormal morphology; they were round and large and 30% of the cells died with a bud. This suggested that Boi1 and Boi2 are required for the assembly or function of structures at the bud site.

Lethality of *boi1*Δ *boi2*Δ mutants is rescued by multicopy plasmids expressing RHO3, RHO4 and the PH domain of Boi1. (Bender, Lo, H. Lee, Kokojan, J. Peterson, et al. 1996a). This result shows that the PH domain of Boi1 is sufficient for Boi essential function. Also, it suggests that Boi1 and Boi2 either promote the activation of Rho3/4 GTPases, or in some other way facilitates some process that is controlled by Rho3 or Rho4.

Boi1 interacts by yeast two hybrid with the GTP-locked form of Cdc42 (Ziman et al. 1991; Bender, Lo, H. Lee, Kokojan, J. Peterson, et al. 1996a). Overexpression of BOI1 impaired growth, causing cells to become unbudded, large, and multinucleate Interestingly, co-overexpression of Boi1 and Cdc42 rescued viability of the first ones (Matsui et al. 1996). One possibility is that Boi1 binds Cdc42 in vivo and cells die when overexpressing Boi1 because it acts as a dominant negative and sequesters all the available Cdc42-GTP.
Interestingly, there is some contradictory data about the double deletion. In the first studies where Boi proteins were related to cell polarity, the double mutant was found to be unviable (Bender, Lo, H.

Lee, Kokojan, V. Peterson, et al. 1996b; Matsui et al. 1996). In contrast, as it was mentioned above, in later studies in which Boi proteins were described as cytokinetic inhibitors, the *boi1*Δ *boi2*Δ strain used was viable and grew at wild-type rates (Norden et al. 2006; Mendoza et al. 2009). In this study I addressed this apparent contradiction by creating a conditional mutant.

2. Aim of my work

Boi1 and Boi2 proteins were first been described as scaffold proteins essential for polarity establishment acting together with Rho3, Rho4 and Cdc42 (Matsui et al. 1996; Bender, Lo, H. Lee, Kokojan, V. Peterson, et al. 1996b). However, little was known about their molecular mechanism of action. Later on, Boi proteins were found to be required for inhibiting abscission through the NoCut pathway. Nevertheless, it was not clear how Boi proteins could affect abscission timing at the bud neck. I set out to investigate the role of Boi proteins in polarized growth and cytokinesis.

The aim of the work presented here is to investigate the following topics:

- Which is the molecular mechanism by which Boi1 and Boi2 regulate polarized growth
- How do these proteins inhibit abscission in the context of NoCut

3. Materials and methods

3.1. Cell growth and synchronization

3.1.1. Cell growth

Yeast cells were grown in YP Dextrose (YPD), YP Galactose (YPG) or YP Raffinose (YPR) media containing 1% bacto-yeast extract (Becton-Dickinson), 2% bacto-peptone (Becton-Dickinson), 2% sugar (Sigma-Aldrich) and 0.004% adenine (Sigma-Aldrich). Solid medium contained 2% agar (Becton Dickinson). Agar, peptone and yeast extract were mixed with water and autoclaved, all other components were filter sterilized.

For selection of auxotrophies, cells were grown on synthetic minimal medium, lacking the aminoacid of choice. Complete synthetic minimal medium is composed by 0.67% Yeast Nitrogen Base without ammonium sulfate (Becton Dickinson), 0.004% adenine (Sigma-

Aldrich), 0.002% uracil (Sigma-Aldrich), 0.002% tryptophan (Sigma-Aldrich), 0.002% histidine (Sigma-Aldrich), 0.003% lysine (Sigma-Aldrich), 0.003% leucine (Sigma-Aldrich) and 0.002% methionine (Sigma-Aldrich).
For Antibiotic selection, 100mg/L nourseothricin (ClonNAT, Werner Bioagents, 51000), 200mg/L Geneticin (G418, Invitrogen, 11811023), 300mg/L hygromycin B (Nucliber, ant-hm-1) or 40mg/L phleomycin (Nucliber, ANT-PH-1) was added to rich medium.

To induce sporulation of diploid yeast, cells were inoculated in minimal sporulation medium (1% potassium acetate (Sigma-Aldrich)) supplemented with 0.002% of each of the 5 amino acids added to synthetic minimal medium.

To stock strains, cells were harvested directly from 1 day old rich solid medium and resuspended in 30% glycerol/70% liquid rich medium. Stocks were kept at -80 °C.

3.1.2. Serial dilutions to assess cell growth

To assess growth rates on solid media, cells were grown to logarithmic phase ($0.2 < OD_{600} > 0.6$) on liquid rich medium, set to an $OD_{600}=0.02$ and serially diluted four times. $OD_{600}=0.02$ corresponds approximately to $2{,}5.10^5$ cells. Cells were plated to appropriate medium (YPD, YPG or SC lacking leucine) and incubated at the indicated temperatures for 48–72h.

3.1.3. Single cell growth assay

Haploid yeast cells were placed on YPG plates with 500μM NAA or DMSO. Single cells were isolated using a dissection microscope (axioskop 40, Zeiss). After 20h at 25ºC the number of cells per microcolony was counted.

3.1.4. Cell synchronization

For synchronization in G1 phase, cells were arrested in 15μg/ml α-factor for 2h at room temperature (1mg/ml stock in methanol stored at -20 °C, α-factor was purified by the in house proteomics facility). To release cells from the α-factor arrest, cells were washed three times with rich medium.

For metaphase synchronization, cells were arrested with 10μl/ml of 2mg/ml Nocodazole (Sigma-Aldrich).

For S-phase synchronization cells were arrested with 200mM of hydroxyurea (HU) (Sigma-Aldrich).

3.2. Yeast strains

3.2.1. Strain background

Saccharomyces cerevisiae strains are derivatives of S288c background. The *ipl1-321* allele was derived from W303 background. The *boi1*Δ *boi2*Δ strain from (Norden et al. 2006) is derived from BF264-15D and is a

gift from E.Bailly (Institut National de la santé et recherché médicale, INSERM).

3.2.2. Strain crosses

Haploid yeast cells of opposite mating type were mixed on YPD plates. Diploid cells grown for 1 day on YPD plates were inoculated in minimal sporulation medium (1% potassium acetate (Sigma-Aldrich). After 3 days tetrads were digested with 0.02mg/ml Zymolyase 100T (Seikagaku Biobusiness, #120493) at room temperature for 5 minutes. Digested tetrads were plated on YPD plates and dissected using a dissection microscope (axioskop 40, Zeiss). Spores of the relevant genotype were selected based on growth on selection plates.

3.3. Strain generation and plasmids

3.3.1. Yeast cell transformation

DNA insertion, gene deletions and gene fusions were generated by transformation of PCR products or plasmids essentially as described in (Janke et al. 2004). Yeast cells were inoculated over night in liquid medium and diluted to an optical density of OD600=0.2 in 15ml rich medium in the morning. Cells were harvested when the cultures reached an optical density of OD600=0.8 by centrifugation at 3500rpm for 2min at room temperature in a 15ml tube. Cells were washed in 1ml transformation buffer (100mM Li acetate, 10mM Tris, 1mM EDTA, pH 8) and resuspended in 72µl transformation buffer.

8µl of freshly denatured, chilled salmon sperm DNA (10mg/ml salmon sperm DNA (Sigma-Aldrich, #D1626); 10min denatured at 95 °C, cooled on ice) were added to the cells. 9µl of PCR product or 1µl plasmid were added to the cells, followed by 500µl of PEG buffer (as transformation buffer, but containing 40% PEG-3350 (Sigma-Aldrich, #P4338) and incubated on a rotating wheel for 30min. After addition of 60µl of DMSO (Sigma-Aldrich, #D2650) cells were heat-shocked for 15min at 42 °C. Cells were harvested by centrifugation at 400g for 2min, resuspended in 200µl of water and plated. Selection for auxotrophic markers was carried out directly on synthetic minimal medium lacking the amino acid of choice. For selection of antibiotic resistances, cells were first plated on rich medium and replicated onto plates containing the antibiotic after 1-2 days.

To check insertions or deletions, genomic DNA of transformants was isolated for analysis by PCR essentially as described in (Harju et al. 2004). For fluorescently tagged proteins strains were checked under the fluorescent microscope (Leica AF6000).

3.3.2. Directed mutagenesis of Exo70

To generate the Exo70^{G388R} mutant allele, the Quick change II XL site-directed mutagenesis (Agilent technologies) was used on the PGal-Exo70 2µ BG1805 plasmid from the Thermo Scientific Yeast ORF library. Mutagenesis product was transformed into E.coli supercompetent cells provided by the kit. To check G338R mutation, four clones were sent to sequencing (GATC biotech). Positive plasmids were digested with BamHI and SacI to clone Exo70 and

Exo70^{G388R} allele into a pRS415 centromeric plasmid digested with the same enzymes.

Endogenous EXO70 was mutated to EXO70^{G388R} by transformation. *Exo70* in strain YMM236 (E.Bailly, Institut National de la santé et recherché médicale, INSERM) was tagged with 6xHA::Nat by PCR and confirmed by sequencing of the EXO70 locus. Using oligos OMM878 and OMM1204 the C-terminal fragment of E*xo70* was amplified from position 1000 to 378bp upstream the stop codon, including the HA tag. The PCR fragment was then transformed into several other strains and positive clones were confirmed by sequencing.

3.3.3. Origin of plasmids and mutants

Template plasmids for PCR mediated deletions and fusion protein generation were all from (Janke et al. 2004) except for the fluorescent proteins mCherry and tdTomato which were amplified from plasmids provided by the lab of Karsten Weiss (University of California, Berkeley). (For the rest of the plasmids see appendix II)

The mutant allele of *ipl1-321* was provided by Yves Barral (ETH Zurich, Switzerland).

3.4. Microscopy

3.4.1. Fluorescence microscopy

Time-lapse analysis of live cells was performed on log phase cells or cells synchronized with either 200mM HU or 10μg/ml alpha-factor, released in fresh medium, and placed in a pre-equilibrated temperature-controlled microscope chamber. Cells were plated in minimal synthetic medium in 8 well Lab-Tek chambers (Nunc). Prior to plating, chambers were coated with concanavalin A (Sigma-Aldrich, #C7275) by incubation of 250μl of 1mg/ml (in PBS) solution for 20min. Before use, the chambers were washed 3x with 1ml minimal synthetic medium.

Imaging of cells in figures 4.2, 4.6.2, and 4.7.2 was performed using a Zeiss cell observer microscope with a Zeiss AxioCam MrX. Time-lapse series of 4μm thick stacks spaced 0.8μm every 5min were acquired.

Andor Revolution XD spinning disc confocal microscope equipped with an Andor Ixon 897E Dual Mode EM-CCD camera, was used for figures 4.8.1, 4.8.2 C, 4.8.3 and 4.9.1. B. Time-lapse series of 4μm spaced 0.3μm every 1-2min were accquired.

For figures 4.3.1, 4.3.2, 4.7.1, 4.8.2. A/B, 4.8.4 and 4.9.1, cells were imaged using Leica AF 6000 wide-field fluorescent microscope with an Andor DU-885K-CSO-#VP. Images of 4μm stacks spaced 0.4-0.5μm were acquired.

3.4.1. High pressure freezing and electron microscopy

Cells were transferred to 3mm diameter and 200μm depth planchettes and immediately cryoimmobilized using a Leica EM HPM 100 high-pressure freezer (Leica, Vienna, Austria) and then stored in liquid nitrogen until further use. They were freeze-substituted over 3 days at –90ºC in anhydrous acetone containing 2% glutaraldehyde and 0.1% uranyl acetate at –90 °C for 72 hours and warmed to room temperature, 5º per hour (EM AFS-2, Leica, Vienna, Austria). After several acetone rinses, cells were incubated with tanic acid in 1% acetone for 1h on ice. Then, cells were washed 4 times for 15min with acetone, and incubated for 1h with OsO4 at 1% acetone on ice. Samples were washed 4 times for 15min with acetone, infiltrated with Epon resin during 2 days and embedded and polymerised at 60ºC during 48 hours. Ultrathin sections were obtained using a Leica Ultracut UC6 ultramicrotome and mounting on Formvar-coated copper grids. They were staining with 2% uranyl acetate in water and lead citrate. Then, sections were observed in a Tecnai Spirirt (FEI Company, The Netherlands) electron microscope equipped with a megaview III CCD camera.

3.5. Image analysis and statistics

Images were analyzed on 2D maximum projections or 3D stacks using ImageJ (http://rsb.info.nih.gov/ij/).

Graphs and statistical analysis (*t*-test allowing for unequal variance) were performed with Prism software (GraphPad) and Excel (Microsoft). On figures, Asterisks indicate $p<0.02$ (*) or $p<0.001$ (**).

3.6. Protein analysis

To fix cells, 1ml of liquid yeast cultures were mixed with 300µl of 85% trichloracetic acid (TCA, Sigma-Aldrich, #T6399) to a final concentration of 20%. After 5 to 60 minutes of fixation at room temperature (RT), cells were collected by centrifugaion at 13000g for 1min, the supernatant was discarded. Centrifugation was repeated to remove most of the supernatant. Cells fixed like this can be stored at -80 °C.

Add 100µl of 1x Sample Buffer (350mM Tris, 0.1M dithiotheritol (DTT), 2% SDS, 4% Glycerol, 0.1% Bromophenolblue, pH 8.8,) and approximately 300µl of acid washed glass beads (Sigma-Aldrich, #G8772). Cells were broken by shaking for 1min in a FastPrep FP120 (Thermo Savant). To reduce foam, tubes were centrifuged at maximum speed for 1 minute. Samples were denatured at 95 °C for 10 minutes. To collect cell lysates, 1.5ml tubes were perforated with a hot needle and placed in a fresh 1.5ml tube for centrifugation. 15 to 25µl of this lysate were run on a 10% acrylamide gel and transferred onto a nitrocellulose membrane using a semi-dry system (BioRad).

Membranes were blocked with 5% milk in TBST (TBS with 0.05% Tween20) for 30min at room temperature or over night at 4 °C. Primary antibodies were diluted in 5% milk/TBST and incubated for

3h at RT or over night at 4 °C. The mouse α-HA antibody (Roche, #11666606001) and the rabbit α-myc (santa cruz, sc-40) and α-Glucose-6-Phosphate dehydrogenase (Sigma, A9521) were used at 1/1000 dilution. The rabbit α-Clb2 antibody (TEBU-BIO, #SC9071) was used at 1/2000 dilution. Primary antibodies were washed away with TBST (3 washes of 5min).

Secondary antibodies were used all used at 1/25000 dilution in TBST: goat α-mouse IgG-HRP (Santa Cruz, #A2312), donkey α-rabbit-HRP (Santa Cruz, #H2912). Secondary antibodies were incubated for 30min at room temperature. After 3x washing with TBST, the membranes were developed by the ECL system (Perkin Elmer).

3.7. Immunoprecipitation assays

Strains expressing Exo70 or Exo70^{G388R} tagged with HA were grown to mid-log phase in YPR at 25°C and shift to YPG for 2h. 200OD units of cells were pelleted and washed with cold lysis buffer (50mM Tris-HCl pH7.4; 200mM NaCl; 1mM EDTA; PIC). Pellets were resuspended in lysis buffer and were lysed by bead beating. Lysates were cleared by centrifugation at 3000rpm for 10min. The supernatant was collected and the membranes were pelleted for 45min at 45000rpm in an L100-XP Beckman ultracentrifuge. The cytosolic fraction was saved to perform the IP and the membranes were solubilised and incubated with 1% NP40; 50mM Tris-HCl pH7.4; 200mM NaCl; 1mM EDTA; PIC for 4h. Then samples were

incubated with dynabeads (invitrogen) and 1:1000 of antiHA 12CA5 (Roche) over night. Samples were washed three times with 1% NP40; 50mM Tris-HCl pH7.4; 200mM NaCl; 1mM EDTA; PIC. Samples to analyze by MS were collected with digestion buffer (6M urea (Bio-Rad Laboratories S.A.), 200mM NH_4HCO_3). Samples to analyze by SDS-PAGE gels were collected with sample buffer and boiled at 95°C for 5min.

3.8. Bgl2 assay

Bgl2 accumulation was performed as described in (Kozminski et al. 2006) with minor modifications. Cells were cultured in YPR at 25°C to logarithmic phase, 2h in YPG and 2h in YPG 500mM NAA or DMSO. Equal cell numbers were harvested by centrifugation at 2000×g for 5min. Cell pellets were resuspended in 10mM NaN3/NaF solution and incubated on ice for 10min, harvested at 10,000×g for 1min, resuspended in fresh pre-spheroplasting buffer (100mM Tris-H_2SO_4, pH 9.4; 50mM β-mercaptoethanol; 10mM NaN3; 10mM NaF) and further incubated on ice for 15min. Cells were harvested as before, washed once in spheroplast buffer without zymolyase (50mM KH2PO4-KOH, pH 7.4; 1.4M sorbitol; 10mM NaN3), resuspended in spheroplast buffer containing 167μg/ml zymolyase 100T (Seikagaku Biobusiness, #120493), and incubated with gentle mixing for 30min at 30°C. Spheroplasts were harvested by centrifugation at 5000×g for 10min and resuspended in sample buffer before

separation by SDS–PAGE and western blotting to detect Bgl2 (a gift from Randy Schekman, University of California, Berkeley) and G6PDH as a loading control (Sigma).

3.9. Sequencing and bioinformatics analysis

The analysis of the sequences was performed in collaboration with Dr. Toni Gabaldón and Leszek Pryszsc (Bioinformatics and genomics program, CRG).

3.9.1. Genomic DNA isolation for sequencing

To extract genomic DNA of wild-type and *boi1 boi2* mutants, 10ml of cells were grown to saturation. Cells were spin down and resuspended with 2% Triton X-100 (Sigma-Aldrich, #T8787), 1% SDS, 100mM NaCl, 10mM Tric-HClpH8 and 1mM Na_2 EDTA. DNA was purified from detergents, proteins, salts and reagents used during cell lysis step with phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma Aldrich).

3.9.2. Genome Assembly

All strains were sequenced at the Ultrasequencing core facility in the CRG using Illumina HiSeq paired-end technology. Reads were trimmed at the base with quality lower than 20 using FASTX-Toolkit (Cold Spring Harbor Laboratory, http://hannonlab.cshl.edu/fastx_toolkit). Subsequently, reads shorter than 31 bases (and their pairs) were discarded. Assemblies for each strain were created de novo using Velvet (ver 1.1.02) (Zerbino &

Birney 2008). The insert size of the paired-end reads was estimated by aligning reads onto the assembly created from single reads. The optimal k-mer length (k) for each strain was chosen using VelvetOptmiser.pl to maximize the total number of bases in contigs longer than 1kb (Lbp). In addition, we used auto-estimation of coverage cut-off and removed contigs shorter than 1kb. Table XX lists detailed information about each resulting assembly.

3.9.3. SNP detection

Filtered genomic reads were aligned onto the reference strain assembly using GEM-mapper (build 544) (Marco-Sola et al. 2012), allowing two mismatches. Paired reads whose mapped distance was higher than 10Kb were removed. Unlike most SNP detection strategies that relay entirely on the reference genome sequence, we could make use of the availability of the original reads used in assembling the reference genome, thus having a way of detecting ambiguous sites. Accordingly SNPs were detected using a double filtering, calling SNPs on aligned discordant sites with high coverage (>=10x) and high unambiguity (>=90% of aligned reads call the same nucleotide at that position) at both the mapped reads of the given mutant strain and the reference. This filtering procedure was able to discard likely false-positive SNPs detected when mapping the reference reads against its own assembly. Flanking regions of possible SNPs (200bp windows) were mapped onto SGD (Cherry et al. 2012) reference (BLAT ver. 3.4) (Kent 2002), in order to evaluate the possibility of non-synonymous mutations. In total, 5411 SNPs were identified (382 to 733 per strain).

3.9.4. Structural variation detection

Assembled contigs of 5 *boi1*Δ *boi2*Δ mutants and 5 wild-types were aligned onto the reference assembly using MUMmer (ver. 3.07, default parameters (Kurtz et al. 2004)). Best reciprocal matches were further processed, but partial overlaps between matches were allowed. Structural variants (SVs) were called only when one query contig overlap the entire event. A minimal alignment length of 65bp was set as a threshold. In addition, cut-off of 200bp was set for deletion detection.

4. Results

4.1. Generation of a Boi1/2 conditional mutant

S.*cerevisiae* contains two boi genes; BOI1 and BOI2. It was previously shown that Boi1 and Boi2 are functionally redundant, and that the Boi proteins are important for cell growth. However, their function is still unclear. It has been reported that boi1 boi2 double deletion is lethal (Matsui et al. 1996). In contrast, later work from the Barral laboratory found that a *boi1*Δ*boi2*Δ strain was viable, but defective in the NoCut pathway (Norden et al. 2006; Mendoza et al. 2009). The reason for this discrepancy was not clear.

Here we revisit the in vivo function of budding yeast Boi1 and Boi2 in conditional mutants. Because the *boi* mutant strain described in (Norden et al. 2006; Mendoza et al. 2009) was generated in a different

genetic background (BFA264-15D; in the lab of Eric Bailly, Institut National de la santé et recherché médicale, INSERM) than the one used in our laboratory, we first set out to determine whether deletion of BOI1 and BOI2 is compatible with viability in our wild-type reference background (S288c, strain YMM1) (see strain list in Apendix I). We first created a haploid *boi1*Δ and a *boi2*Δ single mutant with different mating types. As after transformation of the second deletion to get the double mutant, we did not obtain any colony, we crossed and dissect diploids but we did not get any viable *boi1*Δ*boi2*Δ out of 11 expected double mutants (Michael Maier, data not shown). We conclude that Boi1 Boi2 mutants are inviable in our lab wild type background.

To investigate the function of Boi1 and Boi2 in the s288c background, we then generated a *boi1 boi2* conditional mutant strain through the use of a recently developed auxin-inducible degron (AID) technology (K. Nishimura et al. 2009). BOI1 was deleted from the genome, and subsequently Boi2 was tagged at the C-terminus with the AID peptide. Also, we inserted the Arabidopsis thaliana TIR1 gene (AtTIR1) under the control of the galactose-inducible GAL promoter in the URA3 locus. The AtTIR1 forms a complex with yeast proteins forming an SCF-TIR1 E3 ubiquitin ligase that will recognize the AID tag in the presence of auxin (NAA) in the medium (Figure 4.1 A).

In order to characterize the conditional mutant *boi1*Δ *boi2-AID* Gal1pr-AtTIR1, we followed the degradation kinetics of *Boi2-aid*

protein in liquid cultures and we found that 97% of the protein was depleted after 2h of either 500 or 250 μNAA (Figure 4.1 B,C).

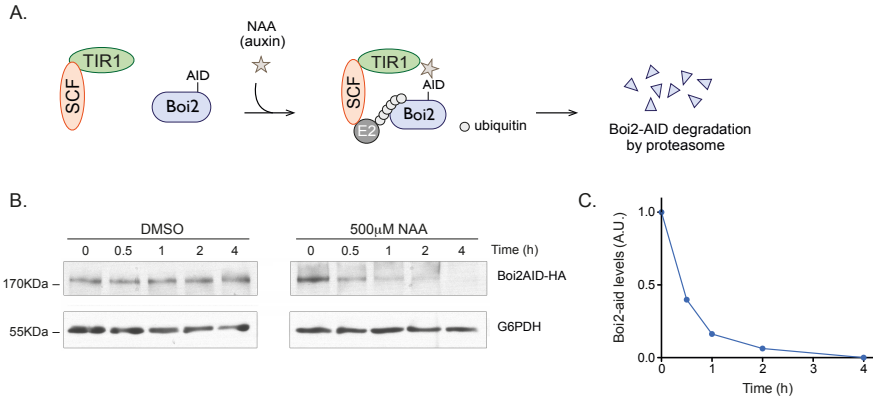

Figure 4.1.1. Construction of a Boi1 and Boi2 auxin-based degron. (A) Schematic representation of the auxin-based degron system. (B) Immunoblotting to detect Boi2-aid-HA and G6PDH with antibodies to HA and G6PDH, respectively. Representative example of three independent experiments. Yeast strains used were initially grown in YPRaffinose (YPR) and then were transferred to YPGalactose (YPG) for 2h. Cells were collected for analysis at indicated time points after addition of 500 μM NAA or DMSO. The same results were observed using 250 μM NAA. (C) Quantification of band intensity in A.U. normalized to time 0.

*PGalTIR1 boi1*Δ *boi2-aid* cells plated on YPG containing 500μM of 1-Naphthaleneacetic acid (NAA) die before forming visible colonies, even though the wild-type strain and the control strains grew well on the same plate (Figure 4.1.2 A, B). In agreement with these results, when measuring the optical density of WT and *boi1*Δ *boi2-aid* cells growing on YPG, we observed a much slower rate of growth of the Boi mutant compared to the WT (Figure 4.1.2 D). As there is a mild

toxicity effect caused by NAA in wild-type cells, we checked whether the viability was lost in boi1 boi2 cells at lower concentrations of NAA (Figure 4.1.2 C). As we observed the same toxicity effect in solid media at concentrations ranging from 250 to 50μM, for certain experiments we used 250μM of NAA.

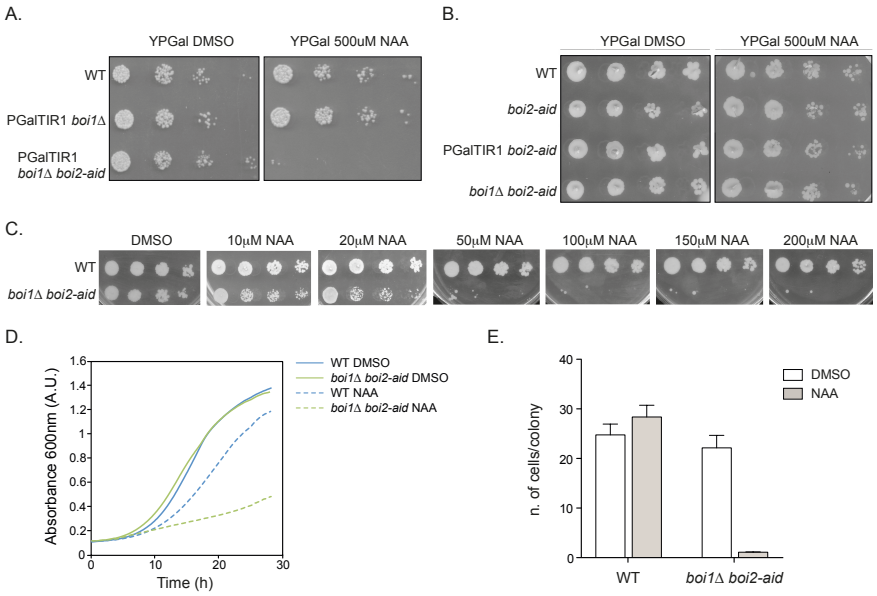

Figure 4.1.2. Lack of Boi1 and Boi2 is lethal for the cells. (A) Serial dilutions of the indicated strains were spotted onto YPG plates containing DMSO or 500μM NAA. Plates were incubated at 30°C. (B,C) Same as (A). Plates were incubated at 25°C. (D) WT and *boi1*Δ *boi2-aid* cells were grown to an optical density at 600nm of 0.1 in YPG at 25ºC and split into flasks that were then incubated either with 500μM of NAA or DMSO. Optical density was measured every 10min. (E) Single cell assay. WT and *boi1*Δ *boi2-aid* cells were arrested with αfactor. Cells were released and plated on YPG with DMSO or NAA using a micromanipulator. After 20h, number of cells per colony were scored. Number of colonies scored; WT DMSO = 21, WT NAA = 13, *boi1 boi2* DMSO = 19, *boi1 boi2* NAA = 11.

To determine the primary defect of *boi1 boi2* cells, we arrested wild-type and *boi1*Δ *boi2-AID* cells in G1 with alpha factor in liquid media for 2h, and then plated them on YPG with DMSO or NAA and isolated single G1 cells using a dissection microscope. Interestingly, examination of microcolony size 20h after plating show that *boi1 boi2* cells did not divide in the presence of auxin, and arrested as unbudded or small budded cells. In contrast, Boi mutants in the absence of auxin, and wild-type cells in either auxin or DMSO had formed colonies of around 25 cells after the same time period (Figure 4.1.2 E). These observations are consistent with an essential role of Boi proteins in polarized cell growth and division.

4.2. Boi1 and Boi2 mutants arrest cell growth

To further characterize the phenotype of Boi mutants, we performed live imaging using differential contrast microscopy (DIC) of wild-type and *boi1 boi2* cells in the presence of NAA or DMSO. Cell volumes were quantified with BudJ plugin from ImageJ (Aldea et al. 1994). Small buds of wild-type cells are able to grow in the presence of DMSO and auxin. In contrast, *boi1*Δ *boi2-AID* cells grow well in DMSO but not in auxin containing media (Figure 4.2 A). While WT cells are able to increase their volume over time, cells lacking Boi1/2 block cell surface expansion. Inhibition of growth was observed in *boi1 boi2* cells over a wide range of bud sizes. (Figure 4.2. B).

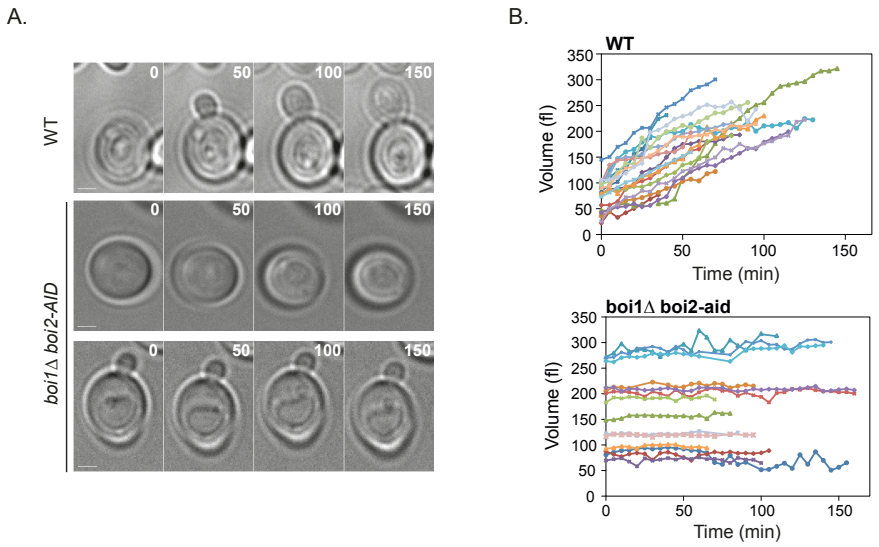

Figure 4.2. Boi defective cells arrest cell growth. (A) DIC time-lapse imaging of WT and *boi1*Δ *boi2-aid* mutants. Cells were grown in YPRaffinose, and changed to YPGalactose for 2h. Then, 500µM NAA was added for 2h more. Images were acquired every 5min with a confocal microscope. Time is represented in minutes. White bars represent 2µm. (B) Single cell traces of quantification of bud cell volumes from (A) using the BudJ plugin from imageJ (Ferrezuelo et al. 2012). n (WT) = 19, n (*boi1 boi2*) = 14.

These results show that one of the earliest consequences of Boi1/2 depletion is the arrest of bud growth. Also, after a prolonged exposure to the hormone (20h), we observed large rounded cells suggesting that after a prolonged time in the absence of Boi1 and Boi2, cells are able to grow albeit in a depolarized manner.

4.3. Boi function is not required for the localization of cell polarity determinants

Cells defective in actin cytoskeleton arrest growth as secretory vesicles cannot be delivered to the cell surface and accumulate in the cytoplasm. This has been observed in experiments done by adding the actin depolymerising drug Latrunculin A (Ayscough et al. 1997; Karpova et al. 2000).

To test whether the phenotype observed in Boi1/2 conditional mutants is caused by defects in actin polarization, we depleted *boi2-aid* for 2h, fixed and incubated the cells with phalloidin-488 to stain F-actin. The quantitation of overall actin polarity is shown in figure 4.3.1 B. Cells were considered polarized if they contained polarized cortical actin patches in the cell cortex or bud neck. Cells that show a dispersed distribution of actin patches are considered depolarized and were counted but are not represented in the plot. I observed no differences between wild-type and *boi1*Δ *boi2-aid* nor between DMSO and auxin samples, suggesting that the primary defect of Boi1/2 mutants is not in the polarization of the actin cytoskeleton.

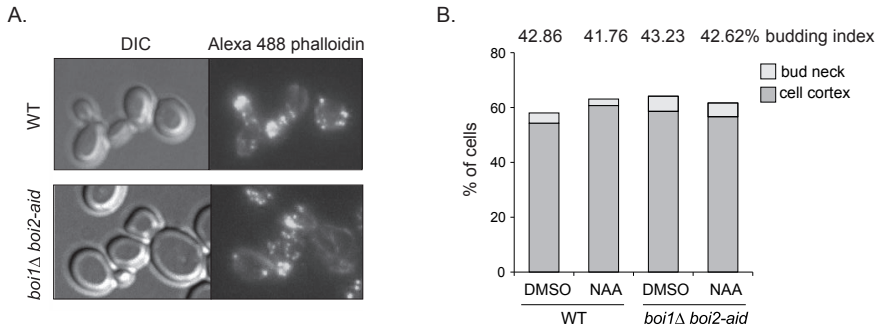

Figure 4.3.1. Actin cytoskeleton is polarized in cells lacking Boi1 and Boi2. (A) Actin localization in wild-type and *boi1*Δ *boi2-aid* cells. Cells were grown in YPR at 25°C, and shifted to YPG for an hour before adding 500μM NAA or DMSO. After 2h in either NAA or DMSO, cells were fixed with formaldehyde and subsequently permeabilized and stained with phalloidin as described in MATERIALS AND METHODS. Images were taken by DIC and fluorescence microscopy. White bars represent 2μm. (B) Quantification of images in (A). Scoring of the percentage of cells with actin polarized at the cell cortex or at the bud neck. Cells that are not polarized are included in the quantification but not represented. The budding index represents the percentage of cells that are budded. n (WT DMSO) = 182, n (WT NAA) = 91, (*boi1 boi2* DMSO) = 192, (*boi1 boi2* NAA) = 61.

To further examine whether proper cell polarity was established in *boi* mutants, I examined the localization of the polarity marker Sec4, a Rab-family GTPase (homolog to mammalian Rab proteins) that is essential for transport and fusion of Golgi vesicles to the plasma membrane (Salminen & Novick 1987; Kabcenell et al. 1990). Localization of Sec4 can be used as specific probe to monitor vesicle delivery defects and the integrity of cell polarity (Schott et al. 2002).

I also checked the localization of other polarized proteins like the Cdc42 GEF Cdc24, the exocyst components Sec3, Exo70 and Exo84, the scaffold protein Bem1, and the daughter cell specific protein Dse1 (Figure 4.3.2 B, C) in Boi1/2 defective cells. Localization of some of these proteins (Exo84, Cdc24, Bem1 and Dse1) depends on actin, in contrast, Sec3 and Exo70 localization depends on interactions with PIP2 and GTPases. Consistent with my observations by phalloidin staining, I found that there is no major change in localization of none of the mentioned proteins we checked either between wild-type and Boi mutants or between auxin samples and DMSO controls (Figure 4.3.2 A, B). Thus, demonstrating that the *boi1*Δ *boi2-aid* mutant has proper cell polarity.

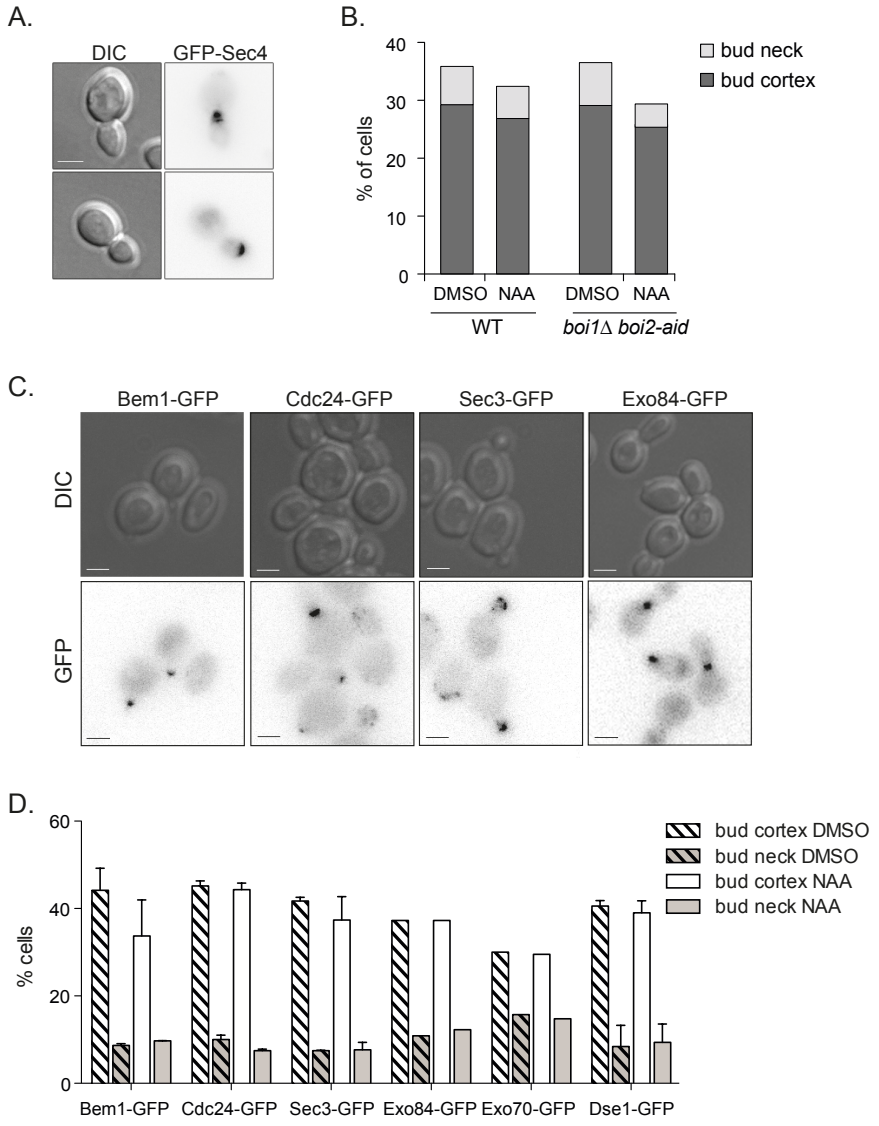

Figure 4.3.2. Localization of polarized proteins in Boi1/2 mutants. (A) Localization of GFP-Sec4. Fluorescence images were taken to visualize Sec4 localization. Cells were grown in YPR, then in YPG for 2h at 25ºC. Pictures were taken 2h after addition of 500μM NAA or DMSO. Bar, 2μm. (B) Quantification of Sec4 localization of cells in (A). n (WT t0) = 106, n (WT 2h) = 108, (*boi1 boi2* t0) = 187, (*boi1 boi2* 2h) = 121. (C) Examples of *boi1*Δ *boi2-aid* cells expressing Bem1-GFP,

Cdc24-GFP, Sec3-GFP or Exo84-GFP. Cells were grown on YPR, for 2h in YPG and 2h hours in the presence of 500μM NAA or DMSO. (D) Quantification of Bem1-GFP, Cdc24-GFP, Sec3-GFP, Exo84-GFP, Exo70-GFP and Dse1-GFP localization in *boi1*Δ *boi2-aid* cells in (C). Over 150 cells for each condition were counted. Error bars indicate SEM.

Taken together, these data, along with the direct staining of the actin itself, demonstrate that the primary cellular defect responsible for the loss of bud growth of *boi1*Δ *boi2-aid* cells, is not in the localization of known components of the polarity machinery.

4.4. Boi1 and Boi2 are required for a late step in exocytosis

Cell growth and surface expansion are mediated by exocytosis. Apart from actin cytoskeleton defects, cell growth arrest can be caused by a block in secretory vesicle fusion to the plasma membrane, preventing the membrane and/or cell wall to expand. To directly determine whether Boi1/2 mutants have a secretory defect, we looked at the internal accumulation of the abundant exoglucanase Bgl2 protein which is normally secreted into the periplasmic space (Harsay & Bretscher 1995). Cells were grown in YPR to logarithmic phase at 25ºC and changed to YPG for 1h before 500μM NAA addition (t_0). After 2h cells were harvested and cell wall was removed. Immunoblots were used to probe the internal fraction of Bgl2 which is secreted rapidly and is nearly undetectable in spheroplasts of wild-type cells. In contrast, *boi1*Δ *boi2-aid* cells accumulate large amounts of Bgl2 in the

cytoplasm after 2h in 500μM NAA (Figure 4.3.3. A, B). Thus, *boi1 boi2* mutants are unable to secrete Bgl2 vesicles.

Consistent with the secretory defects observed for Bgl2, preliminary analysis of Boi1/2 mutant cells by electron microscopy revealed that these cells accumulated 80–100nm vesicles. These vesicles were preferentially distributed in the tips of small /medium sized buds (Figure 4.4.3 C) and are rarely seen in wild-type cells (Jäntti et al. 2002; He, Xi, Jian Zhang, et al. 2007a).

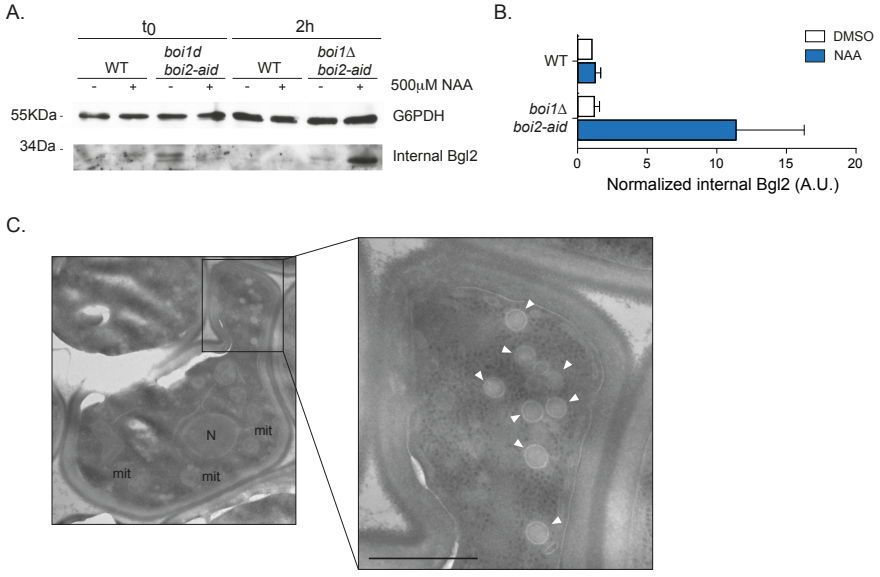

Figure 4.3.3. Bgl2 secretion defects and accumulation of cytoplasmic vesicles after depletion of Boi1/2. (A) Immunoblot of internal fraction of Bgl2. Wild-type and *boi1*Δ *boi2-aid* cells were grown in YPR to logarithmic phase at 25°C. After 1h in YPG, 500μM NAA or DMSO was added. Cells were harvested at time 0 and 2h after addition of NAA or DMSO. Equal cell numbers were treated to generate spheroplasts, washed to remove the cell wall, and analyzed by western blot to

determine the amounts of intracellular Bgl2. (B) Normalized cytoplasmic amounts of Bgl2 in wild-type and *boi1*Δ *boi2-aid* cells. Averages of three independent experiments. Error bars indicate SEM. (C) Electron microscopy images of *boi1*Δ *boi2-aid* mutants. Cells were treated as described in (A). After high pressure freezing, cells were processed for transmission electron microscopy. Arrowheads indicate secretory vesicles, N, nucleus, mit, mitochondria. Scale bar, 500nm.

Together, these results indicate that polarized delivery of exocytic vesicles is not affected in *boi1 boi2* mutants, and support the hypothesis that Boi1/2 are essential for the vesicle-thethering or fusion step after vesicles are transported to the daughter cells.

4.5. Identification of an exocyst gain-of-function mutation as a potential suppressor of Boi1/2

The results described in the previous sections show that Boi1 and Boi2 are essential for bud growth and cell viability. In striking contrast, *boi1*Δ *boi2*Δ cells described previously (Norden et al. 2006) are viable and showed no obvious growth defects. Why are these mutants viable? The viable *boi1 boi2* cells were generated in the BFA264-15D genetic background (Eric Billy laboratory, INSERM) before being crossed into S288c (Caren Norden, personal communication). We first wondered if differences between the two backgrounds could account for the survival of *boi1 boi2* mutants. Single *boi1* and *boi2* deletion mutants, which are viable, were generated by gene replacement and genetic crosses were set up to isolate double mutants in both BFA264-15D (15D) and S288c backgrounds.

Analysis of meiotic products by tetrad dissection (by Michael Maier in our laboratory) showed that *boi1 boi2* double mutants failed to form visible colonies in both backgrounds after ten days (18/18 double mutants in 15D, and 11/11 in S288c). Microscopic examination of the double mutants showed that *S288c boi1 boi2* cells arrested forming microcolonies of 3-4 cells. Interestingly, and unlike S288c mutants, after two weeks 3/18 15D *boi1 boi2* cells arrested as microcolonies containing around 100 cell bodies. These results show that genetic background differences between S288c and 15D might allow the survival of *boi1 boi2* cells for a limited number of division cycles. Furthermore, they suggest that additional suppressor mutations accumulated in some 15D *boi1 boi2* cells might account for their long term survival.

To identify these potential supressors, in collaboration with Leszek Pryszcz and Dr. Toni Gabaldón, we performed whole genome sequencing of the original 15D *boi1*Δ *boi2*Δ isolate (YMM236, (Norden et al. 2006)) and compared it to its wild-type isogenic strain, obtained from the laboratory of Eric Bailly (INSERM).

To assess variation at the single nucleotide level, we mapped all reads from each mutant strain onto the wild-type strain assembly. As a negative control, and to properly set the thresholds, we mapped the wild-type reads onto its own assembly, where no single nucleotide polymorphisms (SNPs) are expected. SNPs were called on positions that were highly covered (>10x) and unambiguous (>90%

consistency) both at the mapped and reference strains. This procedure identfied only 7 SNPs between YMM236 and its parental wild type strain (see table I).

SGD position	Name	Ref. codon	Codon	Mutation	Gene function
chrII:39982	**BNA4**	GAT	AAT	D281N	biosynthesis of NAD from tryptophan
chrIII:59865	**GFD2**	ATA	ATG	I280M	unknown function, suppressor of *dbp5*
chrV:221858	**YER034W**	ACC	AGC	T5S	unknown function
chrVIII:186001	**MSC7**	ATT	GTT	I268V	meiotic recombination
chrX:273982	**EXO70**	GGA	AGA	G388R	exocytosis
chrXII:799068	**NUP2**	TCG	CCG	S547P	Nucleoporin
chrXVI:390476	**SEC16**	GCG	GAG	A1138E	ER vesicle budding

Table I. Non-synonymus exonic SNPs in boi1d boi2Δ (BFA264-15D). List of point mutations that are present in a *boi1*Δ *boi2*Δ and absent in an isogenic wild-type.

To detemine if any of these mutations is linked to the survival of boi1 boi2 cells, we then performed genetic crosses between YMM236 and a wild type strain in the S288c background. As expected, only a fraction of *boi1*Δ *boi2*Δ spores recovered from this cross where able to form colonies. To isolate the putative *boi1 boi2* suppressor(s), one *boi1 boi2* isolate from this cross was then backcrossed six times with S288c wild type. Finally, we performed whole genome sequencing of colonies derived from four viable spores of a *boi1*Δ/BOI1+ *boi2*Δ/BOI2+ zygote after the 6th backcross. Candidate suppressors should be

present in both viable *boi1*Δ *boi2*Δ spores, and absent in the two BOI1+ BOI2+ clones. SNPs fulfilling these criteria are shown in Table II. A large number of SNPs were clustered around specific genomic regions, as expected by a selection sweep effect. Following the criteria described above, 31 SNPs passed the filter, of which 17 corresponded to non-synonymous mutations in 11 protein-coding genes (see table II and Apendix III). Notably, only one of the mutated genes was also found in the original 15D *boi1*Δ *boi2*Δ strain.

Indeed, two SNPs were found in the EXO70 gene, a subunit of the exocyst complex. The first one introduces a change in aminoacid 147 from an I to V, and the second at position 388 from G to R. Interestingly, the G388R mutation was independently isolated in a screen for EXO70 alleles able to rescue viability in Cdc42 and Rho3 mutants. Exo70^{G388R} (hereafter termed as EXO70*) is a gain-of-function allele; it is genetically dominant and its protein product is incorporated into endogenous exocyst complexes (Wu et al. 2010).

SGD position	Name	Ref. codon	Codon	Mutation	Gene function
chrV:297010	**ARG5,6**	AGA	GGA	R535G	arginine biosynthesis
chrVI:191001	**FAB1**	CGC	CAC	R2171H	1-phosphatidylinositol-3-phosphate 5-kinase
chrX:272185	**TRL1**	GAT	AAT	D99N	tRNA ligase
chrX:272557	**YJL086C**	CCC	CGC	P95R	Dubious open reading frame unlikely to encode a protein
chrX:273260	**EXO70**	GCA	GTA	A147V	Subunit of the exocyst complex
chrX:273982		GGA	AGA	G388R	
chrX:275946	**ALY2**	ATT	ACT	I684T	Protein proposed to regulate the endocytosis of plasma membrane proteins
chrX:276447		AAT	AGT	N517S	
chrXIII:24889	**PHO84**	AAA	GAA	K305E	High-affinity inorganic phosphate and low-affinity

					manganese transporter
chrXV:245066	**PSK2**	CTC	CCC	L524P	PAS domain containing S/T protein kinases
chrXVI:432088	**YPL062W**	TTG	TCG	L66S	Dubious open reading frame unlikely to encode a protein
chrXVI:458203		GTG	ATG	V85M	
chrXVI:458439		AGC	AAC	S6N	
chrXVI:459116	**KTR6**	AGG	TGG	R107W	Probable mannosylphosphate transferase
chrXVI:459225		AAA	AGA	K143R	
chrXVI:459327		TTA	TGA	L177*	
chrXVI:461195	**MNN9**	ATC	GTC	I257V	Subunit of Golgi mannosyltransferase complex

Table II. Exonic non-synonymus SNPs after boi1d boi2Δ backcrossing into S288c. List of SNPs that are present in a *boi1*Δ *boi2*Δ and absent in an isogenic wild-type after extensive backcrossing to the s288c background. "SGD position" indicates the "Ref. codon" corresponds to the sequence annotated whereas "codon" is the sequence found in our samples.

Together, our genetic and sequencing data show that the EXO70* allele arose after generation of *boi1*Δ *boi2*Δ strain in the BFA264-15D background and was carried over during crossing into s288c, presumably because it promotes cell viability in the absence of *BOI1* and *BOI2*. I next concentrated in the characterization of this exocyst mutation. However, it should be noted that other mutations identified in *boi1 boi2* mutants are also linked to membrane remodelling and the secretory pathway (like SEC16, FAB1, ALY2). Their possible role as potential *boi1 boi2* suppressors remains to be explored.

4.6. An exocyst gain-of-function mutation is a suppressor of Boi1/2 defects

To determine whether EXO70 is a suppressor of *boi1 boi2*, I introduced EXO70 under the control of a galactose promoter in a centromeric (pRS415) plasmid, and mutagenized aminoacid G388 to an R. A plasmid containing Gal1-EXO70* was sufficient to restore viability of *boi1*Δ *boi2-AID* on auxin plates whereas the wild-type version of the gene or the empty plasmid did not (Figure 4.6.1 A). I next replaced endogenous EXO70 with EXO70* with the same results (Figure 4.6.1 B). Also, overexpression of the endogenous EXO70 did not rescue viability of Boi mutants (Figure 4.6.1 C). Therefore, the G388R mutation in Exo70 can explain the viability of *boi1*Δ *boi2*Δ cells.

The crystal structure of Exo70 from yeast and mice has revealed a conserved rod structure with four different domains A, B, C and D. (Dong et al. 2005) Aminoacid G388 of Exo70 is located at the interface of domains C and D, which potentially cause rearrangements relative to each other within the structure of Exo70. However, the interactions with the rest of the exocyst complex are maintained (Wu et al. 2010). In addition, as EXO70* is a gain-of-function mutation, we tested whether it could rescue defects in other exocyst components and SNARE function. For that, we used the thermosensitive alleles *exo84-102, sec8-6, sec3-2, sec6-4* (encoding exocyst components) and *sec9-4* (encoding a t-SNARE) (Figure 6.4.1 D). We could only see a very

subtle improvement in growth of *exo84-102* and *sec3-4* but there were no major changes in viability of any of the other mutants tested. Interestingly, in *sec8-6* mutants, overexpression of the wild-type version of EXO70 rescues viability slightly better than EXO70*. As positive controls of the rescue we used *exo70-38* and *rho3-1* (Wu et al. 2010). Thus, EXO70* is not able to rescue viability of cells lacking other exocyst subunits apart from Exo70, suggesting that it cannot take over other subunit's function.

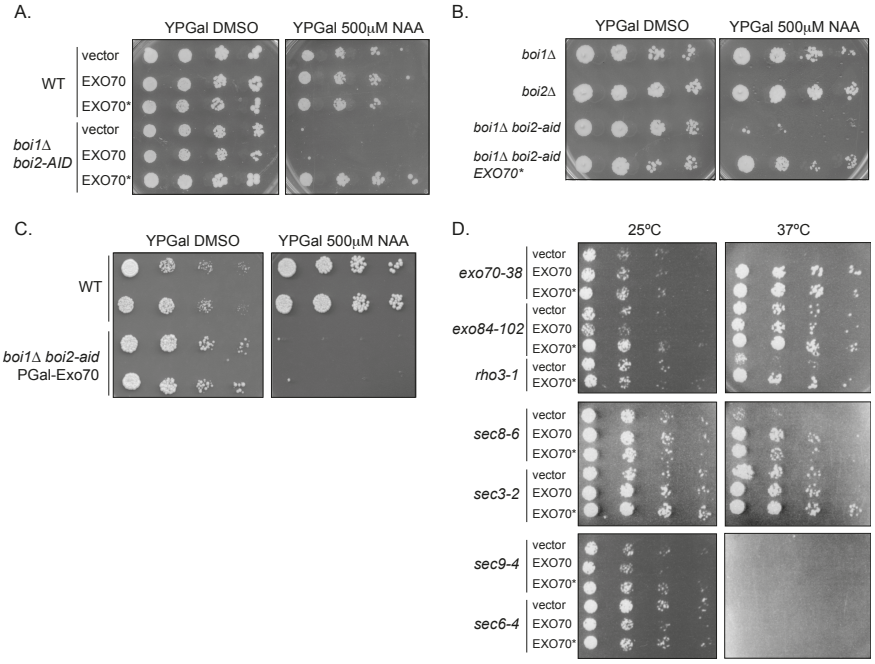

Figure 4.6.1. EXO70* is sufficient to rescue Boi1/2 lethality. (A) Serial dilutions of the indicated cells containing plasmids with EXO70, or EXO70*, were spotted onto YPG Leu- plates containing NAA or DMSO, and incubated for 4 days at 30ºC. (B,C) Serial dilutions of the indicated cells were plated onto YPG plates containing NAA or DMSO, and incubated for 3 days at 30ºC. (D) Serial dilutions of the

indicated cells containing plasmids with EXO70, or EXO70*, were spotted onto YPG Leu- plates containing NAA or DMSO, and incubated for 3 days at 25 or 37ºC.

Thus, these data show that a dominant mutation in the exocyst complex can restore viability in Boi1/2 double mutants.

To characterize the phenotype of EXO70* cells lacking Boi1 and Boi2, I imaged *boi1*Δ *boi2-aid* cells with a plasmid containing EXO70 and EXO70* and in the presence of auxin and DMSO. The bud growth rate was measured in μm/min. Control *boi1*Δ boi2-AID cells with EXO70 showed a growth rate of 0.033μm/min, slightly higher than the ones carrying the empty plasmid (0.028μm/min), whereas in the presence of auxin the growth rate was nearly zero (Figure 4.6.2 B). Control *boi1*Δ *boi2-aid* cells with EXO70* grow at a higher speed than the ones carrying the empty plasmid. In contrast, *boi1*Δ boi2-*aid* cells with EXO70* in the presence of auxin grow at an intermediate velocity.

The fact that boi mutants have a defect in Bgl2 vesicles secretion, and that EXO70* rescues *boi1*Δ *boi2-aid* lethality and bud growth defects, suggest that Boi proteins and the exocyst complex may be involved in the same biological process. Because loss of function of Boi1/2 cause an accumulation of internal Bgl2 (Figure 4.4), we tested whether the exocyst gain of function allele EXO70* could rescue such defects. The quantification is the normalized mean of three assays, and a representative blot is shown (Figure 4.6.2). These results show that

boi1 boi2 defective cells block Bgl2 vesicle secretion, and such block is bypassed by EXO70*.

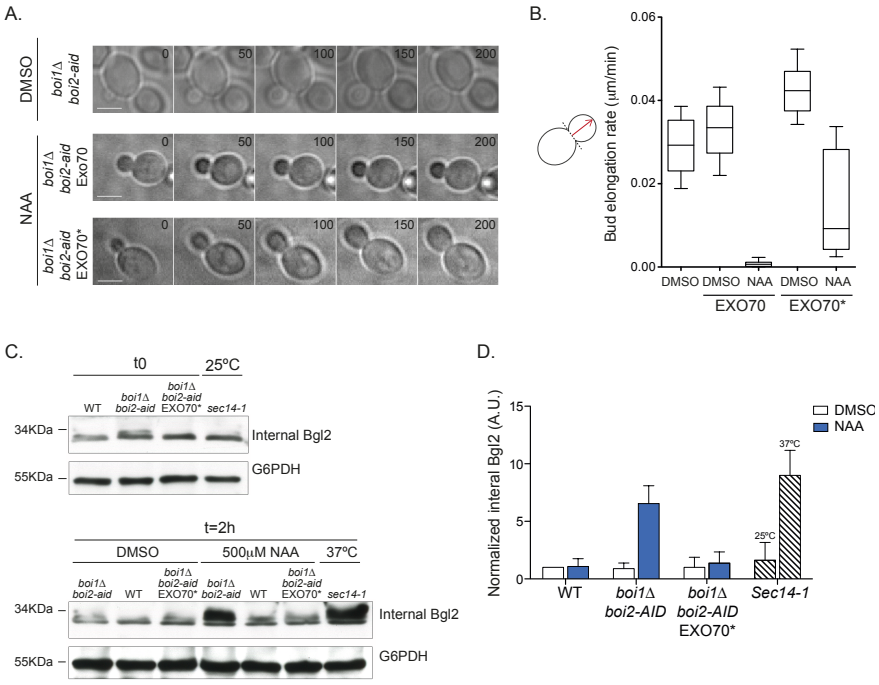

Figure 4.6.2. The exocust gain-of-function allele EXO70* rescues Boi1/2 defects in exocytosis. (A) Differential interference contrast (DIC) time-lapse images of individual *boi1*Δ *boi2-aid* cells. Cells in log phase were imaged in SC-Leu with galactose, 30min after addition of either DMSO or NAA at 30ºC. (B) Quantification of cells in (A). Bud growth rate (µm/min) of *boi1*Δ *boi2-aid* cells transformed with an empty vector or plasmid containing EXO70 or EXO70*. Error bars indicate SEM. n (empty plasmid) = 20, n (EXO70 DMSO) = 21, n (EXO70 NAA) = 14, n (EXO70* DMSO) = 22, n (EXO70* NAA) = 35. (C) Immunoblot of internal fraction of Bgl2. Wild-type and the indicated boi1/2 mutant cells were grown in YPR to logarithmic phase at 25°C. After 1h in YPG, 500uM NAA or DMSO was added. Cells were harvested at time 0, addition of NAA or DMSO, and 2h later. *sec14-1* cells were grown at 25°C and shifted to 37°C for 2 h. Equal cell

numbers were treated to generate spheroplasts, washed to remove the cell wall, and analyzed by Western blot to determine the amounts of intracellular Bgl2. Representative example of two independent experiments. (D) Quantification of cells in (C). Level of Bgl2 was normalized to the wild-type in DMSO. Error bars indicate SEM.

Overall, our results show that the essential function of Boi1 and Boi2 can be bypassed by a gain-of-function allele of an exocyst subunit. This suggests that Boi1/2 function in exocytosis through activation of the exocyst complex.

4.7. Boi1/2 regulate exocyst function through Rho GTPases

How could Boi1,2 activate the exocyst? Boi1/2 defects are suppressed by overexpression of RHO3 and RHO4 GTPases (Matsui et al. 1996). Morover, toxicity caused by Boi1 overexpression is palliated by co-overexpression of Boi1 together with Cdc42 (Bender, Lo, H. Lee, Kokojan, J. Peterson, et al. 1996a). These findings suggested that it is important to maintain stoichiometry of both proteins, otherwise Boi1 sequesters Cdc42 and acts as a dominant negative.

In order to test whether Boi1/2 regulate the master regulator of cell polarity, Cdc42, active state, we used the CRIB-tdTomato as a reporter for active Cdc42 (Tong et al. 2007). We compared localization of the

CRIB domain in wild-type and *boi1*Δ *boi2-aid* after 2h of incubation with 250μM NAA or DMSO. We could see CRIB-tdTomato at the cortex of both unbudded and budded cells, but never at the division site, as previously reported only becomes activated at the adjacent bud site when cells polarize in the next cell cycle (Tong et al. 2007; Atkins et al. 2013). We could not detect any difference in CRIB localization between DMSO and NAA samples (Figure 4.7.1 A, B). However, we observed higher signal intensity of CRIB-tdTomato in *boi1 boi2* mutant cells compared to wild-type (Figure 4.7.1 C), suggesting that there is more Cdc42-GTP at growth sites in *boi1 boi2* mutants even if there is no vesicle fusion. Because at the time of NAA addition there is already a difference in CRIB-tdTomato intensity, one possibility is that Boi1 could be an inhibitor of Cdc42.

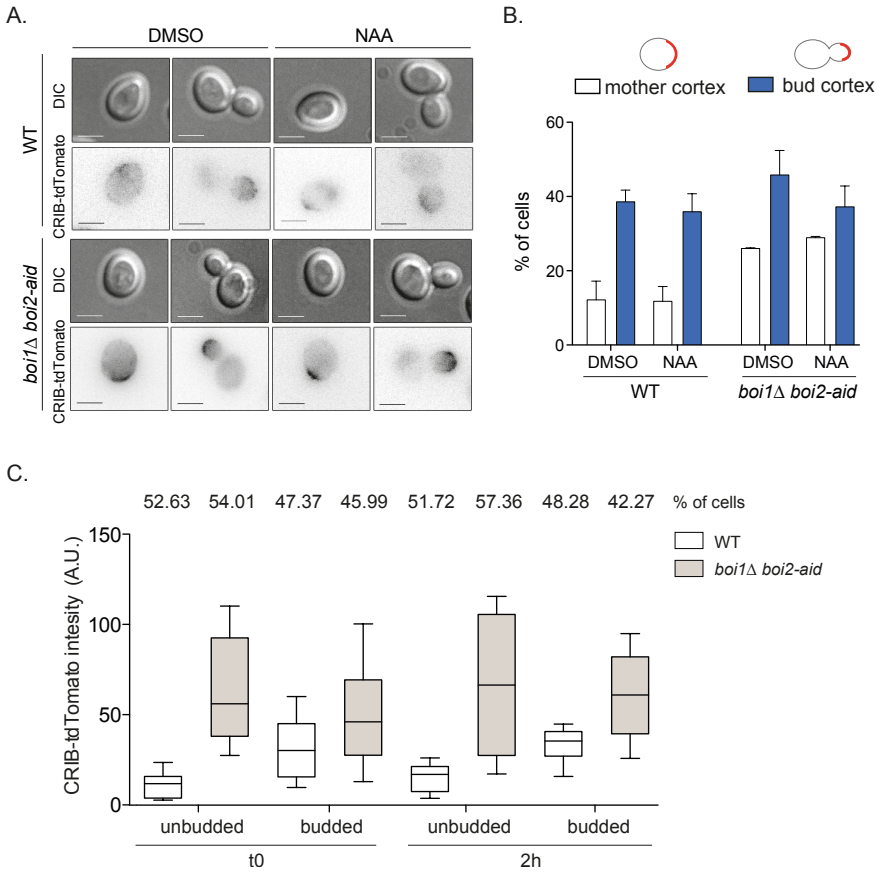

Figure 4.7.1. Cdc42 is active in Boi1/2 mutants. (A) Examples of wild-type and *boi1*Δ *boi2-aid* cells expressing CRIB-tdTomato were grown in YPR, then in YPG for 2h and fluorescent and DIC images were taken after 2h in either 250μM NAA or DMSO, at 25ºC. Scale bar, 5μm. (B) Quantification of the percentage of wild-type and *boi1*Δ *boi2-aid* cells showing CRIB-tdTomato localization at the cell cortex of unbudded (mother cortex) or budded (bud cortex) cells in (A). Error bars indicate SEM. n > 100 in all categories. (C) Quantification of the fluorescence intensity, in arbitrary units, using ImageJ software. Intensity from the area around the fluorscent signal was measured and the cytoplasmic background of the same area was substracted. Numbers represent the percentage of budded/unbudded cells over total population. Error bars indicate SEM. n (WT, unbudded t0) = 11, n (*boi1 boi2*,

unbudded t0) = 20, n (WT, budded t0) = 23, n (*boi1 boi2*, budded t0) = 23, n (WT, unbudded 2h) = 14, n = (*boi1 boi2*, unbudded 2h) = 20, n (WT, budded 2h) = 9, n (*boi1 boi2*, budded 2h) = 20.

We next investigated whether hyperactivation of Rho GTPase activity could rescue Boi1/2 defects. To test this possibility, we used a novel ts allele of the gene encoding the GAP BEM2, *bem2-84* (Atkins et al. 2013). The Bem2-GAP domain promotes GTP hydrolysis by Rho1, Cdc42 and Rho4 (J. Peterson et al. 1994; T. Wang & Bretscher 1995; Marquitz et al. 2002; Gong et al. 2013). However, there is evidence for Rho1 not being hyperactivated by this particular allele of Bem2 (Atkins et al. 2013). Interestingly, the *bem2-84* allele could rescue viability of *boi1*Δ *boi2-aid* mutants in the presence of NAA even at 25ºC (Figure 4.7.2 A). Wild-type, *bem2-84*, *boi1*Δ *boi2-aid*, and double mutants were treated with 250μM NAA and the cell-surface growth of individual cells was observed by microscopy and quantified (Figure 4.7.2 B, C). The buds in *bem2-84 boi1*Δ *boi2-aid* and *bem2-84* cells increased in volume at a rate comparable to wild-type cells, slightly faster in the latter. In contrast, the buds in *boi1*Δ *boi2-aid* cells treated with 250μM NAA did not grow (Figure 4.7.2 C).

Together, these result suggest that Boi1/2 function in bud emergence and bud growth through Rho GTPase-dependent regulation of the exocyst complex.

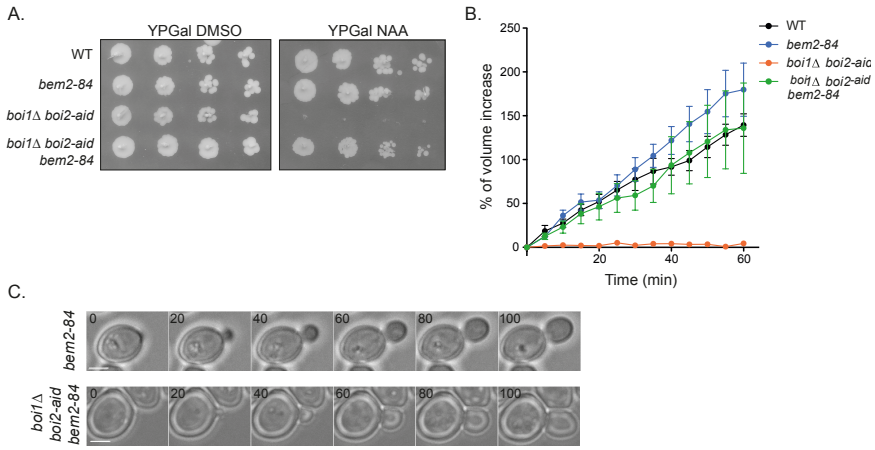

Figure 4.7.2. Rho GTPases hyperactivation rescues growth defects of *boi1 boi2* mutants. (A) Serial dilutions of the indicated strains were spotted onto YPG plates with DMSO and 250µM NAA. Cells were grown at 30ºC. (B) Wild-type, *boi1*Δ *boi2-aid*, *bem2-84* and *boi1*Δ *boi2-aid bem2-84* cells were grown in YPR at 25ºC. Medium was changed to YPG and after 2h, 250 µM NAA was added. 1h after NAA addition cells were imaged on a confocal microscope every 5min. Volume of buds was measured over time. In the plot, the percentage increase in bud surface area over time is represented. The volume of the bud was measured from individual DIC images with BudJ and converted to a percentage increase over time. Buds of different sizes were followed. The error bars indicate SEM where n=19 (WT), n=13 (*bem2-84*), n=14 (*boi1*Δ *boi2-aid*) and n=10 (*bem2-84 boi1*Δ *boi2-aid*). (C) DIC time-lapse images of individual cells showing the rescue of bud growth in *boi1*Δ *boi2-aid* in the presence of 250 µM NAA. Cells were imaged at 25ºC. Time is represented in minutes. (C) Examples of cells quantified in (B).

4.8. Boi1 and Boi2 proteins are inhibitors of abscission

Boi1 and Boi2 were initially described as polarity regulators (Bender, Lo, H. Lee, Kokojan, J. Peterson, et al. 1996a; Matsui et al. 1996). My results show that Boi1 and Boi2 are essential for a late-step of vesicle fusion to the plasma membrane, and as a consequence, those mutants are unable to grow. These results are consistent with previous observations of Boi1 and Boi2 function during interphase, but, what about Boi1/2 function during cytokinesis?

In order to address whether Boi1/2 have a positive function during cytokinesis, we arrested *boi1 boi2-aid* cells in S-phase with 200uM of hydroxyurea (HU). By adding auxin to the arrested cells, *Boi2-aid* was depleted after the daughter cell is already large, since otherwise cells would arrest growth and they would not progress into the cycle. Cells were then released from the S-phase arrest and imaged by time lapse microscopy to assess cytokinesis. The depletion of Boi1/2 did not affect contraction of the plasma membrane at the bud neck, or separation of the daughter cell after cytokinesis, as seen using the GFP-CAAX reporter (Figure 4.8.1 C). Abscission time is defined as the time between contraction and resolution of the membrane at the bud neck (Figure 4.8.1. A). Similar results were observed using myo1-GFP. The actomyosin ring contracts with normal kinetics (Figure 4.8.4 C, D). Therefore, the timing of cytokinesis was not affected by disruption of Boi1/2 function.

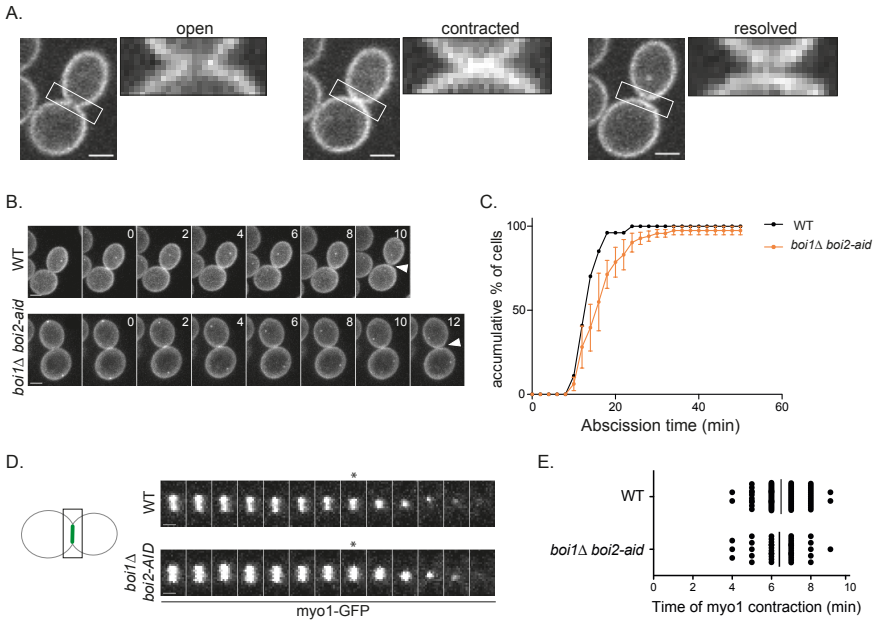

Figure 4.8.1. (A) Images of cells expressing GFP-CAAX and spc42-GFP showing open, contracted and resolved membranes. Scale bar, 2μm. (B) Wild-type and *boi1*Δ *boi2-aid* cells were grown in YPD at 25ºC. After 1h of 200mM HU arrest, 250μM NAA and 36μM β-estradiol was added in the medium. All cells have an estradiol inducible system of the Galacose promoter expression (ADGEV), which allow PGal-TIR1 and PGal-GFP-CAAX expression by adding β-estradiol in the medium. Cells were released into the cycle in the presence of 250μM NAA and 36μM β-estradiol. Time-lapse images were acquired every 2min with a spinning disk confocal microscope. (C) Quantification of abscission time of cells in (B). The time between contraction and resolution of the membranes was quantified. The plot shows the accumulative percentage of cells that undergo abscission in the indicated strains. Error bars indicate SEM. n (WT) = 52, n = (*boi1 boi2*) = 53. (D) Cells in log phase expressing myosin1-GFP were imaged in a spinning disk microscope taking stacks of images every 1min. Cells were grown at 25ºC in YPR. After 2h in YPG, 250μM NAA was added into the medium and 1h later the imaging started. Scale bar, 1μm.

(E) Quantification of ring contraction time of cells in (C). n (WT) = 72, n (*boi1 boi2*) = 40.

Next, I tested whether Boi1/2 are inhibitors of cytokinesis. Lack of Boi1 and Boi2 was shown to suppress abscission delays in mutants like *top2-4*, *ase1*, *ndc10-1* or *ndc80-1* (Norden et al. 2006; Mendoza et al. 2009). Because all the experiments in these previous studies were performed using the *boi1*Δ *boi2*Δ strain, which I showed contains *EXO70** (among other SNPs), I re-investigated whether the inhibition of abscission in these mutants is indeed dependent on Boi function (section 4.5).

As previously described, one way to trigger the NoCut checkpoint is by inactivating topoisomerase II function, generating anaphase chromatin bridges (Figure 4.8.3 A) (Mendoza et al. 2009). First, I confirmed that *top2-4* cells indeed have delays in cytokinesis. Wild-type and *top2-4* cells were arrested with αfactor and released from G1 at the restrictive temperature. Anapahse progression was followed with spc42-GFP signal, and plasma membrane contraction at the bud neck with GFP-CAAX. Interestingly, after 50min of membrane contraction we could not observe any membrane resolution (abscission) occurring in *top2* mutants (Figure 4.8.2 C). In contrast, all wild-types cells finish abscission 20min after contraction. Therefore, chromosomal bridges caused by topoisomerase II defects trigger an abscission delay.

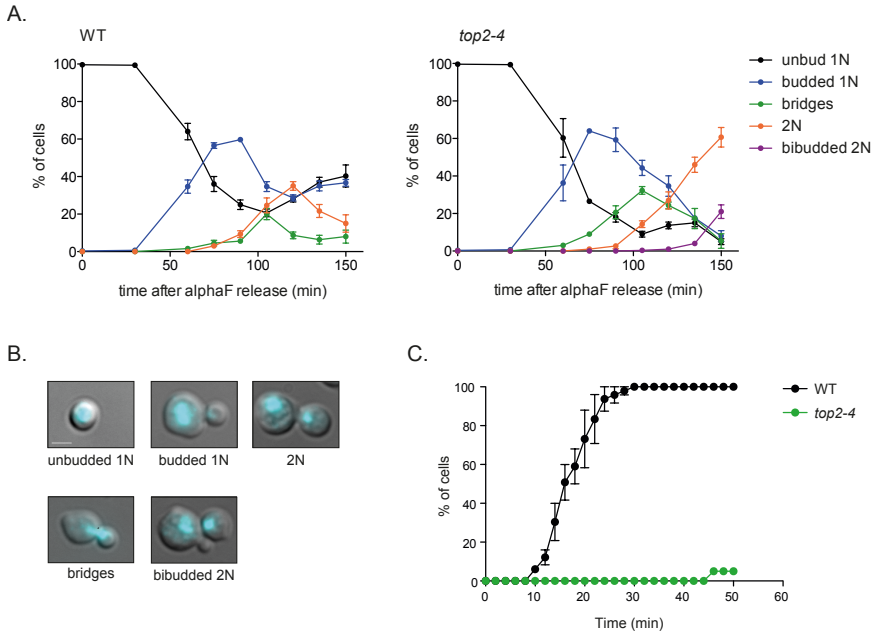

Figure 4.8.2. *top2-4* mutants have chromosome bridges and delay abscission. (A) Quantification of budding and nuclear content in cells were grown in YPDA and arrested for 2h with αfactor at 25ºC. Temperature shift to 37ºC was performed 15min before release. At each time point, cells were fixed with 70% ethanol and stained with DAPI. Then cells were visualized by fluorescent microscopy and DIC. Averages of 3 independent replicas. Error bars indicate SEM. n > 100 cells. (B) Cells quantified in (A). Scale bar, 5µm. (C) Quantification of abscission timing in wild-type and *top2-4* cells expressing GFP-CAAX and spc42-GFP. Cells were arrested with alpha-factor, and the temperature was shifted to 37ºC 15min before release. Imaging started 2h after release. Error bars indicate SEM. n (WT) = 49, n (*top2-4*) = 20.

To assess if inhibition of abscission depends in Boi1/2, the S-phase arrest and release described above was performed in *top2-ts* cells expressing the GFP-CAAX reporter, in the presence and absence of

Boi1/2. We observed that 75% of *top2-4* mutants released from s-phase at the restrictive temperature, inhibited abscission and cell separation for more than 50min after contraction of the membranes in the presence of Boi1/2, but only 20% had defects after Boi1/2 depletion (Figure 4.8.3). Thus, this experiment confirms that Boi1/2 are inhibitors of abscission in cells with chromatin bridges.

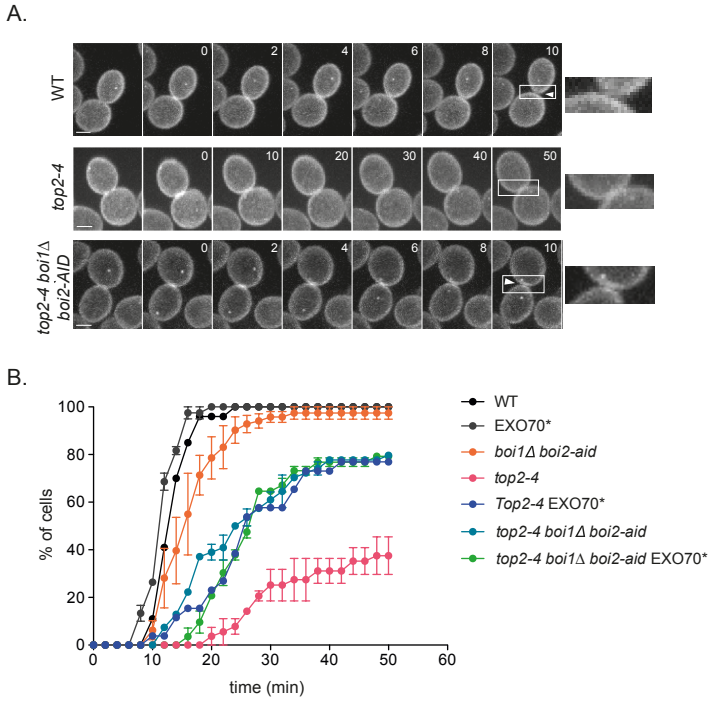

Figure 4.8.3. *top2-4* mutants delay abscission in a Boi1/2 and EXO70* dependent manner. (A) The indicated strains were grown in YPD at 25ºC. After 1h of 200mM HU arrest, 250μM NAA was added in the medium. All cells have the β-estradiol inducible system of Gal promoter expression (ADGEV), which allow PGal-TIR1 and PGal-GFP-CAAX expression. After 1h 30min, cells were released into the cycle in the presence of 250μM NAA and β-estradiol and temperature was shifted to 37ºC. Time-lapse images were acquired with a spinning disk confocal

microscope. Arrowheads indicate that membranes are separated. (B) Quantification of abscission times of the indicated strains treated as in (A). Between 15-25 cells were analysed for each independent experiment. Error bars indicate SEM.

As explained in the introduction (section 1.2.5), the Aurora kinase Ipl1 acts upstream of the NoCut pathway by regulating the localization of Boi1 and Boi2 (Norden et al. 2006; Mendoza et al. 2009). The functional evidence linking Ipl1 and Boi proteins is based on localization experiments. After an αFactor arrest, wild-type and Ipl1 mutant cells were released into the cycle and followed localization of Boi1-GFP over time. It was reported that Boi1-GFP was completely delocalized from the cortex and the bud neck in *ipl1-321* mutants and it was observed in the nucleus instead. However we were unable to reproduce this result, as localization or intensity of Boi1-GFP during cytokinesis is not altered by disruption of Ipl1 function (Figure 4.8.4).

Interestingly, at the restrictive temperature, *ipl1-321* mutants are delayed in budding, which might explain, partially, the reduction of the signal of Boi1-GFP at the bud cortex. This result suggests that Boi1 localization at the neck is not dependent on Ipl1, which raises the question whether they actually work in the same pathway.

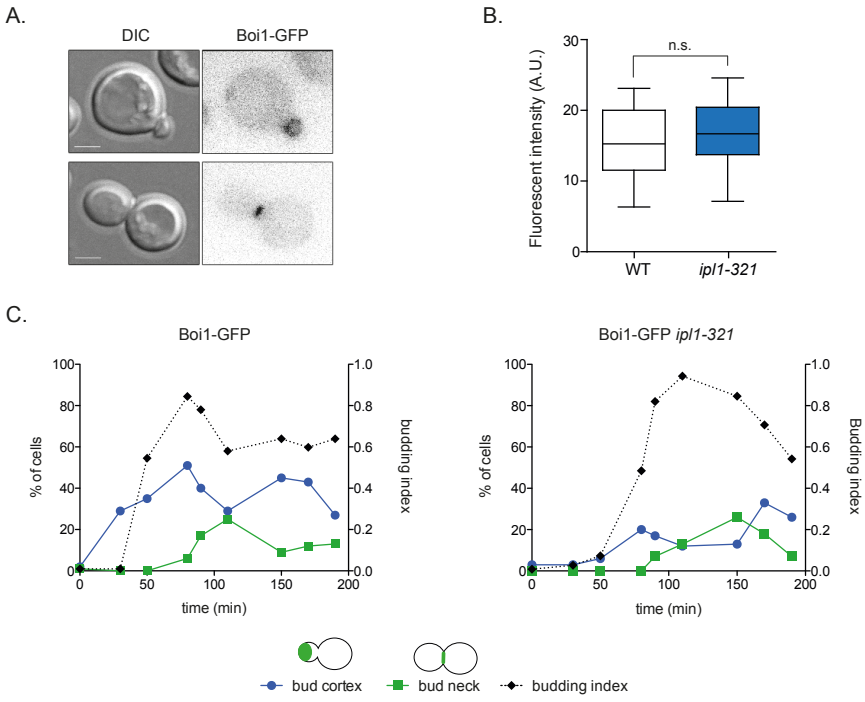

Figure 4.8.4. Ipl1 function is not required for Boi1-GFP localization at the division site. (A) Boi1-GFP localization in wild-type cells. Scale bar, 2μm. (B) Whisk and boxes representation of the fluorescent intensity of Boi1-GFP at the bud neck in wild-type and *ipl1-321* cells 105 and 150min after alpha-factor release from experiment in (C). Intensity was measured with ImageJ. Error bars indicate SEM. n (WT) = 22, n (*ipl1-321*) = 26. P value (t-test) = 0.46. (C) Wild-type and *ipl1-321* cells expressing Boi1-GFP were grown in YPD at 25ºC. Cells were arrested in G1 with αfactor for 2h at 25ºC. The temperature shift to 37ºC was performed 15min before αfactor release. Still pictures were taken at the indicated time points. Number of cells showing Boi1-GFP at the bud cortex or bud neck were quantified. In the right axis the percentage of budded cells is represented (budding index). N > 100.

4.9. The exocyst as an effector of the Nocut pathway

Chromatin bridges caused by topoisomerase II inactivation inhibit abscission in a Boi1/2-dependent manner. Because cytokinesis has been proposed to depend on exocyst function, we characterized abscission in Exo70 loss-of-function and gain of function mutants.

As we hypothesise that Exo70 regulation might be enough to regulate abscission, we first tested if Exo70-deficient cells had any defect in cytokinesis. For that, we used a thermosensitive allele of Exo70, *exo70-38*. In order to test the function of Exo70 only during cytokinesis, we arrested the cells with HU, so that the protein can do its essential function during bud growth. By looking at still images of *exo70-38* cells expressing GFP-CAAX as a membrane reporter, we detected an increase in cells with contracted membranes compared to wild-type, suggesting a defect in abscission (Figure 4.9.1 A).

Inactivation of Exo70 during cytokinesis caused a delay in abscission (Figure 4.9.1 B). By live doing cell imaging of *exo70-38* cells and measuring the time contraction and resolution and there is a 10min delay comparing *exo70-38* cells with wild-type. This difference in penetrance of the phenotype might be due to a difference in the resolution of the microscopes used in each experiment. This result shows that exocyst mutants generate delays in abscission and suggests that the delay in cytokinesis of *top2-4* (Figure 4.8.3 B) could not

depend only in exocyst regulation as the defect is much more pronounced in *top2-4* mutants than in *exo70-38*.

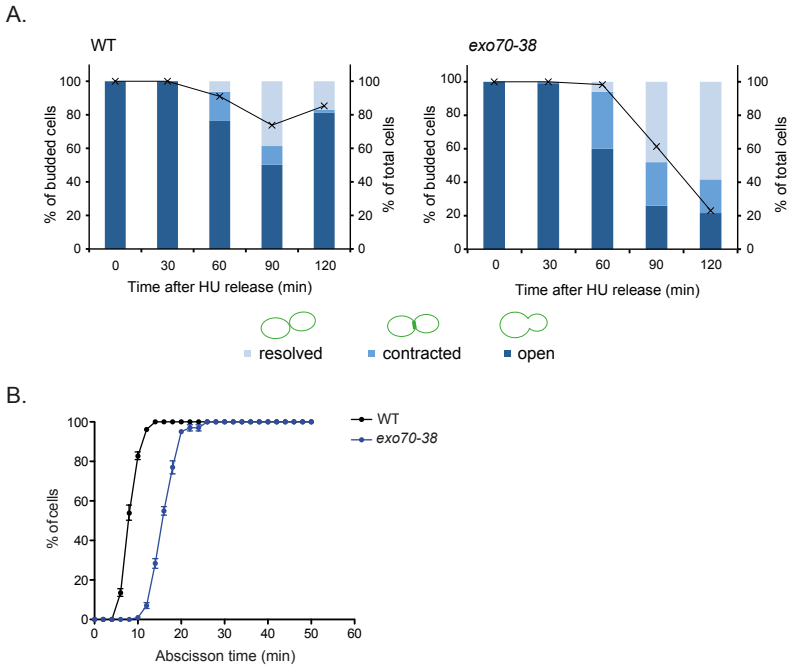

Figure 4.9.1. Abscission defects in exocyst loss of function allele exo70-38. (A) Quantifications of still images of wild-type and *exo70-38* mutants expressing GFP-CAAX and spc42-GFP. Cells were grown in YPD and arrested for 2.5h with 200mM HU at 25ºC. 1h after the arrest, 36 M of β-estradiol was added into the medium to induce GFP-CAAX exoression. At the time of release, temperature was shifted at 37ºC. Pictures were taken every 30min. (B) Quantification of abscission times from a time-lapse of live cells in a confocal spinning disk. Wild-type and *exo70-38* cells were treated as in (A).

In summary, Boi1/2 are required for activation of the exocyst during bud growth, and for cytokinesis inhibition in *top2-4* mutants.

Hyperactivation of the exocyst did not cause faster abscission in unchallenged cells. However, *top2-4* exo70* cells failed to inhibit abscission (Figure 4.8.3 B). One possibility would be that boi1/2 are required for vesicle fusion of only a certain type of vesicles. In that case, those vesicles could contain a certain cargo which could be essential for cytokinesis inhibition. Lack of Boi1/2 in the presence of chromosomal bridges would not allow such vesicles to reach the plasma membrane at the bud neck delaying abscission.

It has recently been shown that in wild-type cells, the exocyst is disassembled during metaphase through a CDK-dependent phosphorylation on Exo84 (Luo et al. 2013), preventing growth until exit of mitosis when growth is restored to finish division. In the light of these results, we hypothesise that Nocut could require inhibition of the exocyst function in order to inhibit cytokinesis.

We performed co-precipitation assays during interphase, anaphase and telophase of wild-type and *top2-4* cells. The cell cycle stage was determined by DAPI staining of cells released from alpha-factor. Interestingly, we could observe that by pulling down Exo84, the amounts of Exo70 that co-immunoprecipitate are slightly reduced in *top2-4* cells at the time of telophase (Figure 4.9.2). However it is likely that the small reduction in disassembly does not account for the cytokinetic inhibition observed in *top2-4* cells. This hypothesis should be further investigated.

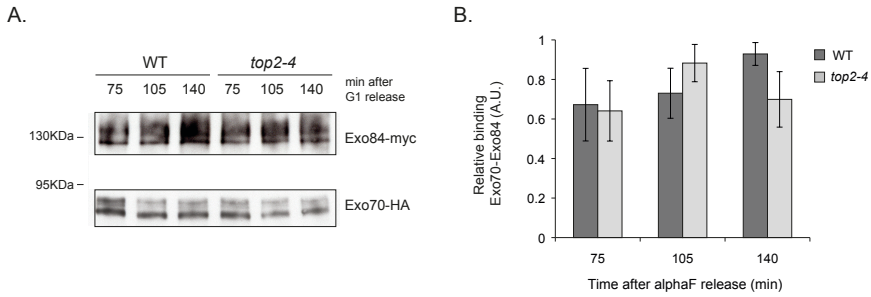

Figure 4.9.2. Exo84-Exo70 co-immunoprecipitation assays of *top2-4* cells. (A) Wild-type and *top2-4* cells expressing Exo84-9myc and Exo70-6HA were grown in YPD at 25ºC. Cells were arrested in G1 with alpha-factor and temperature was shifted to 37ºC 15min before release. Exo84-myc was immunoprecipitated using the anti-myc antibody after 75, 105 and 140min after alpha-factor release. Then, western blots were performed to detect the amounts of coprecipitating Exo70. (B) Quantification of the amounts of Exo70-HA co-immunoprecipitated with Exo84-9myc. Amounts of Exo70 were normalized to Exo84-9myc levels. The plot represents averages of 4 different experiments with SEM.

5. Discussion

In order to investigate the mechanism of action of Boi1 and Boi2 proteins in the context of both cell polarity and the NoCut pathway, we created a conditional mutant using an auxin-based degron. We have shown that Boi proteins are essential for a late-step of exocytic vesicle fusion. Cells lacking Boi1 and Boi2 arrest cell growth. As a secondary effect, these cells become large, rounded and lyse. We have identified an exocyst gain-of-funcion allele that rescues Boi defects. Moreover, hyperactivation of Cdc42/Rho4 GTPases also rescues Boi1/2 mutant phenotypes. In wild-type cells, Boi1/2 do not promote cytokinesis, however, in the presence of chromatin bridges they are necessary for inhibition of abscission through their function in the NoCut checkpoint.

5.1. Boi1 and Boi2 mutants arrest bud growth

By using an auxin-based Boi1/2 conditional mutant I have shown that Boi1 and Boi2 functions are essential for the cell. Deletion of either of them does not affect cell viability, but when depleting Boi2 in a *boi1*Δ background, cells die. In addition, in the absence of Boi1/2 function, cells arrest bud growth. This arrest happens in the first cycle after Boi2 depletion, because after G1 release, *boi1 boi2* cells growing in the presence of NAA do not form microcolonies but arrest at the 1 or 2 cell stage (Figure 4.1.2 E). I have demonstrated that the cause for this bud growth arrest is a block in exocytic vesicle fusion with the plasma membrane. Cells lacking Boi1/2 accumulate Bgl2 secretory vesicles as seen by immunoblotting the cytoplasmic fraction of Bgl2 and by electron microscopy (Figure 4.4.). There are at least two types of secretory vesicles in budding yeast. We have not tested whether secretion of the cargo in the other main type of secretory vesicles, invertase, is defective in Boi1/2 mutants, but it would be very interesting to find out. However, we anticipate that Boi proteins are not required for all exocyst-dependent secretion events, because cytokinesis (which requires the exocyst), is normal in Boi-deficient cells.

By live cell imaging, we observed that *boi1 boi2* cells arrest bud growth, but also cycle progression. For instance, unbudded cells do not duplicate SPBs; and budded cells with duplicated SPBs show short spindles that never elongate. In the literature we find other mutants

like *cdc24-ts*, or wild-type cells under stress, that also show bud growth and G2 arrest. Even though these cells complete DNA replication, they arrest in G2 (Lew & Reed 1995a; Beck et al. 2001). This cell cycle arrest in G2 is mediated by the Morphogenesis checkpoint, which monitors bud formation and delays nuclear division in order to prevent the formation of multinucleated cells (Lew & Reed 1995a; Lew 2003; Keaton & Lew 2006). From the *boi1 boi2* phenotype, it is likely that the arrest in growth is mediated by the morphogenesis checkpoint. In this case, it would be easy to test. On one hand we should observe hyperphosphorylation of Swe1, the kinase that mediates the checkpoint, by western blot and on the other, multinucleated cells when deleting Swe1. If it was the case, it explains why in *boi1 boi2* mutants we see an arrest in growth and cells do not go into mitosis.

Moreover, I have also shown that the defect in exocytosis of *boi1 boi2* cells is not caused by actin cytoskeleton malfunctioning. As seen by phalloidin staining of F-actin (Figure 4.3.1), polarity is maintained at the bud tip and bud neck. In addition, localization of cell polarity determinants like Cdc24, Bem1 or exocyst subunits, is not changed compared to wild-type (Figure 4.3.2). Therefore, suggesting that in *boi1 boi2* mutants vesicles are transported to the vicinity of the growth sites, but they are not fused to the plasma membrane. In the initial description of Boi1/2 mutants, *boi1*Δ *PGal-Boi2* cells were growing in YPD for 24h, and by then the cells looked large, rounded and showing depolarized actin. For that reason, Boi1/2 were described as

determinants of cell polarity (Matsui et al. 1996). However, my results indicate that this is a secondary defect of Boi mutants.

5.1.1. Identification of supressors in *boi1*Δ *boi2*Δ cells

Because others and us have shown that Boi1/2 function is essential for cell viability (Matsui et al. 1996; Bender, Lo, H. Lee, Kokojan, J. Peterson, et al. 1996a), we wondered why a *boi1*Δ *boi2*Δ strain could grow with normal rates (Norden et al. 2006; Mendoza et al. 2009). We presumed that identifying suppressor mutations in Boi mutants could help us in the understanding of their function.

The SNPs identified in the *boi1*Δ *boi2*Δ segregate together with BOI1 and BOI2 deletions, but we do not know if and how all of them contribute in the viability of Boi mutants. Most of them do not have any obvious relationship with exocytosis or cytokinesis, but some of them are related to internal membranes or the cell wall:

- FAB1 encodes the kinase that synthesize PI(3,5)P2, which is required for proper trafficking of endocytic cargo from the late endosomes to the vacuole.
- Sec16 is a COPII vesicle coat protein required for budding of vesicles from the endoplasmic reticulum (ER) and it is essential for the formation of the ER exit sites.
- Aly2 is an alpha-arrestin that promotes endocytosis of membrane proteins.

- KTR6 and MNN9 are related to mannan biosynthesis, which is the main component of the yeast cell wall.

Even if it has not been tested, it could be that one or more of these alterations can rescue viability of *boi1 boi2* cells. Or, it could also be that their function is not sufficient to bypass Boi1/2 function, but combinations of them might improve the fitness of Boi mutants.

We focused our work exclusively in one SNP: $Exo70^{G388R}$ (EXO70*). The EXO70* might had aroused during the very initial passages of *boi1*Δ *boi2*Δ cells, because it is not found in the wild-type parental strain (YMM235). However, it is determinant for viability of Boi1/2 mutants as after *boi1*Δ *boi2*Δ backcrossing to the s288c background it was the only SNP that was conserved from the sequencing of the parental strains.

5.1.2. EXO70* rescues *boi boi2* defective cells

I have shown that EXO70* is able to rescue the exocytosis defect and viability of *boi1*Δ *boi2-aid* cells. These results show that, genetically, Boi function is upstream of the exocyst. EXO70* is an exocyst gain-of-function allele which was previously identified as a suppressor for Rho3 and Cdc42 defective cells (Wu et al. 2010), and we know that on one hand overexpression of Rho3/4 rescue lethality of *boi1 boi2* cells, and co-overexpression of Boi1 and Cdc42 rescues lethality of

overexpression of either of them (Bender, Lo, H. Lee, Kokojan, J. Peterson, et al. 1996a; Matsui et al. 1996). All together, these results suggest that Boi proteins could be working together with Rho GTPases in order to regulate exocytosis.

It has been proposed that Rho GTPases might regulate exocyst function by releasing an autoinhibitory inhibition of the complex in a process analogous to the GTPase regulation of formins (Goode & Eck 2007; Lu et al. 2007). The structure of Exo70 is formed by 4 domains (A, B, C, D). The interaction with the plasma membrane is mediated by domain D, that binds to PIP2, whereas the interaction to Rho3 is mediated by domain C (He, Xi, X. Zhang, et al. 2007b). In a basal state, Exo70 might be folded in particular way that does not allow interactions with other subunits of the complex. In contrast, upon binding to Cdc42 or Rho3-GTP, a change in conformation would allow Exo70 interaction with the rest of the complex being now active and would increase rates of vesicle tethering and fusion to the plasma membrane (Figure 5.1). Thus, the GTPase activity would regulate exocyst catalytic function to control rates of docking and vesicle fusion with the plasma membrane (Wu et al. 2008).

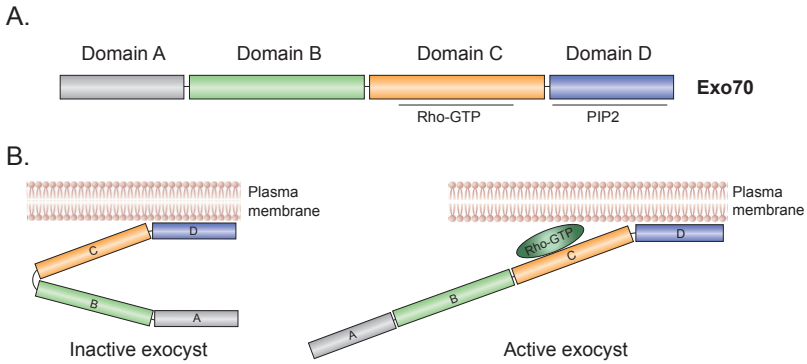

Figure 5.1. Excocyst function regulation by Rho GTPases. (A) Structure of Exo70. (B) Model of exocyst regulation by GTPases. Adapted from (Wu et al. 2008)

The G388R mutation in Exo70 is located at the interface of domains B and C. The substitution of glycin for an arginine, would presumably create modifications in the structure of Exo70, as glycin is a very small aminoacid whereas arginine has a large and electrically charged side chain. These modifications might, in turn, facilitate Exo70 interactions with the other components of the exocyst bypassing the need for Rho3-GTPbinding.

Putting together this model with the Boi1/2 results described here, one possible model would be that Boi proteins mediate Exo70 interaction with Rho GTPases. There is evidence that on one hand, overexpression of Rho3/4 rescues Boi1/2 defects (Matsui et al. 1996; Bender, Lo, H. Lee, Kokojan, J. Peterson, et al. 1996a), on the other, EXO70* rescues both, Boi1/2 (our work) and Rho3/Cdc42 defects

(Wu et al. 2010). Thus, in the absence of Boi1/2, there is no vesicle fusion to the plasma membrane because there is no functional interaction between Rho GTPases and the exocyst complex, and as a result cells arrest growth. In contrast, in *boi1 boi2* mutants expressing EXO70*, the interaction between Rho GTPases and the exocyst would take place anyway, and consequently there is cell growth (Figure 5.2). In Bem2 defective cells, the increase of GTP-bound fraction of Rho GTPases (Rho4/Cdc42) would facilitate the interaction of Rhos with the exocyst resulting in vesicle fusion even in the absence of Boi1/2. One way to show the functional regulation of Boi1/2 and Rho GTPases directly could be by doing immuno-precipitation experiments in which first we test whether Exo70-Rho3/Cdc42 interaction depends on Boi1/2 function, and if it is the case, whether Boi function is bypassed by the expression of EXO70*.

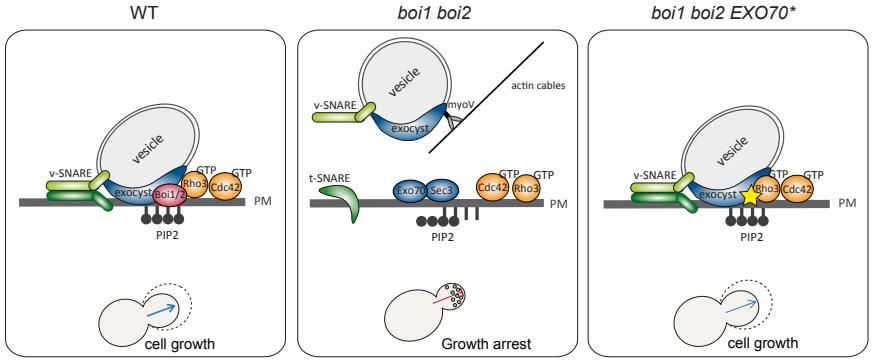

Figure 5.2. Model. In a wild-type cell, Boi1 and Boi2 proteins mediate exocyst function through Rho GTPase activity. Secretory vesicles that are transported toward the plasma membrane through actin cables are tethered at exocytic sites and SNARE proteins mediate the fusion step allowing the cell to expand its surface. In *boi1 boi2* mutants, Cdc42 and Rho3/4 cannot activate the exocyst is not functional

and vesicles accumulate at the vicinity of the bud cortex but they do not fuse causing the cells to arrest growth and presumably activating the morphogenetic checkpoint. In cells lacking Boi1/2 but expressing EXO70* (yellow star), the exocyst can function independently of GTPases, thereby rescuing cell growth. Based on (Wu et al. 2008).

In mammalian cells, regulation of the exocyst at the level of Exo70 is also of vital importance. It has been demonstrated that exocytosis is a determinant process for cancer cell invasion, and specifically the exocyst complex is involved in invadopodia formation by mediating secretion of matrix metalloproteinases (MMPs) (Liu et al. 2009; Sakurai-Yageta et al. 2008). The extracellular signal-regulated kinases 1 and 2 (ERK1/2) regulate, among other processes, exocytosis by phosphorylation of Exo70 in response to epidermal growth factor (EGF). Thus, in tumor cells, blocking Exo70 phosphorylation inhibits MMPs secretion and cell invasion (J. Ren & Guo 2012).

It is important to notice that the EXO70* rescue of Boi1/2 defects is not complete, which suggest that the function of Boi1/2 proteins might be more complex than mediating functional interactions between the exocyst and one Rho. They probably regulate exocytosis by integrating different signals and regulating GTPase active states and functional interactions with the exocyst in a complex way that will be definitely interesting to investigate. One way to test whether Boi proteins regulate the active state of Rho3/4 GTPases is to test directly the amounts of GTP-bound Rho3/4 by looking at a fluorescent-

tagged Rho binding domain (RBD) from the effector protein Rhotekin (in the case of Rho3) (X. D. Ren et al. 1999; Claret et al. 2011; Atkins et al. 2013). However, if Boi1/2 only regulate the activity of Rho GTPases on exocytosis and not on actin polarization, it might be difficult to detect by this method.

5.1.3. The interplay between Rho GTPases and Boi1/2

In cells lacking Boi1 and Boi2, we do not observe changes neither in actin polarity nor in localization of proteins that are normally polarized. That is not the case for example in complete loss-of-function Cdc42 mutants (*cdc42-1*, *cdc42-201*, *cdc42-13*), that lose polarized localization of proteins such as Sec3, Sec8 or Sec4 (X. Zhang et al. 2001; Adamo et al. 2001). Interestingly, there is a Cdc42 GTPase mutant that allows separation of functions, *cdc42-6*. This mutant has polarized actin cytoskeleton and it is only defective in a late-step in exocytosis. *cdc42-6* cells, like *boi1 boi2* mutants, show normal localization of Sec4 and exocyst subunits, but are defective in vesicle fusion to the plasma membrane (Adamo et al. 2001). A Rho3 mutant that is defective in the interaction with Exo70, *rho3-v51*, shows analogous phenotypes to *cdc42-6* cells. *rho3*-v51 has a defect in exocytosis, but not in actin polarization. Therefore, one possibility is that Boi1 and Boi2 function work together with the Rho GTPases Cdc42 and Rho3 in order to promote vesicle fusion specifically through their effector in exocytosis, the exocyst complex.

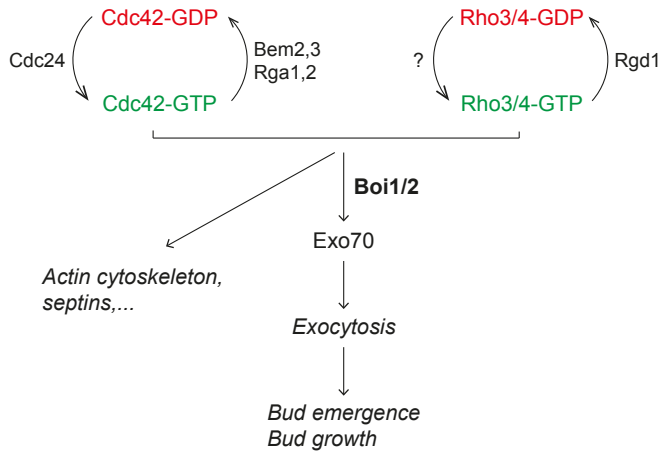

Figure 5.3. Model proposed for Boi1/2 mechanism of action during cell growth. Boi1 and Boi2 might regulate the action of Rho GTPases (Cdc42 and Rho3/4) on the exocyst in order to promote fusion of exocytic vesicles to the plasma membrane that results in bud emergence and bud growth.

The fact that partial inactivation of Bem2 by the *bem2-84* allele rescues *boi1*Δ *boi2-aid* cells (Figure 4.7.2) and that GTP-bound Cdc42 is increased in *boi1 boi2* (Figure 4.7.1), supports the hypothesis of Boi proteins and Rho GTPases working in the same pathway. Interestingly, Bem2 has GAP activity for Cdc42, Rho1 and Rho4. The *bem2-84* allele was described in (Atkins et al. 2013). Even if in this study it was shown that Rho1 activity was not altered in *bem2-84* mutants, we cannot rule out the possibility that the rescue of *boi1 boi2* cells is due to a hyperactivation of Rho4. And actually, if that was the case, it would be consistent with data showing that overexpression of Rho3 and Rho4 rescues viability of Boi defective cells, whereas Cdc42

overexpression does not (Bender, Lo, H. Lee, Kokojan, J. Peterson, et al. 1996a).

In *boi1*Δ *boi2-aid* cells, we did not observe defects in localization of active Cdc42 by expression of a CRIB-tdTomato construct (Figure 4.7.1). Nevertheless, we detected a higher intensity of this fluorescent quimera in *boi1* mutants compared to wild-type, even before NAA addition. These results show that there is more GTP-bound Cdc42 at growth sites of Boi1 mutants, even though it cannot mediate vesicle fusion. It was previously observed that cooverexpression of Boi1 and Cdc42 rescues Boi1 overexpression defects (Matsui et al. 1996; Bender, Lo, H. Lee, Kokojan, J. Peterson, et al. 1996a), suggesting that the balance in the amounts of Boi1 and Cdc42 are essential for viability. One interpretation is that Boi proteins are important to regulate the inactivation of Cdc42 in order for Rho3 to function at the same sites.

The interplay between Cdc42 and Rho3 is not well understood. Even though Rho3 can suppress *cdc42-6* defects and Cdc42 can rescue *rho3*Δ and *rho3-v51* cells (Adamo et al. 1999; Adamo et al. 2001), analysis of mutant cells supports the idea that Cdc42 acts predominantly during bud emergence and Rho3 during bud growth (Matsui & Toh-E 1992; Imai et al. 1996; Adams et al. 1990). If that was the case, Rho3 would act in the same sites where Cdc42 had acted previously. Because Rho3 can suppress Boi1/2 defects and, when overexpressed, Rho3 exacerbates the bud emergence defects caused by overexpression of Boi1 (Bender, Lo, H. Lee, Kokojan, J. Peterson, et al. 1996a), it is

possible that Boi1 (and perhaps Boi2) promotes, directly or by recruiting GAPs, inactivation of Cdc42 from a site where Rho3 would act subsequently.

5.2. Boi1 and Boi2 as NoCut effectors

*boi1*Δ *boi2*Δ cells abolish abscission delays in cells with chromosome segregation or midzone defects (*ase1*Δ*, top2-4*, *ndc10-1*) (Norden et al. 2006; Mendoza et al. 2009). We have found that *boi1*Δ *boi2*Δ cells present several SNPs that account for their viability. One possibility was that the cytokinetic rescue depended on one of the suppressor mutations and not on Boi1/2 function. For that reason we used the conditional mutant *boi1*Δ *boi2-aid* to revisit the role of Boi1 and Boi2 in NoCut.

We have shown that *top2-4* cells, which present chromosome bridges, indeed, delay cytokinesis in a Boi1/2 dependent manner (Figure 4.8.3). This had been suggested before by examining the state of the plasma membrane at the bud neck of *top2-4* cells, and we have confirmed it by doing live cell imaging (Mendoza et al. 2009). How do Boi1 and Boi2 inhibit abscission in cells with chromatin bridges? Our data suggest that Boi proteins allow Rho GTPases to activate the tethering of specific vesicles by the exocyst complex. Indeed, secretion of Bgl2 vesicles requires Boi1/2 and exocyst activity; in contrast, secretion of Chs2 requires the exocyst, but as *boi1 boi2* mutants have normal

cytokinesis, might not need Boi function. Boi1/2 proteins might thus regulate the exocyst-dependent tethering of specific vesicles. The notion that the exocyst can discriminate between different types of vesicles is supported by the finding that a ts allele of EXO70 is defective in secretion of Bgl2 but not invertase (He, Xi, Jian Zhang, et al. 2007a; Wu et al. 2010). An attractive possibility is that in top2 mutants, abscission is inhibited by the synthesis or activation a putative inhibitor, that requires Boi-dependent tethering for its exocytosis at the bud neck. In order to find candidates important for abscission inhibition in a Boi1/2 dependent manner, it would be interesting to perform a screen with a library of GFP tagged proteins and look for bud neck localization of proteins in *top2-4* cells that would not be present at the bud neck of *top2-4 boi1 boi2* mutants.

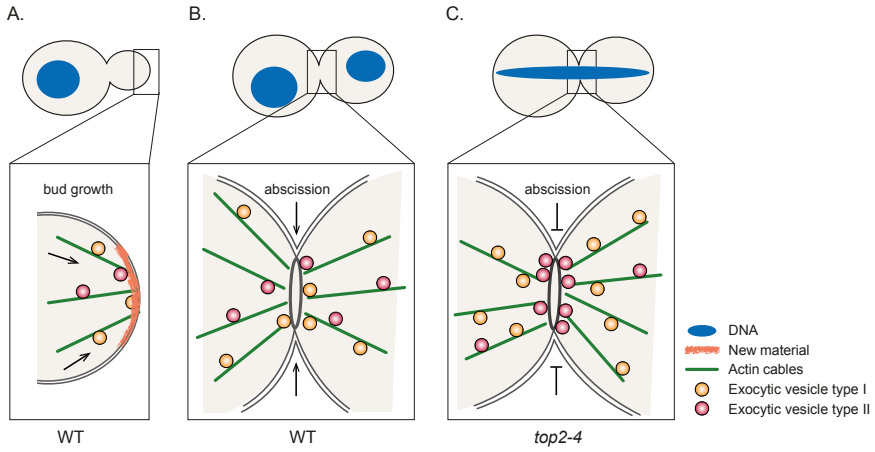

Figure 5.4. Model of Boi1 and Boi2 function during bud growth and abscission. (A) During bud growth, Boi1 and Boi2 promote vesicle fusion by facilitating functional interactions between the exocsyt complex and GTPases. (B) During normal cytokinesis, Boi1/2 promote vesicle fusion at the bud neck. (C) In

the presence of chromatin bridges, like in *top2-4*, NoCut is active and signals a specific inhibitor to be delivered at the bud neck in a Boi1/2 dependent manner.

Recently, some studies showed that regulation of Rho GTPases is important for cytokinesis. In particular, it has been proposed that Cdc42 is inactive and in contrast, Rho1 is active during abscission and secondary septum deposition (Atkins et al. 2013; Onishi et al. 2013). Artificial activation of Cdc42 at the division site results in misslocalization of the cytokinetic proteins Iqg1 and Inn1 and abnormal septa. It is unlikely that regulation of Cdc42 is affected in boi mutants during cytokinesis, because I have shown that Boi mutants are competent in cell division. However, other experiments can be done to address this question. The way to show that hyperactivation of Cdc42 cause defects in cytokinesis was by using a Bem2-ts, but I have mentioned that it is likely that downregulation of Bem2 cause defects in Rho4 too. Therefore, it would be interesting to check if hyperactivation of Cdc42 by other means (for example by inhibiting other Cdc42 GAPs) also cause cytokinetic defects.

Interestingly, EXO70* alone could rescue *top2-4* abscission delays (Figure 4.8.3). This result shows that elevated exocyst activity can bypass Boi1/2-dependent abscission inhibition. Further, it raises the possibility that exocyst function is regulated upon chromosome segregation defects. Consistent with this hypothesis, defects in exocyst function inhibit cytokinesis (Dobbelaere & Barral 2004; Liu et al. 2009).

To answer the latter option, I performed co-immunoprecipitation assays to investigate whether the exocyst complex was formed during *top2-4* abscission delay. In (Luo et al. 2013) exocyst complex assembly was assessed by Exo70-Exo84 co-immunoprecipitation assays. They found that during mitosis, Exo84 was phosphorylated in a Cdk-Clb2 dependent manner, and the exocyst disassembled, stopping growth. We could not detect major differences in amounts of Exo70 and Exo84, suggesting that the complex is formed during *top2-4* delay in abscission. However, we cannot rule out the possibility that the exocyst is inactivated by other means that we cannot detect by this method, for example through inactivation of GTPases at the bud neck. The hypothesis of the exocyst being regulated upon chromosome segregation defects requires further investigation.

I have shown that Boi1-GFP localization does not depend on Ipl1 function. However, inactivation of Ipl1 results in a partial rescue of the abscission defects of *top2-4* cells (unpublished results by Alexandre Vendrell and Nuno Amaral in our laboratory) similar to the effect of Boi1/2 depletion (my results). These results raise the possibility that Ipl1 and Boi1/2 regulate abscission through independent pathways in cells with chromosome segregation defects. In order to discard or confirm this hypothesis it would be interesting to image *top2-4* cells defective in both Ipl1 and Boi function. These results leave other open questions such as the existence of the NoCut pathway the way we know now. We do not discard the possibility that more than a pathway

it is more like a network of proteins that respond upon chromosome segregation defects.

6. Conclusions

From the work presented in this study, the following are the main research contributions:

- Boi1/2 function is essential for the exocyst dependent tethering of secretory vesicles to the plasma membrane, required during cell growth.

- An exocyst gain-of-function allele and inactivation of the Bem2 GAP for Cdc42/Rho1/Rho4 GTPases can rescue the essential Boi function, suggesting that Boi1/2 work through GTPase activity on the exocyst complex.

- *boi1 boi2* mutants do not show cytokinesis defects suggesting that they are not essential for the exocyst-dependent delivery

of vesicles required during cytokinesis. However, Boi1/2 function is essential for inhibition of abscission in *top2* mutants, suggesting that they may be important for the delivery of a cytokinetic inhibitor.

- An exocyst gain-of-function allele of Exo70 can rescue cytokinetic defects of *top2-4* mutants to the same extent that Boi1/2. This result suggests that regulation of the exocyst function could be important for the abscission delay of *top2-4* mutants.

7. Future directions

The results of this study open up more questions that would be interesting to follow up.

- There are at least two types of exocytic vesicles in budding yeast. We have shown that Bgl2 vesicle fusion is defective in Boi1/2 mutants, however there are several cytokinetic factors that are transported to the division site by membrane trafficking that are properly delivered as cytokinesis is not affected in Boi mutants. Which types of vesicles are Boi1/2 regulated? For that, checking secretion of other cargoes like invertase or the CPY peptide would help in the understanding of Boi function.

- How Boi1/2 function regulates GTPase activity in exocytosis? The inactivation of the Cdc42/Rho1/Rho4 GAP Bem2 results

in the rescue of Boi defects. As Bem2 has pleiotropic activity, modification of one Rho GTPase activity at the time might tell us if the rescue of Boi defects is due to an increase of one particular activity or it is the combination of several of them. Also, looking at the amounts of GTP-bound Rho by expressing a fluorescently tagged Rho-GTP-binding domain, like we did for Cdc42, we could see if another Rho activity is affected in *boi1 boi2* mutants.

- Boi1/2 regulate vesicle fusion at the bud cortex during most of the cycle. Do they mediate exocytosis of specific cargoes at the bud neck too? To get a mechanistic insight of the *top2-4* delay in abscission, it would be very informative to do a genetic screen looking for changes in localization of bud neck proteins in *top2-4* mutants with and without Boi function. Also, to test whether there is vesicle accumulation in *top2-4* mutants, we could do EM microscopy in those mutants with and without Boi1/2 function.

- Inactivation of the exocyst function results in abscission defects. Also, an exocsyt gain-of-function allele rescues abscission delays in *top2-4*. One possibility is that the exocyst complex might be regulating the abscission delay in *top2-4* cells. The other possibility is that the exocyst gain-of-function allele rescues abscission delays in a passive manner. We could promote vesicle fusion at the neck by increasing SNARE

function to discard the second possibility. It could also be interesting to test whether any of the exocyst subunits is regulated by post-translational modifications during abscission inhibition of *top2-4* mutants.

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Appendix I: list of strains

In the following table there are all the strains used in this sudy.

NUMBER	STRAIN	GENOTYPE
1	Wild-type	MATa *ura3-52 his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63*
2	Wild-type	MATalpha *ura3-52 his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63*
113	*top2-4*	MATa *top2-4 ura3-1 leu2,3-112 his3 trp1 ade2*
235	Wild-type	MATa *ade1 his2 leu2-3,112 trp1-1a ura3Δns* BFA264-15D background. (Eric Bailly, INSERM)
236	*boi1*Δ *boi2*Δ	MATalpha *boi1*Δ*::LEU2 boi2*Δ*::URA3 ade1 his2 trp1-1a* BFA264-15D background. (Eric Bailly, INSERM)
423	Boi1-GFP *Ipl1-321*	MATa boi1-GFP *ipl1-321 ura3-52 his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63*
429	Boi1-GFP	MATa boi1-GFP *ura3-52 his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63*
1307	*boi1*Δ *boi2*Δ	MATa From 3rd backcross of YMM236 with S288c
1308	*boi1*Δ *boi2*Δ	MATa From 3rd backcross of YMM236 with S288c
1317	*boi1*Δ	MATalpha *boi1*Δ*::ura his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63his3-Δ200*
1335	Gal1:GFP-CAAX GAL4.ER.VP16 (ADGEV) Spc42-GFP	MATa GAL4.ER.VP16 (GEV)::URA3 Gal1:GFP-CAAX::HIS3 spc42GFP::HphMX
1485	*boi1*Δ *boi2-aid*	MATalpha *boi1*Δ*::ura Boi2AID::Kan his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63his3-Δ200*

1521	Boi2-aid	MATa *boi2AID::kan ura3-52 his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63*
1523	PGalTIR1 *boi2-aid*	MATa *GalAtTIR1::ura boi2AID::kan his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63*
1531	PGalTIR1 *boi1Δ boi2-aid*	MATa *PGalAtTIR1::ura boi1Δ::TrpHA boi2AID::kan his3Δ200 leu2 lys2-801 ade2-101*
1725	PGalTIR1 boi1Δ boi2-aid-6xHA	MATa *PGalAtTIR1::ura boi2AID-6xHA::His boi1Δ::TrpHA leu2 lys2-801 ade2-101*
1827	Exo70-GFP	MATa exo70-GFP::his *ura3-52 leu2 lys2-801 ade2-101 trp1Δ63*
2082	GalAtTIR1 PGal-GFP-caax *Boi2-aid* spc42-GFP *boi1Δ* ADGEV	MATa GalAtTIR1::leu GFPcaax::His *Boi2-aid::Kan boi1Δ::TrpHA* ADGEV::ura spc42GFP::hyg *lys2-801 ade2-101*
2102	GalAtTIR1::ura boi2AID boi1Δ::TrpHA exo70-GFP	MATa GalAtTIR1::ura *boi2-aid::kan boi1Δ::TrpHA exo70-gfp::his leu2 lys2-801 ade2-101*
2106	GalAtTIR1 *boi2-aid boi1Δ exo70*-HA*	MATa GalAtTIR1::ura *boi2-aid::kan exo70*-HA::Nat boi1Δ::TrpHA his3Δ200 leu2 lys2-801 ade2-101*
2221	*sec14-1*	MATa *Sec14-1::NatMx4 His3Δ his2 trp1Δ63 ade2-101 lys-801 ura3-52*
2251	PGal-GFP-caax spc42-GFP ADGEV *Exo70**	MATa GFPcaax::His spc42GFP::hyg ADGEV::ura Exo70*::Nat *lys2-801 ade2-101*
2274	GalAtTIR1::ura *boi2-aid boi1Δ::TrpHA* exo84-GFP	MATa GalAtTIR1::ura *boi2-aid::kan boi1Δ::TrpHA* exo84-GFP::His *leu2 lys2-801 ade2-101*
2378	*top2-4* ADEGV caax-GFP spc42-GFP	MATa *top2-4* ADEGV::ura caaxGFP::His spc42GFP::hyg
2469	GalAtTIR1 PGal-GFP-caax *top2-4 Boi2-aid boi1Δ* spc42-GFP ADGEV	MATa GalAtTIR1::leu GFPcaax::His Boi2AID::Kan spc42GFP::hyg boi1Δ::TrpHA ADGEV::ura *top2-4 lys2-801 ade2-101*

2494	GalAtTIR1 PGal-GFP-caax *top2-4* spc42-GFP *boi1*Δ ADGEV *Exo70*-HA Boi2-aid*	MATa GalAtTIR1::leu GFPcaax::His Boi2AID::Kan spc42GFP::hyg boi1Δ::TrpHA ADGEV::ura Exo70-G388R-HA::Nat *top2-4 lys2-801 ade2-101*
2500	GalAtTIR1 *boi2-aid boi1*Δ cdc24-GFP	MATa GalAtTIR1::ura *boi1*Δ*::TrpHA boi2AID::kan* cdc24-GFP::his *leu2 lys2-801 ade2-101*
2501	GalAtTIR1 *boi2-aid boi1*Δ sec3-GFP	MATa GalAtTIR1::ura *boi1*Δ*::TrpHA boi2AID::kan* sec3-GFP::his *leu2 lys2-801 ade2-101*
2502	GalAtTIR1 *boi2-aid boi1*Δ dse1-GFP	MATa GalAtTIR1::ura *boi1*Δ*::TrpHA boi2AID::kan dse1-GFP::his leu2 lys2-801 ade2-101*
2504	GalAtTIR1 *boi2-aid boi1*Δ bem1-GFP	MATa GalAtTIR1::ura *boi1*Δ*::TrpHA boi2AID::kan* bem1-GFP::his *leu2 lys2-801 ade2-101*
2523	PGal-GFP-caax spc42-GFP *top2-4* ADGEV *Exo70**	MATa GFPcaax::His spc42GFP::hyg ADGEV::ura Exo70*::Nat *top2-4 lys2-801 ade2-101*
2537	*sec3-2*	MATa *sec3-2::kan his3^1 leu2^0 ura3^0 met15^0*
2539	*exo70-38*	MATa *exo70-38::kan his3^1 leu2^0 ura3^0 met15^0*
2540	*exo84-102*	MATa *exo84-102::kan his3-1 leu2^0 ura3^0 met15^0*
2541	*sec8-6*	MATa *sec8-6::kan his3^1 leu2^0 ura3^0 met15^0*
2544	*sec6-4*	MATa *sec6-4::kan his3^1 leu2^0 ura3^0 met15^0*
2660	exo84-9xmyc exo70-6xHA	MATa exo84-9xmyc::his exo70-6xHA::Nat *ura3-52 leu2 lys2-801 ade2-101 trp1Δ63*
2665	PGal-GFP-caax spc42-GFP ADGEV *Exo70-38*	MATalpha ADEGV::ura caaxGFP::His spc42GFP::hyg *exo70-38::kan leu2 lys2-801 ade2-101 trp1Δ63*

2668	*top2-4* exo84-9xmyc exo70-6xHA	MATa *top2-4* exo84-9xmyc::his exo70-6xHA::Nat *ura3-52 leu2 lys2-801 ade2-101 trp1Δ63*
2709	Sec3-GFP	MATa Sec3-GFP::His *ura3-52 his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63*
2711	exo84-GFP	MATa Exo84-GFP::His *ura3-52 leu2 lys2-801 ade2-101 trp1Δ63*
2764	Dse1-GFP::His	MATa Dse1-GFP::His *ura3-52 leu2 lys2-801 ade2-101 trp1Δ63*
2765	bem1-GFP	MATa Bem1-GFP::His *ura3-52 leu2 lys2-801 ade2-101 trp1Δ63*
2848	Myo1-GFP	MATalpha myo1-GFP::his *top2-4 ura3 leu2 lys2-801 ade2-101*
2851	Myo1-GFP *boi1Δ boi2-aid* PGal-TIR1	MATalpha myo1-GFP::his PGalTIR1::ura *boi1Δ::trp boi2-aid::kan leu2 lys2-801 ade2-101*
2877	*bem2-84*	MATa *bem2-84::leu ura3-52 his3Δ200 lys2-801 ade2-101 trp1Δ63*
2879	*GalAtTIR1 boi2-aid boi1Δ bem2-84*	MATa GalAtTIR1::ura *boi1Δ::TrpHA boi2-aid::kan bem2-84::leu his3Δ200 lys2-801 ade2-101*
2886	CRIB-tdTomato	MATa CRIB-tdTomato::ura *his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63*
2888	GalAtTIR1 *boi2-aid boi1Δ* CRIB-tdTomato	MATa GalAtTIR1::leu *boi1Δ::TrpHA boi2AID::kan* CRIB-tdTomato::ura *his3Δ200 lys2-801 ade2-101*

Note: all strains are from the S288c background except when noted.

Appendix II: list of plasmids

In the following table there are all the plasmids used in this sudy.

Number	Name	Reference
148	pRS415 CEN6/ARSH4 LEU2	ATTC
195	ADGEV	Gift from F. Posas, UPF
240	pRS315 PSec4-GFP-Sec4-3' UTR Sec4	(McCusker et al. 2012)
269	pRS415 PGalExo70-G388R-6xHis LEU2	This work
270	pRS415 PGalExo70 6xHis LEU2	This work
347	pRS305 *bem2-84*	Douglas Jhonson, University of Vermont
370	ylP211-Gic2-PBD-tdTomato-URA3	(Tong et al. 2007)

Appendix III

Single nucleotide polymorphisms in *boi1*Δ *boi2*Δ

Table III. List of SNPs found in *boi1*Δ *boi2*Δ cells (BF264-15D background) compared to its isogenic wild-type.

Position	gene name	SNP	Function
chrII:39982	BNA4	D281N	biosynthesis of NAD
chrII:494902	YBR129C	A144A	unknown function
chrIII:59865	GFD2	I280M	unknown function
chrV:221858	YER034W	T5S	unknown function
chrVIII:186001	MSC7	I268V	unknown function,
chrVIII:345839	intergenic		
chrX:273982	EXO70	G388R	exocytosis
chrX:378209	intergenic		
chrXII:20050	intergenic		
chrXII:318225	intergenic		
chrXII:665060	intergenic		
chrXII:737638	intergenic		
chrXII:799068	NUP2	S547P	Nucleoporin
chrXIII:236402	intergenic		
chrXIV:295923	intergenic		
chrXIV:716060	intergenic		
chrXIV:724846	intergenic		
chrXV:797427	intergenic		
chrXVI:390476	SEC16	A1138E	ER transport vesicle budding

Table IV. List of SNPs found in *boi1*Δ *boi2*Δ strain after intensive backcrossing into the S288c background compared to its isogenic wild-type.

Position	Gene name	SNP	Gene function
chrII:116491	YBL055C	H114H	3'-->5' exonuclease and endonuclease
chrII:74909	intergenic		
chrV:297010	ARG5,6	R535G	arginine biosynthesis
chrVI:191001	FAB1	R2171H	phosphatidylinositol (3,5)P2
chrX:272185	TRL1	D99N	tRNA ligase
chrX:272557	YJL086C	P95R	Dubious open reading frame partially overlaps the verified genes EXO70 and TRL1
chrX:272841	EXO70	I7I	Exocytosis
chrX:273260		A147V	
chrX:273955		L379L	
chrX:273982		G388R	
chrX:275946	ALY2	I684T	Endocytosis
chrX:276447		N517S	
chrXIII:24889	PHO84	K305E	inorganic phosphate (Pi) transporter
chrXV:245066	PSK2	L524P	protein kinases; regulates sugar flux and translation
chrXVI:432088	YPL062W	L66S	Dubious open reading frame
chrXVI:458203	KTR6	V85M	Probable mannosylphosphate transferase
chrXVI:458408		S16S	
chrXVI:458439		S6N	
chrXVI:458476	intergenic		
chrXVI:458632	intergenic		
chrXVI:459031	OAZ1	R78R	Regulator of ornithine decarboxylase
chrXVI:459116		R107W	
chrXVI:459225		K143R	
chrXVI:459327		L177*	

chrXVI:459601		R268R	
chrXVI:459681	intergenic		
chrXVI:459926	intergenic		
chrXVI:460599	intergenic		
chrXVI:460610	intergenic		
chrXVI:460758	intergenic		
chrXVI:461195	MNN9	I257V	Subunit of Golgi mannosyltransferase