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Bmh1/Bmh2 Redundancy in the Control of the Spindle Assembly Checkpoint in Yeast Cells

Nat Jones

THE FLORIDA STATE UNIVERSITY

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BMH1/BMH2 REDUNDANCY IN THE CONTROL OF THE SPINDLE ASSEMBLY CHECKPOINT IN YEAST CELLS

By

NAT JONES

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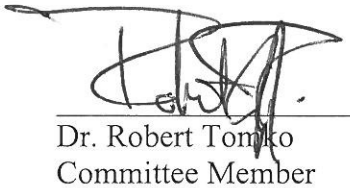
Dr. Yanchang Wang
Thesis Director

Dr. Hong-Guo Yu
Committee Member

Dr. Tim Megraw
Committee Member



Dr. Robert Tomko
Committee Member

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Abstract

The spindle assembly checkpoint (SAC) monitors errors in chromosome attachment, but the regulation of the SAC is not fully understood. Previous research shows that the budding yeast 14-3-3 protein Bmh1 serves a sequestering function for Fin1, which prevents the kinetochore recruitment of Fin1-PP1 and prolongs the SAC. After chromosome segregation, Bmh1 detaches from Fin1, allowing the Fin1-PP1complex to bind to the kinetochore and remove SAC proteins from the kinetochore. *bmh1*Δ mutant cells show chromosome missegregation and early dephosphorylation of SAC proteins in the presence of tension defects, which indicates premature anaphase entry. We found that Bmh2, a homologue of Bmh1 that is less prevalent within cells, has some redundant function with Bmh1. By creating a *bmh1*Δ *GAL-BMH2* cell line, we were able to overexpress the Bmh2 protein in the absence of Bmh1. This new strain grew poorly on glucose medium when Bmh2 expression is suppressed, which confirmed the synthetic growth defect of the *bmh1*Δ *bmh2*Δ double mutant. When this strain was grown in galactose medium to induce Bmh2 overexpression, we observed suppressed sensitivity to microtubule poison and decreased Fin1 kinetochore localization. Therefore, we concluded that Bmh1 and Bmh2 play redundant roles in cell cycle regulation.

Introduction

Correct mitotic chromosome segregation is essential for accurate transmission of genetic information between generations. If there are defects in this eukaryotically-conserved process, aneuploidy and cancer can result. The kinetochore-based spindle assembly checkpoint (SAC) monitors defects in chromosome attachment in yeast and other eukaryotic cells and blocks anaphase onset when defects are present (Lara-Gonzalez et al., 2012; Musacchio, 2015). Once all chromosomes achieve correct bipolar attachment, the SAC needs to be silenced to allow anaphase onset. However, some aspects of SAC silencing are not fully understood.

An example of a well-understood mechanism of the SAC concerns the Anaphase Promoting Complex (APC). Once activated after binding to Cdc20, the APC complex degrades securin, thereby freeing and activating separase. Separase then cleaves cohesin proteins, which allows for sister chromatid separation and cellular progression into anaphase. When there are errors in chromosome attachment, such as monotelic or syntelic attachments, the Mitotic Checkpoint Complex (MCC) binds to and segregates the APC complex, thereby preventing the cell from prematurely progressing into anaphase (Herzog et al., 2009; Alfieri et al., 2016). Another known aspect of the SAC concerns the tension generated by bipolar attachment of chromosomes. Correct attachments trigger Dam1 dephosphorylation to silence the SAC in yeast cells (Jin and Wang, 2013; Bokros et al., 2016). When that tension is not present, Ipl1/Aurora B kinase and centromere protein Sgo1 work together to prevent SAC silencing and delay anaphase onset (Biggins and Murray, 2001; Indjeian et al., 2005; Jin et al., 2012). Mutations in these proteins lead to early SAC silencing and premature anaphase onset.

The mutation of certain proteins can also prohibit SAC silencing, including the mutation of Spc105's Protein Phosphatase 1(PP1)-binding motif, which indicates the importance of PP1-

Spc105 interaction in SAC silencing (Rosenberg et al., 2011). PP1 also promotes SAC disassembly during anaphase by binding to Fin1, a verified S-phase CDK substrate (Loog and Morgan, 2005). The dephosphorylation of Fin1 by Cdc14 phosphatase leads to kinetochore localization of the Fin1-PP1 complex, which facilitates SAC disassembly (Bokros et al., 2016).

Phosphorylation of Fin1 promotes its interaction with the yeast 14-3-3 proteins Bmh1 and Bmh2 (Akiyoshi et al., 2009; Mayordomo and Sanz, 2002; Bokros et al., 2016). These two proteins have 90% similarity according to the Basic Local Alignment Search Tool. It is known that the former protein, Bmh1, binds to and sequesters the phosphorylated Fin1-PP1 complex, thereby preventing the kinetochore association of Fin1-PP1 and upregulating SAC activity (Fig. 1). *bmh1*Δ cells show obvious viability loss with syntelic attachments, while the phenotype of the *fin1*Δ*bmh1*Δ double mutant is less severe (Bokros et al., 2016). This indicates that Fin1 acts downstream of Bmh1, and the phenotype of the *bmh1*Δ mutant is likely due to the loss in Fin1 sequestering by Bmh1.

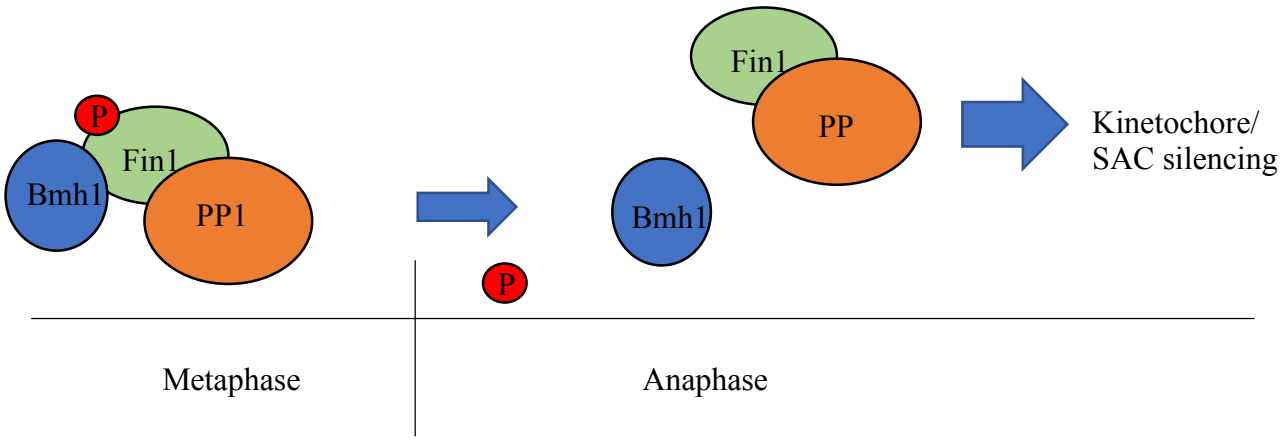

Figure 1. Current model: Bmh1 sequesters Fin1-PP1
By binding to phosphorylated Fin1, Bmh1 prevents Fin1-PP1 from localizing at the kinetochore and disassembling SAC machinery. After Cdc14 dephosphorylates Fin1, Bmh1 releases the Fin1-PP1 complex, which then localizes at the kinetochore to silence the SAC. Figure adapted from Michael Bokros's design.

Less is known about the role of Bmh2 in the SAC process. Although it is known that Bmh2 also binds to Fin1, whether it has a redundant function to Bmh1 in terms of sequestering Fin1-PP1 from the kinetochore is not well-studied (Akiyoshi et al., 2009). Bmh2 is less abundant than Bmh1 in yeast cells, but a *bmh1*Δ*bmh2*Δ double mutation is lethal (van Heusden et al., 1995; Gelperin et al., 1995). This implies some redundant function between the two 14-3-3 proteins. To further study the relationship between Bmh1 and Bmh2, we constructed the strain *bmh1*Δ *GAL-BMH2*, wherein the Bmh2 protein is conditionally overexpressed in the presence of galactose but not expressed at all in glucose. With this strain, we examined if Bmh2 overexpression suppresses the phenotypes of *bmh1*Δ mutant cells. Our results support the conclusion that Bmh1 and Bmh2 play a redundant role in regulating SAC activity (Fig. 2).

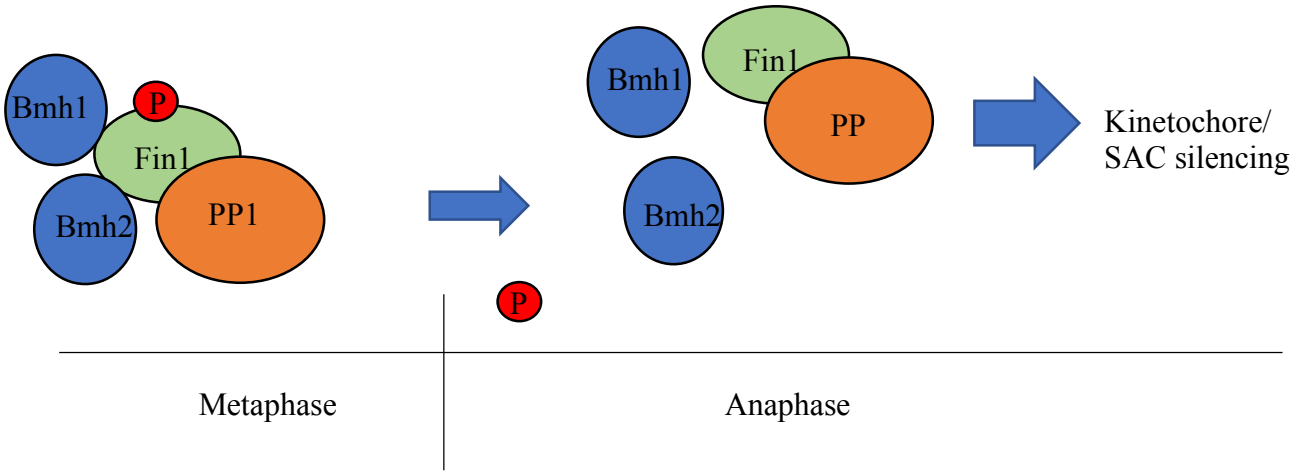

Figure 2. Proposed model: Bmh1 and Bmh2 play redundant roles in SAC regulation
We hypothesize that by binding to Fin1, both Bmh1 and Bmh2 prevent Fin1-PP1 from localizing at the kinetochore and disassembling SAC machinery. Figure adapted from Michael Bokros's design.

Materials and Methods

1. The confirmation of *bmh1*Δ *bmh2*Δ lethality by tetrad dissection

   A *bmh1::HIS3* strain and a *bmh2::KAN* strain were crossed to confirm the lethality of the double mutant. The haploid cell lines were mated on a yeast extract-peptone-dextrose (YPD) plate, grown at 30º C, inoculated into sporulation media, and grown at 30º C before the tetrad dissection was performed.

2. Creation of *bmh1*Δ *HIS3-*P_{GAL}*-HA-BMH2* strain (YNJ-001)

   A pair of primers was designed to insert the *HIS3-*P_{GAL}*-HA* cassette in front of the *BMH2* gene in haploid *bmh1::KAN* cells in order to induce Bmh2 overexpression. The GAL-Bmh2-F4 primer had a melting temperature of 64.5º C and a sequence of 5' ATT ATC AAA TCA ACA AAA AGT ACC CGT TAC AAC AAA AAA AGA ATT CGA GCT CGT TTA 3'. The GAL-Bmh2-R3 primer had a melting temperature of 67.4º C and a sequence of 5' CTA ATT TAG CTA GGT AAA CAG AAT CTT CAC GAG TTT GGG AGC ACT GAG CAG CGT AAT 3. The PCR mixture was 47µl ddH2O, 50µl 2x Taq, 1µl F primer, 1µl R primer, and 1µl 1/50 *HIS-GAL-HA* template DNA at an annealing temperature of 47º C. A transformation was performed so that the PCR products could insert in front of the *BMH2* gene in the *bmh1*Δ strain. 240µl PEG 3350, 35µl 1M LiAc, 25µl ssDNA, and 30µl PCR DNA were added to the cell mixture, which was incubated at 30º C for 30 min, heat-shocked at 42º C for 25 min, and put on ice for 1 min. The mixture was spread on a HIS-GAL plate. Colony #4 grew on a His-GAL plate but not on a simple HIS- plate, and was selected for further examination. To confirm the insertion, colony PCR was performed. We designed a F-Bmh2 check primer with a melting temperature of 54.5º C and a sequence of 5' TAC GAG GAA AAA GTC GGT

CG 3'. The *HIS3*-Rev primer had a melting temperature of 52.7º C and a sequence of 5' AAG AAT CCA ATT CCC GTG TC 3'. The PCR mixture was 8µl ddH2O, 10µl 2x Taq, 0.5µl F, 0.5µl R, 1µl zymolase, and a scoop of cells from the plate at an annealing temperature of 50º C. This protocol was simultaneously performed on the wild-type strain as a negative control.

3. Determination of YNJ-001 rescue phenotype

The growth patterns were determined by diluting log-phase cultures to an OD of 1 and then performing a 10-fold serial dilution with 30µl into 270µl ddH2O spotted onto YPD, YEP-GAL, and 15 µg/mL benomyl-GAL plates. The plates were incubated at 30º C for two days before scanning.

The nocodazole sensitivity was determined by diluting log-phase cells to an OD of 0.2 (in YEP galactose medium) and then taking time-point samples after adding 20 µg/mL nocodazole to the cell cultures. 6µl of cells were plated onto a YEP-GAL plate for each time point at 0, 2, and 4 hours. After overnight incubation at 30º C, the micro-colonies were examined.

4. Fin1 kinetochore localization

To create the necessary strains, YNJ-001 cells were crossed with *cdc13-1* cells to generate the strain *cdc13-1 bmh1*Δ *HIS3-*P_{GAL}*-HA-BMH2* (3569-1-4). High temperature growth conditions were used to select the correct strain. Then, the Fin1-GFP plasmid was transformed into 3569-1-4 using transformation protocol of 1mL H_2O, 2 scoops of cell culture, and 1µl pSB1252 plasmid before they were heat shocked and then spread on a Leu-GAL plate. This plate transformation procedure of Fin1-GFP was repeated for *cdc13-1*, *cdc13-1 bmh1*Δ, *cdc13-1 bmh2*Δ cell lines. To examine the Fin1 localization of

these cell lines, each strain was grown to log-phase in Leu-GAL media before they were temperature-arrested for 2h at 34º C. 1mL cells were collected and fixed in paraformaldehyde before their Fin1 localization was examined. This experiment was repeated three times.

To examine the Fin1 localization in these yeast strains without the galactose-induced overexpression of Bmh2, the cells were grown to log-phase in Leu-GAL media before 20% glucose was added, which the cells then preferentially used instead of galactose. Following the addition of glucose, the cells were arrested for 2h at 34º C. 1mL cells were collected and fixed in paraformaldehyde before their Fin1-GFP localization was examined.

Results

1. The confirmation of *bmh1*Δ *bmh2*Δ lethality by tetrad dissection

To determine if the *bmh1*Δ *bmh2*Δ double mutation is lethal, we crossed *bmh1::HIS3* and *bmh2::KAN* haploid cells. After mating these cells, the resulting diploid cells were sporulated and subjected to tetrad dissection. Some of the resulting spores could not form colonies. There were no viable colonies that were both Kan resistant and HIS+. After examining the genotypes of all the spores, we found that all the inviable spores were Kan resistant and HIS+ (Fig. 3). Because the parental haploid strains were *bmh1::HIS3* and *bmh2::KAN*, we concluded that the *bmh1*Δ *bmh2*Δ double mutation is synthetically lethal, confirming previous reports from other labs (Bokros et al., 2016).

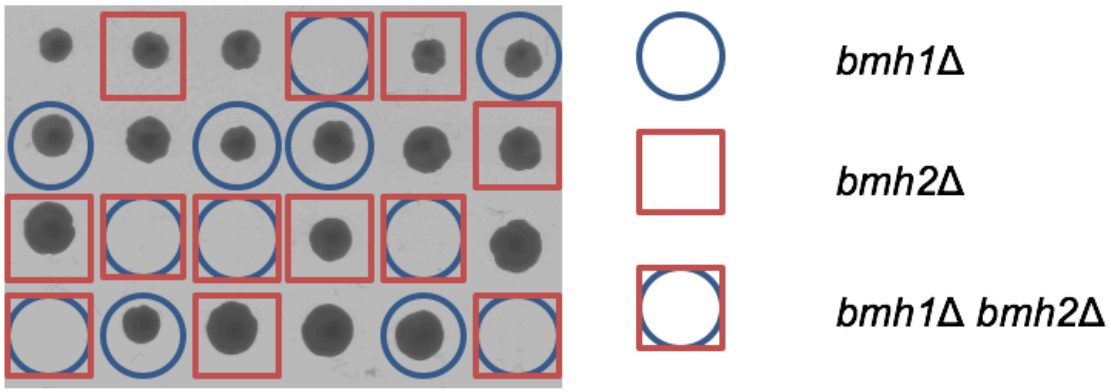

Figure 3. The *bmh1*Δ *bmh2*Δ cells are lethal
The *bmh1*Δ *bmh2*Δ cells were not viable. The genotypes are shown as a compilation from the original YPD plate, a copied HIS- plate, and a copied Kan plate.

2. Creation of *bmh1*Δ *HIS3-*P_{GAL}*-HA-BMH2* strain (YNJ-001)

To overexpress Bmh2 in *bmh1*Δ cells and determine if Bmh2 overexpression suppresses the phenotype of *bmh1*Δ due to redundant function of the two 14-3-3 proteins, we designed a pair of primers to insert a *HIS3-*P_{GAL}*-HA* cassette into the genome of haploid *bmh1::KAN* cells in front of the *BMH2* gene. Using the primers, we performed PCR to amplify the *HIS3-GAL-HA* template and then transformed the DNA product into the *bmh1*Δ strain. The growing colonies on HIS-GAL plates (HIS-) were selected. We then designed primers to examine the location of the insertion in the selected colonies using PCR. After running a DNA gel on this PCR product, a band was visible at the expected size of around 500 base pairs for the selected colony, but not for control cells (Fig. 4). This indicates the successful insertion of the *GAL* promoter at the correct locus and the creation of a *bmh1*Δ *HIS3-*P_{GAL}*-HA-BMH2* strain, YNJ-001, which conditionally overexpresses Bmh2 in the presence of galactose but expresses no Bmh2 without galactose.

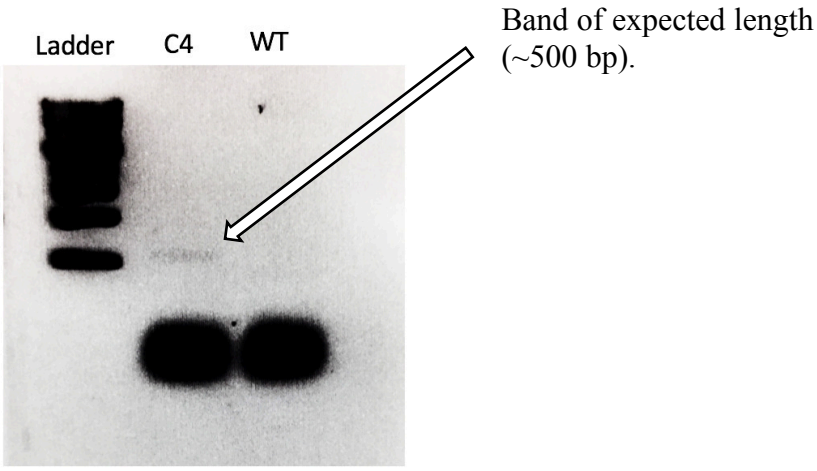

Figure 4. Insertion of a GAL promoter before the *BMH2* gene in *bmh1::KAN* cells
The YNJ-001 yeast cells contain the expected *GAL* promoter before the *BMH2* gene. The shown electrophoresis gel indicates the presence of a band of expected length (~500 bp) for Colony #4 but not the wild-type cells. A 1 kb ladder was used, with the primers forming dimers at an expected length of 30 bp.

3. Determination of YNJ-001 rescue phenotype

To determine if overexpression of Bmh2 suppresses the sensitivity of *bmh1*Δ mutants to spindle poison, we subjected YNJ-001 cells to the microtubule-depolymerizing agent benomyl and compared the growth patterns to wild-type, *bmh1*Δ, and *bmh2*Δ cells on plates containing glucose and galactose. After performing a 10-fold serial dilution experiment, we found a significant growth difference between YNJ-001 and *bmh1*Δ cells under different growth conditions (Fig. 5). First, we found that *bmh1*Δ P_{GAL}*-HA-BMH2* cells grew poorly on glucose plates (YPD), which supports the synthetic growth defect of the *bmh1*Δ *bmh2*Δ double mutant because of the inhibition of Bmh2 expression in YNJ-001 strain by glucose. Second, YNJ-001 cells grew well on YEP-GAL plates, indicating the galactose-induced production of Bmh2. In addition, the benomyl sensitivity of *bmh1*Δ cells was suppressed by Bmh2 overexpression in YNJ-001 cells on galactose plates, which demonstrates Bmh2's ability to fulfill the function of Bmh1 when overexpressed in the face of microtubule defects.

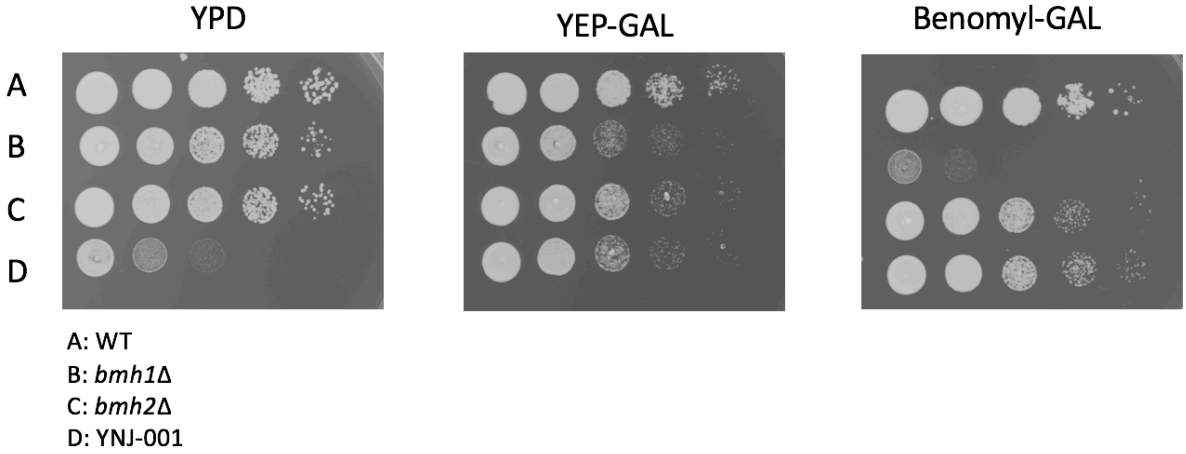

Figure 5. Bmh2 overexpression suppresses the benomyl sensitivity of *bmh1*Δ cells
The YNJ-001 cells have restored viability on galactose plates in microtubule-disrupting conditions. The scan of each dilution plate is shown.

As a secondary confirmation of the rescue phenotype of YNJ-001, we grew the four strains in the presence of galactose and the microtubule-depolymerizing agent nocodazole in a time-course experiment and then plated the cells on a YEP-GAL plate. We then compared their viability losses by counting the percentage of living versus dead cells. We found that the growth patterns of YNJ-001 cells were similar to the wild-type and *bmh2*Δ cells as compared to the sharp drop to 50% viability observed in the *bmh1*Δ cells (Fig. 6). This further indicates that YNJ-001 cells behave like cells with functioning Bmh1 in the face of microtubule disruption as a result of nocodazole treatment.

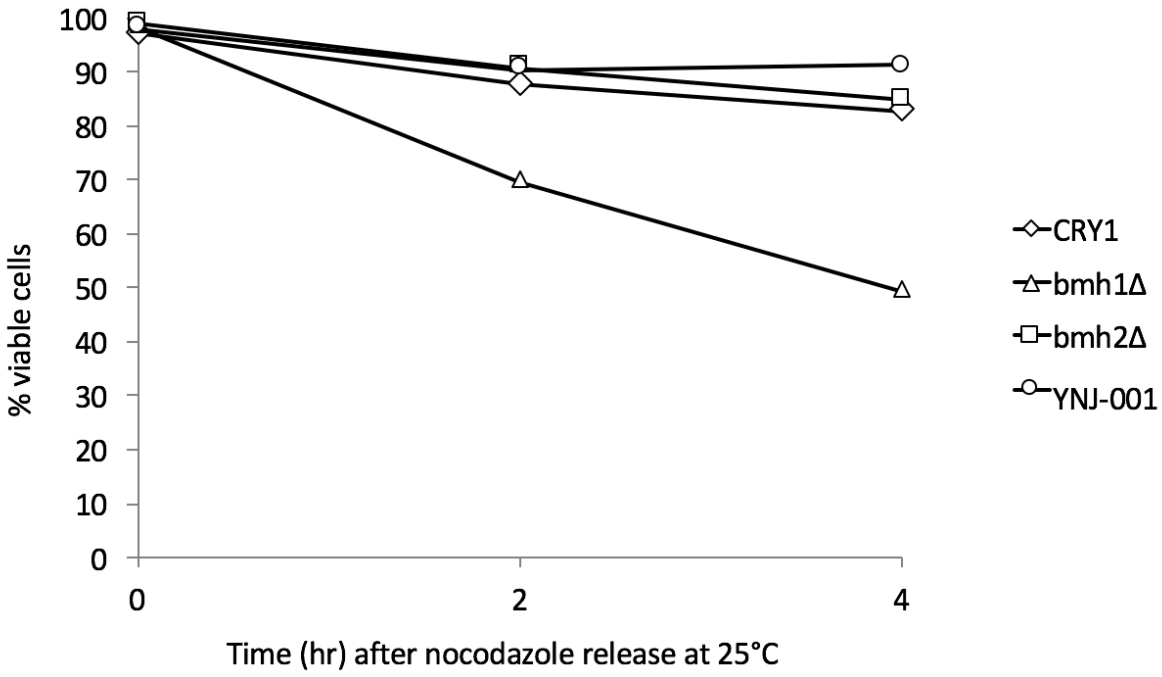

Figure 6. The YNJ-001 cells have restored viability in media containing galactose and nocodazole
The YNJ-001 cells have restored viability in microtubule-disrupting conditions in the presence of galactose. The viability for each strain is shown in the form of a line graph.

4. Fin1 kinetochore localization

Previous results from this lab show that *bmh1*Δ cells exhibit premature kinetochore localization of the Fin1 protein (Bokros et al., 2016). In order to test the Fin1 localization in YNJ-001 cells, we created a *cdc13-1 bmh1*Δ *HIS3-*P_{GAL}*-HA-BMH2 FIN1-GFP* strain that would arrest at pre-anaphase when grown at 34º C due to uncapped telomeres activating the DNA damage checkpoint. With the strains created, the Fin1 localization in cells with overexpressed Bmh2 in high-temperature conditions could be elucidated. The results of this experiment are depicted in Figure 7 as a ratio out of 100 cells, with the bars representing the number of cells without kinetochore-localized Fin1-GFP. The YNJ-001 data indicates that Bmh2 overexpression restores the non-kinetochore localization of Fin1 in *bmh1*Δ cells arrested in pre-anaphase.

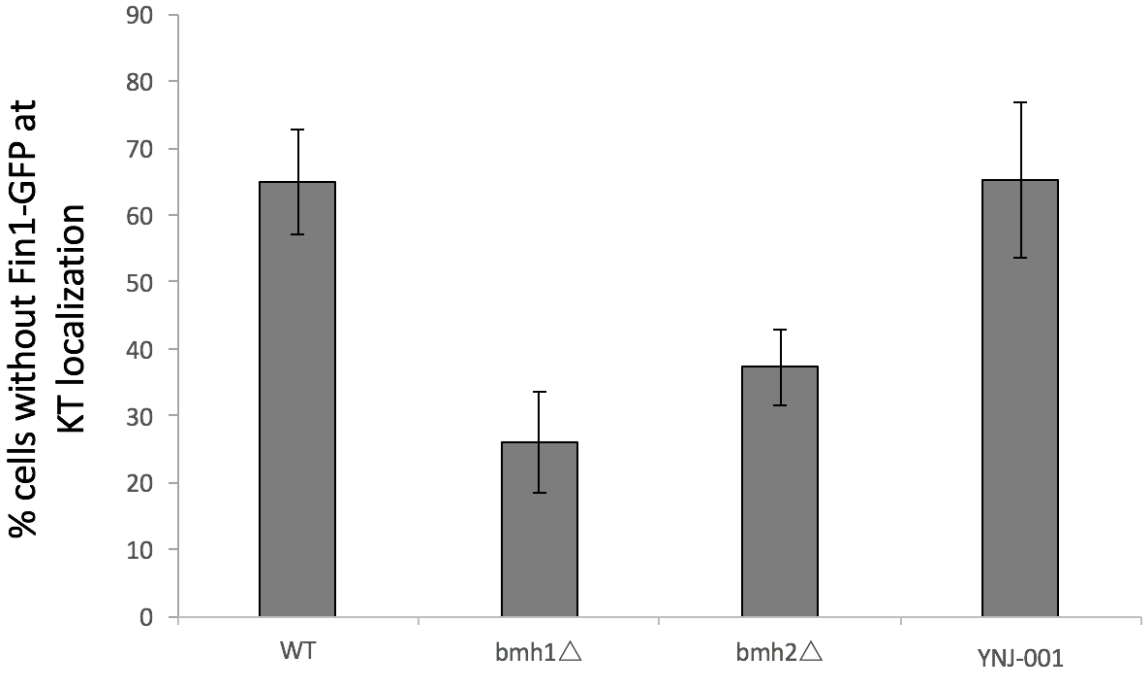

Figure 7. The YNJ-001 cells show decreased kinetochore Fin1 localization in galactose media

Examination of Fin1 kinetochore localization in YNJ-001 cells overexpressing Bmh2. The first experiment had WT:*bmh1*Δ:*bmh2*Δ: *bmh1*Δ*HIS3-*P_{GAL}*-HA-BMH2* non-KT-localized values of 56:18:31:53, the second 69:33:42:76, and the third 70:27:39:67. These values averaged out to 65:26:37:65, and are plotted in the form of a bar graph.

The Fin1 localization was also investigated in glucose conditions in order to determine the galactose-inducible effect of the YNJ-001 cells. This was accomplished through the addition of glucose to the same conditions as the prior Fin1 experiment, which would shut down Bmh2 expression in YNJ-001 cells. As reported previously in this lab, the absence of Bmh1 allows premature kinetochore localization of the Fin1 protein, and the same is true for *bmh2*Δ mutant cells. In cells lacking both Bmh1 and Bmh2 (YNJ-001), we found even more kinetochore localization of Fin1 (Fig. 8). Therefore, this observation also supports the notion that Bmh1 and Bmh2 play redundant roles in regulating the kinetochore localization of the Fin1 protein.

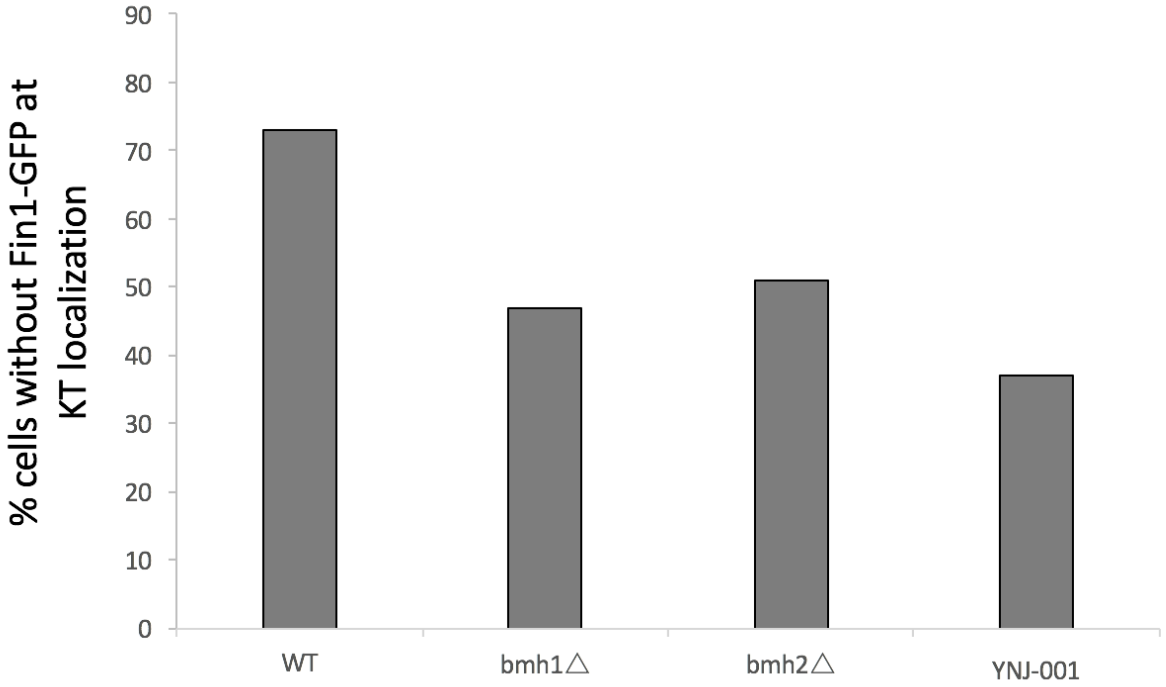

Figure 8. The YNJ-001 cells show more dramatic Fin1 kinetochore localization in glucose media that represses Bmh2 expression
Premature Fin1-GFP kinetochore localization in YNJ-001 cells without Bmh2 expression. The experiment had WT:*bmh1*Δ:*bmh2*Δ:*bmh1*Δ *HIS3-*P_{GAL}*-HA-BMH2* non-KT-localized values of 73:47:51:37. These values are plotted in the form of a bar graph.

Discussion

Our data demonstrate that the overexpression of Bmh2 suppresses the phenotype of *bmh1*Δ mutants, which indicates the redundant roles of Bmh1 and Bmh2 in cell cycle regulation. For this investigation, we generated the *bmh1*Δ *HIS3-*P_{GAL}*-HA-BMH2* strain YNJ-001, which allows for Bmh2 overexpression in galactose medium. In the presence of galactose, these cells were more resistant to treatment with microtubule disruption agents nocodazole and benomyl than *bmh1*Δ cells. Moreover, Bmh2 overexpression suppresses the premature kinetochore localization of Fin1 in *bmh1*Δ cells. By contrast, the absence of Bmh2 expression in *bmh1*Δ cells causes more dramatic kinetochore localization of Fin1. These pieces of evidence support the conclusion that both Bmh1 and Bmh2 regulate Fin1 kinetochore localization.

A missing result in this project is the confirmation of galactose-induced HA-Bmh2 expression in the YNJ-001 strain by way of a Western Blot. Also, the Fin1-GFP localization of YNJ-001 cells in glucose medium must be repeated to obtain standard deviations and average values. In order to ensure that there is no Bmh2 remaining in the YNJ-001 cells after the addition of glucose, the use of an auxin-inducible degron system should be explored. For all Fin1-GFP experiments, analyzing the GFP signal immediately after sampling would eliminate any systemic error associated with paraformaldehyde fixation. Alternatively, the use of an anti-GFP antibody may provide a more accurate visualization of Fin1-PP1 location. Finally, we must investigate chromosome segregation behavior after exposure to the spindle poison nocodazole using the *bmh1*Δ *HIS3-*P_{GAL}*-HA-BMH2 CEN4-GFP TUB1-mCherry* (3564-1-2) strain that I constructed.

Further paths of inquiry that relate to this investigation include the following questions: why do yeast cells require both Bmh1 and Bmh2 proteins? Do Bmh1 and Bmh2 have distinct functions? Why do Bmh1 and Bmh2 regulate Fin1 localization during the cell cycle? How do

these two proteins affect the mechanism that allows for SAC reactivation in anaphase before Fin1-PP1 kinetochore localization (which depends on the activation of the mitotic exit pathway)?

In summary, our results indicate another layer of SAC regulation by Bmh1 and Bmh2 that prevents premature kinetochore association of Fin1-PP1 and SAC silencing. Untimely activation of this particular pathway leads to premature SAC silencing and chromosome segregation errors. Such alterations of the expression of 14-3-3 proteins, the Bmh1 and Bmh2 homologues in human cells, have been associated with several human cancers, with the downregulation of 14-3-3σ being associated with a multitude of human epithelial cancers (Wilker and Yaffe, 2004). Further examination into the specific roles of Bmh1 and Bmh2 in addition to investigating their overlap in function in a yeast model may thus advance our understanding of the molecular mechanisms of cancer development.

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