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ROLE OF MICROTUBULES IN BUDDING YEAST CYTOKINESIS

by

SU YOUNG PARK

A THESIS

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Approved by

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ABSTRACT

Cytokinesis is an important step to finish cell cycle events and separate into two daughter cells. This event is driven by actomyosin ring contraction. In budding yeast, the mitotic exit network (MEN) controls completion of ana/telophase by signaling events. Bub2 is a mitotic exit network regulator and binds to Tem1p which is an important component in MEN. Mad2 is a component of a separate spindle checkpoint. Mad2 dependent pathway prevents the metaphase to anaphase transition under spindle damage. Because yeast cells have cell walls, septum formation follows actin ring contraction. Here it was investigated how microtubule defects affect cytokinesis in budding yeast. It was examined various mutant cells. Data shows that, bub2 deletion or overexpression leads to a higher rate of myosin contraction. Moreover, these mutants showed chain morphology after depolymerizaion of microtubules by nocodazole. This phenomenon is caused by cell septation defects not cell cytokinesis defects.

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1. INTRODUCTION

1.1 CYTOKINESIS EVENTS AMONG CELL SYSTEMS

Cytokinesis is the final step of cell division resulting in one cell divided into two cells. Cytokinesis events must be coordinated with separation of sister chromatids to make sure that daughter cells have the right genetic information. Therefore, components of the cytokinesis machinery are controlled at the exact time to perform accurate functions during this cell cycle event (Mishima et al., 2004, Petronczki et al., 2007).

Cytokinesis in animal cells is accomplished by an actomyosin-based contractile ring, which forms late in the cell cycle and constricts the plasma membrane (Balasubramanian et al., 2004). A similar contractile system is highly conserved in Yeast cells, including *Saccharomyces pombe* and *Saccharomyces cerevisiae* (Field et al., 1999). The actomyosin ring in budding yeast is assembled as cells progress through the cell cycle. These proteins are translocated to the bud neck site chronologically from late G1 phase to M phase. In late G1 phase, myosin II heavy chain (Myo1p) and light chain (Mlc2p), a regulator of heavy chain, locate to the bud neck (Luo, J. et at., 2004). Septins are required for Myo1 localization (Bi et al., 1998). After that in S phase, Mlc1p which is an essential light chain for myosin II comes to the neck (Shannon and LI, 2000).

Hof1p/Cyk2p follows in S/G2 phase (Lippincott, J. et al., 2000). Iqg1p/Cyk1p, a formin Bni1p, and finally actin and Cyk3p are located to the bud neck in late anaphase (Bi, E., 2001, Lippincott and Li, 1998, Korinek et al., 2000). Even though a lot of components that are involved in actin ring function arrive at the bud neck earlier, actomyosin ring forms that is ready to contract in late M phase (Balasubramadian et al., 2004). After contraction, actomyosin ring is finally disassembled and the narrow cytoplasmic bridge exists between the two cells. That bridge should be solved by abscission to complete cytokinesis (Norden, C et al., 2006). Because yeast cells have a cell wall, septation occurs at the same time as plasma membrane contraction (Cid V. J. et al., 1998). It is known that at least three different classes of proteins are involved in budding yeast cell division. First there are proteins devoted to function of actomyosin ring only. Second, there are proteins that have functions for both actomyosin ring activity and septum formation and lastly, proteins that could organize these two processes (Balasubramanian et al., 2004).

Even though yeast cells are a good model for cytokinesis, at least three differences exist between animal cells, *Saccharomyces pombe* and *Saccharomyces cerevisiae* (Vallen et al., 2000). First, initiation and site selection mechanisms for cytokinesis are different. In case of budding yeast, *Saccharomyces cerevisiae*, the site for dividing is determined from the beginning of cell cycle when the bud emerges (Pringle et

al., 1995 and Drubin and Nelson, 1996). In contrast, fission yeast, *Saccharomyces pombe*, chooses the division site after onset of mitosis and the premiotic nucleus plays a role (Chang and Nurse, 1996 and Chang et al., 1996). In animal cells, at anaphase F-actin and myosin II are collected at the presumptive cleavage site, determined by mitotic spindle position (Satterwhite and Pollard, 1992 and Rappaport, 1996). Second, unlike animal cells, *Saccharomyces pombe* and *Saccharomyces cerevisiae* have a cell wall. Therefore septum formation is required for cytokinesis (Vallen et al., 2000). Finally, the actomyosin contractile ring is not essential in budding yeast, *Saccharomyces cerevisiae* as it is in fission yeast, and in animal cells (Vallen et al., 2000). Regardless of these differences, a number of components that are involved in cell cytokinesis are conserved among the system (Lee et al., 2002).

1.2 BUDDING YEAST, SACCHAROMYCES CEREVISIAE, AS A MODEL SYSTEM

The budding yeast, *Saccharomyces cerevisiae*, is an attractive model system to study the temporal regulation of cytokinesis events since these cells divide by a contractile ring, an actin and myosin rich structure which is analogous to the cleavage furrow in animal cells. *Saccharomyces cerevisiae* also provides several advantages as a

model system including ease of genetic manipulations, that the genome sequence is complete, and that previous studies have yielded a well characterized cell cycle as well as several classes of cytokinesis mutants (Vallen et al., 2000, Bi 2001, Schmidt et al., 2002).

1.3 MITOTIC EXIT NETWORK IN BUDDING YEAST

Cytokinesis only happens after chromosome segregation in all cells, therefore, the nuclear cycle should be related to cytokinesis (Hu et al., 2001). Mitotic exit network (MEN) likely has a role in coordination of cytokinesis and nuclear cycle. MEN genes from *Saccharomyces cerevisiae* are conserved with many other eukaryotes (Bettignies and Johnston, 2003). The MEN signaling pathway starts from activation of Tem1, a GTPase, and terminates with protein phosphatase Cdc14p (Simanis, 2003). A small G protein, Tem1p is a main switch of this cascade. There are two regulators for it. One is a two complex molecule made of Bub2 and Bfa1 and they inactivate Tem1p. The other one is Lte1, a putative exchange factor, and Lte1p activates Tem1p. Once Tem1p is activated, it activates Cdc15, a protein kinase. Activated Cdc15 is phosphorylated and also activates another protein kinase Dbf2 (Jaspersen et al., 1998, Shou et al., 1999 and Visintin et al., 1999). “Another protein, the Cdc5 polo-like kinase, is involved at several steps in the cascade” (Bettignies and Johnston, 2003). Net1p is phosphorylated during MEN cascade

and triggers release of Cdc14p from the nucleolus (Shou et al., 1999 and Visintin et al., 1999). Swi5p, a transcription factor, is dephosphorylated after Cdc14p has been released from the nucleolus as soon as level of Sci1p which is an inhibitor of the Cdc28p-cyclin B kinases is increased. Cdc14p also acts on Cdh1p by dephosphorylation and allows Cdh1p to work together with the anaphase promoting complex (APC). This interaction triggers degradation of cyclin (Simanis, 2003, Visintin et al., 1998, Gray et al., 2003). Low level of Cdc28p-cyclin B by action of Sic1p and APC/Cdh1p causes mitotic exit (Balasubramanian et al., 2004).

The MEN also plays a role in cytokinesis distinguished from the mitotic exit role. All components in MEN machinery are located to spindle pole bodies (SPB) starting from S/G2 phase to the end of anaphase. The SPB is the yeast equivalent of the centrosome. At the end of mitosis, some MEN proteins are translocated to the bud neck and remain there to function at cytokineisis (Simanis, 2003). Many studies indicate that MEN proteins have a role in cytokinesis. Some mutants of MEN proteins permit not cytokinesis but mitotic exit event (Jimenez et al., 1998). For instance, in deficient mutant of Net1p that led to bypass the mitotic exit, interrupting function of Temp1p causes cytokinesis defects. Therefore, Tem1p plays an important role in cytokinesis independently from mitotic exit (Lippincott et al., 2001). Cdc15, which is a kinase

involved in MEN network, also has a role in cytokinesis (Lim et al., 2003). To determine this function, they studied a Cdc15 mutant which is a temperature-sensitive mutant that causes mitotic arrest at 37℃ but is normal to exit in mitosis at 25℃. This mutant showed cell cytokinesis defect in semi-permissive temperature, 30℃ (Lim et al., 2003).

1.4 BUB2 AND MAD2 PROTEINS RESPOND TO MICROTUBULE DEFECTS IN SEPARATE PATHWAYS

Cell cycle checkpoints check that all cell cycle events are completed and control further progression by regulation of Cdk activity. These actions ensure that cell division takes place correctly (Murray, 1994 and Elledge, 1996). By microtubule mutation or drugs like nocodazole, spindle assembly is interrupted, preventing exit from mitosis and entry to the next cell cycle (Rudner et al., 1996 and Straight, 1997). Bub1, 2 and 3, Mad1, 2, and 3 were first identified by genetic analysis in budding yeast. Mutations in those genes lead to failure of cell cycle arrest after inhibition of microtubule function (Li et al., 1991, Hoyt et al., 1991 and Weiss et al., 1996). Bub2 was originally found in budding yeast, *Saccharomyces cerevisiae*, by screening. Bub2 mutant gene could not arrest the cell cycle in does of benomyl which is a microtubule-depolymerizion drug (Hoyt et al., 1991). In eukaryotes an abnormal spindle results in mitotic arrest in metaphase. This

checkpoint requires MAD and BUB genes. Recently, many studies have revealed that Bub2 is a novel branch to the spindle assembly checkpoint which blocks mitotic exit (Alexandru et al., 1999, Fesquet et al., 1999, Fraschini et al., 1999 and Li, 1999). This check point arrests the cell cycle by inhibition of the MEN protein Tem1p. Bub2 and Bfa1 physically join together for the entire cell cycle and interact with Tem1 during mitosis and early G1 (Lee et al., 2001). Once Bub2/Bfa1 complex binds to Tem1, it prevents exiting mitosis (Bardin et al., 2000, Bloecher et al., 2000 and Pereira et al., 2000). Bub2 is not a phosphorylated protein. In contrast, Bfa1 is phosphorylated at many sites during cell cycle especially at mitosis. Bfa1 phosphorylation is dependent on Bub2 binding (Lee et al., 2001).

MAD2 (mitotic arrest deficient 2) exists on the chromosome as one of the kinetochore proteins during cell division. It is involved in making sure all chromosomes are attached to the mitotic spindle before the beginning of anaphase. During mitosis (and meiosis), the attachment of sister chromatids is monitored by the spindle checkpoint. If the sister chromatids do not attach to opposite poles, the spindle checkpoint is activated. Mad2 is associated with Mad1 to act as a complex. This complex binds to the APC (anaphase-promoting complex) to create APC/Mad2 complex. When Mad2 binds to the APC, APC can not bind with Cdc20p to make APC/Cdc20 complex which is necessary to

enter anaphase. The binding of Mad2 proteins to unattached kinetochores effectively prevents anaphase until all of the chromatids are appropriately attached to opposite spindle poles (Lew et al., 2003). Figure 1.1 illustrates two pathways of the spindle check point.

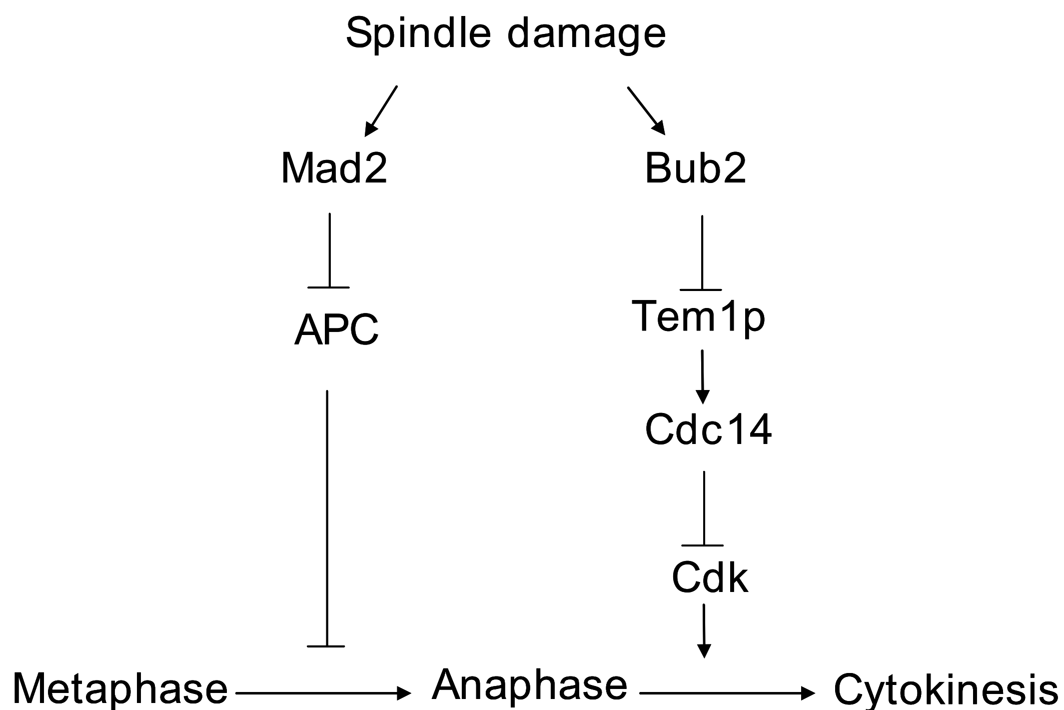


Figure1.1 Spindle assembly checkpoint. There are two spindle damage checkpoint pathways that are regulated by Mad2 and Bud2.

1.5 DO BUB2 AND MAD2 AFFECT CYTOKINESIS? DOES MICROTUBULE PLAY A ROLE?

The goal of this study was to determine whether Bub2, Mad2 and/or microtubules play a role in cytokinesis in budding yeast. To investigate this, Bub2 and Mad2 deletion mutants were used to allow cells to progress through the cell cycle, exit mitosis, and enter a new cycle in the absence of microtubules. Nocodazole is used for depolymerization of microtubules. It was investigated actin ring formation, myosin contraction, and cell morphology under these conditions. Previous studies from Lee et al. 2001 hypothesized that in metaphase cells Bub2 is crucial to restrain downstream events such as actin ring formation, emphasizing the importance of the Bub2 pathway in the regulation of cytokinesis. However, data indicates that actin ring formation occurs at the same time without Bub2 or Mad2. It means Bub2 does not negatively regulate actin ring formation.

Myosin ring contraction showed slightly faster rate in bub2 deletion mutants. However, rate of contraction is even faster in bub2 deletion with depolymerization of microtubules than in normal. This data suggests that both bub2 and microtubules may regulate actomyosin ring contraction.

According to the previous studies from Norden C. et al., Mad2 deletion mutant failed to complete cytokinesis without microtubules, suggesting that microtubules are required for cytokinesis in yeast as well as animal cells. However, data showed Mad2 and

Bub2 deletion mutant with microtubules depolymerized by nocodazole can complete cytokinesis, but they have septation defects. Therefore, microtubules are not required for completing cytokinesis in budding yeast but are essential for the completion of cell septation events. It was also investigated cells having overexpressed Bub2 condition. These cells also showed cell septation defects, not cytokinesis defects. Like in Bub2 deletion mutant, Myo1-GFP contraction is if faster not normal condition in overexpressed Bub2 but slower than bub2 deletion with condition of microtubule depolymerization.

2. RATIONALE AND DESIGN

2.1 INTRODUCTION

Cytokinesis is the final stage of cell division driven by atomyosin ring contraction and septum formation in yeast. Role of actomyosin contractile rings in cell division is highly conserved among eukaryotic organisms (Lippincott et al., 2001). In the budding yeast, *Saccharomyces cerevisiae*, mitotic exit network controls the completion of ana/telophase by specific signaling pathway to enter cytokinesis event (Tolliday et al., 2001). The key molecule of this network is Tem1p, a small G protein.

Bub2 is a mitotic exit network regulator which interacts with Bfa1p to form GTPase-activating complex (GAP) that binds Tem1p. If a cell has damage to spindle or mis-aligned spindle, the Bub2/Bfa1 complex blocks progression of cell cycle (Frashcini et al., 1999 and Preira et al., 2000). Moreover, much research proposed that the Bub2p-Bfa1p controlled the novel branch of the spindle checkpoint pathway distinguished from pathway that involving Bub1p, Bub3p, Mad1p, Mad2p, Madp, and Mps1p, which may prevent premature chromosome disjunction (Hardwick et al., 1999 and Alexandru et al., 1999). Bub2 has been hypothesized to prevent premature actin ring formation (Lee SE et al., 2001), an idea that tested in experiments.

MAD2 is a non-essential gene that encodes Mad2p, a component of the spindle checkpoint. Mad2p makes a complex with Mad1p, another spindle checkpoint protein, across the cell cycle (Chen et al., 1999). Mad2p forms a complex with Cdc20p as well. It is known that Mad2p/Cdc20p complex activates Mad3p and APC (anaphase promoting complex). These reactions are dependent on the presence of Mad1p (Hwang et al., 1998). As mentioned before, Bub1p, Bub3p, Mad1p, Mad2p, Mad3p, and the protein kinase Mps1p function in a branch of the spindle checkpoint pathway that acts by inhibition APC to prevent the metaphase to anaphase transition (Alexandru et al., 1999).

In yeast, septum formation will follow ring contraction because they possess cell walls. After recruitment and contraction of the actomyosin ring, a septum is formed by vesicle fusion with the plasma membrane (Longtine et al., 1996 and Faty et al., 2002). Formation of the primary septum is dependent on actomyosin ring contraction (Schmidt et al., 2002).

It has been investigated how microtubule defects effect cytokinesis in budding yeast. Previous studies proposed that microtubules were required for abscission, the final stage of cytokinesis (Maiato, H. et al., 2004). To reveal this, nocodazole was used to depolymerize microtubules and monitored actomyosin ring formation as well as myosin ring contraction. To investigate myosin ring contraction, Myo1-GFP fusion strain was

made. Experiments tested in Bub2, a MEN regulator, deleted and overexpressed mutants. Interestingly, disruption of microtubule function leads to cell morphology defects that are not because actomyosin ring failed contraction but due to failure to complete septation. According to the Myo1-GFP imaging, timing of ring contraction was a little faster in mutant cells than in control cells. It might be explained as septum formation can not be able to catch up the speed of actomyosin contractile ring that led to septum defection so eventually showed chain form morphology cells. However, further study will be necessary to verify that.

2.2 MATERIALS AND METHODS

2.2.1. Strains, Media, and DNA Methods. All *S. cerevisiae* strains were derived from KSY3 (a ura3 leu2 his3 trp1 ade2 Δbar1) (Li). All yeast strains that were used for this study listed in Table 2.1. Yeast cells were grown in YPD (yeast extract peptone dextrose), YPR (Yeast extract peptone ribose), YPGR (Yeast extract peptone ribose and galactose) liquid medium, or on – HIS, – LEU, – TRP or – URA drop-out solid and/or liquid medium at 30 ◦C (Kaiser et al., 1994). To create Myo1-GFP expressing strains, pKT36 (MYO1 tagged with GFP) TRP1 plasmid (Lippincott et al., 2001) was digested with *AgeI* enzyme and transformed into KSY3, KSY19, KSY47 and KSY51 strains.

To create Bub2 overexpressed mutant, pKT1315 (Ro et al., 2001) was digested with *NcoI* and transformed into KSY3, KSY19 and KSY51.

2.2.2 Yeast Transformation. Yeast transformations were performed by modified method from the lithium acetate methods (Gietz et al., 1992). After 30 minutes incubation at 30℃, 50 µl of DMSO (dimethyl sulfoxide) was added and mixed. Heat shock was given at 42℃ for 15 minutes. Cells were pelleted briefly in microcentrifuge and resuspended in 500 µl of 1X TE (10 mM Tris-HCl, 1mM Na_2EDTA, pH8.0). Resuspension was plated on appropriate media and incubated at 30℃ for two-three days.

2.2.3. Staining in Yeast for Actin Ring Formation. KSY3, KSY19, KSY47, KSY51, KSY111 and KSY112 were grown in YPD overnight. α-factor (100ug/ml) was added to synchronize cell for 2 hours. Cells were washed with dH_2O three times and resuspended in YPD or YPD with nocodazole (15ug/ml). Cells released in YPD were incubated for 60 to 100 min, during this period at every 10 minute, 5ml of cells were taken and fixed with 670ul 37% formaldehyde for 2 hour in room temperature. Otherwise, cells released in YPD+Nocodazole were incubated for 60 to 240 min. at each 60, 80, 100, 140, 180, 220 240 minutes time points, 5ml of cell was collected and fixed with 670ul 37% formaldehyde for 2 hours in room temperature.

Table 2.1 Strains used in this study. All strains are W303 background.

Strain	Genotype	source
KSY3	a ura3 leu2 his 3 trp1 ade2 Δbar1	R Li (Tolliday N. et al., 2003)
KSY19	a ura3 leu2 his3 trp1 ade2 Δbar1 Δbub2:LEU2	R Li (Tolliday N. et al., 2003)
KSY47	a ura3 leu2 his3 trp1 ade2 Δbar1:HIS3 Δbub2:LEU2 Δmad2:URA3	K. Shannon
KSY51	a ura3 leu2 his3 trp1 ade2 Δbar1:HIS3 Δmad2:LEU2	J. E. Park
KSY70	a ura3 leu2 his3 trp1 ade2 Δbar1 MYO1-GFP:TRP1 (pKT36 int)	This work
KSY71	a ura3 leu2 his3 trp1 ade2 Δbar1 Δbub2:LEU2 MYO1-GFP (pKT36 int)	This work
KSY72	a ura3 leu2 his3 trp1 ade2 Δbar1:HIS3 Δbub2:LEU2 Δmad2:URA3 MYO1-GFP (pKT36 int)	This work
KSY73	a ura3 leu2 his3 trp1 ade2 Δbar1:HIS3 Δmad2:URA3 MYO1-GFP (pKT36 int)	This work
KSY110	a ura3 leu2 his3 trp1 ade2 Δbar1:HIS3 Δbub2:LEU2 Δbfa1:URA3	K. Shannon
KSY111	a ura2 leu2 his3 trp1 ade2 Δbar1:HIS3 Δmad2:LEU2 Δbfa1:URA3	K. Shannon
KSY129	a ura2 leu2 his3 trp1 ade2 Δbar1 pKT1315 (GAL-BUB2, URA)	This work
KSY130	a ura2 leu2 his3 trp1 ade2 Δbar1 Δbub2:LEU2 pKT1315 (bub2, URA)	This work
KSY131	a ura2 leu2 his3 trp1 ade2 Δbar1:HIS3 Δmad2:LEU2 pKT1315 (bub2, URA)	This work
KSY132	a ura2 leu2 his3 trp1 ade2 Δbar1 MYO1-GFP (pKT36 int) pKT1315 (GAL-BUB2, URA)	This work

These cells were stained with Alexa 568 (Invitogen) or rhodamine (Cytoskelecton Inc. Denver, CO) phalloidin as described (Lippincott and Li, 1998).

For the staining, polylysine-coated slides were prepared by adding 15ul of 1mg/ml polylysine to each well of the multitest slide 10-well (MP Biomedicals, LLC). Incubate for 10 minutes. Aspirate off the liquid until only a thin film of the liquid is left. Let the wells air dry completely. Rinse slides with ddH2O. Air dry again. Transfer cells into an eppendorf tube (Should use a small amount otherwise phalloidin will be limiting).

Spin shortly and remove supernatant. Wash cell one time with 1X PBS+0.2%TX100. Spin shortly and remove supernatant. Resuspend the cells in 1X PBS+0.2%TX100. Add Alexa or rhodamine phallodin. Incubate at room temperature for 40 minutes in the dark. Wash the cells 2 times with 1X PBS+0.2%TX100. Resuspend the cells in 1X PBS. Put the cells in each well of a lysine-coated slide. Incubate in the dark for 10minutes. Aspirate off the cells until only a thin film of liquid is left. Let the wells dry completely in the air. Wash each well one time with 1X PBS. Add a drop of mounting solution to each well and put on the cover slip. Squeeze out and then aspirate off excess mounting solution. Seal the slide with nail polish.

2.2.4 Image Capture and Analysis. Cells were observed by microscopy on Olympus IX51 inverted microscope at 1,000X total magnification using a UPLSAPO 100X NA 1.4 objective. Texas-red filter was used to see actin ring. Images were captured with a Hamamatsu ORCA285 CCD camera. Shutters, filters, and camera were controlled using Slide Book software (Intelligent Imaging Innovations, Denver, CO). The deconvolved images were transferred to a Dell computer, and analyzed.

2.2.5 Time-Lapse Microscopy. Cells were grown overnight in selective medium and placed on agarose pads (Waddle et al., 1996). Because YPD gives high background fluorescence, 0.2 g of agarose were melted in 1 ml -TRP media by boiling at 100 ºC. Lab

tape was attached onto the glass slides to make it about 0.1 cm thicker. Two taped slides were placed parallel to each other and then, one empty glass slide was placed in between the two taped glass slides. 170 µl of melted agarose was placed on the empty glass slide and then, pressed by another clean glass slide. After 3 to 5 minutes drying, taped slides and covered slide were removed. 15ul of cell, was placed on the agarose pad and the pad was covered with cover slip and then, sealed with valap (1:1:1 mixture of Vaseline, lanolin, and paraffin). Living cells were viewed using an Olympus IX51 inverted microscope at 1,000X total magnification using a UPLSAPO 100X NA 1.4 objective. FITC (EX 482/35 506DM EM 536/40) filter was used (Brightline). Images were captured with a Hamamatsu ORCA285 CCD camera. Shutters, filters, and camera were controlled using Slide Book software (Intelligent Imaging Innovations, Denver, CO).

Images were collected with exposure (100 ms to 2000 ms) to fluorescent light every 1 minute.

2.2.6 Morphological Observations. All wild type and mutant cells were treated both with and without zymolyase before observing phenotypic changes (Popolo et al., 1997). 670 µl of 37% formaldehyde was added to 5 ml overnight culture of each deletion mutant strain and then, cells were incubated at room temperature on the roller for one hour. Cells were washed twice with 1 ml 1X PBS and then, washed one time with 1 ml

Sorbitol Buffer (1 M Sorbitol in 50 mM KPO_4, pH 7.5). Cells were resuspended in 1 ml Sorbitol Buffer with 0.2 mg/ml zymolyase 20T (Seikagaku Kogyo Co, Tokyo,. Japan) and 2 mM DTT (dithiothreitol). Cells were incubated at 37℃ for ten minutes and then, immediately put on ice. Morphology was observed and cells were counted under Olympus CH2, objective EA40 NA0.65.

2.2.7 Calcofluor Staining. Calcofluor staining based on a method by Pringle et al. (1991). Cells in growth medium, add Calcofluor (Fluorescet brightener 28: Sigma F3397) to a final concentration of 100ug/ml. Incubate 5 minutes or more, wash twice with H_2O, and observe with a DAPI filter set.

2.2.8 T-test. Use Microsoft exel to pdrform standard T-test. 2 teriled, type 3 standard deviation was calculated using Microsoft exel.

3. RESULTS

3.1 BUB2 IS NOT A NEGATIVE REGULATOR FOR ACTIN RING FORMATION

Previous studies observed that deletion of Bub2 causes actin ring formation in cells arrested in mitosis (Lee et al., 2001). This leads to the hypothesis that Bub2 negatively regulates actin ring formation. To test this, we observed actin ring formation in normal or in depolymerized microtubule condition by nocodazole. Cells were synchronized with alpha factor for 2 hours in room temperature. After that, cells were released in normal YPD or YPD+nocodazole media. After treatment with nocodazole, cells were arrested at mitosis dependent on the activity of both Bub2 and Mad2. However, deletion mutants of either Bub2 or Mad2 delayed, but did not arrest and continued to complete the cell cycle. Data show that actin ring was formed at the same time in cells without Bub2 or Mad2 (Figure 3.1). Therefore, the Bub2 branch of the pathway doesn't specifically regulate actin ring formation. The double mutant with nocodazole treatment does not delay cell cycle, and forms actin ring at same time (80 minutes) as wild type without nocodazole treatment (Figure 3.2). Therefore, cells without Bub2 do not form actin rings earlier than wild type cells.

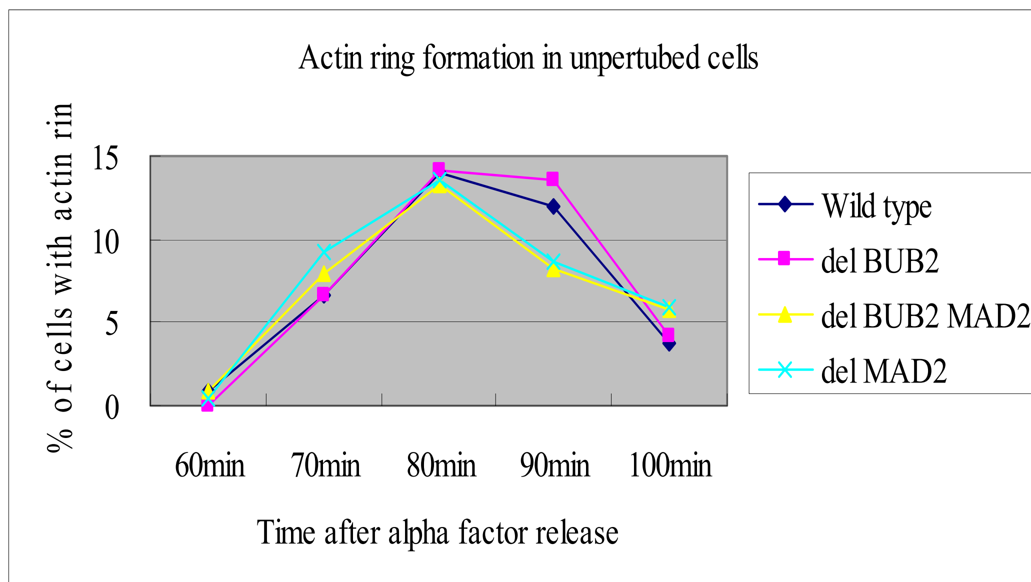


Figure 3.1. Actin ring formation in Bub2, Mad2 and double deletion mutants. Bub2 does not affect the timing of actin ring formation under normal condition.

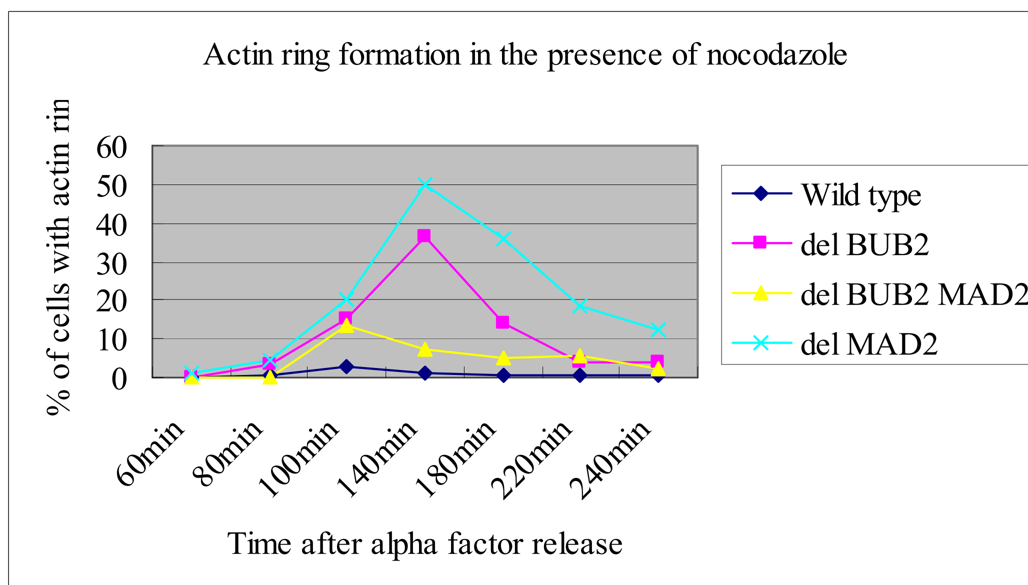


Figure 3.2. Actin ring formation in the presence of nocodazole. Bub2 does not interrupt actin ring formation with depolymerization of microtubule.

3.2. ACTIN RING FORMATION IN BOTH BUB2/BFA1 AND MAD2/BFA1 DELETION MUTANT

As mentioned before, Bub2 and Bfa1 work as a complex. However, it has been reported that Bfa1 can regulate Tem1 independently of Bub2 (Ro et al., 2002). It was curioused about whether deletion of Bfa1 affects on actin ring formation or not. To test this, both Bub2/Bfa1 and Mad2/Bfa1 deletion mutants were used. It is examined actin ring formation in synchronized cells under both normal conditions and after depolymerization of microtubules by nocodazole as before. Data showed actin ring formation occurs at same time in wild type and Bfa1 deletion mutant in unperturbed cells. After nocodazole treatment, mutants exhibited cell cycle delay, but formed actin rings at same time as single Bub2 or Mad2 mutants (Figure 3.3 and Figure 3.4). Therefore, Bfa1 does not restrain actin ring formation during normal cell cycle, or during cell cycle delays due to microtubule depolymerization.

3.3 BUB2 AND MYOSIN CONTRACTION

A question arose on how deletion of Bub2 would affect the timing of a myosin contraction. Therefore, Myo1-GFP was transformed into KSY3, a normal cell line and KSY19, a Bub2 deletion cell line. Myo1-GFP was imaged with or without nocodazole condition (Figure 3.5).

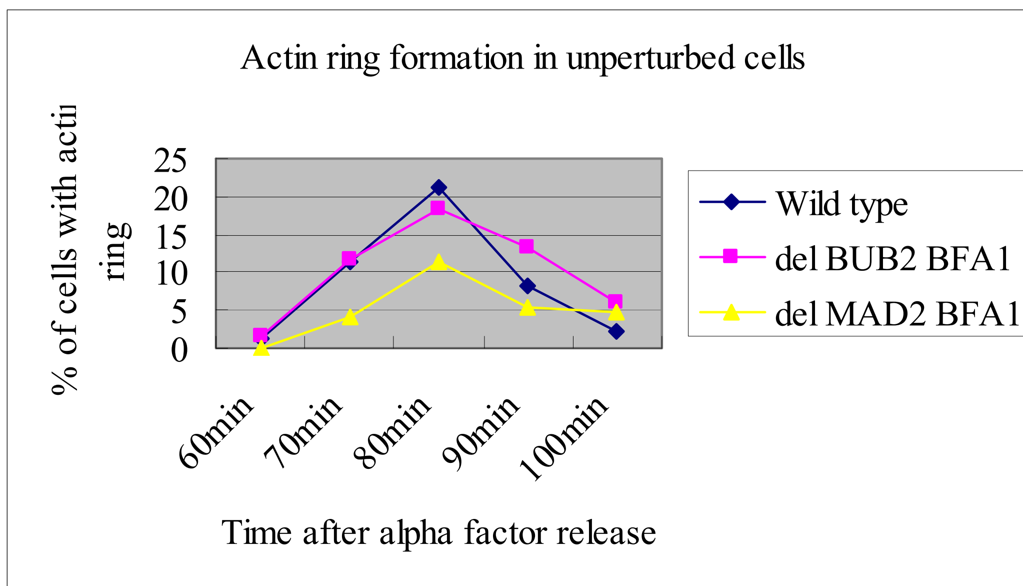


Figure 3.3. Actin ring formation in Bub2/Bfa1 and Mad2/Bfa1 mutants. Deletion of Bfa1 does not affect the timing of actin ring formation under normal condition.

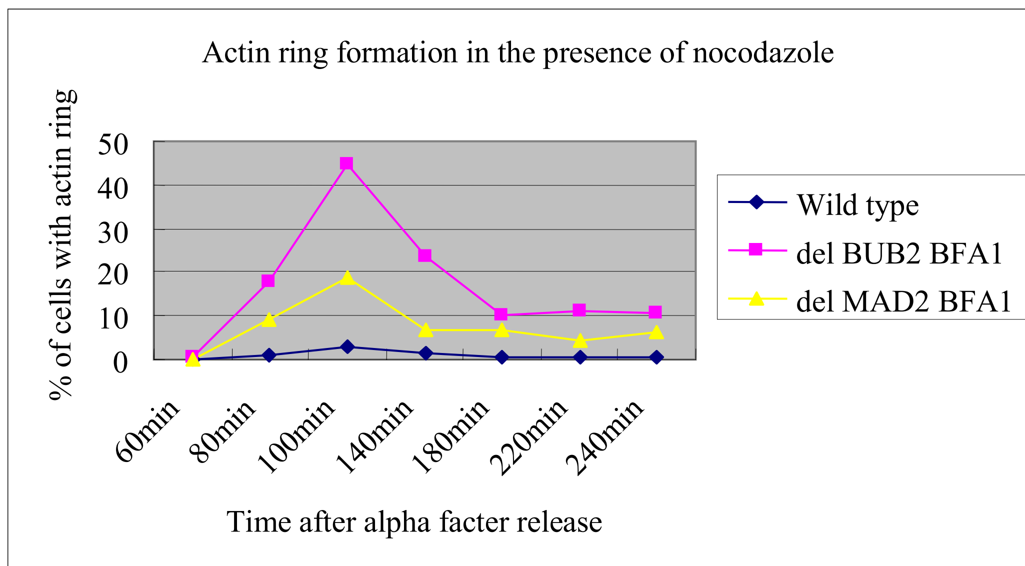


Figure 3.4. Actin ring formation in the presence of nocodazole. Deletion of Bfa1 does not change time relative to deletion of Mad2 or deletion of Bub2 alone

In normal cells, myosin contraction took 7.5 minutes average without nocodazole. In Bub2 deletion mutant without nocodazole, myosin contraction took 5.5 minutes average. In contrast, in Bub2 deletion mutant with nocodazole, myosin contraction lasted only 5minutes on average (Table 3.1). This data suggests that Bub2 deletion causes an increase in the rate of myosin contraction, and that microtubules depolymerization causes a further increase in rate.

Investigate the rate of myosin contraction in overexpressed Bub2 condition were also wanted. A cell line which contains Bub2 under the inducible GAL1 promoter was used. This promoter is repressed by glucose and activated by galactose. Cells were arrested in G1 phase and Bub2 transcription was induced by addition of galactose. Cells were then released from G1 phase and followed through M phase. Myo1-GFP was imaged under the fluorescence microscope (Figure 3.5).

At first, it was expected that overexpressed Bub2 might cause delay of rate of myosin contraction. However, myosin contraction in this condition showed faster that wild type but not as fast as Bub2 deletion mutants both with and without nocodazole condition. The average rate of contraction is 6.3 minutes (Table 3.1).

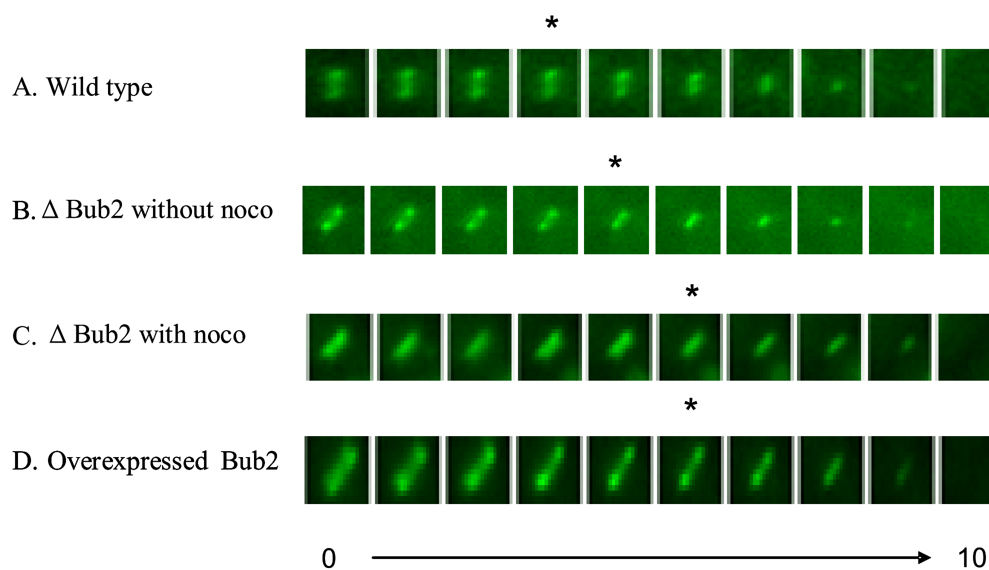


Figure 3.5. Myosin ring contraction. Asterisks indicate the onset of myosin contraction. Images were taken at one-minute intervals.

Table 3.1. Timing of myosin contraction in different cell lines. Asterisk represents T test indicated significance (P < .05). +/- represents standard deviation.

Strain	Time of contraction (Ave.)	Rate of contraction
Wild type	7.5 minutes +/- 1.3	.19um/min +/- .05
Deletion Bub2 without nocodazole	5.5 minutes +/- 1.8	.24um/min +/- .04
Deletion Bub2 with nocodazole	5 minute +/- 1 *	.27um/min +/- .05 *
Overexpressed Bub2	6.3 minute +/- .49	.21um/min +/- .02

3.4 CELL CYTOKINESIS AND CELL SEPTATION DEFECTS IN BUB2 DELETION MUTANT

Since deletion of Bub2 indicated faster rate of myosin ring contraction, we thought that this could cause either cell cytokinesis defection or cell septation defection. To test this, we grew the both wild type, Bub2, Mad2 and both Bub2 and Mad2 deletion mutants with or without nocodazole for 15 hours. Cells were fixed and examined cell morphology and counted the percentage of cells with chains of cell bodies (3 or more) with and without zymolase treatment (Figure 3.6). Zymolase digests the cell wall, leaving plasma membrane intact. Bub2 deletion mutant showed higher percentage of chains of cell bodies both with and without nocodazole compared to wild type. This chain phenotype disappeared after treatment of zymolase that means Bub2 deletion mutants showed septation defects not cytokinesis defects. This phenomenon might be due to septum formation could not be able to complete cell wall synthesis in such a high rate of myosin contraction. Previous studies have shown that septum formation and cytokinesis is linked (Maiato, H. et al., 2004).

3.5 CELL CYTOKINESIS AND CELL SEPTATION DEFECTS IN BUB2 OVEREXPRESSED MUTANT

Because Bub2 overexpressed cells indicated a faster rate of myosin contraction like Bub2 deletion mutant, this phenomenon could also be expected to cause cell cytokinesis defects or cell septation defects. To test this, cells were grown at YPGR to induce overexpressed BUB2 for 15 hours. Cell morphology was examined and counted

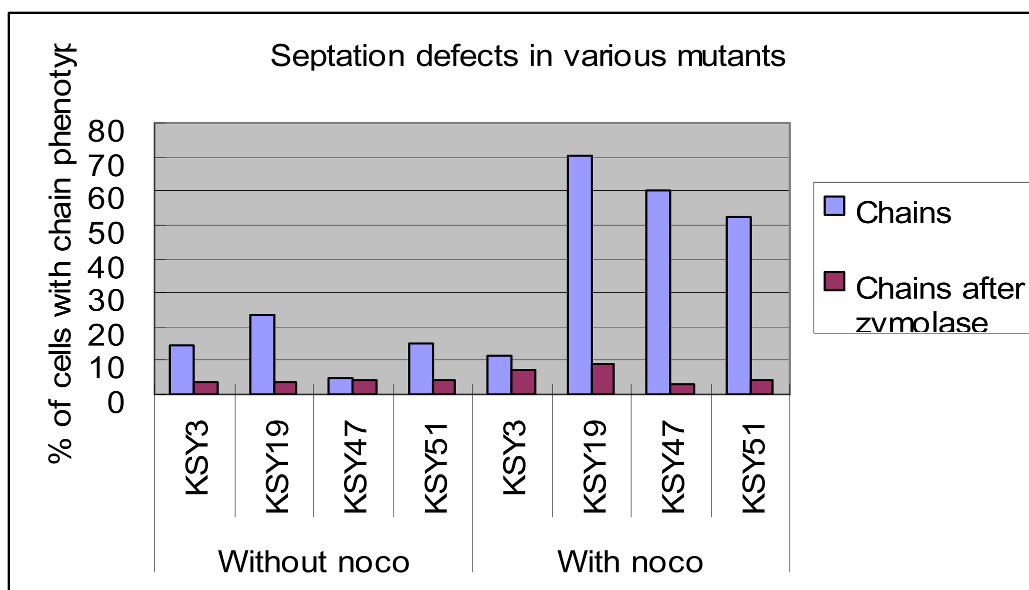


Figure 3.6. Chain morphology in cells with and without nocodazole before and after zymolase treatment. KSY3, KSY19, KYS47 and KSY51 indicate wild type, BUB2 deletion mutant, both BUB2 and MAD2 deletion mutant and, MAD2 deletion mutant respectively. All deletion mutants reveal a septation defects. Data from single experiment, which was repeated 3 times with similar results. 300 cells were counted for each treatment.

cells which have chains of cell bodies (3 or more). Morphology was examined both with and without zymolase treatment (Figure 3.7). Morphology were also taken under the microscope (Figure 3.8) As indicated in Figure 3.7 and Figure 3.8, overexpressed Bub2 represented high percentage of chain morphology. This chain form morphology disappeared after zymolase treatment, indicating that overexpression of Bub2 leads a cell separation defects not a cytokinesis defects like Bub2 deletion mutant. It is also related fact that myosin contraction rate is faster than wild type. Septum formation would not be able to complete with this faster contraction in these cells.

3.6 CALCOFLUOR CONFIRMED CELL SEPTATION DEFECTS IN BUB2 DELETION AND OVEREXPRESSION MUTAINTS

Calcofluor stains cell walls. This staining picture confirmed septation defects caused by Bub2 deletion (Figure 3.9). As shown in Figure 3.9, B, deletion mutants cells showed chain penototype with an open bud neck in the condition of depolymerization of microtubules by nocodazole. In contrast, wild type arrested at the G2 phase by nocodazole. Like deletion mutants, separation defects in Bub2 overexpressed cells also confirmed by staining (Figure 3.10). As indicated in figure 3.10, B, in cells showed chain form with connected opened bud neck.

Therefore, either deletion of Bub2 or overexpression of Bub2 leads to cell septation defects.

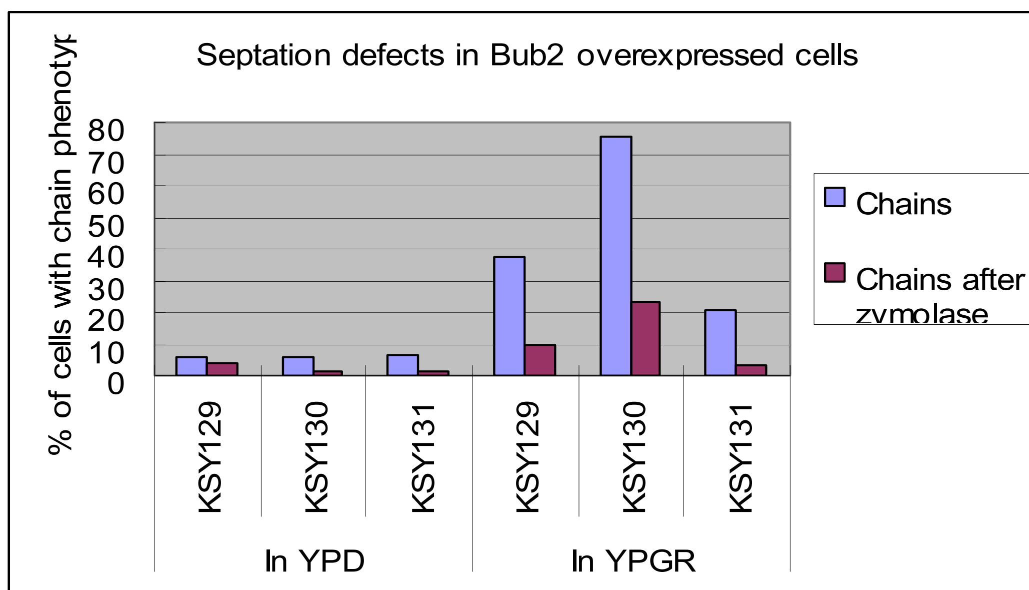
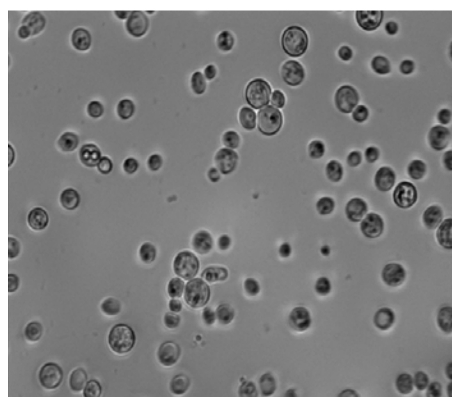
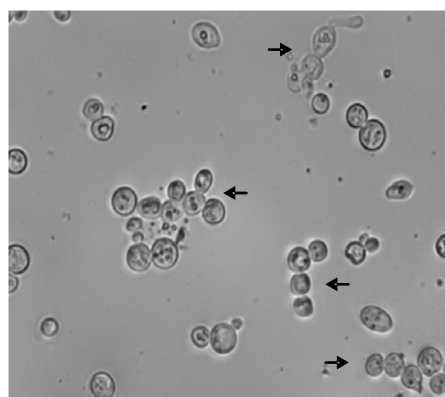


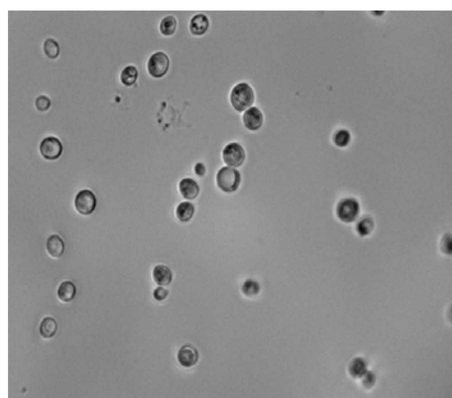
Figure 3.7. Chain morphology in cells with and without overexpressed Bub2 before and after zymolase treatment. KSY129, KSY130, and KSY131 indicate BUB2 overexpressed in wild type, Bub2 deletion mutant and MAD2 deletion respectively. In YPD Bub2 was not overexpressed. In contrast Bub2 was overexpressed in YPGR respectably. All overexpressed Bub2 mutants showed a septation defects.



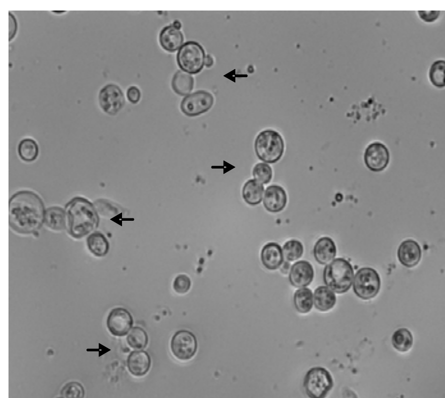
KSY129 in YPD



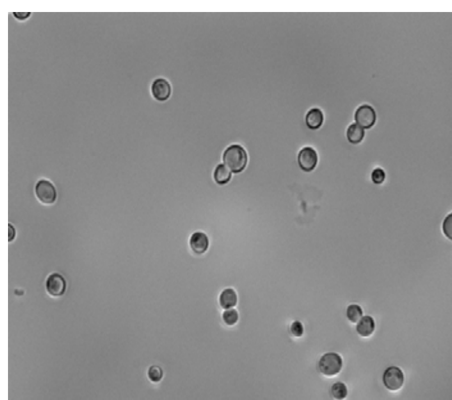
KSY129 in YPGR



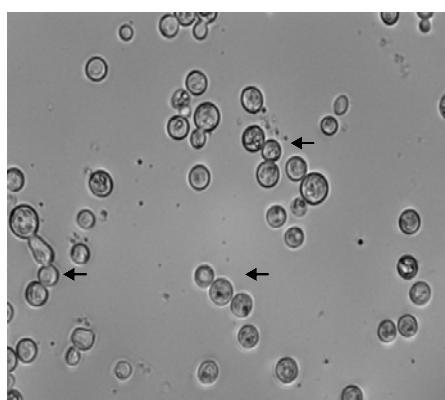
KSY130 in YPD



KSY130 in YPGR



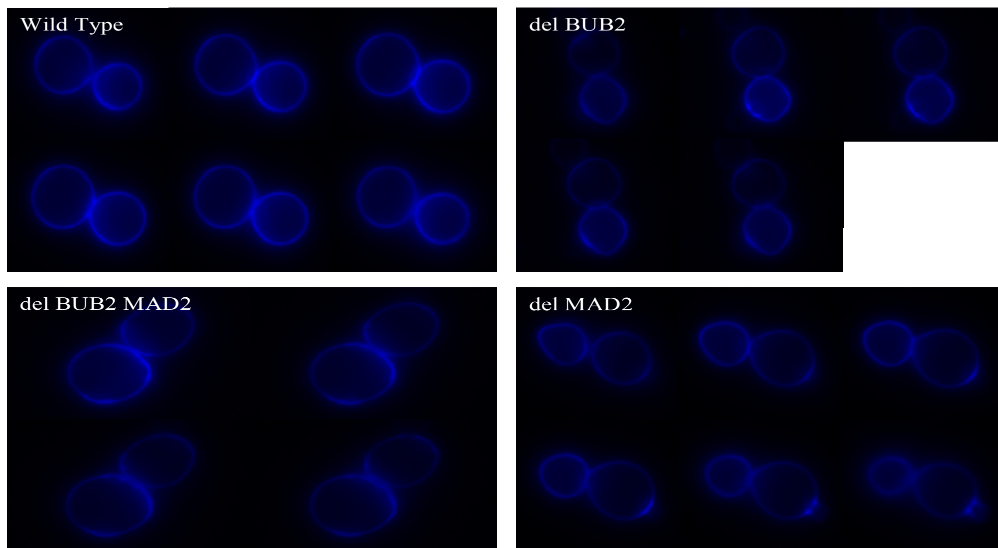
KSY131 in YPD



KSY131 in YPGR

Figure 3.8. Chain morphology cell picutures were taken under the microscope. Arrows indicate chain phenotype cells (3 or more cell bodies). All these chain form disappeared after zymolase treatment (data not shown).

A. Without nocodazole



B. With nocodazole

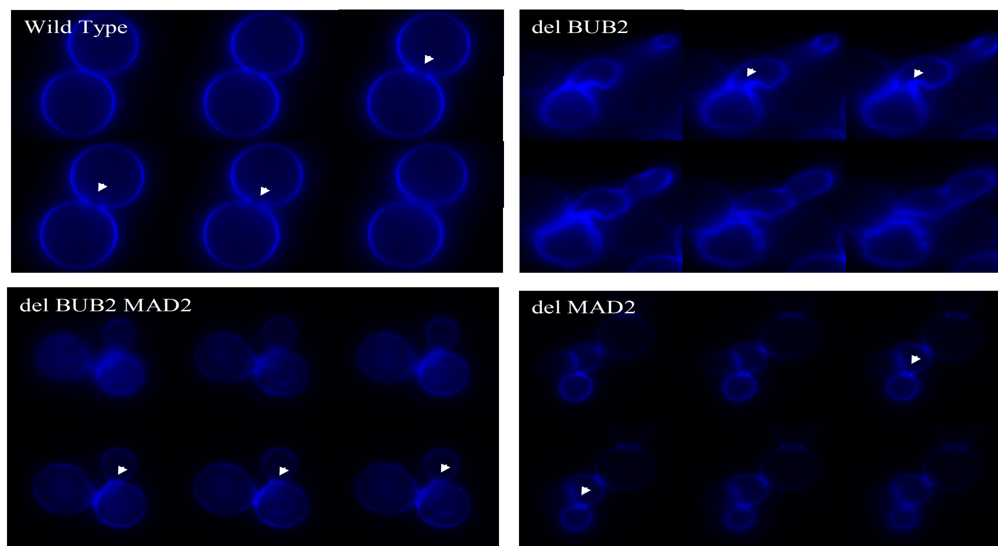
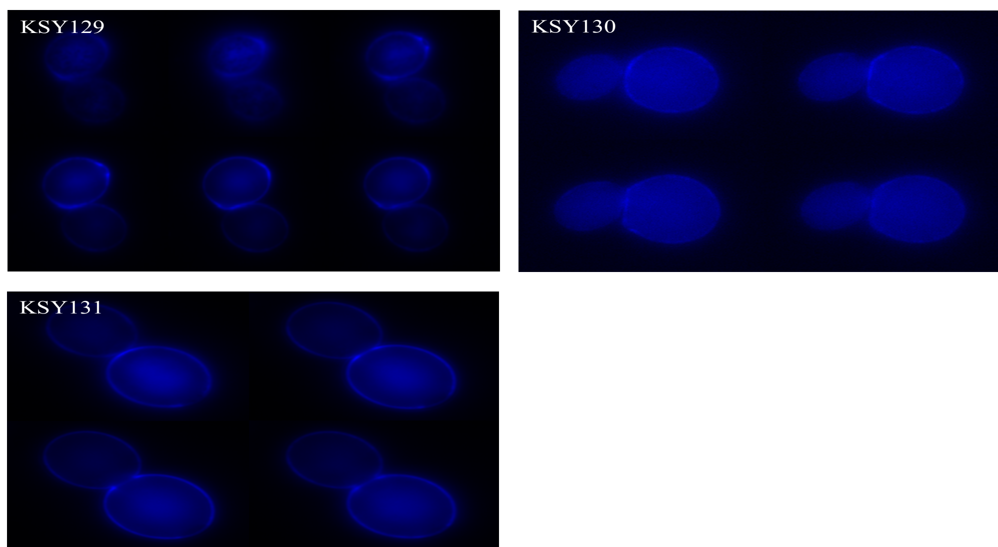


Figure 3.9. Calcofluor staining in Bub2 and Mad2 deletion mutants. To investigate septum formation, we stained cells with calcofluor. If myosine is depolymerized by certain condition, cell could not be able to make complete septin wall. Therefore cells were remained in chain form abnormally. A represented septum formation without nocodazole treatment. B represented with nocodazole treatment. Arrowhead indicated connected budneck because of failure complete septum formation.

A. In YPD



B. In YPGR

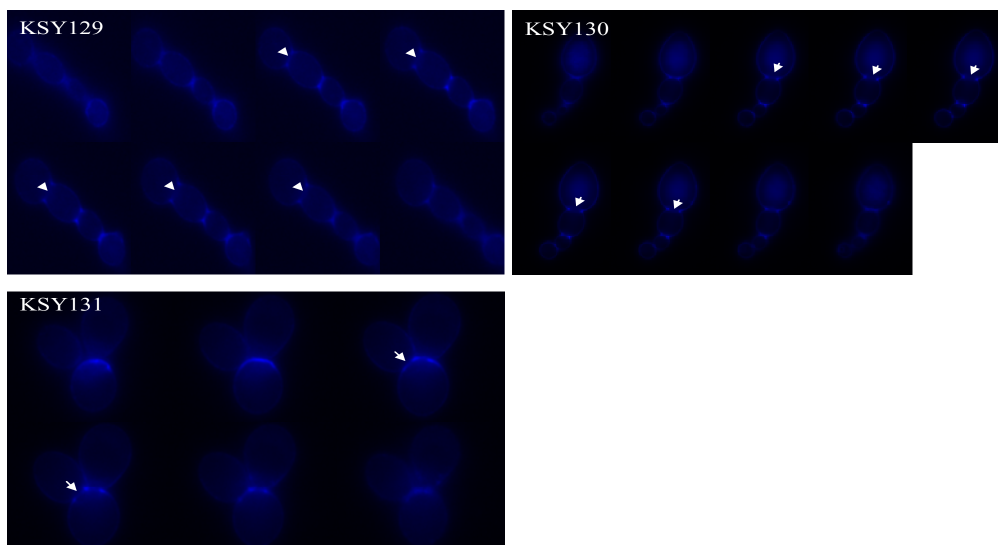


Figure 3.10. Calcofluor staining in Bub2 overexpressed mutants. To indicate septum formation in Bub2 overexpressed condition, we made a yeast strain which has a GAL-BUB2 plasmid. In YPGR, Cells showed septum defection. Therefore, overepressed Bub2 caused septum defection resulting in chain form morphology. A is normal condition and B is the Bub2 overexpressed condition, respectively. Arrows indicated uncompleted septum wall between mother and daughter cell.

4. CONCLUSIONS

Cell cytokinesis is the final step for cell division. Since this step is critical, many components are involved in this signaling. In budding yeast, MEN signaling regulates mitotic exit. Bub2 is a negative regulator of MEN signaling. Our data suggest that Bub2 and the MEN regulate the rate of myosin contraction.

Here, it was wanted to examine role of microtubules and Bub2 in budding yeast cytokinesis. To test this nocodazole was used which causes depolymerization of microtubules. This was also examined in various mutant cell lines, since wild type cells arrest prior to cytokinesis after nocodazole treatment.

Bub2 deletion did not affect the timing of actin ring formation with or without depolymerization of microtubule condition. Both Bub2 and Bfa1 deletion mutant showed same result. However, timing of rate contraction of myosin ring increased in Bub2 deletion mutant compared to wild type. Rate of contraction is even faster in Bub2 deletion mutant with depolymerization of microtubules, showing an additional role for microtubules.

Moreover, cells which have various mutants revealed chain phenotype of cell bodies. These chains disappeared after zymolase treatment. This means there were

septation defects in these cells instead of cell cytokinesis defect. This observation was confirmed by calcofluor staining. We hypothesized that septation defects result due to inability of cell wall synthesis to keep pace with the higher rate of myosin contraction in these cells. However, further study is needed.

Therefore, further study for this project will be monitoring of cell septation defects. To confirm septum formation could not be completed, we might make Chs2-GFP cell line and observe phenomenon under the fluorescence microscope. Chs2 is required for formation of primary septum. Indeed there are a lot of components are involved in septum formation. Therefore, it could be examined which components of septum formation involved this septum defection, expression level of those proteins in high rate of myosin ring contraction strain will be compared with wild type.

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