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Regulation of the mitotic cyclin Clb2 in the budding yeast *Saccharomyces cerevisiae*

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Regulation of the mitotic cyclin Clb2 in the budding yeast
Saccharomyces cerevisiae

Cody R. Richardson

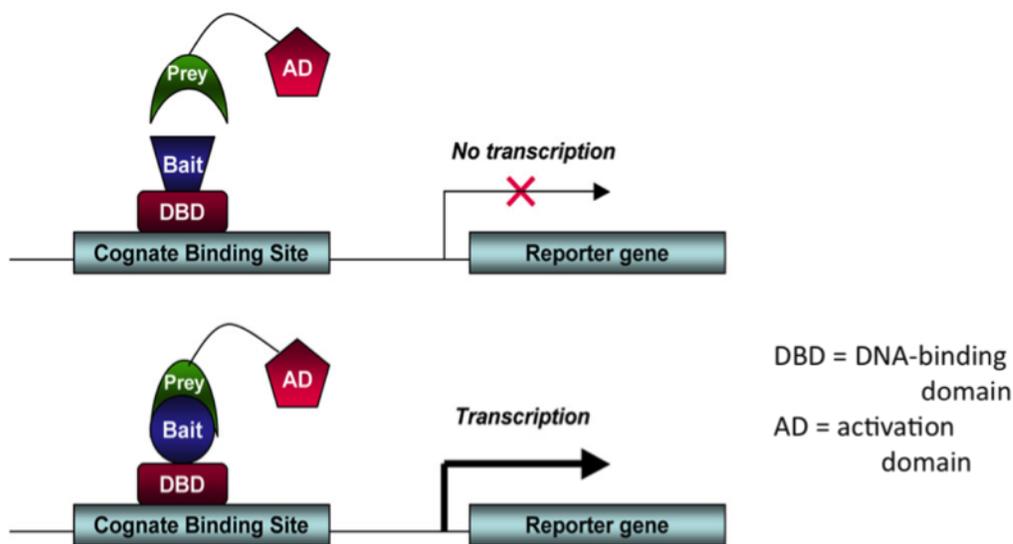
Advisor: Ana A. Kitazono
May 2010

INTRODUCTION

In all eukaryotes, cyclin-dependent kinases (CDKs) direct the cell cycle. As the nomenclature implies, activation requires binding to a cyclin to form a CDK-cyclin complex, which then directs the different phases of the cell cycle. Cdc28 is the primary CDK in the budding yeast *Saccharomyces cerevisiae*, and its activation is dependent on binding to one of nine cyclins found at specific times during the cell cycle as well as phosphorylation of Thr169 by the CDK-activating kinase CAK1. The formation of different Cdc28-cyclin complexes at specific times throughout the cell cycle allows different functions to be accomplished at specific times.

The cyclin Clb2 has been identified as the main mitotic cyclin through genetic testing (Grandin 1993). In combination with either $\Delta clb1$ or $\Delta clb3$ null mutants, $\Delta clb2$ is synthetically lethal, but $\Delta clb1,3,4$ null mutants show no discernible phenotype and remain viable (Mendenhall 1998). However, Clb2 is not essential; *clb2* mutants are viable but have elongated cells and delayed entry into mitosis. Clb2 binds tightly to Cdc28 via two well-characterized domains called the cyclin boxes. Though much is known about Clb2, many of its regulators and substrates are still unknown.

In this study, we seek to identify proteins that interact with Clb2 in a manner that is independent of Cdc28. A two-hybrid screen was conducted using a bait plasmid expressing a Clb2 fragment devoid of the cyclin boxes (Clb2-222N). The yeast two-hybrid system uses transcriptional activation to report protein-protein interactions. The protein of interest, the “bait”, is fused to the Gal4 DNA-binding domain, while the interacting protein, the “prey”, is fused to the Gal4 activation domain (James 1996). Any protein that binds to the bait will activate transcription of a downstream reporter gene, such as *ADE2* or *HIS3*. Plasmids harboring fragments that code for interacting proteins can be isolated by growing the cells on medium that selects for expression of the reporter gene(s).



V. Ratushny & E. Golemis - Biotechniques 2008; 44(5):655-662

Figure 1. Depiction of the yeast two-hybrid assay to test for protein-protein interactions.

In the initial screen, one protein was found to interact with Clb2-222N: Muk1. In an attempt to identify regulators that are specific for the mitotic function of Clb2, the two-hybrid screen was repeated in the presence of benomyl, a microtubule toxin that inhibits mitotic progression. In this screen, Ndc80, Zds2, Cin5, Fob1, and Srs2 were identified as interacting proteins. These results show that the two-hybrid strategy followed has been successful in identifying proteins that interact with Clb2.

MATERIALS AND METHODS

Strains, Media, and Transformations: For two hybrid analysis, the Gal4-deficient *Saccharomyces cerevisiae* PJ69-4A strain containing *HIS3* and *ADE2* reporters under the control of *GAL* promoters with the following genotype was used: *MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*. The plasmids used were pGAD and pGBDU. The pGBDU plasmid contains the Gal4DBD and *URA3* gene for selection on plates lacking uracil, whereas the pGAD plasmid contains the Gal4AD and *LEU2* gene for selection on plates lacking leucine. Both contain the Amp^R gene encoding β-lactamase, which confers resistance to Ampicillin for selection of *E. coli* transformants. For the two-hybrid screen, the tester strain harborin pGBDU-Clb2-222N was transformed with genomic libraries in vectors pGAD-C1, pGAD-C2, and pGAD-C3. The following plasmids were transformed into yeast for spot testing: (1) pGAD + pGBDU, (2) pGAD + pGBDU-Clb2, (3) pGAD + pGBDU-Clb2-222N, (4) pGAD-Cdc28 + pGBDU, (5) pGAD-Cdc28 + pGBDU-Clb2, (6) pGAD-Cdc28 + pGBDU-Clb2-222N, (7) pGAD-Muk1 + pGBDU, (8) pGAD-Muk1 + pGBDU-Clb2, and (9) pGAD-Muk1 + pGBDU-Clb2-222N. All transformations were performed using the following lithium acetate (LiOAc) protocol: Yeast cells inoculated in 50 ml YPD broth containing yeast extract, peptone, and dextrose and incubated with gentle shaking overnight at 25 °C. To harvest cells, culture centrifuged at room temperature for 2 minutes at 3000 rpm and supernatant discarded. Cells resuspended in 5 ml 1X Lithium Acetate (LiOAc)/TE. Then, cells centrifuged at 3000 rpm for 2 minutes, supernatant discarded, and LiOAc/TE treatment repeated once. To 10 μl of boiled salmon sperm DNA, 350 μl of cell suspension added. 1-5 μl of pGAD or pGBDU plasmid DNA added to cells and vortexed to mix. 1 ml 50% PEG 3350 (Sigma) added to cells and vortexed. Cells incubated for one hour at 25°C with rotation. Immediately following incubation, cells heat shocked at 42 °C in hot water bath for 20 minutes and allowed to cool to room temperature. Transformed cells centrifuged at 14000 rpm for 30 seconds and supernatant discarded. 1 ml YPD added to resuspend. Transformation mixes were then plated on selective media (-UL, -ULA, -ULH, and -ULH + 3-aminotriazole) and incubated at 30 °C. Media containing 10 μg/ml benomyl was used where indicated.

For plasmid amplification, *E. coli* DH5α strain was used. Transformations were performed in the following manner: 10 μl of ligation mix added to 250 μl of DH5α competent cells and placed in ice for 30 minutes. Mixtures were heat shocked in 42 °C in hot water bath for 90 seconds, and immediately placed back on ice for 2 minutes. 1 ml of 2X YT was added to promote cell growth and expression of β-lactamase. Cell mixtures incubated at 37 °C for 30 minutes before collecting cells by centrifugation at 14000 rpm for 5 minutes. Approximately 900 μl of supernatant discarded, and the cell pellet resuspended in remaining solution before plating on agar plates containing Luria broth and ampicillin for selection.

Construction of pGBDU-Clb2-222N: Polymerase chain reaction (PCR) was used to amplify the first 222 codons of CLB2 to avoid the cyclin boxes and introduce flanking *BamHI* and *EcoRI* endonuclease sites. Each reaction contained 2 μl genomic DNA, 0.8 μl Accuzyme/Bio-X-Act (3:1) DNA polymerase, 5 μl 10X Accubuffer, 1 μl 20μM CLB2EIFW2Hyb forward primer (5'-GGCCGAATTCTCCAACCCAATAGAA AACACAG-3'), 1 μl 20μM CLB2cdn222BamHI reverse primer (5'-CGTAGGATCCCTACCATGAAT GGATC-3'), 0.5 μl 25mM dNTPs, 1 μl 50mM MgCl₂,

and 38.7 μl dH_2O for a final volume of 50 μl per reaction. Production of the expected 660 bp product was checked by comparing bands of PCR product against a DNA ladder on a 0.8% agarose gel run at 110 V for 40 minutes. Amplified Clb2-222N PCR product and pGBDU vector digested in separate 35 μl reactions containing 12 μl DNA, 1 μl *EcoR1* endonuclease, 3.5 μl 10X Buffer E, 0.35 μl 10 mg/ml BSA, and 18.15 μl H_2O . Clb2-222N digested in a 40 μl reaction containing 15 μl plasmid DNA, 1 μl *BamH1* endonuclease, 4 μl 10X Buffer E, 0.4 μl 10 mg/ml BSA, and 19.6 μl H_2O . pGBDU vector digested in a 40 μl reaction containing 20 μl plasmid DNA, 1 μl *BamH1* endonuclease, 4 μl 10X Buffer E, 0.4 μl 10 mg/ml BSA, and 14.6 μl H_2O . Reactions incubated at 37 °C for 2 hours and chased with 1 μl enzyme after 1 hour. Digestion verified by comparing bands of digested and undigested DNA on a 0.8% agarose gel run at 110 V for 40 minutes.

Following each round of digestion, both Clb2-222N and pGBDU vector treated with phenol/chloroform to denature any proteins and enzymes, followed by ethanol precipitation and phosphatase treatment. For phenol/chloroform treatment, 1.5 volumes of TE added to the DNA followed by 4/3 ratio phenol-chloroform to DNA. Mixture vortexed for 1 minute, centrifuged at 14000 rpm for 5 minutes at room temperature, and top layer of supernatant retained. For ethanol precipitation, 1/3 volume 7.5 M ammonium acetate was added to DNA along with 2 volumes cold 100% ethanol. Mixture incubated in dry ice for 10 minutes, centrifuged at 14000 rpm for 15 minutes at room temperature, and supernatant discarded. Then, mixture rinsed with 500 μl of 90% ethanol (v/v), centrifuged 2-3 minutes at 14000 rpm at room temperature, supernatant discarded, and rinse repeated. Ethanol was allowed to evaporate, and DNA resuspended in 50 μl TE. Phosphatase treatment completed in 50 μl reaction containing: 5 μl DNA in TE, 5 μl 10X Antarctic phosphatase buffer, 1 μl Antarctic phosphatase enzyme, and 44 μl dH_2O . Reactions incubated at 37 °C for 30 minutes followed by a 15 minute incubation at 65 °C to inactivate the phosphatase enzyme.

To clone the Clb2-222N fragment in the pGBDU vector, 25 μl ligation reaction prepared under following conditions: 4 μl Clb2-222N, 2 μl pGBDU vector, 2.5 μl 10X ligation buffer, 1 μl ligase enzyme, and 15.5 μl dH_2O . Ligation was run overnight at 16 °C.

Preparation of Plasmid DNA: Following transformation in *E. coli* as previously described, plasmids were isolated using the following miniprep protocol: 3 ml culture centrifuged for 15 minutes at 3500 rpm to room temperature and supernatant discarded. Cells resuspended in 1 ml dH_2O to wash, vortexed to resuspend, and centrifuged at 14000 rpm for 5 minutes at room temperature. Supernatant discarded, 100 μl cold GTE buffer added, and cells resuspended by vortexing. 200 μl SDS/NaOH added, mixed by inversion, and allowed to stand at room temperature for 3 minutes allowing SDS to degrade membranes and NaOH to denature genomic DNA. Next, 150 μl 7:3 KOAc (3M K^+ , 5M OAc^-)/phenol added to precipitate and separate genomic DNA. Mixtures were immediately inverted 3-4 times, vortexed for 1 minute, and centrifuged for 5 minutes at 14000 rpm. Supernatant collected, 1 ml 100% ethanol added, and placed on dry ice for 15 minutes to precipitate plasmid DNA. Two ethanol washes performed by adding 500 μl 90% ethanol (v/v), mixed by vortexing, centrifuged for 1 minute at 14000 rpm, and decanted of supernatant. Ethanol was allowed to evaporate and DNA resuspended in 35 μl TE with 20 $\mu\text{g}/\text{ml}$ RNaseA (DNase free) added. Incubated at 37 °C for 30 minutes.

Two-hybrid Screen and Spot Tests: The two-hybrid tester strain harboring pGBDU-Clb2-222N was transformed with each of with the pGAD-C1, pGAD-C2, and pGAD-C3 genomic libraries and the transformation mixes plated on -UL media. The selected transformants were replica plated to -ULA, -ULH, and -ULH + 3-aminotriazole media. Screens with 10 $\mu\text{g}/\text{ml}$ benomyl conducted using SE media. All media contained glucose as a carbon source. Results were collected and are recorded below.

For spot tests, cells were cotransformed as described. Next, cultures were grown overnight, cell densities normalized using OD600nm readings, and cell suspensions of similar densities serially diluted 5 fold. The dilutions were then spotted on media lacking uracil and leucine (-UL), lacking uracil,

leucine, and histidine with 5mM 3-aminotriazole added (-ULH + 3-aminotriazole), and lacking uracil, leucine, and adenine (-ULA).

RESULTS

In preparation for the two-hybrid screen, a bait plasmid expressing a Clb2 fragment devoid of the C-terminal cyclin boxes (Clb2-222N) was constructed in order to avoid identifying proteins interacting with Cdc28. PCR was used to construct the fragment by introducing flanking *EcoR1* and *BamH1* endonuclease sites in-frame with the construct, producing a 660 base pair fragment. Then, each endonuclease was used to digest the PCR fragment as well as the pGBDU vector as described. Following digestion, the fragment was ligated into the pGBDU vector to complete the bait plasmid, and it was transformed into the PJ69-4A two-hybrid tester yeast strain as described. Transformation was confirmed by growth on media lacking uracil. Growth indicated successful transformation and expression of the *URA3* gene carried by the pGBDU vector.

To make sure that the interaction with Cdc28 was eliminated, a two-hybrid screen was set up expressing Clb2-222N as the bait and Cdc28 as the prey with wild-type Clb2 and empty vector as a control. Yeast were transformed as described in the following combinations: (1) pGAD + pGBDU, (2) pGAD-Cdc28 + pGBDU, (3) pGAD + pGBDU-Clb2, (4) pGAD-Cdc28 + pGBDU-Clb2, (5) pGAD + pGBDU-Clb2-222N, and (6) pGAD-Cdc28 + pGBDU-Clb2-222N. To each a five-fold serial dilution was performed, and all were spotted on -UL, -ULH + 3-aminotriazole, and -ULA media. The results are shown in Figure 2. All combinations grew on -UL media, indicating that all transformations successfully obtained both plasmids. Clb2-222N did not demonstrate any interaction with Cdc28 on either -ULH + 3-aminotriazole or -ULA restrictive media, whereas wild-type Clb2 demonstrated strong interaction with Cdc28 on both restrictive media.

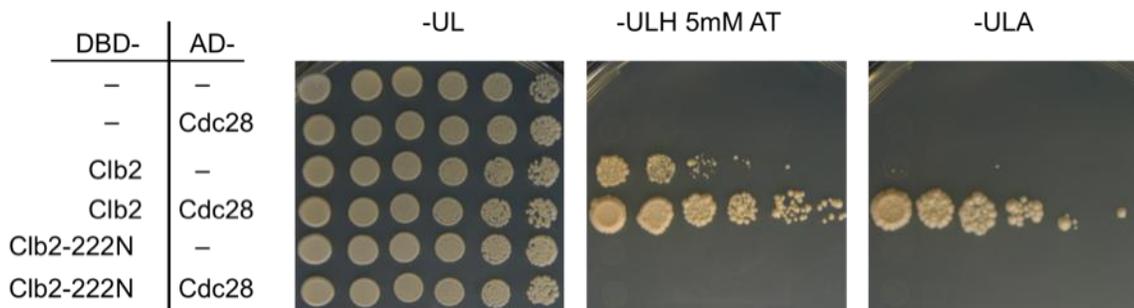


Figure 2. Spot tests from two-hybrid checking interaction with Cdc28. Growth on -UL indicates presence of both plasmids. Growth on medium lacking histidine or adenine indicates that the protein fusions are interacting to cause transcription of the respective reporter gene. No interaction was detected between Clb2-222N and Cdc28, while interaction between Clb2 and Cdc28 still observed.

Armed with our cyclin box free Clb2 fragment, the two-hybrid screen was begun. Yeast cells were transformed as described, expressing Clb2-222N as the bait and the respective pGAD-C1, pGAD-C2, or pGAD-C3 genomic library as the prey. When plated on -UL media, growth of approximately 90,000 colonies was recorded. Replica plating was performed from each -UL plate to -ULH + 3-aminotriazole and -ULA selective media. Six colonies were identified after growth on the restrictive media. Each of the six candidates was treated with fluoroorotic acid (FOA) to evict the bait plasmid

harboring the *URA3* marker, leaving only the prey plasmid. The prey plasmid was isolated and amplified as described and sent for nucleotide sequencing. Sequencing analysis identified Muk1 as an interacting protein. To verify the interaction and see if it occurs with wild-type Clb2, a two-hybrid assay was set up expressing either Clb2-222N or wild-type Clb2 as the bait and Muk1 as the prey with empty vector as a control. Yeast cells were transformed as described in the following combinations: (1) pGAD + pGBDU, (2) pGAD-Muk1 + pGBDU, (3) pGAD + pGBDU-Clb2, (4) pGAD-Muk1 + pGBDU-Clb2, (5) pGAD + pGBDU-Clb2-222N, and (6) pGAD-Muk1 + pGBDU-Clb2-222N. Each was diluted as described and spotted on -UL, -ULH + 3-aminotriazole, and -ULA media. The results are shown in Figure 3. All combinations grew on -UL media, to similar levels. Muk1 demonstrated interaction with both Clb2-222N and wild-type Clb2.

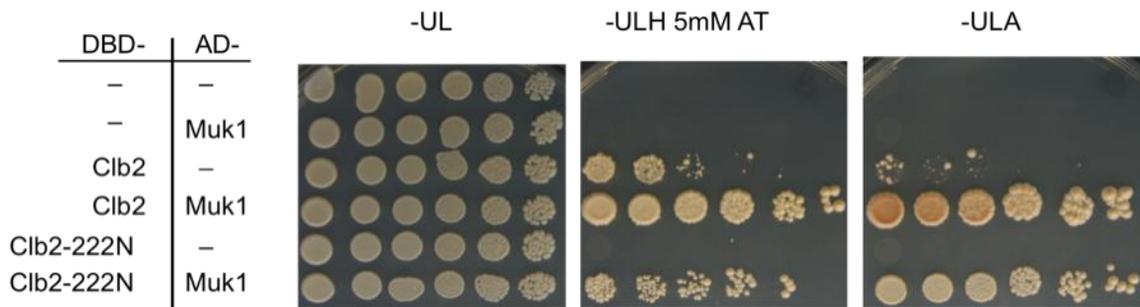


Figure 3. Spot tests from initial two-hybrid screen without benomyl. Growth on -UL indicates both plasmids transformed into cell. Growth on restrictive media indicates that the protein fusions are interacting to cause transcription of the reporter gene.

The two-hybrid screen was repeated with the addition of 10 $\mu\text{g/ml}$ benomyl to all media preparations. Benomyl was added in order to favor the identification of proteins that interact with Clb2 in conditions that activate the spindle assembly checkpoint. Benomyl works by inhibiting microtubule polymerization and thus, preventing formation of the mitotic spindle. This activates the spindle assembly checkpoint, which halts mitotic progression at metaphase to prevent chromosome separation. Clb2, first appearing in G2, reaches maximum levels during M phase, just before its degradation (Grandin 1993), so the addition of benomyl should maintain high levels of Clb2 expression. As before, yeast cells were transformed as described, expressing Clb2-222N as the bait and pGAD-C1, pGAD-C2, or pGAD-C3 genomic library as the prey. When plated on -UL media, approximately 108,000 colonies were identified. Replica plating was performed from each -UL plate to -ULH + 3-aminotriazole and -ULA selective media, both containing benomyl (Figure 4). Twenty-five colonies were identified after growth on the restrictive media. Each of the twenty-five candidates was treated with FOA to evict the bait plasmid, leaving only the prey plasmid. The prey plasmid was then isolated from each as described and sent for sequencing. Sequencing analysis identified Ndc80, Zds2, Cin5, Fob1, and Srs2 as interacting proteins.

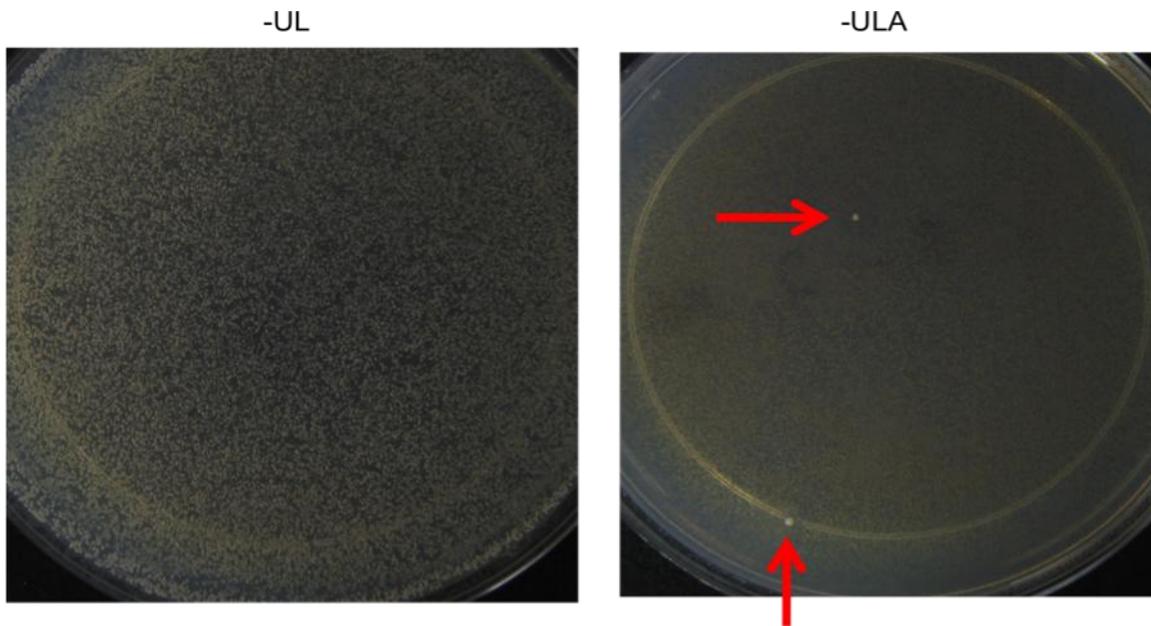


Figure 4. Initial steps of two-hybrid screen with benomyl. (Left) All cells successfully transformed with both plasmids grew on –UL media. Approximately 9000 colonies were present in this plate. (Right) Same plate was replica plated to –ULA restrictive media. Two colonies appeared after three days of growth.

DISCUSSION

Cyclin-dependent kinases (CDKs), CDK-activating kinases (CAKs), and cyclins function in unison to regulate cell cycle progression. In the budding yeast *Saccharomyces cerevisiae*, regulation occurs through the phosphorylation of Cdc28 by Cak1 and the binding of one of the nine cyclins to form Cdc28-cyclin complexes at specific times throughout the cell cycle. Clb2 is the most important mitotic cyclin, but several of its regulators and substrates remain to be identified. The two-hybrid screen was employed to aid in the identification of novel interactions with Clb2. For this reason, the Clb2-222N fragment was constructed devoid of the cyclin boxes, preventing interaction with Cdc28.

Initial screening revealed that Muk1 was an interacting protein (Figure 3). Little is known about Muk1, but it has been identified as a putative substrate of Cdc28 with a possible role in transcriptional regulation (Samanta 2003). Two-hybrid screening with the addition of benomyl was carried out to identify interactions occurring under conditions of mitotic arrest due to activation of the spindle assembly checkpoint. Five proteins were identified from this screen: Ndc80, Zds2, Cin5, Fob1, and Srs2. Ndc80 forms a complex at the spindle pole body that plays a role in chromosome segregation and condensation (Wigge 2001). Zds2 has been identified in several screens as indicated by the nomenclature: zillion different screens. Therefore, it is not surprising to see it show up in this screen, since further, genetic interactions between *zds2* and *cdc28* mutations have been reported. The interaction with Cin5 is believed to be non-specific and most likely related to activation of the reporter gene due to the transcriptional regulation capability of Cin5.

Fob1 is a replication fork blocking protein that acts as a *cis* element for condensin recruitment to the rRNA gene repeats (rDNA) (Johzuka 2009). Transcription of these genes takes place from S phase to anaphase, and condensin is believed to help stabilize these long repeats, preventing improper recombination activity. It is possible that Fob1 is a substrate of Clb2 or the Cdc28-Clb2 complex that is acted on solely when the spindle assembly checkpoint is activated.

The most interesting interactor is Srs2. Studies indicate that Cdc28-cyclin complexes promote DNA double strand break (DSB) repair by homologous recombination during G2 in dividing yeast cells (Wohlbold 2009). Srs2 is one of the proteins that requires phosphorylation by Cdc28 to be active (Saponaro 2010). Srs2 is a DNA helicase related to the UvrD family (Palladino 1992) with the ability to unwind duplexed DNA. Its function in DSB repair is dismantling D-loop structures to ensure accurate homologous recombination (Saponaro 2010). Based on interaction with this protein, it appears that the Cdc28-Clb2 complex functions in DSB repair by promoting accurate homologous recombination using Srs2.

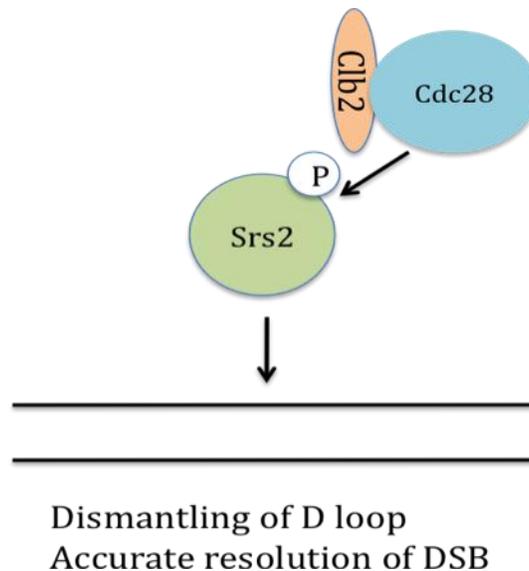


Figure 5. Proposed model of the role of Clb2 in DSB repair. Phosphorylation of Srs2 by Cdc28-Clb2 complex leads to activation of Srs2 helicase. In the presence of active Srs2, D loop can be broken down, leading to the accurate resolution of the DSB.

REFERENCES

- Grandin N, Reed SI (1993) Differential function and expression of *Saccharomyces cerevisiae* B-type cyclins in mitosis and meiosis. *Mol Cell Biol.* 13: 2113-2125.
- James P, Halladay J, Craig EA (1996) Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics.* 144: 1425-1436.
- Johzuka K, Horiuchi T (2009) The *cis* element and factors required for condensin recruitment to chromosomes. *Molecular Cell.* 34: 26-35.
- Mendenhall MD, Hodge AE (1998) Regulation of Cdc28 cyclin-dependent protein kinase activity during the cell cycle of the yeast *Saccharomyces cerevisiae*. *Microbiol Mol Biol Rev.* 62: 1191-1243.

- Palladino F, Klein HL (1992) Analysis of meiotic and mitotic defects *Saccharomyces cerevisiae srs2* DNA helicase mutants. *Genetics*. 132: 23-37.
- Ratushny V, Golemis E (2008) Resolving the network of cell signaling pathways using the evolving yeast two-hybrid system. *Biotechniques*. 44(5): 655-662.
- Samanta MP, Liang S (2003) Predicting protein functions from redundancies in large-scale protein interaction networks. *Proc Natl Acad Sci USA*. 100(22): 12579-12583.
- Saponaro M, *et al* (2010) Cdk1 targets Srs2 to complete synthesis-dependent strand annealing and to promote recombinational repair. *PLoS Genetics*. 6(2): e1000858.
- Wigge PA, Kilmartin JV (2001) The Ndc80p complex from *Saccharomyces cerevisiae* contains conserved centromere components and has a function in chromosome segregation. *J Cell Biol*. 152(2): 349-360.
- Wohlbold L, Fisher RP (2009) Behind the wheel and under the hood: functions of cyclin-dependent kinases in response to DNA damage. *DNA Repair*. 8: 1018-1024.