APC/C Independent Function of the Spindle Assembly Checkpoint

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This work is dedicated to my family

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Abstract

The spindle assembly checkpoint (SAC) maintains genomic stability by delaying cell division until conditions for accurate chromosome segregation are established. The SAC delays anaphase onset by inhibiting APC/C^{Cdc20} activity until all sister kinetochores acquire bipolar microtubule attachment. We found that the SAC is required for the timely accumulation of key M-phase proteins in meiotic *ndt80*Δ *ama1*Δ cells. Specifically, deleting SAC genes or inhibiting the SAC kinase Mps1 dramatically reduces protein levels of *CLB2*-cluster proteins. The phenotype is not due to elevated APC/C activity since it is not rescued in various loss-of-function APC/C mutants. We found that the SAC is required for the activation of transcription of *CLB2*-cluster genes. Deleting SAC genes prevents the transcriptional up-regulation of *CLB2*-cluster genes in M-phase that usually occurs in wild-type strains. We observed that the SAC proteins Mad1 and Mad2 interact with histone H3 and that the interaction depends on the conformation of Mad2. The *mad2-*Δ*C* mutation that locks Mad2 in open conformation reduces Mad2 binding to histones. Moreover, expression of *CLB2*-cluster genes is reduced in the *mad2-*Δ*C* mutant, which suggests that Mad2 needs to bind histones to regulate transcriptional activation. Taken together, our results show that we have uncovered a noncanonical function of the SAC, which is required for the timely expression of key M-phase genes. Therefore, the SAC regulates both the entry and the exit from M-phase via modulating both gene expression and protein degradation of M-phase proteins. The molecular mechanism of transcriptional regulation by the SAC remains to be investigated in more details.

1. Introduction

Understanding cell division is one of the central topics of biological research. Inheritance of complete copies of genomic information by daughter cells is central to the process of cell division. How sister genomes are segregated with high fidelity is also of a great concern for medical research. Defects in genome segregation are hallmarks of human cancer and a major cause of birth defects. Therefore, understanding how genome transmission is regulated could form the basis for therapeutic intervention of human diseases.

1.1. Understanding cell division

The cell cycle is the series of events that take place in a dividing cell leading to duplication and segregation of the genome and cell division, which give rise to two genetically identical daughter cells. The cell cycle has four discrete stages that are G1, S, G2, and M phase (or mitosis). The cell cycle starts with the G1 phase when cells make a decision whether to divide or not, depending on nutrients, temperature, and other environmental cues. If cells are committed to division, they will synthesize nucleic acids and proteins required for DNA replication. After all conditions are met, cells enter S phase synthesizing DNA and duplicating chromosomes. Chromosomes are not separated immediately after duplication, but they are held together by a large protein complex, known as cohesin, for an extended period of time until conditions for chromosome segregation are met (Nasmyth, 2002). The S phase is usually followed by a long gap, called G2, when cells continue to grow and synthesize proteins to prepare for mitosis. Afterwards, cells enter M phase and undergo a dramatic and coordinated change in cellular architecture to segregate sister chromatids. M phase can be further separated into prophase, metaphase, and anaphase. In prophase, chromosomes condense and a multisubunit protein complex known as the kinetochore is assembled around the centromere region of chromosomes.

The kinetochore is the platform that mediates microtubule attachment in metaphase and a signal-processing center that couples microtubule attachment to anaphase onset. In metaphase, microtubules growing from microtubule organizing centers (centrosome in animal cells and spindle pole body in yeast cells) attach and pull sister kinetochores towards opposite spindle poles. When all sister chromatids have acquired bipolar attachment, the cohesin complexes are destroyed and sister chromatids are segregated towards opposite poles, which allows two daughter cells to inherit exactly the same copy of the genetic information. This marks the beginning of anaphase. After chromosome segregation, cells undergo cytokinesis to physically divide the cytoplasm into two daughter cells.

1.2. The cell cycle is driven by the cyclin-dependent kinase 1 (Cdk1) and the anaphase-promoting complex/cyclosome (APC/C)

It is generally accepted that the cell cycle oscillation is driven by the negative-feedback loop of Cdk1-APC/C: Cdk1 activates APC/C, which leads to cyclin destruction and Cdk1 inactivation (King et al., 1996; Novak et al., 2007; Rahi et al., 2016; Yang and Ferrell, 2013). While high kinase activity is required for DNA replication and chromosome segregation, low kinase activity is required for exit from mitosis and maintenance of the G1 phase. Therefore, the cell cycle could be viewed as a series of transitions between high and low kinase states. Cdk1 controls diverse cell cycle events by phosphorylating a variety of protein substrates. Cdk1 becomes active only when it is bound by a cyclin activator. Therefore, Cdk1 activity is mainly regulated by the availability of different cyclins (Andrews and Measday, 1998; Morgan, 1997). For example, in *Saccharomyces cerevisiae*, there is only one Cdk homologous to Cdk1 in animals and Cdc2 in *S. pombe*, which is Cdc28 (Surana et al., 1991). When bound with S-phase cyclins Clb5 and Clb6 (Epstein and Cross, 1992), Cdc28 triggers DNA replication, and when bound with M-phase cyclins Clb1, Clb2, Clb3, and Clb4, Cdc28 triggers entry into metaphase and activation of APC/C^{Cdc20} (Rudner and Murray, 2000;

Surana et al., 1991), which leads to chromosome segregation. APC/C^{Cdc20} lowers Cdk1 activity by initiating the destruction of cyclins. Cyclins degradation is completed by activation of APC/C^{Cdh1} (Peters, 2006; Zachariae and Nasmyth, 1999). Low Cdk1 activity also activates the Cdk1 inhibitor Sic1, which further inhibits Cdk1 activity. The protein phosphatase Cdc14 plays an important role in the mitotic exit at least in budding yeast by reversing Cdk1 phosphorylation and activating Sic1 and Cdh1 (Queralt and Uhlmann, 2008; Visintin et al., 1998).

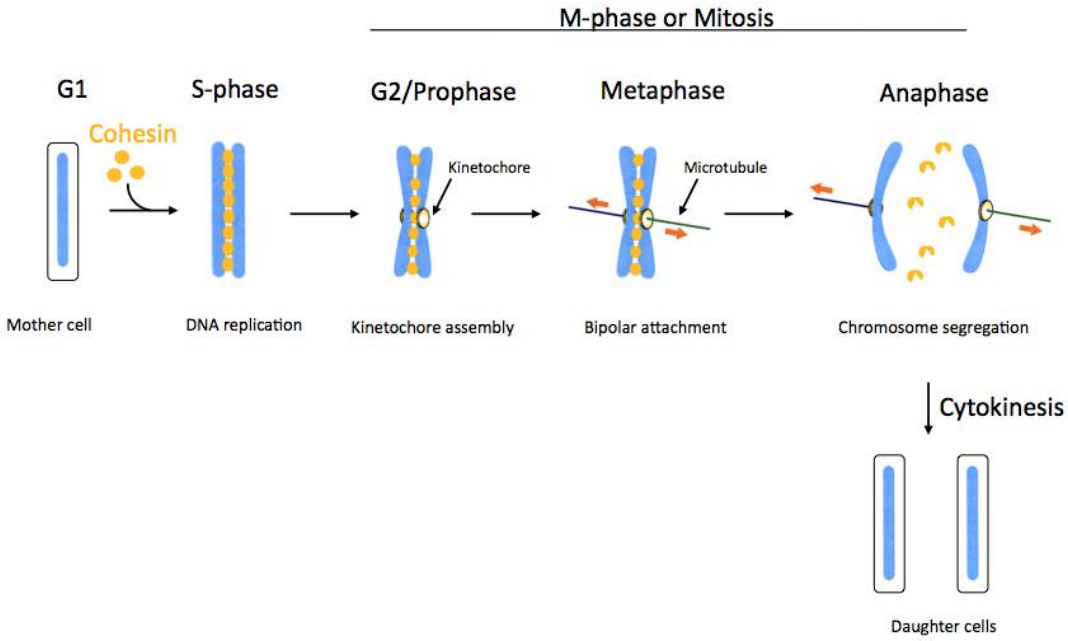

Figure 1. Overview of key events of chromosome segregation in the cell cycle. When the decision to proliferate is made, cells start to grow and prepare for DNA replication by synthesizing proteins during G1. Cells replicate DNA and duplicate all chromosomes during S phase. Sister chromatids are held together by the cohesin complex to prevent premature separation. During G2/prophase, kinetochore complexes are assembled around the centromere region on each sister chromatid, which later in metaphase mediate microtubule attachment. When all sister chromatids are attached to microtubules from opposite spindle poles, cohesin complexes are cleaved to allow chromosomes segregation in anaphase. Finally, cells physically divide in a process called cytokinesis, which produces two daughter cells with identical chromosome set.

1.3. The *CLB2*-cluster genes

Almost two decades ago, the sequencing of the *S. cerevisiae* genome and the invention of DNA microarray technology have enabled the genome-wide identification of cell-cycle regulated genes (Cho et al., 1998; Spellman et al., 1998). Particularly, Spellman et al., identified about 800 transcripts showing expression periodicity within the cell cycle, by combining three different synchronization methods (alpha factor arrest, elutriation and a *cdc15* temperature-sensitive mutant) with mathematical algorithms. They classified these fluctuating genes by phasing and clustering. Phasing refers to classifying genes according to when they reach peak expression with respect to cell cycle stages (G1, S, G2, M, and M/G1 phases). For example, G1 cyclin *CLN2* and M cyclin *CLB2* reach peak expression in G1 phase and M phase, respectively. This method is useful to correlate the timing of expression of a gene to its cellular function. Another way to group genes is using a clustering algorithm to identify co-regulated genes. Common promoter elements can be identified by analyzing the 5' regions of genes in the same cluster, which provides an important mechanism of how these genes can be regulated transcriptionally. In this way the *CLB2*-cluster was discovered. The *CLB2*-cluster contains 35 genes that peak in M phase and show strong co-regulation. The transcription of *CLB2*-cluster genes can be strongly induced by *CLB2* overexpression from the *GAL* promoter. Most *CLB2*-cluster genes encode important mitotic proteins. Clb2 and Clb1 are B-type cyclins that activate Cdk1 activity promoting entry into M-phase. Cdc20 is an activator of APC/C that triggers the metaphase to anaphase transition. The polo-like kinase Cdc5 has multiple roles in regulating mitotic progression, ranging from mitotic entry, to cohesion cleavage and mitotic exit. Swi5 and Ace2 are two closely related transcription factors that activate expression of genes in M/G1 and G1 phases.

Gene name	Main function in the cell cycle	APC/C substrate
CLB2	CDK regulatory subunit, G2/M phase transition	Yes
CLB1	CDK regulatory subunit, G2/M phase transition	Yes
CDC20	APC/C activator, anaphase transition	Yes
CDC5	Polo-like kinase, exit from mitosis	Yes
SWI5	Transcription factor of genes expressed at the M/G1 phase boundary and in G1 phase	No
ACE2	Transcription factor of genes expressed at the M/G1phase boundary and in G1 phase	No
ASE1	Microtubule-associated protein, spindle elongation and stabilization	Yes
SPO12	Mitotic exit, activation of Cdc14 phosphatase	Yes
BUD4	GTP-binding protein involved in septin ring organization and axial bud site selection	No
HOF1	Mitotic cytokinesis	No
RAX2	Maintenance of bud site selection during budding	No

Table 1. A list of representative *CLB2*-cluster genes, their essential functions and whether or not they are APC/C substrates.

1.4. Transcriptional activation of *CLB2*-cluster genes at G2/M

Analysis of the promoter sequences reveals that most of *CLB2*-cluster genes have a consensus binding motif for the transcription factors Mcm1 and Fkh2 (Althoefer et al., 1995; Lydall et al., 1991; Maher et al., 1995). The MADS box transcriptional factor Mcm1 is required for the cell cycle-regulated periodicity of these genes and forms a ternary complex with Fkh2 on the upstream activating sequence (UAS) of *CLB2*-cluster genes. It was also shown that Fkh2 assembles into a ternary complex with Mcm1 and was required for the transcriptional periodicity (Kumar et al., 2000; Pic et al., 2000). Intriguingly, the Mcm1-Fkh2 complex occupies the promoter region of *CLB2*-cluster genes throughout the cell cycle indicating that its binding to the promoter cannot explain the periodic expression of these genes. Therefore, an additional transcriptional activator should be involved and its own activity is subject to cell cycle regulation. Ndd1 was shown to be such a transcriptional activator for *CLB2*-cluster genes. *NDD1* gene was discovered as a high-dosage suppressor of the *cdc28-1N* mutation, which progresses through G1 and S phases normally but fails to undergo nuclear division (Loy et al., 1999). As an essential gene, *NDD1* is required for the normal expression of *CLB2*, *CLB1*, and *SWI5* (Loy et al., 1999). Although Ndd1 has no DNA binding activity, it can associate with the promoter region of *CLB2*-cluster genes in a Mcm1- and Fkh2-dependent manner, as determined by chromatin immunoprecipitation (Koranda et al., 2000). Both mRNA and protein levels of *NDD1* are strictly regulated and fluctuate within the cell cycle. The transcriptional factor Hcm1 activates *NDD1* transcription during S phase (Pramila et al., 2006). Ndd1 is an unstable protein, targeted for degradation by APC/C^{Cdh1} and SCF in mitosis (Edenberg et al., 2015; Sajman et al., 2015) and by APC/C^{Ama1} in meiosis (Okaz et al., 2012). Ndd1 is also regulated by post-translational modification. Ndd1 is phosphorylated by Cdk1-Clb in a cell cycle-dependent manner and the phosphorylation triggers interaction of Ndd1 with Fkh2 through its FHA domain (Darieva et al., 2003; Reynolds et al., 2003). Either mutating the Cdk1 phosphorylation sites or the FHA domain abolishes the cell cycle-regulated

transcription of *CLB2*-cluster genes. Additionally, Ndd1 can be phosphorylated by Cdc5 and protein kinase C (Darieva et al., 2006; Darieva et al., 2012). Therefore, the phosphorylation-dependent interaction of Ndd1 and Fkh2 is thought to be the main mechanism regulating G2/M transcription of *CLB2*-cluster genes.

1.5. Transcriptional repression of *CLB2*-cluster genes at G1, S and M/G1

As discussed previously, the molecular mechanism of the positive regulation of *CLB2*-cluster genes in M phase has been studied extensively. However, less is known about how exactly the transcription of these genes is repressed in G1, S, and M/G1. A hint was provided by Zhu et al. who assayed *SWI5* transcript levels in mitotic time course in both wild-type and *fkh2*Δ *fkh1*Δ double deletion strains (Zhu et al., 2000). They found that *SWI5* mRNA transcripts were undetectable in G1 and S phase, increased markedly in G2/M phase, and then dropped in M/G1 phase in wild-type cells. In *fkh2*Δ *fkh1*Δ double deletion cells, however, the *SWI5* transcripts remained at basal levels throughout the cell cycle. Interestingly, the *SWI5* mRNA levels at G1 and S phase were higher in *fkh2*Δ *fkh1*Δ cells than in wild-type cells. Therefore, this indicates that Fkh2 and its redundant paralog Fkh1 have a negative effect on *SWI5* transcription. It seems they repress expression of *SWI5* gene at G1 and S phases. Another hint is that the inviability of *ndd1*Δ mutants can be rescued by deletion of *FKH2*, which implies that the sole essential function of Ndd1 is to antagonize the repressive activity of Fkh2. Recently, it was proposed that Fkh2 acts as a scaffolding factor and dictates the transcriptional timing of *CLB2*-cluster genes by recruiting transcriptional activators and repressors. It was shown that Fkh2 recruits the chromatin remodeling factor Isw2 (Sherriff et al., 2007), the histone deacetylase complex Sin3-Rpd3 (Veis et al., 2007), and the histone deacetylase Sir2 (Linke et al., 2013) to *CLB2*-cluster gene promoters to repress transcription. Additionally, *CLB2*-cluster genes are repressed at G1, S, and M/G1 by modulating Ndd1 expression and activity. Firstly, *NDD1* is an S phase gene (Pramila et al., 2006). Secondly, Ndd1 protein is not active until Cdk1-Clb activity rises at G2/M

(Darieva et al., 2003; Reynolds et al., 2003). Thirdly, Ndd1 is an unstable protein. It is targeted for degradation by APC/C^{Cdh1} at G1 (Sajman et al., 2015) and by SCF^{Grr1} at M phase (Edenberg et al., 2015). Lastly, Rad53 restricts the activity of Ndd1 in response to DNA damage (Edenberg et al., 2014; Yelamanchi et al., 2014).

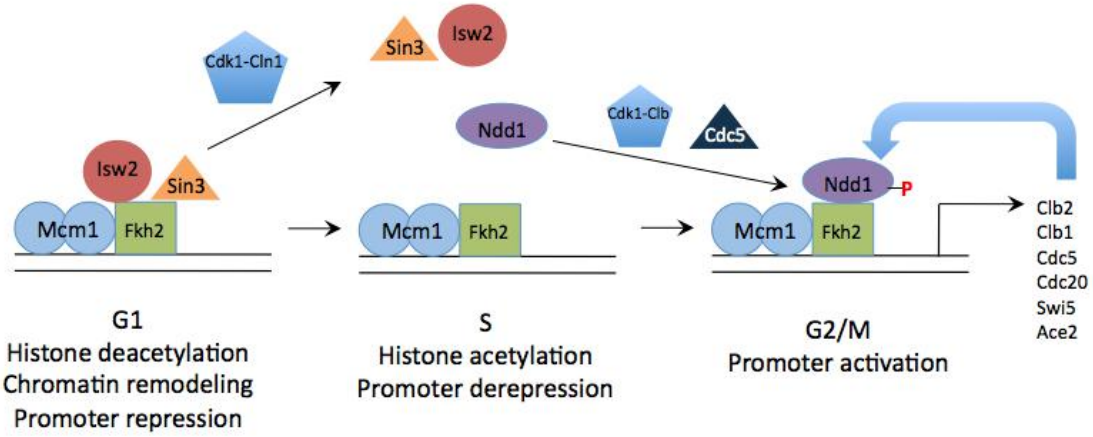

Figure 2. Regulation of *CLB2*-cluster gene expression. The transcription factors Mcm1 and Fkh2 bind to the promoters of *CLB2*-cluster genes cooperatively throughout the cell cycle. During G1, Fkh2 recruits the histone deacetylase Sin3 and the chromatin remodeler Isw2 to promoters of *CLB2*-cluster genes, which makes the chromatin environment transcriptionally repressive. During S phase, rising Cdk1 activity releases Sin3 and Isw2 from the chromatin, and the transcription activator Ndd1 starts to be expressed. When phosphorylated by Clb-Cdk1, Ndd1 interacts with Fkh2 and activates transcription of *CLB2*-cluster genes including *CLB2*, *CLB1*, and *CDC5*, which further increase phosphorylation of Ndd1 and thereby augment their own expression.

1.6. The spindle assembly checkpoint

The major goal of the mitotic cell cycle is to distribute duplicated chromosomes equally to daughter cells so that each cell inherits exactly the same copy of the genetic information. Missegregation of chromosomes generates aneuploidy, a major cause of birth defects and miscarriages and a hallmark of human cancers (Fang and Zhang, 2011). Eukaryotic cells have developed two strategies to protect the fidelity of chromosome segregation (Nasmyth, 2002). The first tool is sister chromatids cohesion. Instead of being separated immediately after DNA replication, sister chromatids are held together by the cohesin complex until all sister kinetochores are attached to microtubules from opposite spindle poles, which is also called bipolar attachment. Since the microtubule-kinetochore attachment is not always correct, a second tool is in place to monitor the attachment status. The spindle assembly checkpoint (SAC) monitors the microtubule-kinetochore attachments and delays chromosome segregation in the presence of erroneous attachments earning enough time for Ipl1/Aurora kinase-dependent error correction (Foley and Kapoor, 2013; Musacchio, 2015). The essential SAC components were initially identified in budding yeast *S. cerevisiae* including Mad1, Mad2, Mad3 (BubR1), Bub1, Bub3, Mps1, Ipl1/Aurora B, and Glc7/PP1.

Proteins	Essential features	Main role in the SAC	Binding partners
Aurara B /Ipl1	S/T protein kinase	Recruitment of Mps1, inhibition of recruitmen of PP1	Other CPC subunits
Bub1	S/T protein kinase	Kinetochore recruitment of BubR1:Bub3 and Cdc20	Bub3, Cdc20, P-MELT
Mad3/ BubR1	Pseudokinase	Component of MCC	Bub3, Mad2, Cdc20, Bub1:Bub3 complex
Bub3	Phosphoaminoacid adaptor	Component of MCC	Bub1R1, Bub3, P-MELT
Cdc20	β-propeller, adaptor for degrons	APC/C activator, component of MCC	APC/C, BubR1, Mad2, Bub1, Cyclin B, Securin
Mad1	Coiled-coil rich	Component of Mad1:C-Mad2 complex	Mad2
Mad2	HORMA domain	Component of Mad1:C-Mad2 complex	Mad1 and Cdc20
Mps1	S/T protein kinase	Phosphorylation of MELT repeats of Knl1	Ndc80 for kinetochore recruitment
$p31^{comet}$	HORMA domain	Dissociation of MCC by binding to C-Mad2, capping of Mad1:C-Mad2 template	C-Mad2, Trip13
PP1/Glc7	S/T phosphatase	SAC silencing, counteracting Mps1 and Aurora B	Knl1

Table 2. List of SAC proteins and their essential functions (adapted from Musacchio, 2015).

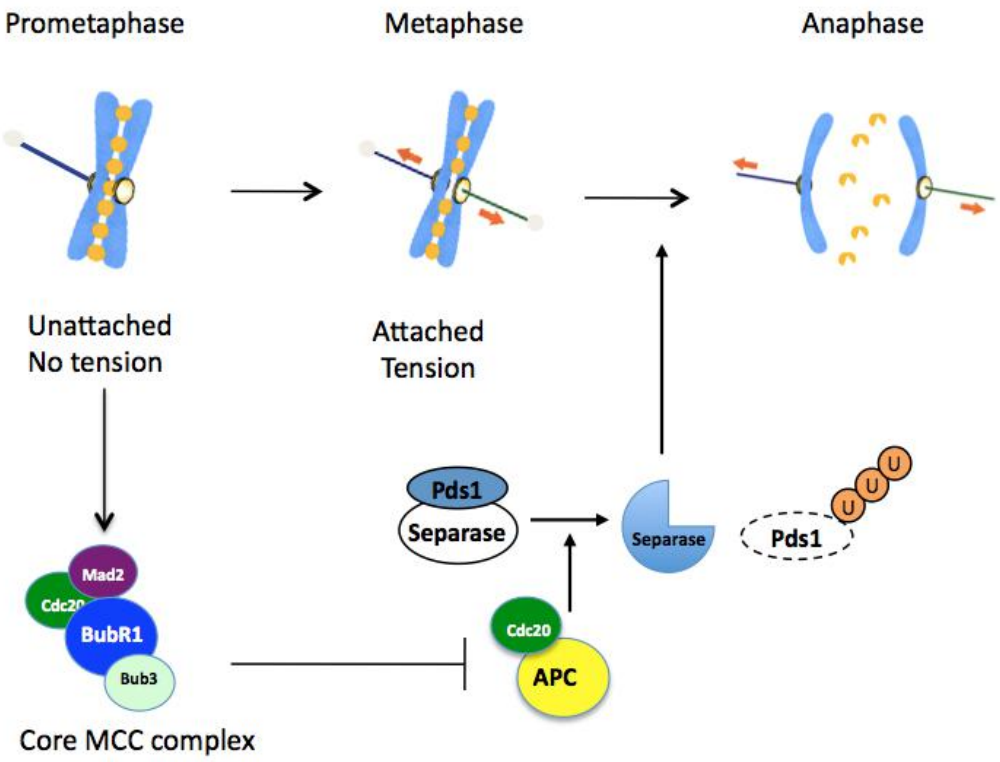

Figure 3. How the SAC couples chromosome segregation with kinetochore-microtubule attachment. Chromosome segregation in anaphase is triggered by activation of APC/C^{Cdc20}. APC/C^{Cdc20} targets securin/Pds1 for degradation, which allows separase-dependent cleavage of cohesin complexes and separation of sister chromatids. However, microtubule attachment is an error prone process. The presence of unattached kinetochores activates the SAC by triggering assembly of the mitotic checkpoint complex (MCC), which inhibits APC/C activity and chromosome segregation until all kinetochores are attached to microtubule.

1.7. The mitotic checkpoint complex (MCC)

The initiation of anaphase is triggered by the E3 ubiquitin ligase APC/C, which targets cyclins and securin/Pds1 for proteasome-dependent degradation (Cohen-Fix et al., 1996; Irniger et al., 1995; King et al., 1995; Sudakin et al., 1995; Zachariae et al., 1998; Zachariae et al., 1996). The role of the SAC is to inhibit APC/C activity as long as incorrectly attached chromosomes persist. To do this, a diffusible inhibitor of APC/C is assembled on the unattached kinetochores, which is known as the mitotic checkpoint complex or the MCC (Musacchio, 2015). The MCC is a heterotetramer of Cdc20, Mad2, Mad3/BubR1, and Bub3 (Fraschini et al., 2001; Hardwick et al., 2000; Sudakin et al., 2001). Mad3/BubR1 has an N-terminal KEN box, a motif that is normally recognized by APC/C^{Cdc20} as a degron. Acting as a pseudo-substrate inhibitor, however, the KEN box of Mad3/BubR1 competes with other substrates for Cdc20 binding (Burton and Solomon, 2007; Sczaniecka et al., 2008). Structural analysis of the MCC complex revealed that the MCC obstructs substrate recognition by Cdc20 and displaces Cdc20 away from the APC/C subunit Apc10 to disrupt the formation of a substrate-recognition site (Chao et al., 2012). Mad2 stabilizes the complex by optimally positioning the KEN box of Mad3/BubR1 to bind Cdc20, while P31^{comet} competes with Mad3/BubR1 for Mad2 binding, thereby antagonizing the MCC formation (Chao et al., 2012). Mad2 also directly interacts with and inhibits Cdc20. Mad2 binds to Cdc20 at a site that also binds APC/C. Therefore, Mad2 competes with APC/C for Cdc20 binding. Accordingly, a Cdc20 mutant that cannot bind to Mad2 abrogates SAC signaling (Hwang et al., 1998). Consistent with the notion that Mad2 directly inhibits Cdc20, artificially tethering Mad2 to Cdc20 arrests budding yeast cells in metaphase independently of other checkpoint proteins (Lau and Murray, 2012). Additionally, APC/C- and MCC-dependent ubiquitination drives the high turnover of Cdc20 during SAC activation, which is required for the maintenance and high responsiveness of the SAC (Foster and Morgan, 2012; Ge et al., 2009; Mansfeld et al., 2011; Nilsson et al., 2008; Pan and Chen, 2004).

1.8. MCC assembly at unattached kinetochores

The presence of unattached kinetochores leads to a hierarchical recruitment of SAC proteins to the kinetochore, which assemble a catalysis platform generating APC/C^{Cdc20} inhibitors (Foley and Kapoor, 2013). The upstream event in the signaling cascade is the recruitment of Mps1, resulting in phosphorylation of the kinetochore protein Spc105/Knl1 on its conserved MELT repeats, which serve as kinetochore receptors for Bub1 and Bub3 (London et al., 2012; Primorac et al., 2013; Shepperd et al., 2012; Yamagishi et al., 2012). The kinetochore-localized Bub1-Bub3 complexes, in turn, recruit Mad2/Mad1 (Moyle et al., 2014). Additionally, Mps1 can also phosphorylate Bub1, which is required for the Bub1-Mad1 interaction (London and Biggins, 2014).

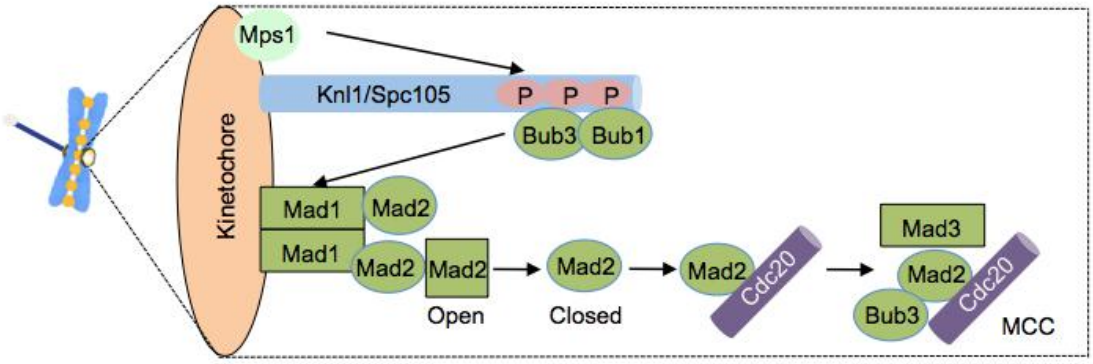

Figure 4. SAC activation at the kinetochore. The SAC signaling is established and amplified through the hierarchical recruitment of various SAC proteins. Unattached kinetochores recruit the SAC kinase Mps1, which phosphorylates the kinetochore protein Knl1 (Spc105 in budding yeast) on the conserved MELT domains. Multiple Phospho-MELT repeats further recruit SAC proteins Bub3 and Bub1 to the kinetochore. Recruitment of Mad1-Mad2 dimers depends on Bub1 in budding yeast and *C. elegans*. Kinetochore-bound Mad1-C-Mad2 dimers catalyze the switch of O-Mad2 to C-Mad2. As the active form of Mad2 protein, C-Mad2 binds Cdc20 and triggers the formation of the MCC complexes, which finally inhibit APC/C activity (Figure adapted from Foley and Kapoor, 2013).

1.9. Conformational switch of Mad2, and the template model

The most striking event of downstream SAC signaling is the conformational switch of the Mad2 protein. The Mad2 protein adopts two distinct conformational states (Luo et al., 2002; Luo et al., 2004). When unbound, Mad2 forms an open conformation (O-Mad2) and when bound with Mad1 or Cdc20, it forms the active closed conformation (C-Mad2). Only C-Mad2 can bind and inhibit Cdc20. Most Mad2 molecules adopt an open conformation. The conformational change of Mad2 entails large activation energy and is therefore extremely slow to the point of being rate-limiting for SAC activation (Simonetta et al., 2009). The question is how unattached kinetochore allows a fast conformational switch of Mad2. The template model is proposed to solve this problem. Mad1 recruits C-Mad2 to kinetochores lacking microtubule attachment. Acting as a template, kinetochore Mad1:C-Mad2 binds cytosolic free O-Mad2, and catalyzes the conformational switch of O-Mad2 to C-Mad2 (De Antoni et al., 2005; Simonetta et al., 2009). Indeed, artificial targeting of Mad1 to kinetochore can recruit Mad2 to the kinetochores and sustain a robust SAC response in cells with normal microtubule attachments (Kuijt et al., 2014). Additionally, FRAP experiments identified two distinctive pools of Mad2 on the kinetochores. One pool of Mad2 stably associates with the kinetochores (Mad1:C-Mad2) while the other pool constantly recycles on the kinetochores (cytosolic O-Mad2 converted to C-Mad2) (Howell et al., 2004; Shah et al., 2004).

1.10. SAC silencing

When all sister chromatids have acquired bipolar attachment, the SAC has to be completely silenced to allow activation of APC/C^{Cdc20} and chromosome segregation. As discussed earlier, the Mps1 kinase plays a pivotal role in SAC signaling via phosphorylation-dependent recruitment of SAC proteins to the kinetochore. Therefore, Mps1 activity has to be down-regulated to silence the SAC. Ji et al., and Hiruma et al., showed that the Ndc80 complex is a kinetochore receptor of Mps1, and microtubules compete with Mps1 for Ndc80 binding

(Hiruma et al., 2015; Ji et al., 2015). Therefore, the microtubule-kinetochore interaction weakens Mps1 activity. An alternative mechanism of Mps1 silencing is provided by Aravamudhan et al., who proposed that end-on microtubule attachments to the kinetochores physically separate Mps1 from its kinetochore substrate Spc105/Knl1, hereby weakening downstream SAC signaling (Aravamudhan et al., 2015). Glc7/PP1 plays a significant role in SAC silencing (Pinsky et al., 2009; Rosenberg et al., 2011; Vanoosthuyse and Hardwick, 2009), presumably by counteracting Mps1-dependent phosphorylation of Spc105/Knl1 (London et al., 2012). Proteins that already localize on the kinetochores when microtubule attachment occurs have to be removed to prevent further generation of the MCC complexes. In higher eukaryotes, the microtubule motor protein dynein is responsible for stripping SAC proteins Mad1, Mad2, and Mps1 from the kinetochores (Howell et al., 2001). MCC has to be disassembled to re-activate APC/C^{Cdc20}. It was shown that checkpoint inactivation is an energy consuming event involving APC/C dependent multi-ubiquitination of Cdc20, which leads to disassembly of the MCC complex in human cells (Foster and Morgan, 2012; Reddy et al., 2007; Uzunova et al., 2012). Additionally, $p31^{comet}$ antagonizes the ability of Mad2 to inhibit APC/C^{Cdc20} by structurally mimicking O-Mad2 and blocking Mad1 assisted Mad2 conformational switch (Xia et al., 2004) (Yang et al., 2007). In addition to blocking Cdc20-C-Mad2 interaction, $p31^{comet}$ also disassociates Mad2 from MCC in an ATP-dependent manner (Eytan et al., 2014; Teichner et al., 2011; Westhorpe et al., 2011). It has been shown that Cdk1-Clb activity is required for SAC function (Kamenz and Hauf, 2014; Rattani et al., 2014; Vazquez-Novelle et al., 2014). Therefore, the drop of Cdk1-Clb activity during anaphase silences the SAC.

1.11. Functions of SAC proteins beyond APC/C^{Cdc20} inhibition

1) Regulation of nuclear transport by the SAC protein Mad1

Nuclear pore complexes (NPC) provide selective barriers for the trafficking of macromolecules between the nucleus and the cytoplasm (Wente and Rout,

2010). It is thought that SAC proteins only localize to kinetochore during SAC activation. However, the SAC proteins Mad1 and Mad2 were shown to associate with NPCs during interphase (Campbell et al., 2001; Iouk et al., 2002). Additionally, NPCs in interphase cells function as scaffolds for generating APC/C inhibiting Mad1/Mad2 complexes (Lee et al., 2008; Rodriguez-Bravo et al., 2014). Therefore, both kinetochores and NPCs emit “wait anaphase” signals that preserve genomic integrity. Interestingly, the SAC protein Mad1 can also regulate nuclear transport (Cairo et al., 2013b). Cairo et al. showed that kinetochore-microtubule detachment arrests nuclear import mediated by the transport factor Kap121 through a mechanism that requires Mad1 cycling between unattched metaphase kinetochores and binding sites at the NPC (Cairo et al., 2013a).

2) Cross-talk between the SAC and the DNA Damage Response (DDR)

DNA damage and chromosome missegragation pose continuous threats to genomic integrity. In the eukaryotic cell cycle, genotoxic insults and chromosome-microtubule attachment errors lead to activation of the DDR and the SAC, respectively, which prevent cell-cycle progression. The two checkpoints are thought to function independently. However, accumulating evidence suggests that there are cross-talks between the SAC and the DDR. It was shown that the important DDR regulators Chk1 kinase and the Fanconi anemia proteins are required for optimal SAC signalling (Eliezer et al., 2014; Nalepa et al., 2013; Zachos et al., 2007). DNA damaging agents even induce SAC activation in a ATM and ATR kinases-dependent machanism (Kim and Burke, 2008). SAC proteins are also required for proper DDR. A single double strand break (DSB) not only trigers the DDR but also the SAC signalling, and Mad2 is required for the prolonged arrest induced by DSB (Dotiwala et al., 2010). In mammalian oocytes, DNA damage also induces meiotic arrest by activating the SAC (Collins et al., 2015; Marangos et al., 2015). In summary, the DDR and the SAC function in synergy to protect genomic integrity.

1.12. Meiosis and the entry into meiosis I

Sexually reproducing organisms undergo meiosis to produce haploid gametes, which include sperms and eggs in multi-cellular organisms and spores in the unicellular yeast. In higher eukaryotes, meiosis is triggered by a hormonal signal (Bowles and Koopman, 2010). In yeast, however, meiosis is triggered in the absence of a fermentable carbon source and nitrogen (van Werven and Amon, 2011). Meiosis begins with one round of DNA replication, thus producing two sister chromatids for the maternal and two sister chromatids for the paternal copy of each chromosome. These four chromatids are then distributed into four different nuclei through two consecutive nuclear divisions, called meiosis I and meiosis II (Figure 5). Homologous maternal and paternal chromosomes must segregate in opposite directions at meiosis I. Sister chromatids are then segregated at meiosis II, which leads to the formation of haploid nuclei (Petronczki et al., 2003). The initiation of DNA replication in meiosis requires not only Cdk1 but also the meiosis-specific kinase Ime2 (Dirick et al., 1998). Ime2 activity leads to the degradation of Cdk1-inhibitor Sic1, which allows Cdk1 bound with Clb5 and Clb6 to activate DNA replication (Benjamin et al., 2003). At the end of the meiotic S-phase, sister chromatids are held together by cohesin. The meiotic form of the complex contains the Rec8 subunit, instead of the mitotic subunit Scc1.

Although entry into M phase is universally driven by Cdk1 bound to cyclin B (Cdk1-Clb), the timing of this event differs dramatically in mitosis and meiosis. Mitotic cells activate Cdk1-Clb shortly after S phase, whereas meiotic cells activate Cdk1-Clb after a long prophase during which homologous chromosomes undergo recombination. Mitotic and meiotic M phases in yeast are initiated by two different transcription factors. Mitotic cells enter M phase by activating the transcriptional factor Ndd1 (Loy et al., 1999), whereas meiotic cells enter M phase by activating Ndt80, a meiosis-specific transcription factor that triggers spindle formation by promoting the accumulation of M-phase cyclins Clb1 and Clb4 and the polo kinase Cdc5 (Chu and Herskowitz, 1998; Sourirajan and

Lichten, 2008). Abrupt accumulation of Ndt80 at exit from prophase I is regulated by two positive feedback loops; the *NDT80* gene is activated by itself and is repressed by Sum1, which is inhibited, in turn, by the Cdk1 activity that results from Ndt80's appearance (Pak and Segall, 2002; Shin et al., 2010). In the presence of double strand breaks, however, accumulation of Ndt80 is blocked by the meiotic recombination checkpoint (RC) (Tung et al., 2000). Since mitotic and meiotic factors can both trigger entry into M phase, how activation of mitotic factor Ndd1 is prevented in meiosis has been elusive. Recently, Okaz et al. showed that APC/C^{Ama1} suppresses mitotic cell-cycle controls during prophase I by triggering the degradation of Ndd1, M phase cyclins, and Cdc5 (Okaz et al., 2012). This is essential for an extended prophase I that is controlled by the RC and for proper homolog segregation at meiosis I. Mathematical modeling in that study also supports a crucial role for APC/C^{Ama1} in the irreversible transition from prophase I to metaphase I.

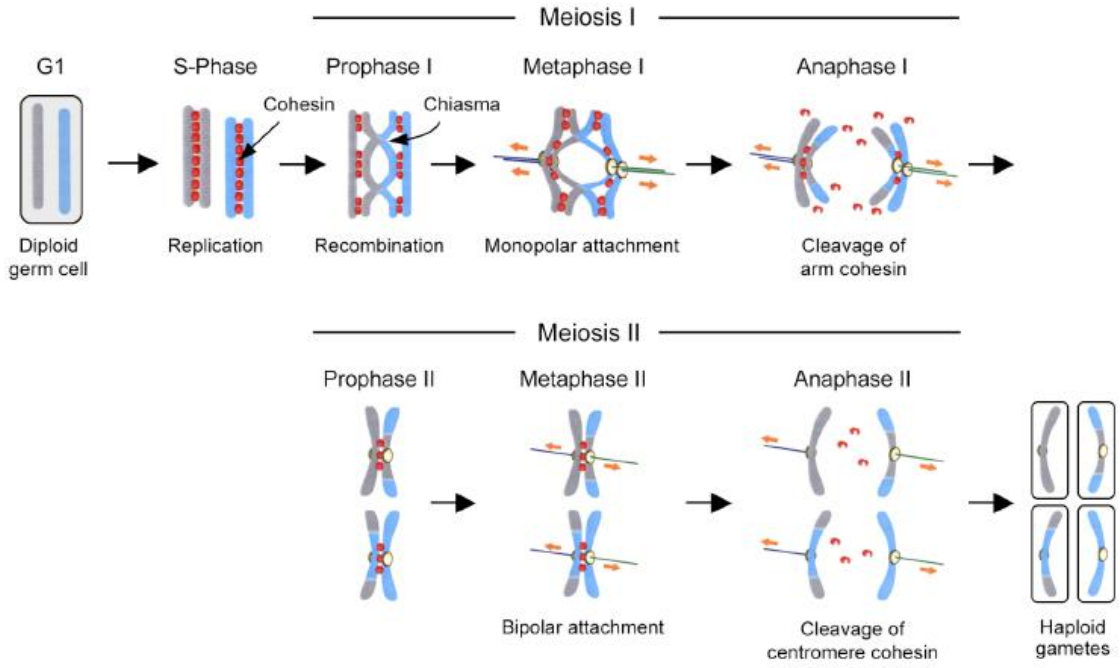

Figure 5. Chromosome segregation in meiosis

During pre-meiotic DNA replication, cohesin containing the meiosis-specific Rec8 subunit is loaded on chromatin, which is followed by the segregation of homologous chromosomes in meiosis I and the disjunction of sister chromatids in meiosis II. In prophase I, reciprocal recombination between homologous non-sister chromatids creates crossovers (also called chiasmata), which link maternal and paternal chromosomes through sister chromatid cohesion on chromosomal arms. In metaphase I, monopolar attachment of sister kintetochores ensures that maternal and paternal centromeres are pulled to opposite spindle poles. Cleavage of Rec8 on chromosome arms at the onset of anaphase I triggers segregation of homologs. Cohesion around centromeres is protected from cleavage in meiosis I, which is required for bipolar attachment of sister kinetochores in metaphase II. Cleavage of centromeric cohesion finally triggers segregation of sister chormatids and the generation of haploid gametes (Okaz, 2010).

1.13. Aim of this study

Spindle formation and accumulation of M-phase proteins in meiotic *ndt80*Δ *ama1*Δ cells are triggered by the mitotic transcriptional activator Ndd1 (Okaz et al., 2012). Moreover, these cells accumulate B-type cyclins Clb4, Clb1, and Clb2 in two waves, which resembles mitotic cells (Fitch et al., 1992; Richardson et al., 1992). Interestingly, however, although meiotic *ndt80*Δ *ama1*Δ cells enter a high-kinase state, these cells fail to disassemble spindles, to degrade Pds1 and M-phase proteins and to undergo nuclear divisions (Okaz et al., 2012). Since the high-kinase state normally activates APC/C^{Cdc20}, the metaphase I arrest of these cells suggests that APC/C^{Cdc20} is somehow inhibited. This thesis work began with exploring strategies to activate APC/C^{Cdc20} and anaphase of meiotic *ndt80*Δ *ama1*Δ cells. We tried to initiate anaphase of *ndt80*Δ *ama1*Δ cells by deleting *MAD2*, *SWE1* and *RAD9,* which are capable of inducing an arrest at metaphase. However, none of these strategies worked. Nevertheless, we discovered a surprising phenotype of *ndt80*Δ *ama1*Δ cells lacking *MAD2.* These cells failed to accumulate key M-phase proteins. The SAC protects genomic stability by inducing a mitotic arrest in the presence of chromosome attachment errors by inhibiting APC/C-dependent degradation. However, it is not clear whether the SAC also regulates the expression of these M-phase proteins. If it is the case, the SAC would be a master regulator of M-phase, regulating both gene expression and degradation of key M-phase proteins. Considering the significant conceptual importance of this possibility, we decided to investigate how the SAC regulates M-phase gene expression. Therefore, this thesis presents results testing this hypothesis by using meiotic *ndt80*Δ *ama1*Δ cells as an experimental system.

1.14. Contribution

I carried out all the experiments described in this thesis.

2. Results

2.1. SAC genes are required for accumulation of *CLB2*-cluster proteins in *ndt80*Δ *ama1*Δ cells

Spindle formation and accumulation of M-phase proteins in meiotic *ndt80*Δ *ama1*Δ cells are triggered by the mitotic transcriptional activator Ndd1 (Okaz et al., 2012). Moreover, these cells accumulate B-type cyclins Clb4, Clb1, and Clb2 in two waves, which resembles mitotic cells (Fitch et al., 1992; Richardson et al., 1992). Interestingly, however, although meiotic *ndt80*Δ *ama1*Δ cells enter high kinase state, they fail to disassemble spindles, degrade Pds1 and M phase proteins and fail to undergo nuclear division (Okaz et al., 2012). Since the high kinase state normally activates APC/C^{Cdc20}, the metaphase I arrest of these cells suggests that APC/C^{Cdc20} is somehow inhibited. The SAC is an established inhibitor of APC/C^{Cdc20} (Musacchio, 2015). Therefore, we investigated whether silencing the SAC would allow *ndt80Δ ama1Δ* cells to activate APC/C^{Cdc20} and to enter anaphase. *MAD2, MAD1, MAD3, BUB1, BUB3, and MPS1* are all essential genes for SAC activity in *S. cerevisiae* (Musacchio, 2015). We observed that deletion of the *MAD2* gene did not lead to degradation of Pds1 or nuclear division in *ndt80Δ ama1Δ* cells (Figures 6A and 6B). Deleting the other essential SAC genes *MAD1, MAD3,* and *BUB1* or inhibiting the Mps1 kinase activity did not allow Pds1 degradation or nuclear division in *ndt80Δ ama1Δ* cells either (Figures 7A, 7B, 8A, 8B, 9A, 9B and 10A, 10B,). Taken together, these results showed that the SAC was not responsible for inhibiting APC/C^{Cdc20} activity in *ndt80Δ ama1Δ* cells. Unexpectedly, however, whereas *CLB2*-cluster proteins Clb1, Clb2, Cdc20, Cdc5, and Swi5 accumulated at a high level after 6 hours into meiosis in *ndt80Δ ama1Δ* cells, they accumulated at a much lower level in cells lacking *MAD2* (Figure 6A). This effect was specific as the S-phase protein Dbf4 and the M-phase proteins Clb4, Cin8, Kip1, which are not *CLB2*-cluster proteins, accumulated normally in cells either having or lacking *MAD2* (Figure 6A).

Additionally, the meiotic specific proteins Rec8, Ime2, and Spo13 also accumulated normally (Figure 6A), showing that the phenotype was not due to poor induction of meiosis in *mad2Δ ndt80Δ ama1Δ* cells. A similar phenotype was observed in *ndt80Δ ama1Δ* cells lacking *MAD1 or BUB1* gene or upon Mps1 inhibition. In these cells, *CLB2*-cluster proteins were significantly down regulated while other proteins were largely unaffected (Figures 7A, 8A, 9A). Interestingly, a milder phenotype was observed in *mad3Δ* cells, in which Clb1, Clb2, Swi5, and Cdc5 but not Cdc20 were strongly down regulated (Figure 10A). The slightly different phenotype of *mad3Δ* and *mad2Δ* was consistent with the fact that Mad2 and Mad3 inhibited APC/C activity either cooperatively or independently (Burton and Solomon, 2007; Izawa and Pines, 2012; Lau and Murray, 2012). Taken together, these results suggested that the SAC was required for accumulation of *CLB2*-cluster proteins in meiotic *ndt80Δ ama1Δ* cells.

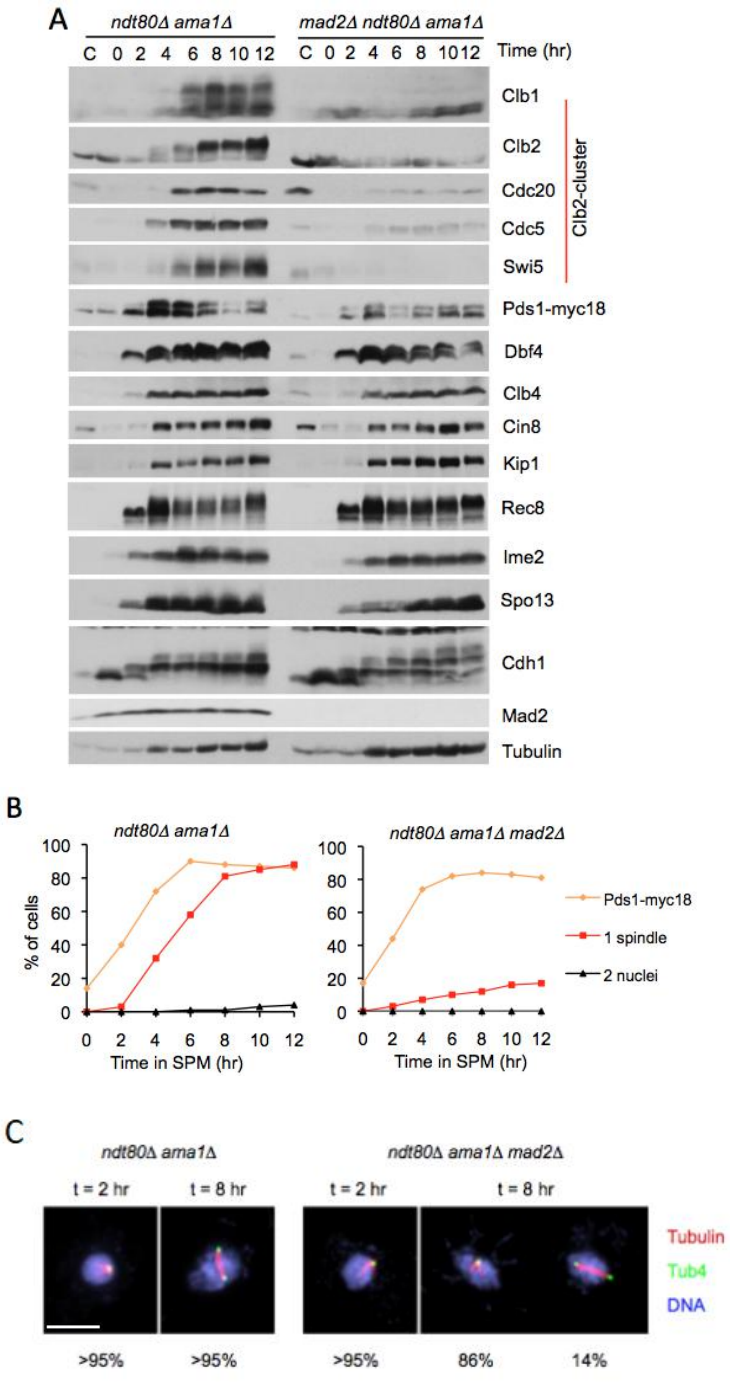

Figure 6. Deletion of *MAD2* reduces the accumulation of *CLB2*-cluster proteins and spindle formation in *ndt80*Δ *ama1*Δ cells. Meiosis was induced in synchronized cultures of *ndt80*Δ *ama1*Δ (Z20094) and *ndt80*Δ *ama1*Δ *mad2*Δ (Z20225) cells. After transfer to sporulation medium (SPM), samples for immunofluorescence and TCA protein extraction were collected every 2 hours. (A) Immunoblotting of protein levels. C means samples from proliferating cells. (B) Quantification of meiotic progression by immunofluorescence detection of Pds1-myc18, spindles (α-tubulin), and divided nuclei (DAPI) in fixed cells. Percentage of cells per time point is shown. (C) Representative immunostained cells of selected time points. Percentages of cells represented by the image. Scale bar, 5 μm.

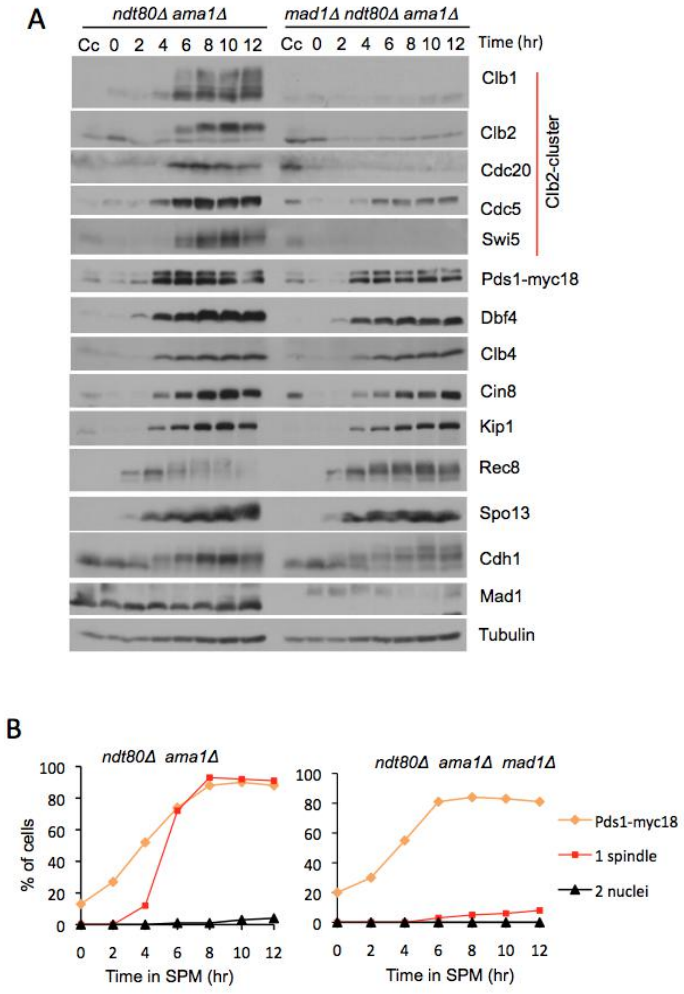

Figure 7. Deletion of *MAD1* reduces the accumulation of *CLB2*-cluster proteins and spindle formation in *ndt80*Δ *ama1*Δ cells. Meiosis was induced in synchronized cultures of *ndt80*Δ *ama1*Δ (Z20094) and *ndt80*Δ *ama1*Δ *mad1*Δ (Z20488) cells. After transfer to sporulation medium (SPM), samples for immunofluorescence and TCA protein extraction were collected every 2 hours. (A) Immunoblot detection of protein levels during the time course. Cc means samples from proliferating cells. (B) Quantification of meiotic progression by immunofluorescence detection of Pds1-myc18, spindles (α-tubulin), and divided nuclei (DAPI) in fixed cells. Percentages of cells per time point is shown.

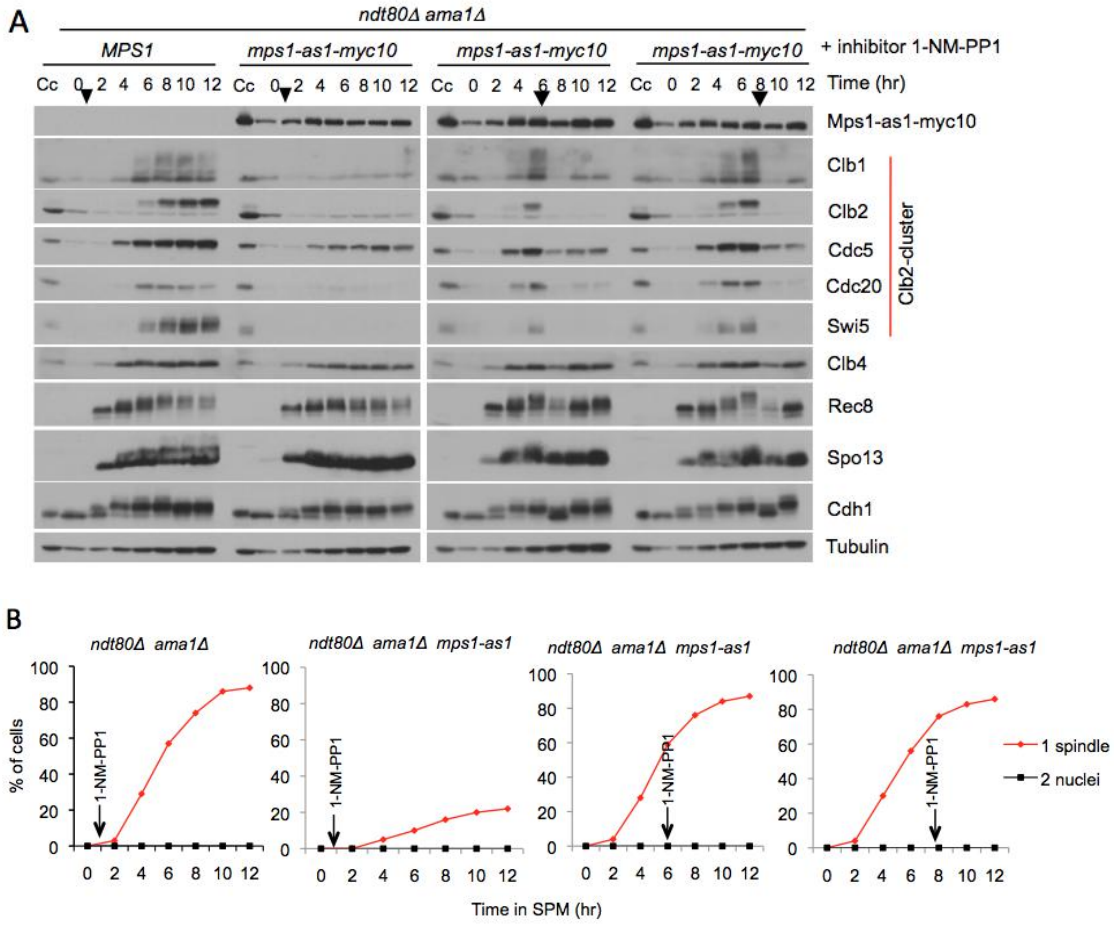

Figure 8. Inhibition of Mps1 kinase activity reduces the accumulation of *CLB2*-cluster proteins and spindle formation in *ndt80*Δ *ama1*Δ cells. Meiosis was induced in synchronized cultures of *ndt80*Δ *ama1*Δ (Z19545) and *ndt80*Δ *ama1*Δ *mps1-as1* (Z20948) cells. After transfer to SPM, 10 μM 1NM-PP1 was added to the culture at 1 hour, 6 hours or 8 hours after induction of meiosis as indicated, Samples for immunofluorescence and TCA protein extraction were collected every 2 hours. (A) Immunoblot detection of protein levels during the time course. Cc means samples from proliferating cells. (B) Quantification of meiotic progression by immunofluorescence detection of spindles (α-tubulin), and divided nuclei (DAPI) in fixed cells.

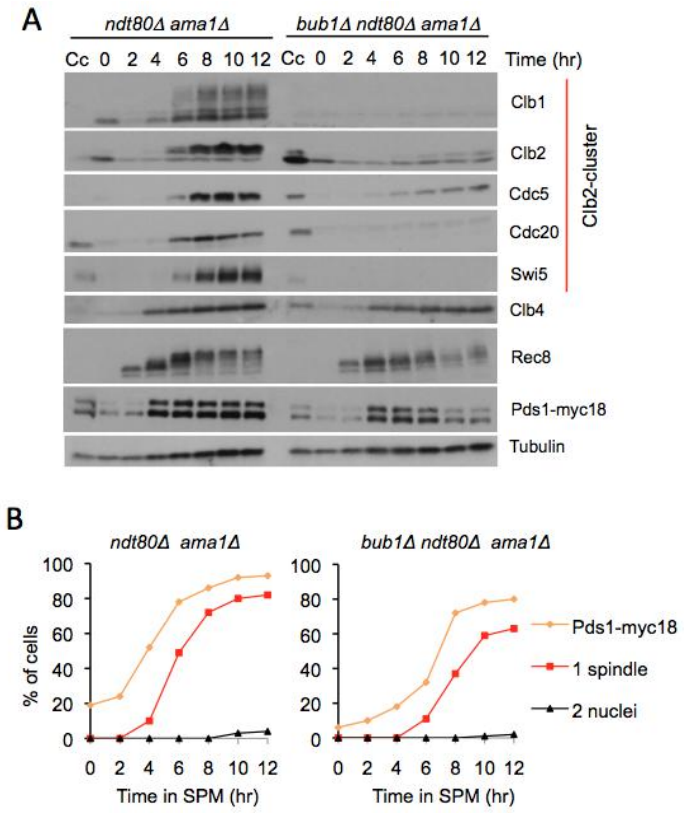

Figure 9. Deletion of *BUB1* reduces the accumulation of *CLB2*-cluster proteins in *ndt80*Δ *ama1*Δ cells. Meiosis was induced in synchronized cultures of *ndt80*Δ *ama1*Δ (Z20094) and *ndt80*Δ *ama1*Δ *bub1*Δ (Z21546) cells. After transfer to SPM, samples for immunofluorescence and TCA protein extraction were collected every 2 hours. (A) Immunoblot detection of protein levels during the time course. Cc means samples from proliferating cells. (B) Quantification of meiotic progression by immunofluorescence detection of Pds1-myc18, spindles (α-tubulin), and divided nuclei (DAPI) in fixed cells. Percentages of cells per time point is shown.

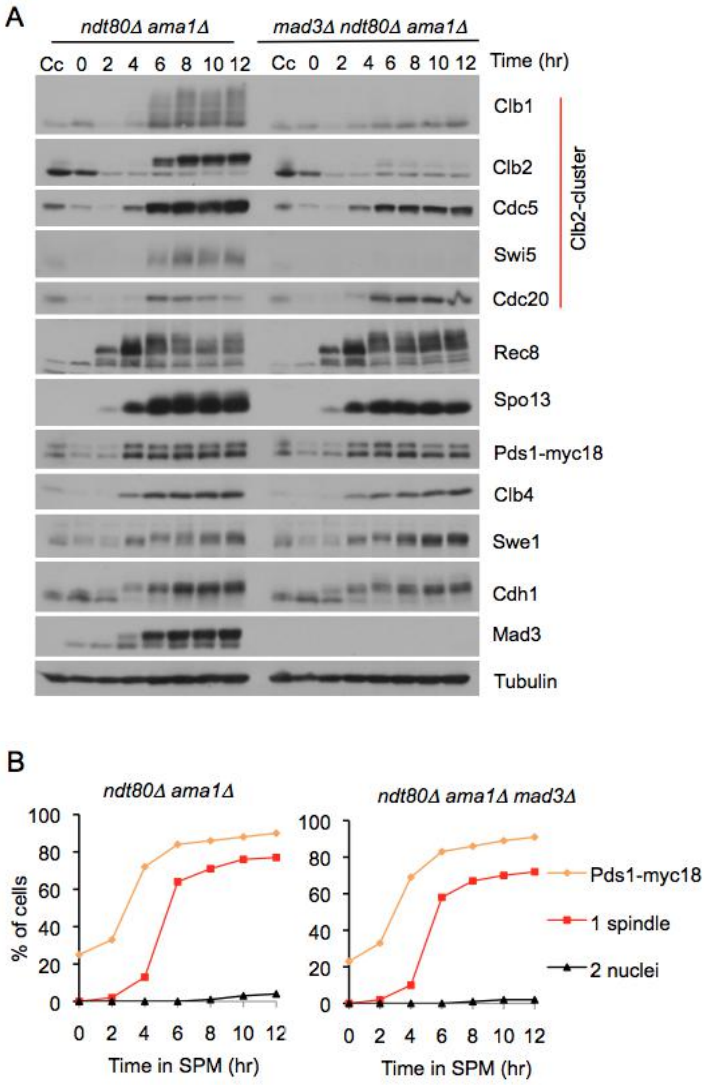

Figure 10. Deletion of *MAD3* reduces the accumulation of *CLB2*-cluster proteins in *ndt80*Δ *ama1*Δ cells. Meiosis was induced in synchronized cultures of *ndt80*Δ *ama1*Δ (Z20094) and *ndt80*Δ *ama1*Δ *mad3*Δ (Z20818) cells. After transfer to SPM, samples for immunofluorescence microscopy and TCA protein extraction were collected every 2 hours. (A) Immunoblot detection of protein levels during the time course. Cc means sample from proliferating cells. (B) Quantification of meiotic progression by immunofluorescence detection of Pds1-myc18, spindles (α-tubulin), and divided nuclei (DAPI) in fixed cells. Percentages of cells per time point is shown.

2.2. Down-regulation of *CLB2*-cluster proteins in cells lacking *MAD2* is not due to APC/C dependent protein degradation in the absence of the SAC.

To investigate why the SAC was required for the timely accumulation of *CLB2*-cluster proteins in *ndt80Δ ama1Δ* cells, we hypothesized that APC/C became hyperactive and thus prevented the accumulation of *CLB2*-cluster proteins by targeting them to proteasome-dependent degradation when individual SAC genes were deleted. To test this hypothesis, we sought to test whether inactivating APC/C activity would allow accumulation of *CLB2*-cluster proteins in *mad2Δ ndt80Δ ama1Δ* cells. APC/C is an essential ubiquitin E3 ligase containing over a dozen of subunits, which assemble as a 1.5 MDa complex (Peters, 2006). The activity of APC/C strictly relies on its essential co-activators, Cdc20 and Cdh1 in mitotic cells and Ama1 in meiotic cells, which are required for substrate recognition. Therefore, one can use conditional mutants of either APC/C subunits or co-activators to study the loss of function phenotype of APC/C.

Firstly we utilized a mutant that expressed *CDC20* from a mitosis specific promoter (P_{HSL1}-*CDC20*). The Cdc20 protein was depleted so that APC/C^{Cdc20} was presumably not active. However, the accumulation of *CLB2*-cluster proteins was not restored in P_{HSL1}-*CDC20 mad2Δ ndt80Δ ama1Δ* cells (Figure 11A). One possibility was that although the level of Cdc20 was below the detection limit of western blotting technique in P_{HSL1}-*CDC20* cells, trace level of Cdc20 were still active enough to prevent the accumulation of *CLB2*-cluster proteins. To address this possibility, we took advantage of *cdc20-3* (Shirayama et al., 1998), a temperature-sensitive allele of *CDC20* that offered temperature-dependent conditional regulation of Cdc20 activity. *cdc20-3 mad2Δ ndt80Δ ama1Δ* cells were maintained at 25°C to allow normal growth. 2 hours after induction of meiosis, the temperature was shifted to 34°C to inactivate Cdc20 activity. However, *CLB2*-cluster proteins were still significantly downregulated in *cdc20-3 mad2Δ ndt80Δ ama1Δ* cells (Figure 12A). Together, the P_{HSL1}-*CDC20* and the *cdc20-3* experiments suggested that APC/C^{Cdc20} activity was not responsible for

the low level of *CLB2*-cluster proteins in *mad2Δ ndt80Δ ama1Δ* cells.

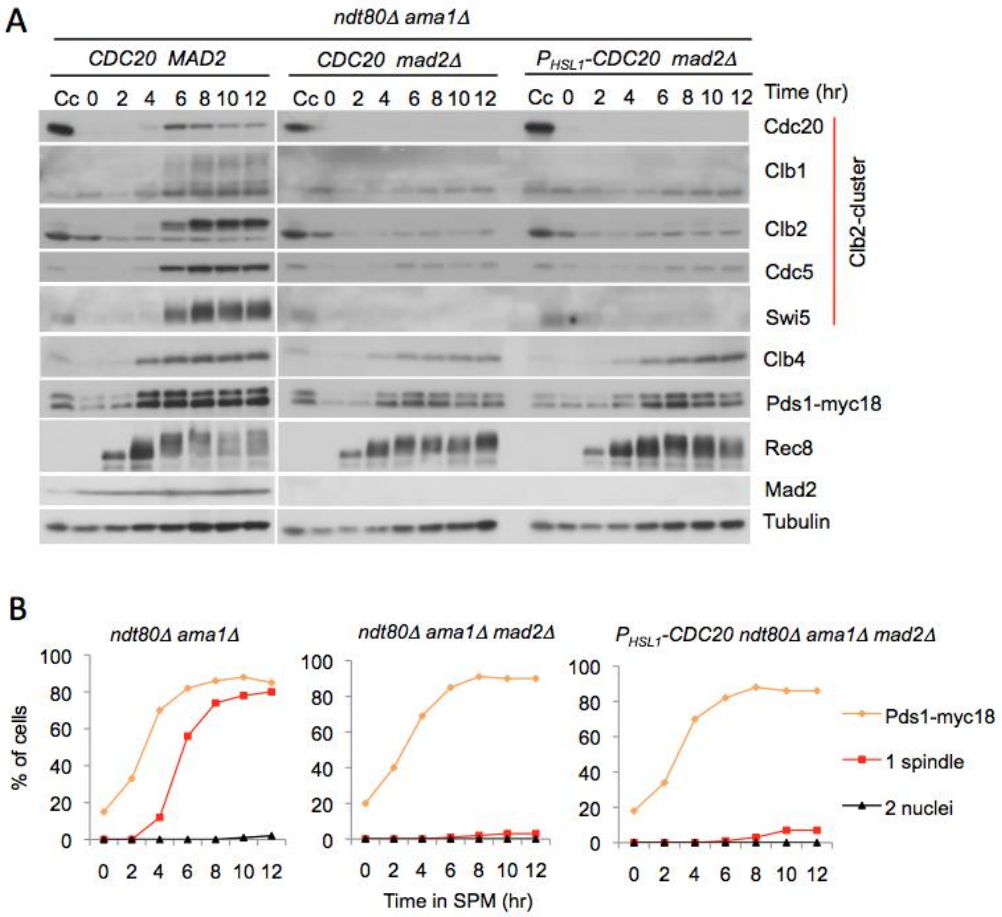

Figure 11. Depletion of *CDC20* does not restore accumulation of *CLB2*-cluster proteins in *ndt80Δ ama1Δ mad2Δ* cells. Meiosis was induced in synchronized cultures of *ndt80*Δ *ama1*Δ (Z20094), *ndt80*Δ *ama1*Δ *mad2*Δ (Z20225) and P_{HSL1}-*CDC20 ndt80*Δ *ama1*Δ *mad2*Δ cells (Z21076). After transfer to SPM, samples for immunofluorescence and TCA protein extraction were collected every 2 hours. (A) Immunoblot detection of protein levels during the time course. Cc means samples from proliferating cells. (B) Quantification of meiotic progression by immunofluorescence detection of Pds1-myc18, spindles (α-tubulin), and divided nuclei (DAPI) in fixed cells. Percentages of cells per time point is shown.

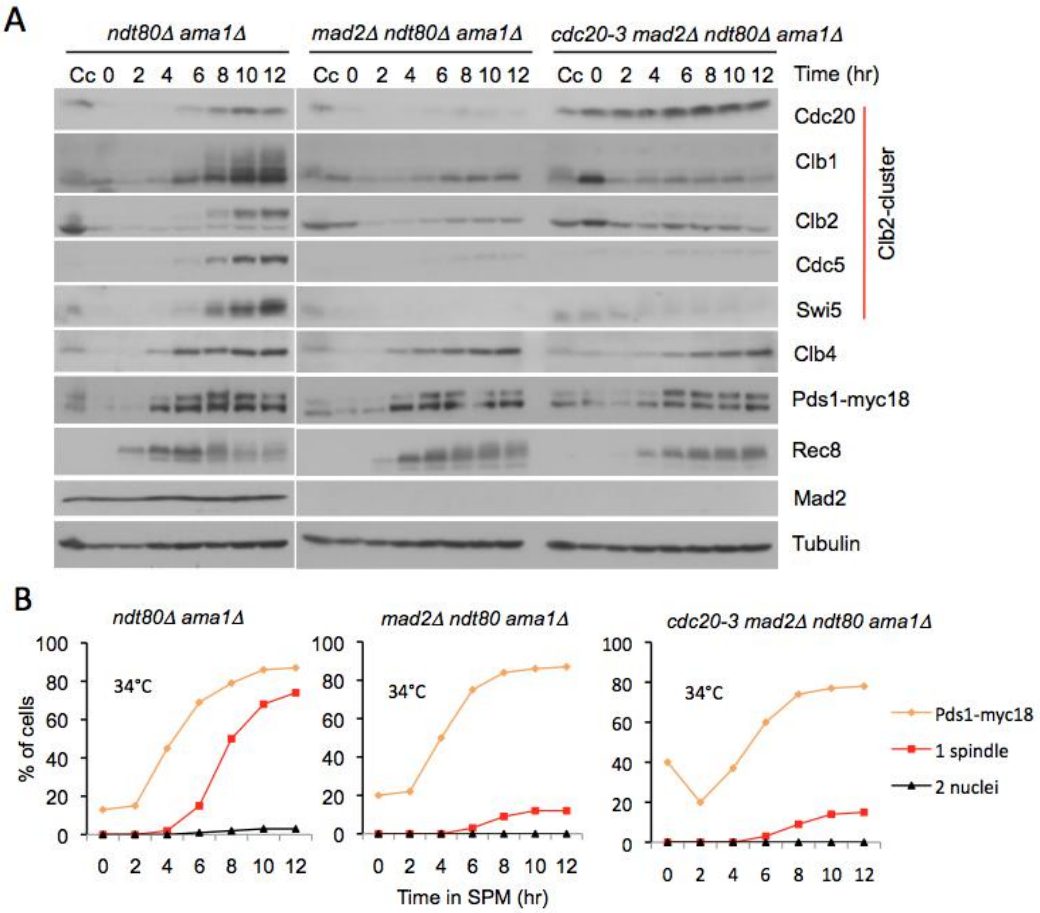

Figure 12. Inhibition of Cdc20 activity does not restore accumulation of *CLB2*-cluster proteins in *ndt80Δ ama1Δ mad2Δ* cells. Meiosis was induced in synchronized cultures of *ndt80*Δ *ama1*Δ (Z20094), *ndt80*Δ *ama1*Δ *mad2*Δ (Z20225) and *cdc20-3 ndt80*Δ *ama1*Δ *mad2*Δ cells (Z21979) at 25°C. Temperature of the culture was shifted to 34°C 1 hour after transfer to SPM, and samples for immunofluorescence and TCA protein extraction were collected every 2 hours. (A) Immunoblot detection of protein levels during the time course. Cc means samples from proliferating cells. (B) Quantification of meiotic progression by immunofluorescence detection of Pds1-myc18, spindles (α-tubulin), and divided nuclei (DAPI) in fixed cells. Percentage of cells per time point is shown.

One might argue that depletion and temperature-sensitive mutants both have limitations in studying protein function in meiotic yeast cells. Depletion might not completely make the target protein disappear and the meiotic process is temperature sensitive itself, and thus might be influenced by temperature shift. Therefore, we sought to use other methods to confirm our results. APC/C^{Cdh1} is active when Cdk1 activity is low. Therefore, although APC/C^{Cdh1} is not the primary target of the SAC, it is possible that APC/C^{Cdch1} becomes hyperactive and degrades *CLB2*-cluster proteins in *mad2Δ ndt80Δ ama1Δ* cells. It has been difficult to study the function of Cdh1 in meiosis because of slow growth of *cdh1Δ* cells and lack of conditional mutants. To address these two problems, we decided to inactivate activity of APC core enzyme using the newly developed anchor away (AA) technique (Haruki et al., 2008). The AA technique takes advantage of rapamycin-dependent dimerization of FKBP12 and FRB domain of mTOR. The protein of interest tagged with FRB domain would interact with the ribosome subunit Rpl13a tagged with FKBP12 and move from the nucleus to the cytoplasm and thus fail to exert its nuclear function in the presence of rapamycin. To inactivate APC/C enzyme activity, we added a FRB tag to Apc2, which forms the catalytic core together with Apc11. We asked whether the *apc2-AA* mutant would stabilize APC/C substrates upon adding rapamycin in normal meiosis. In control strains having wild-type *APC2* and *RPL13A*, APC/C substrates Pds1, Dbf4, Cdc5, Clb4, Ndt80, and Cdc20 accumulated in meiosis I and then became degraded and finally disappeared at the end of meiosis II because of activation of APC/C (Figures 13A, B). By contrast, in cells lacking two of the APC/C co-activator Ama1 and Cdc20, all APC/C substrates accumulated at much higher level in meiosis I and remained stable in meiosis II suggesting that APC/C activity was completely inhibited. In *apc2-AA* strain (*APC2-FRB, RPL13A-FKBP12*), all APC/C substrates accumulated at a much higher level in meiosis I than WT strain after we added rapamycin to the culture at 1 hour into meiosis. Although APC/C substrates persisted longer in *apc2-AA* strain, they appeared to be degraded from time point 10, 2 hours later than in wild-type cells. The activation

of APC in the end of the time course might be due to high accumulation of Cdc20 in *apc2-AA* strain, which was consistent with other observations that APC/C targets Cdc20 for degradation (Foster and Morgan, 2012; Ge et al., 2009; Mansfeld et al., 2011; Nilsson et al., 2008; Pan and Chen, 2004). Moreover, the effect indeed depended on dimerization of *Apc2-FRB* with *Rpl13a-FKBP12* because strain having *APC2-FRB* and wild-type *RPL13A* did not stabilize APC/C substrates when rapamycin was added (Figures 13A, B). Our results showed that anchoring away Apc2 was an effective and fast way to inhibit APC/C activity in meiosis I.

Having the tool in hand, we asked whether inhibiting APC/C activity would allow the accumulation of *CLB2*-cluster proteins in *mad2Δ* cells. Meiotic *ndt80Δ* cells are known to arrest in prophase (Xu et al., 1995). Deleting *AMA1* or inhibiting APC/C activity would allow *ndt80 Δ* cells to produce M-phase proteins, to assemble bipolar spindles and eventually to arrest in a metaphase I-like state (Okaz et al., 2012). *ndt80Δ* cells accumulated Pds1 but failed to accumulate *CLB2*-cluster proteins and to assemble spindles showing that APC/C was active in these cells. In the *apc2-AA* strain, however, cells accumulated *CLB2*-cluster proteins and formed spindles from time-point 10 onward showing APC/C activity was inhibited. And deleting *MAD2* in *APC2-AA* cells downregulated *CLB2*-cluster proteins even in the absence of APC/C activity. This suggested that Mad2 was required for accumulation of *CLB2*-cluster proteins independent of APC/C activity (Figures 14A, B).

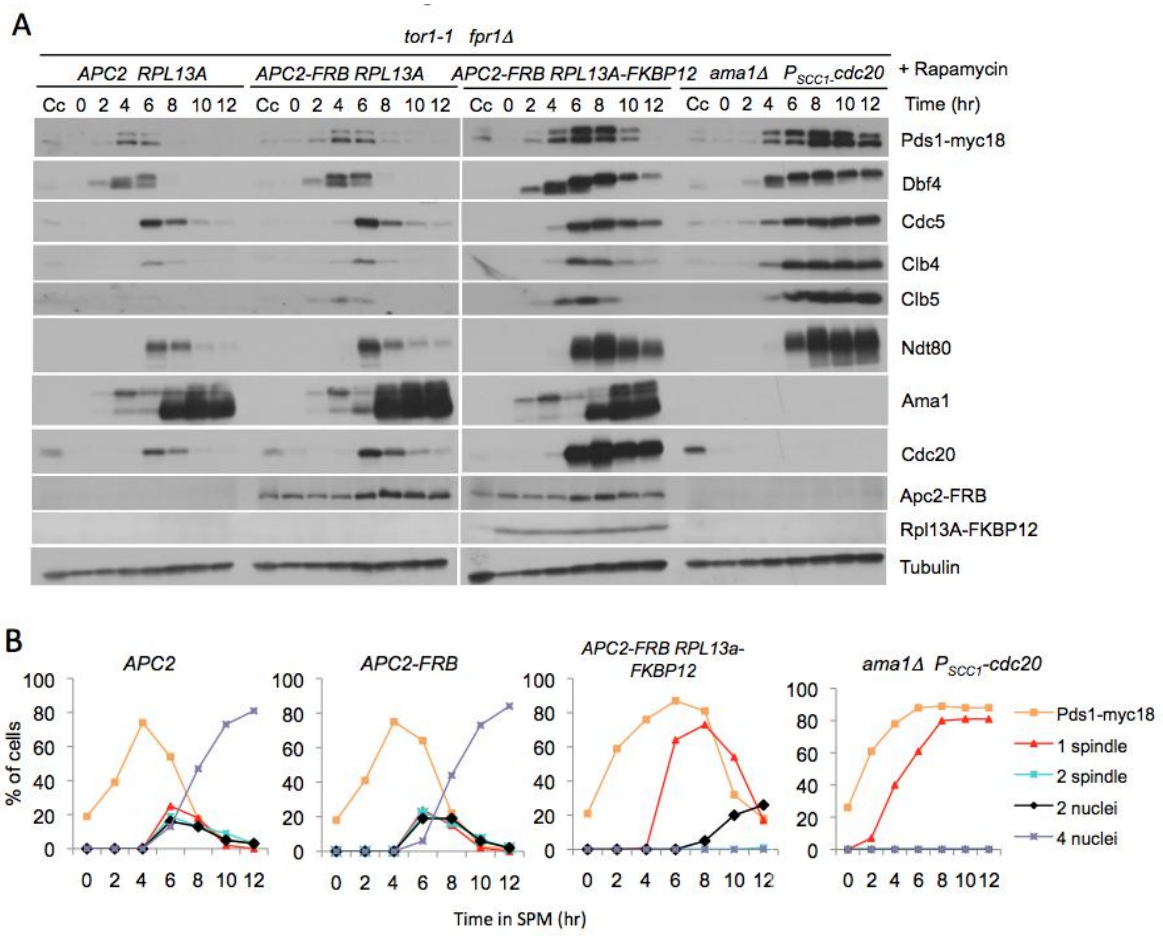

Figure 13. Anchoring-away the APC/C subunit Apc2 inhibits the APC/C-dependent degradation in meiosis. Meiosis was induced in synchronized cultures of WT (Z23831), *APC2-FRB* (Z23832), *APC2-FRB RPL13A-FKBP12* (Z23829) and P_{SCC1}*-CDC20 ama1*Δ (Z27793) cells. 1 hour after transfer to SPM, 10μg/ml rapamycin was added to the culture and samples for immunofluorescence and TCA protein extraction were collected every 2 hours. (A) Immunoblot detection of protein levels during the time course. Cc means samples from proliferating cells. (B) Quantification of meiotic progression by immunofluorescence detection of Pds1-myc18, spindles (α-tubulin), and divided nuclei (DAPI) in fixed cells. Percentages of cells per time point are shown.

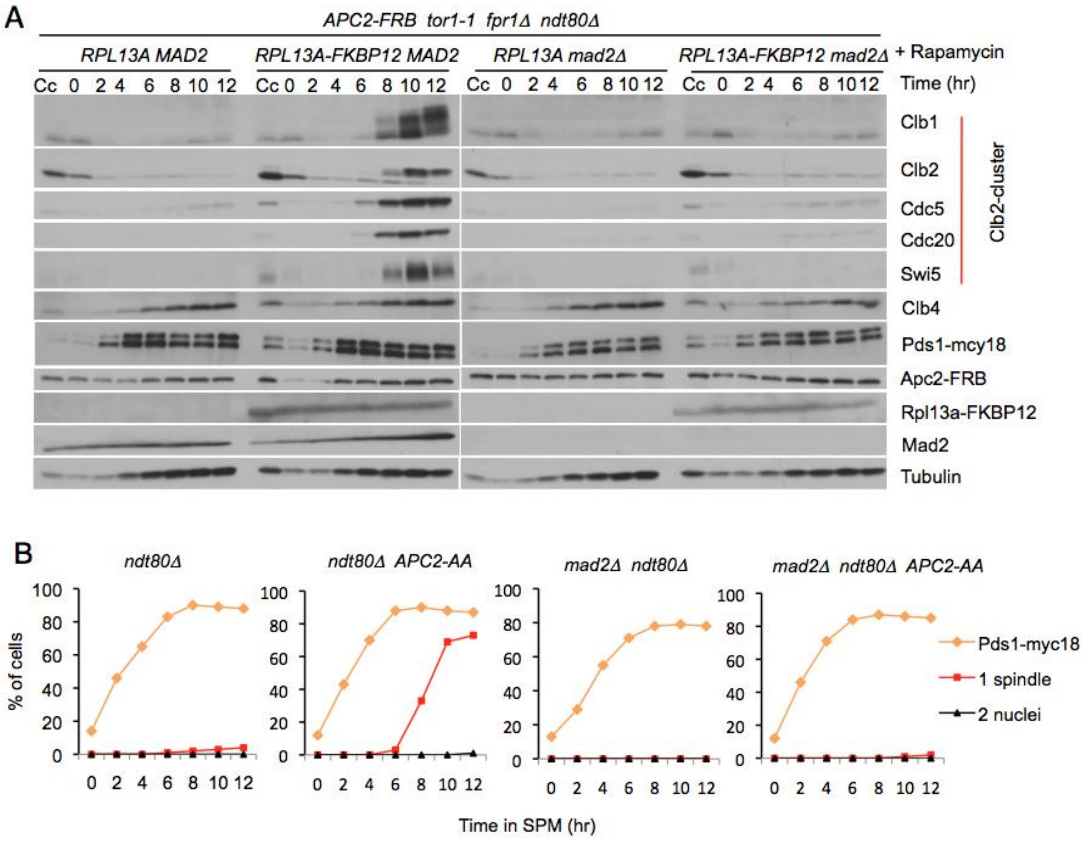

Figure 14. Anchoring-away the APC/C subunit Apc2 does not restore the accumulation of *CLB2*-cluster proteins in *ndt80Δ mad2Δ* cells. Meiosis was induced in synchronized cultures of *APC2-FRB ndt80*Δ (Z25473), *RPL13A-FKBP12 APC2-FRB ndt80*Δ (Z25475), *mad2*Δ *APC2-FRB ndt80*Δ (Z25474) and *APC2-FRB RPL13A-FKBP12 mad2*Δ *ndt80*Δ (Z25476) cells. 1 hour after transfer to SPM, 10μg/ml rapamycin was added to the culture and samples for immunofluorescence and TCA protein extraction were collected every 2 hours. (A) Immunoblot detection of protein levels during the time course. Cc means samples from proliferating cells. (B) Quantification of meiotic progression by immunofluorescence detection of Pds1-myc18, spindles (α-tubulin), and divided nuclei (DAPI) in fixed cells. Percentages of cells per time point are shown.

If *CLB2*-cluster proteins were degraded as a result of hyperactive APC/C in *mad2Δ ndt80Δ ama1Δ* cells, the stability of these proteins should be significantly reduced. To measure the half-life of *CLB2*-cluster proteins, we added cycloheximide to meiotic cultures 8 hours after induction of meiosis. To make sure the starting amounts of proteins in *MAD2* and *mad2Δ* cells are equal, we expressed either *CLB1* or *CLB2* from the *DMC1* promoter. The stability of the Clb1 proteins in *MAD2 ndt80Δ ama1Δ* strain was roughly 20 minutes (Figure 15A). Mutating the D-box and the KEN-box of Clb1 (Clb1-ND) completely stabilized the proteins, showing that the degradation was APC/C dependent. Unexpectedly, however, Clb1 in *mad2Δ ndt80Δ ama1Δ* cells was even more stable with a half-life of more than 80 minutes. Different from Clb1, Clb2 was relatively stable in both *MAD2* and *mad2Δ* strain (Figure 15B). Both experiments suggested that protein stability of *CLB2*-cluster proteins was not reduced in the *mad2Δ ndt80Δ ama1Δ* strain. Therefore, downregulation of these proteins was not due to protein degradation.

The steady state level of a protein is determined by protein degradation and gene expression. Our data show that the low levels of *CLB2*-cluster proteins in *mad2Δ* is not due to protein degradation. This prompted us to ask whether it was due to reduced level of gene expression. If it was true that Mad2 was required for the expression of *CLB2*-cluster genes, we would expect that expressing these genes from a promoter that is not regulated by Mad2 would bypass the requirement of Mad2 for the expression. To test this hypothesis, we expressed *CLB1, CLB2* and *CDC5* from the *DMC1* promoter, which is a meiosis specific promoter. Clb1 was expressed at 2 hours into meiosis, when the *DMC1* promoter is known to be active. Clb1 was expressed at the same time and at the same level in *mad2Δ* as in *MAD2* cells (Figure 16A). Similarly, Clb2 and Cdc5 expressed from *DMC1* promoter accumulated equally well in *MAD2* and *mad2Δ* strains (Figures 16B, C). These observations supported our hypothesis that downregulation of *CLB2*-cluster proteins in *mad2Δ* cells was due to reduced expression rather than degradation.

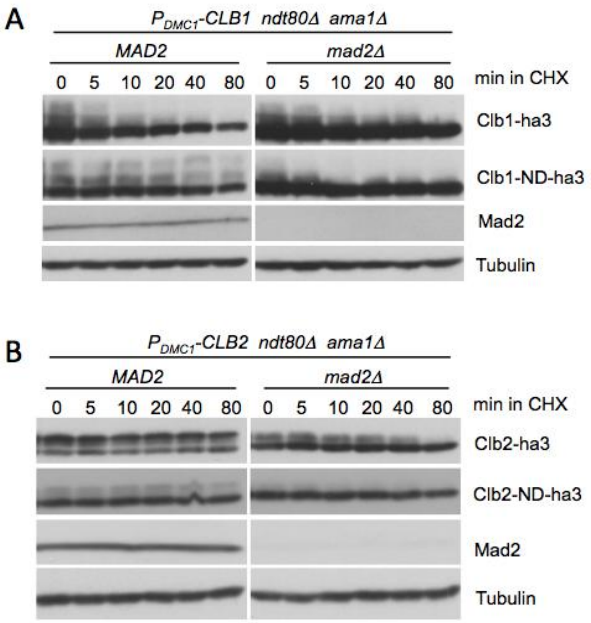

Figure 15. Stability of Clb1 and Clb2 expressed from *DMC1* promoter is not significantly reduced in *ndt80Δ ama1Δ* cells lacking *MAD2*. Meiosis was induced in synchronized cultures. 8 hours after transfer to sporulation medium, 0.5mg/ml of cycloheximide was added to the culture and samples for TCA protein extraction were collected 5 mins, 10 mins, 20 mins, 40 mins, and 80 mins after addition of cycloheximide. Immunoblot detection of protein levels during the time course. ND is short for non-degradable version of the protein. (A) P_{DMC1}-*CLB1 ndt80*Δ *ama1*Δ (Z21099), P_{DMC1}-*CLB1 ndt80*Δ *ama1*Δ *mad2*Δ (Z21100), P_{DMC1}-*CLB1-ND ndt80*Δ *ama1*Δ *(*Z21548*) and* P_{DMC1}-*CLB1-ND ndt80*Δ *ama1*Δ *mad2*Δ (Z21549) cells. (B) P_{DMC1}-*CLB2 ndt80*Δ *ama1*Δ (Z21101), P_{DMC1}-*CLB2 ndt80*Δ *ama1*Δ *mad2*Δ (Z21102), P_{DMC1}-*CLB2-ND ndt80*Δ *ama1*Δ *(*Z21331*) and* P_{DMC1}-*CLB2-ND ndt80*Δ *ama1*Δ *mad2*Δ (Z21330) cells.

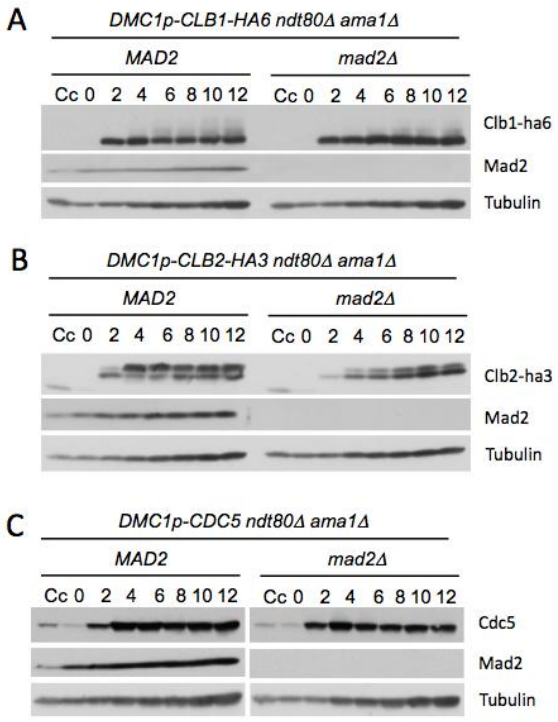

Figure 16. Expression from the *DMC1* promoter restores the level of *CLB2*-cluster proteins in *ndt80Δ ama1Δ* cells lacking *MAD2*. Meiosis was induced in synchronized cultures. After transfer to sporulation medium (SPM), samples for immunofluorescence and TCA protein extraction were collected every 2 hours. Immunoblot detection of protein levels during the time course. Cc means sample from proliferating cells. (A) P_{DMC1}-*CLB1 ndt80*Δ *ama1*Δ (Z21099) and P_{DMC1}-*CLB1 ndt80*Δ *ama1*Δ *mad2*Δ (Z21100) cells. (B) P_{DMC1}-*CLB2 ndt80*Δ *ama1*Δ (Z21101) and P_{DMC1}-*CLB2 ndt80*Δ *ama1*Δ *mad2*Δ (Z21102) cells. (C) P_{DMC1}-*CDC5 ndt80*Δ *ama1*Δ (Z27605) and P_{DMC1}-*CDC5 ndt80*Δ *ama1*Δ *mad2*Δ (Z27608) cells.

2.3. Loss of *CLB2*-cluster proteins in SAC mutants is not due to impaired Clb-Cdk1 activity.

The transcription coactivator Ndd1 tightly regulates expression of CLB2-cluster genes. Their expression is inhibited in the absence of Ndd1 at G1 and S phase. When Ndd1 is produced and subsequently phosphorylated by Cdk1-Clb at G2/M phase, it binds to Fkh2 and Mcm1 to form an active transcriptional activator complex that activates expression of *CLB2*-cluster genes. We thought that the activity of Ndd1 might be affected by deletion of the *MAD2* gene. Therefore, we investigated the level, the modification, and the nuclear localization of Ndd1 by western blotting and immunofluorescence microscopy. We observed that in *MAD2* cells, Ndd1 was strongly induced and highly phosphorylated at 4 hours when cells entered M-phase, and concomitantly all *CLB2*-cluster proteins started to accumulate (Figure 17A). Surprisingly, although Ndd1 level was upregulated, it was not phosphorylated at all in *mad2*Δ cells and, as a result, *CLB2*-cluster proteins failed to accumulate (Figure 17A). However, nuclear localization of Ndd1 was not affected in *mad2*Δ cells. We observed around 60% of cells having nuclear localization of Ndd1 at 4 hours in both strains (Figures 17B, C). Phosphorylation of Ndd1 by Cdk1 plays an essential role in activating *CLB2*-cluster gene transcription. The lack of Ndd1 phosphorylation in *mad2*Δ strains therefore suggested that Cdk1 activity was low in these cells (Figures 17A, B).

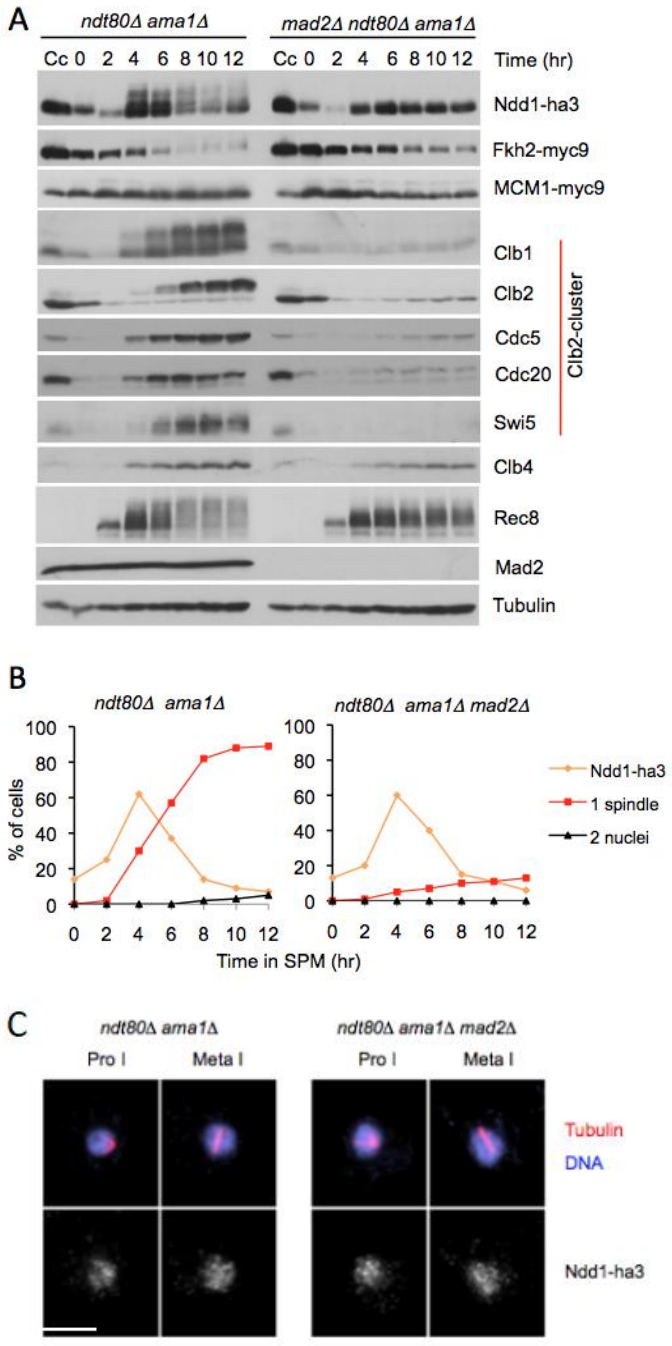

Figure 17. Deletion of *MAD2* reduces Ndd1 phosphorylation in *ndt80Δ ama1Δ* cells. Meiosis was induced in synchronized cultures of *ndt80*Δ *ama1*Δ *NDD1-HA3 FKH2-myc9* (Z21470) and *ndt80*Δ *ama1*Δ *mad2*Δ *NDD1-HA3 FKH2-myc9* (Z21471) cells. After transfer to sporulation medium (SPM), samples for immunofluorescence and TCA protein extraction were collected every 2 hours. (A) Immunoblot detection of protein levels during the time course. Cc means samples from proliferating cells. (B) Quantification of meiotic progression by immunofluorescence detection of Ndd1-ha3, spindles (α-tubulin), and divided nuclei (DAPI) in fixed cells. Percentage of cells per time point is shown. (C) Representative immunostained cells of selected time point. Scale bar, 5 μm.

Next, we asked why Clb-Cdk1 was not active in the absence of Mad2. The activity of Cdk1 is tightly regulated to make sure it is active at the right time and the right place. Cdk1 is phosphorylated and activated by Cdk1-activating kinase 1 (Cak1) (Espinoza et al., 1996; Kaldis et al., 1996). Binding to one of the cyclin activators further activates Cdk1. The activity of Cdk1 during M-phase also requires Cks1 (Tang and Reed, 1993). Cdk1 is inhibited by phosphorylation on its tyrosine residue by Wee1/Swe1 (Lim et al., 1996; Sia et al., 1996). Sic1 binding further inhibits Cdk1 (Schwob et al., 1994). We asked whether Mad2 was required for any of these events. First, we investigated whether Mad2 was required for interaction of Cdk1 with its M-phase activators Clb4 and Cks1 by immunoprecipitation (IP) (Figure 18A). Clb4 or Cks1 was tagged with HA3 epitope to enable the IP process. Clb4-ha3 was immunoprecipitated by anti-HA antibody with an equal efficiency in *MAD2* and *mad2Δ* strains. Cdc28 (budding yeast Cdk1) started to interact weakly with Clb4-ha3 from 2 hours, and the interaction increased significantly from 4 hours, which was consistent with its role in M-phase. Surprisingly, the level of Cdc28-Clb4 interaction was even higher in *mad2Δ* strain, which might be due to less competition for Cdc28 binding from other cyclins. We investigated the role of Mad2 on the interaction of Cdc28 and Cks1 using the same strategy (Figure 18B). Both Cdc28 and Cks1 were expressed from the beginning of the time course and the levels remained constant in *MAD2* strains. The level of Cks1 and Cdc28 proteins were equally constant in *mad2Δ* although the level of *CLB2*-cluster proteins Clb1 and Cdc5 were significantly reduced. Cks1-ha3 was immunoprecipitated with equal efficiency in both *MAD2* and *mad2Δ* strains. Cdc28 interacted with Cks1 constitutively in *MAD2* cells suggesting they were able to bind to each other efficiently. However, the interaction was not affected by deleting *MAD2*. Cdc28 interacted with Cks1 strongly from the beginning until the end of the time course in *mad2Δ*, as robustly as in the *MAD2* cells. Therefore, our results showed that Mad2 was not required for assembly of the active Cdc28-Cks1-Clb4 complex.

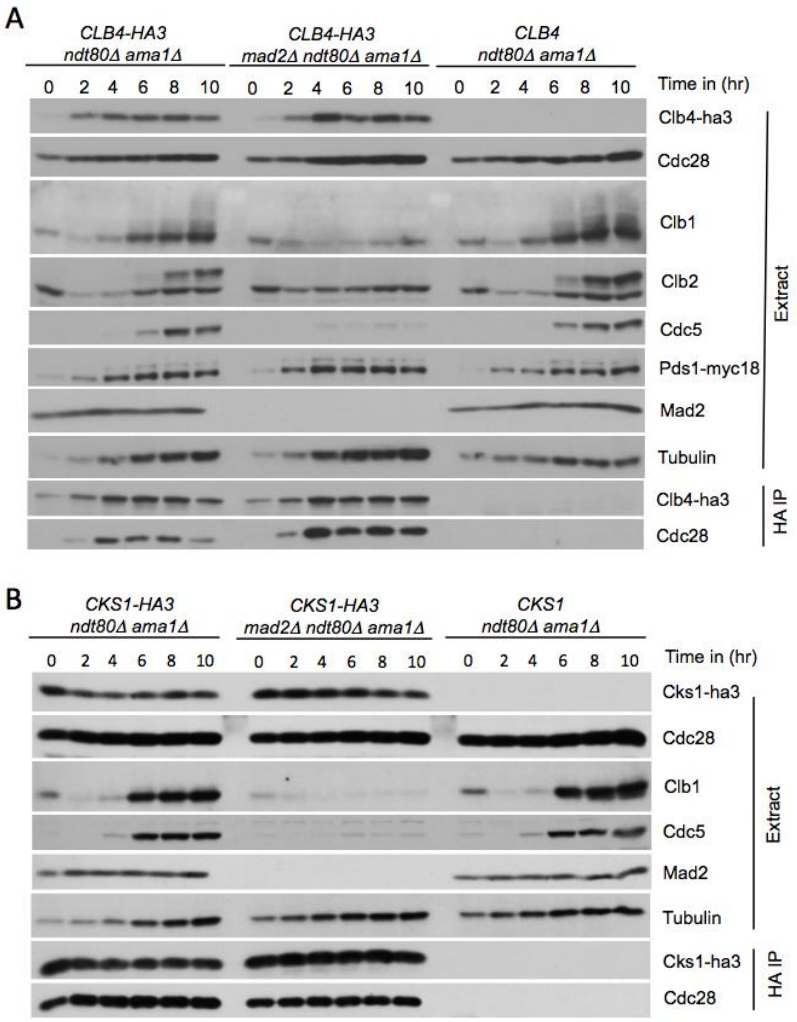

Figure 18. Deletion of *MAD2* does not disrupt Clb4-Cks1-Cdc28 complex formation. Meiosis was induced in synchronized cultures. After transfer to sporulation medium (SPM), samples for immunoprecipitation were collected every 2 hours. Immunoblot detection of proteins from anti-HA immunoprecipitates and whole cell extract. (A) *ndt80*Δ *ama1*Δ *CLB4-HA3* (Z21872), *ndt80*Δ *ama1*Δ *mad2*Δ *CLB4-HA3* (Z21873) and *ndt80*Δ *ama1*Δ *(*Z20094*)* cells. (B) *ndt80*Δ *ama1*Δ *CKS1-HA3* (Z26627), *ndt80*Δ *ama1*Δ *mad2*Δ *CKS1-HA3* (Z26628) and *ndt80*Δ *ama1*Δ *(*Z20094*)* cells.

Next, we asked whether Mad2 was required for the activating or inhibitory phosphorylation of Cdc28. We detected Cdc28-pT and Cdc28-pY by western blotting using monoclonal antibodies raised against these modifications (Figure 19). In *MAD2* cells, we observed that the Cdc28-pT signal was constant throughout the time course, which was consistent with the notion that the responsible kinase Cak1 is constitutively active. The signal of Cdc28-pY only became obvious from 4 hours into meiosis in *MAD2* cells, likely due to the fact the responsible kinase Swe1 was upregulated from 4 hours. The modifications of Cdc28 in *mad2Δ* cells appeared similar compared with *MAD2* cells as Cdc28-pT being constant and Cdc28-pY being induced from 4 hours. Therefore, Mad2 was not required for Cdc28 phosphorylation.

Sic1 is a cyclin-dependent kinase inhibitor (CKI) that regulates G1 to S phase transition by inhibiting Cdk1 (Schwob et al., 1994). Upon entry into S-phase, Sic1 is degraded in a Cdk1 dependent manner to allow DNA replication (Verma et al., 1997). We reasoned that if Mad2 was required for the degradation of Sic1, Sic1 should become stable and continually inhibit Cdk1 activity and prevent M-phase entry of *mad2Δ ndt80Δ ama1Δ* cells. To investigate the requirement of Mad2 for Sic1 stability, we blotted Sic1 protein level to check its stability. However, we found that Sic1 appears to be degraded completely after 4 hours into meiosis that corresponds to S-phase in both wild-type and *mad2Δ* strains (Figure 20). Therefore, we concluded that Mad2 did not regulate Sic1 stability.

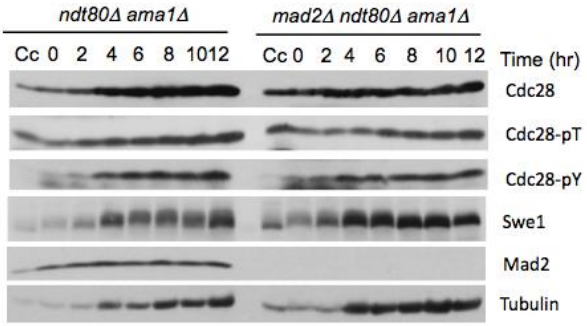

Figure 19. Deletion of *MAD2* does not affect phosphorylation of Cdc28. Meiosis was induced in synchronized cultures of *ndt80*Δ *ama1*Δ (Z20094) and *ndt80*Δ *ama1*Δ *mad2*Δ (Z20225) cells. After transfer to sporulation medium (SPM), samples for TCA protein extraction were collected every 2 hours. Immunoblot detection of protein levels during the time course. Cc means samples from proliferating cells.

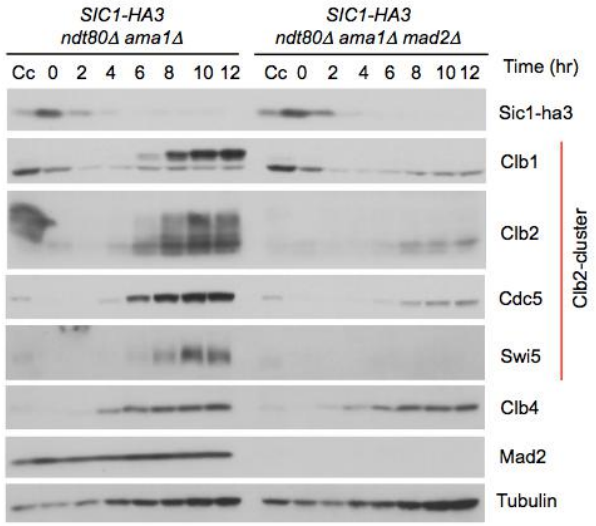

Figure 20. Deletion of *MAD2* does not stabilize Sic1. Meiosis was induced in synchronized cultures of *ndt80*Δ *ama1*Δ *SIC1-HA3* (Z22414) and *ndt80*Δ *ama1*Δ *mad2*Δ *SIC1-HA3* (Z22416) cells. After transfer to sporulation medium, samples for TCA protein extraction were collected every 2 hours. Immunoblot detection of protein levels during the time course. Cc means samples from proliferating cells.

To further interrogate the hypothesis that Mad2 was required for Cdk1 activation, we measured Clb4-Cdc28 activity in an *in-vitro* kinase assay using recombinant histone H1 as a substrate. The reason we chose Clb4-Cdc28 instead of other M-phase Cdk1 complexes was that Clb4 was the only M-phase cyclin that was not downregulated in *mad2Δ* strains (Figure 6A). In *MAD2* cells, we observed that the intensity of histone H1 phosphorylation was low at 0 and 2 hours and then suddenly increased by 7-fold at 4 hours (Figure 21). The change of phosphorylation level was consistent with the protein level of Clb4. Meanwhile, we did not observe any signal in the control experiment, in which Clb4 was not purified, showing the assay was specific. Histone H1 phosphorylation was initially low but increased significantly from 4 hours onward in *mad2Δ* cells, reaching a level that was comparable to that of the *MAD2* cells. In summary, *in-vitro* kinase assays showed that Mad2 was not required for activation of Clb4 associated Cdk1 activity.

Taken together, both *in-vivo* and *in-vitro* evidence did not support the hypothesis that the SAC protein Mad2 regulates Cdk1 activity. Phosphorylation of Cdk1 substrates is balanced by Cdk1 activity and counteracting phosphatase activity (Wurzenberger and Gerlich, 2011) The phosphatase Cdc14 plays a pivotal role in mitotic exit by reversing phosphorylation of Cdk1 substrates (Visintin et al., 1998). Cdc14 activity is tightly regulated by its cellular localization. Before anaphase, inactive Cdc14 is sequestered in the nucleolar RENT (regulator of nucleolar silencing and telophase) complex. At the metaphase-to-anaphase transition, Cdc14 becomes released and active by the FEAR and MEN networks (Stegmeier and Amon, 2004). It might be possible that Cdc14 was prematurely active so that Ndd1 phosphorylation level was low in *mad2Δ* strain. Therefore, we checked the localization of Cdc14 as an indicator of its activity by immunofluorescence microscopy. In *ndt80Δ ama1Δ* strain, we observed around 98 % of cells had nuclear staining of Cdc14, which showed that in the majority of cells Cdc14 was inactive (Figure 22A, B). This was consistent with the fact that *ndt80Δ ama1Δ* cells arrest in metaphase and Cdc14 is not active until metaphase

to anaphase transition. Similarly, in *mad2*Δ strain around 98% of cells had nuclear staining of Cdc14 throughout the time course arguing against the possibility that Cdc14 was active (Figure 22A, B). Therefore, deletion of *MAD2* did not affect activation of either Cdk1 kinase or Cdc14 phosphatase.

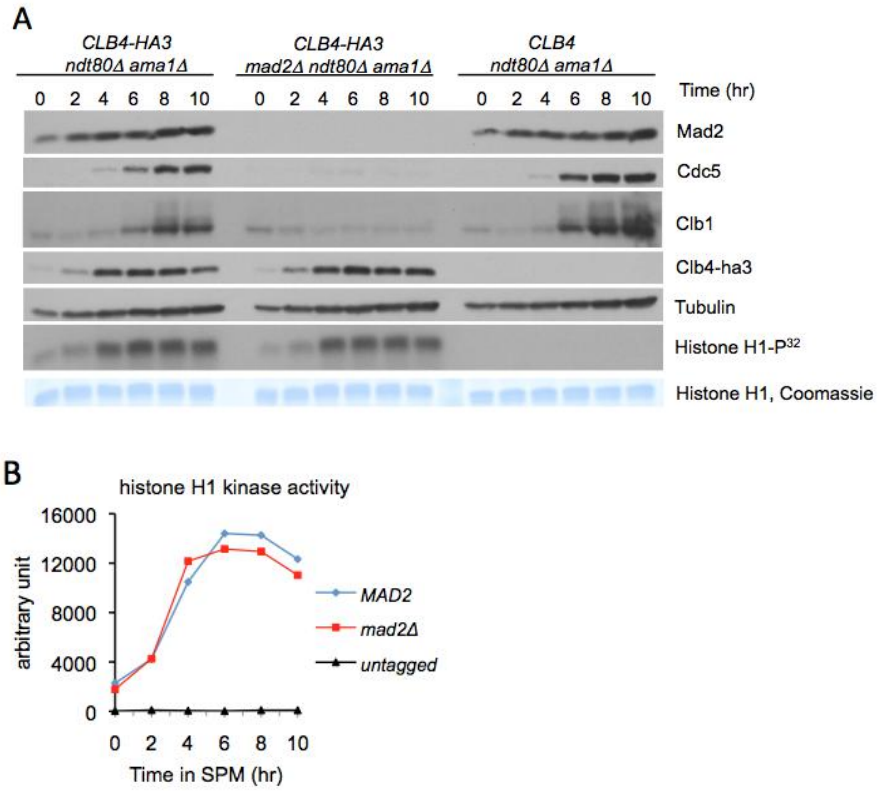

Figure 21. Deletion of *MAD2* does not reduce Clb4-Cdk1 kinase activity. Meiosis was induced in synchronized cultures of *ndt80*Δ *ama1*Δ *CLB4-HA3* (Z21872), *ndt80*Δ *ama1*Δ *mad2*Δ *CLB4-HA3* (Z21873) and *ndt80*Δ *ama1*Δ (Z20094) cells. After transfer to sporulation medium, samples for immunoprecipitation were collected every 2 hours. Immunoprecipitation was performed using an anti-HA antibody. Immunoprecipitates were then subject to radioactive kinase assay using recombinant histone H1 as substrate. Kinase activity was measured by autoradiography. Coomassie staining of histone protein and immunoblot detection of the whole cell extract are also shown.

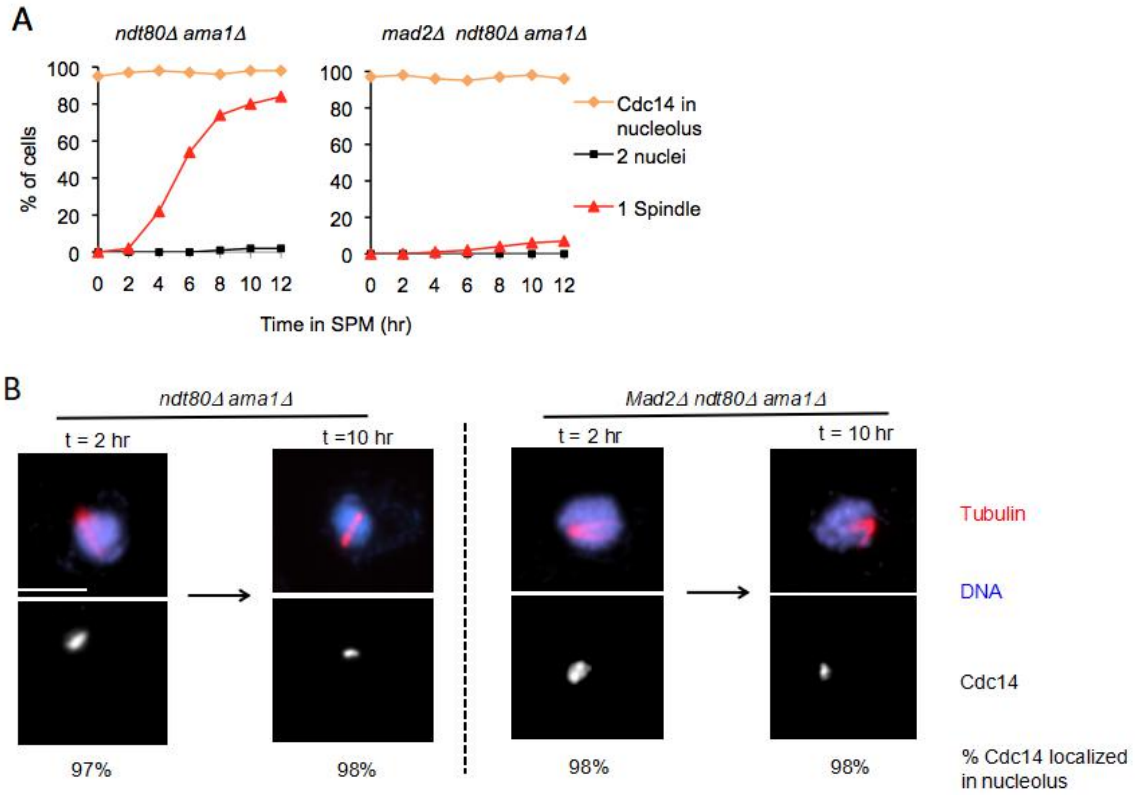

Figure 22. Deletion of *MAD2* does not affect Cdc14 localization in the nucleus. Meiosis was induced in synchronized cultures of *ndt80*Δ *ama1*Δ (Z20094) and *ndt80*Δ *ama1*Δ *mad2*Δ (Z20225) cells. After transfer to sporulation medium (SPM), samples for immunofluorescence were collected every 2 hours. (A) Quantification of meiotic progression by immunofluorescence detection of Cdc14, spindles (α-tubulin), and divided nuclei (DAPI) in fixed cells. Percentage of cells per time point is shown. (B) Representative immunostained cells of selected time point. Percentages of cells having nucleolar Cdc14 signal is shown. Scale bar, 5 μm.

2.4. The SAC is required for transcription of *CLB2*-cluster genes independent of APC/C activity

Steady state protein levels are determined by both proteolysis and gene expression. Our data showed that the SAC inactivation prevented the accumulation of *CLB2*-cluster proteins independent of APC/C-mediated proteolysis. Moreover, the defect was not due to reduced Cdk1-Clb activity. Therefore, we reasoned that Mad2 might directly regulate the Ndd1-dependent transcription of *CLB2*-cluster genes. To test this possibility, we took advantage of a mutant strain (*ama1Δ* P_{SCC1}*-cdc20*), in which *AMA1* was deleted and *CDC20* was depleted. These cells entered meiotic M phase by producing both Ndd1 and Ndt80 (Figures 23A and 24A). Because they have different target genes, one can distinguish the differential regulation of their activity. Consistent with our previous observation (Figures 18-21), deletion of *MAD2* or *MAD1* gene did not reduce Cdk1-Clb activity judged by spindle formation or Ndd1 phosphorylation (Figures 23 and 24). Therefore, the defects of spindle formation and Ndd1 phosphorylation in *ndt80Δ ama1Δ* cells were most likely indirect effects of SAC silencing. Strikingly, deleting *MAD2* gene or *MAD1* gene prevented the accumulation of Ndd1 target proteins Ace2-myc18, Swi5 and Clb2, whereas accumulation of Ndt80 target proteins Cdc5 and Clb1 and meiotic specific proteins Rec8 were not affected (Figures 23A and 24A). This suggests that SAC proteins Mad1 and Mad2 are specifically required for Ndd1 dependent expression of *CLB2*-cluster genes.

To confirm our hypothesis, we determined transcription level of *CLB2*-cluster genes by RT-qPCR (Figures 25 and 26). We observed that from 4 hours (metaphase), RNA level of *CLB2*-cluster genes *CDC5, CLB2, and SWI5* increased by 3 to 6 fold compared to the level in 0 hour (G1). On the contrary, RNA level of these metaphase genes remained low throughout the whole time course in *mad2*Δ cells (Figure 25). However, *PDS1* and *CLB4,* which are not *CLB2*-cluster genes, accumulated to comparable levels in both *MAD2* and *mad2*Δ cells. This showed Mad2 was indeed required for transcription of

CLB2-cluster genes. To confirm this observation, we checked transcription of *CLB2*-cluster genes in cells lacking another essential SAC gene *MAD1* (Figure 26). Although transcription of *CLB2*-cluster genes was strongly induced in *MAD1* cells, transcriptional up-regulation of these genes was completely lost in *mad1*Δ cells. This effect was specific for *CLB2*-cluster genes as transcription of the S-phase gene *PDS1* and the M-phase gene *CLB4* were comparable between *MAD1* and *mad1*Δ cells (Figure 26). Then we asked whether the down-regulation of gene expression of *CLB2*-cluster genes in SAC mutants was due to elevated APC/C activity. We checked whether transcription was still reduced in SAC mutant when APC/C activity was inhibited using the anchor-away technology as described previously (Figure 14). Inhibiting APC/C activity by anchoring away Apc2 (*apc2-AA*) allowed transcription of *CLB2*-cluster genes in prophase-arrested *ndt80*Δ cells (Figure 27). However, deletion of *MAD2* prevented the expression of these genes even when APC/C was inactivated in *apc2-AA* strain. Therefore, down-regulation of gene expression in *mad2*Δ cells was not due to APC/C activity. In summary, SAC is involved in regulating *CLB2*-cluster gene expression independent of its canonical function, which is to inhibit APC/C activity.

Mad2 was recently reported to directly associate with methylated H3K4 in *in-vitro* GST pull-down assay (Schibler et al., 2016). Since H3K4me is a marker of active gene transcription in all eukaryotes (Bernstein et al., 2002; Santos-Rosa et al., 2002; Schneider et al., 2004; Schubeler et al., 2004), it suggests that Mad2 could regulate gene expression by “reading” H3K4me marks on the histone. Therefore, we sought to investigate whether Mad2 associated with histone H3 in vivo in budding yeast cells. Indeed, we observed an interaction between H3 histone and Mad2 although the interaction was weak (Figure 28A). The interaction was regulated because histone H3 and Mad2 started to associate only from 3 hours onward although their protein levels were already high at the starting point of the experiment. Additionally, we observed that the SAC protein Mad1 also interacted with histone H3 and the interaction only appeared strongly from 6 hours (Figure

28B). After we showed that SAC proteins Mad2 and Mad1 associated with histone H3 in vivo, we asked whether binding to histone H3 was required for SAC proteins to regulate gene expression. Mad2 was reported to binds to histone H3 in a conformation-dependent manner. Specifically, C-Mad2 binds to histone H3 much better than O-Mad2 in *in-vitro* GST pull-down assay (Schibler et al., 2016). To confirm this observation in vivo, we took advantage of a Mad2 mutant that the c-terminal tail (6 residues in the c-terminus) was deleted. Since the C-terminal tail is required for Mad2 switching from open to closed form, *mad2-ΔC* proteins only adopt open conformation. Consistent with Schibler et. al, we observed that histone H3 interacted better with wild type Mad2 proteins, which adopt both closed and open conformations, than with *mad2-ΔC* proteins (Figure 29A). Additionally, the transcription of *CLB2*-cluster genes was completely blocked in the *mad2-ΔC* mutants (Figure 29B), suggesting that transcriptional regulation of Mad2 depended on its interaction with histone proteins. In summary, our experiments raise the possibility that the SAC regulates expression of Clb2-cluster genes by associating with histones.

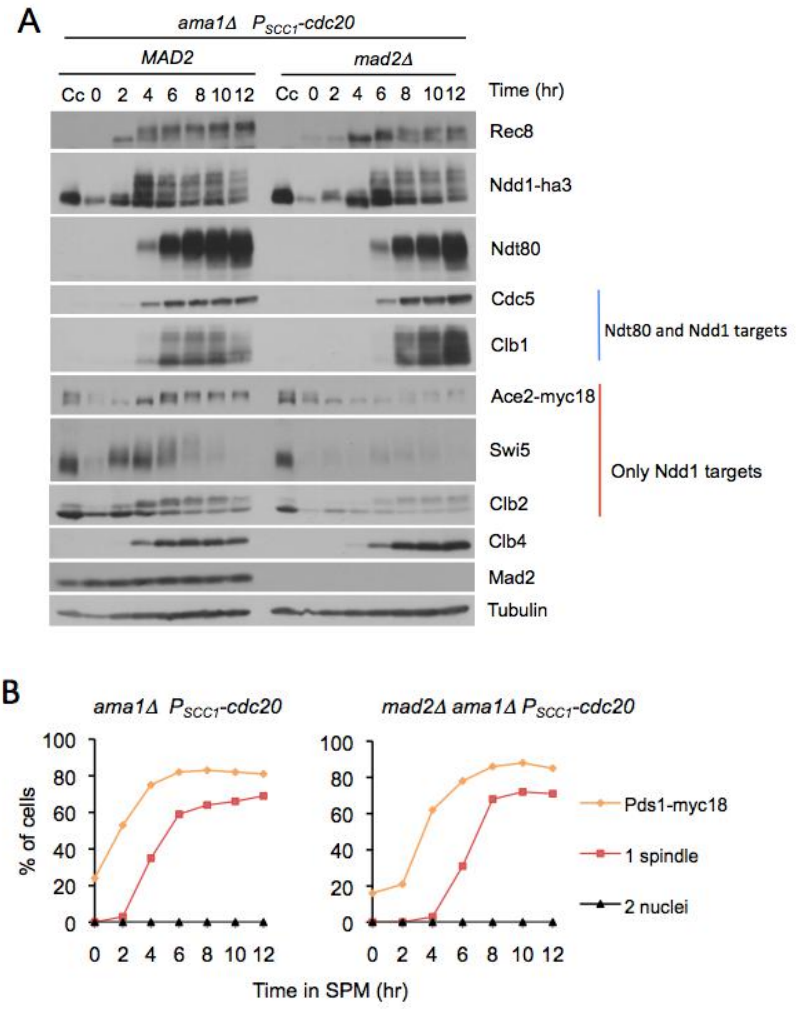

Figure 23. Mad2 is required for optimal expression of Ndd1 target genes in *ama1Δ PSCC1-CDC20* cells. Meiosis was induced in synchronized cultures of *ama1Δ PSCC1-CDC20* (Z28032) and *mad2Δ ama1Δ PSCC1-CDC20* (Z27796) cells. After transfer to SPM, samples for immunofluorescence and TCA protein extraction were collected every 2 hours. (A) Immunoblot detection of protein levels during the time course. Cc means samples from proliferating cells. (B) Quantification of meiotic progression by immunofluorescence detection of Pds1-myc18, spindles (α-tubulin), and divided nuclei (DAPI) in fixed cells. Percentage of cells per time point is shown.

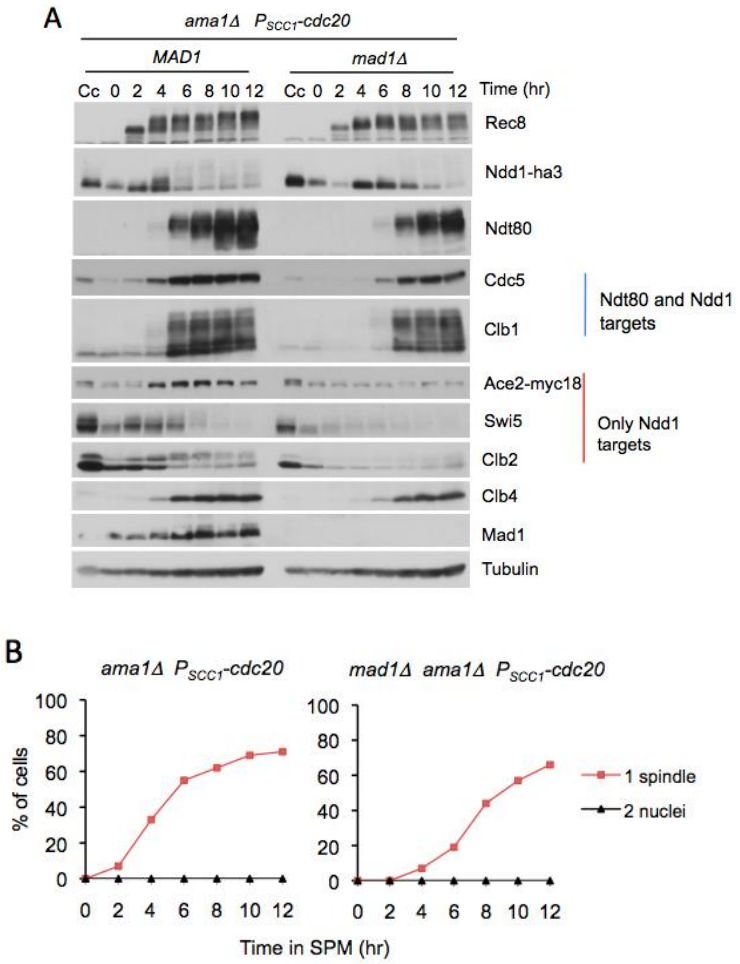

Figure 24. Mad1 is required for optimal expression of Ndd1 target genes in *ama1Δ PSCC1-CDC20* cells. Meiosis was induced in synchronized cultures of *ama1Δ PSCC1-CDC20* (Z29446) and *mad1Δ ama1Δ PSCC1-CDC20* (Z29447) cells. After transfer to SPM, samples for immunofluorescence and TCA protein extraction were collected every 2 hours. (A) Immunoblot detection of protein levels during the time course. Cc means samples from proliferating cells. (B) Quantification of meiotic progression by immunofluorescence detection of Pds1-myc18, spindles (α-tubulin), and divided nuclei (DAPI) in fixed cells. Percentage of cells per time point is shown.

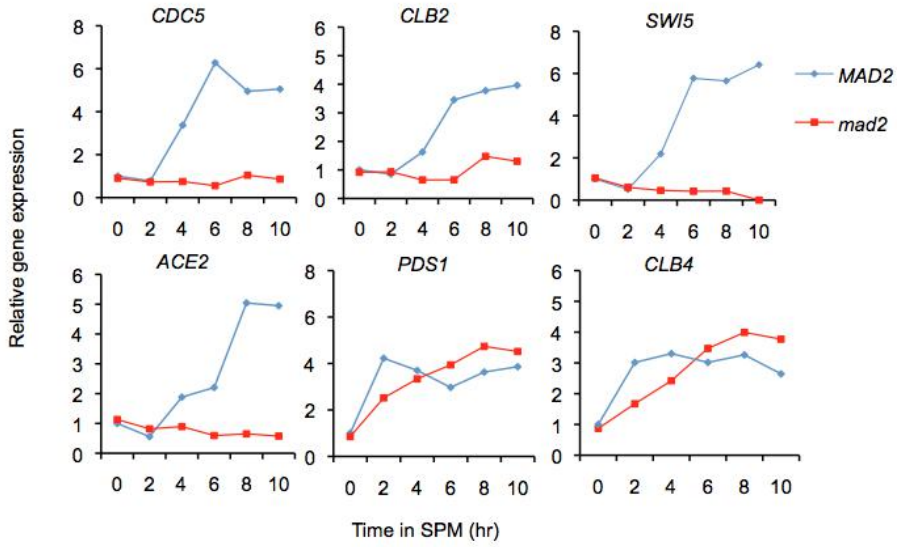

Figure 25. Deletion of *MAD2* reduces transcription of *CLB2*-cluster genes. Meiosis was induced in synchronized cultures of *ndt80*Δ *ama1*Δ (Z20094) and *ndt80*Δ *ama1*Δ *mad2*Δ (Z20225) cells. After transfer to SPM, samples for RNA extraction were collected every two hours. Total RNA was converted to cDNA by reverse transcription. Transcription levels of selected genes were measured by real-time PCR.

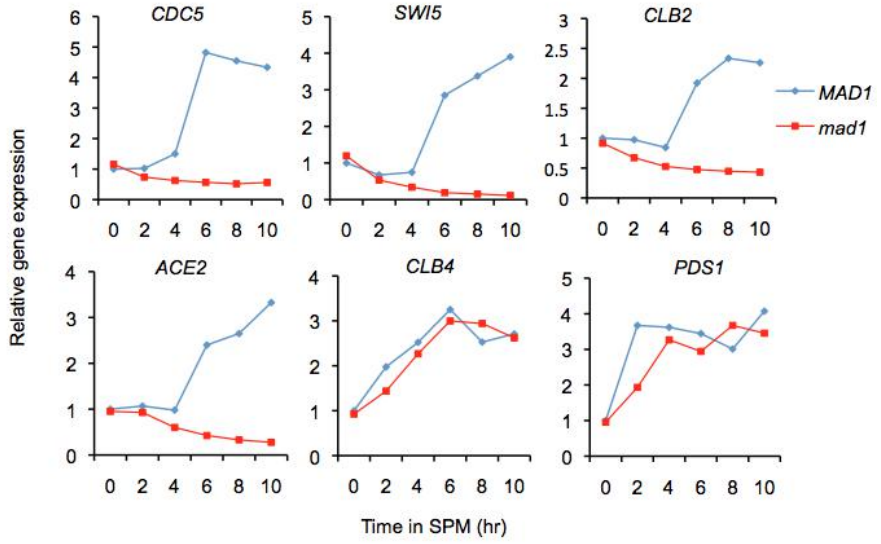

Figure 26. Deletion of *MAD1* reduces transcription of *CLB2*-cluster genes. Meiosis was induced in synchronized cultures of *ndt80*Δ *ama1*Δ (Z20094) and *ndt80*Δ *ama1*Δ *mad1*Δ (Z20488) cells. After transfer to sporulation medium (SPM), samples for RNA extraction were collected every two hours. Total RNA was converted to cDNA by reverse transcription. Transcription levels of selected genes were measured by real-time PCR.

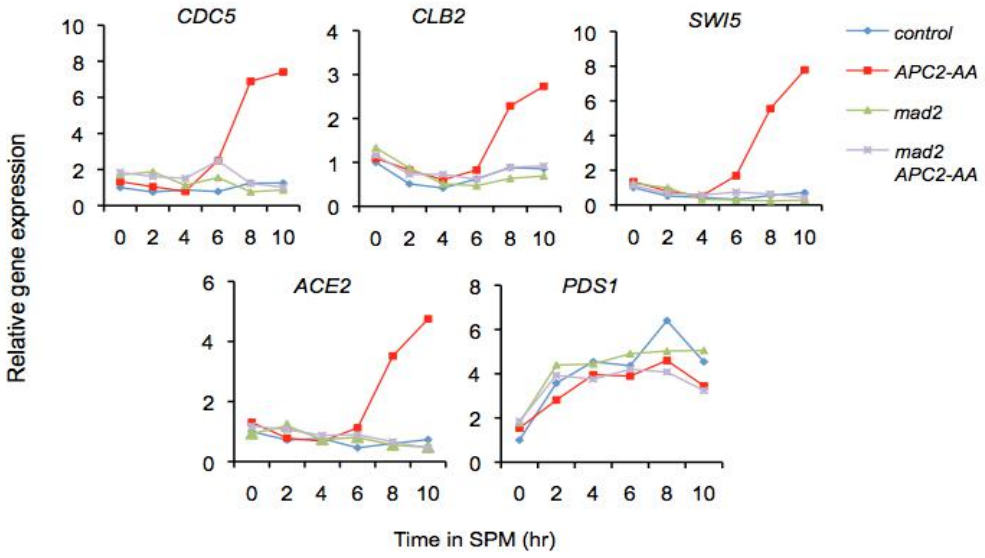

Figure 27. Deletion of *MAD2* reduces transcription of *CLB2*-cluster genes independent of APC/C activity. Meiosis was induced in synchronized cultures of *ndt80*Δ (Z25473), *ndt80*Δ *APC2-AA* (Z25475) mad2Δ *ndt80*Δ (Z25474) *and* mad2Δ *ndt80*Δ *APC2-AA* (Z25476) *c*ells. After transfer to SPM, samples for RNA extraction were collected every two hours. 10 μM of rapamycin was added to each cell culture to inhibit APC/C nuclear activity after 1 hour into meiosis. Total RNA was converted to cDNA by reverse transcription. Transcription levels of selected genes were measured by real-time PCR.

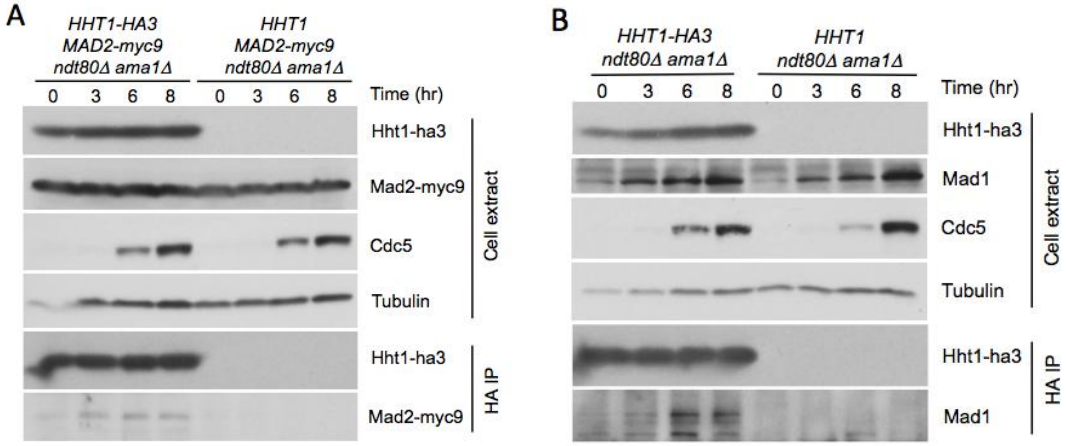

Figure 28. SAC proteins interact with histone H3. Meiosis was induced in synchronized cultures. After transfer to SPM, samples for immunoprecipitation were collected at indicated time points. Immunoblot detection of proteins from anti-HA immunoprecipites and whole cell extract are shown. (A) *ndt80*Δ *ama1*Δ *HHT1-HA3 MAD2-myc9* (Z29955) and *ndt80*Δ *ama1*Δ *MAD2-myc9* (Z29956) cells (B) *ndt80*Δ *ama1*Δ *HHT1-HA3* (Z29955) and *ndt80*Δ *ama1*Δ (Z29956) cells.

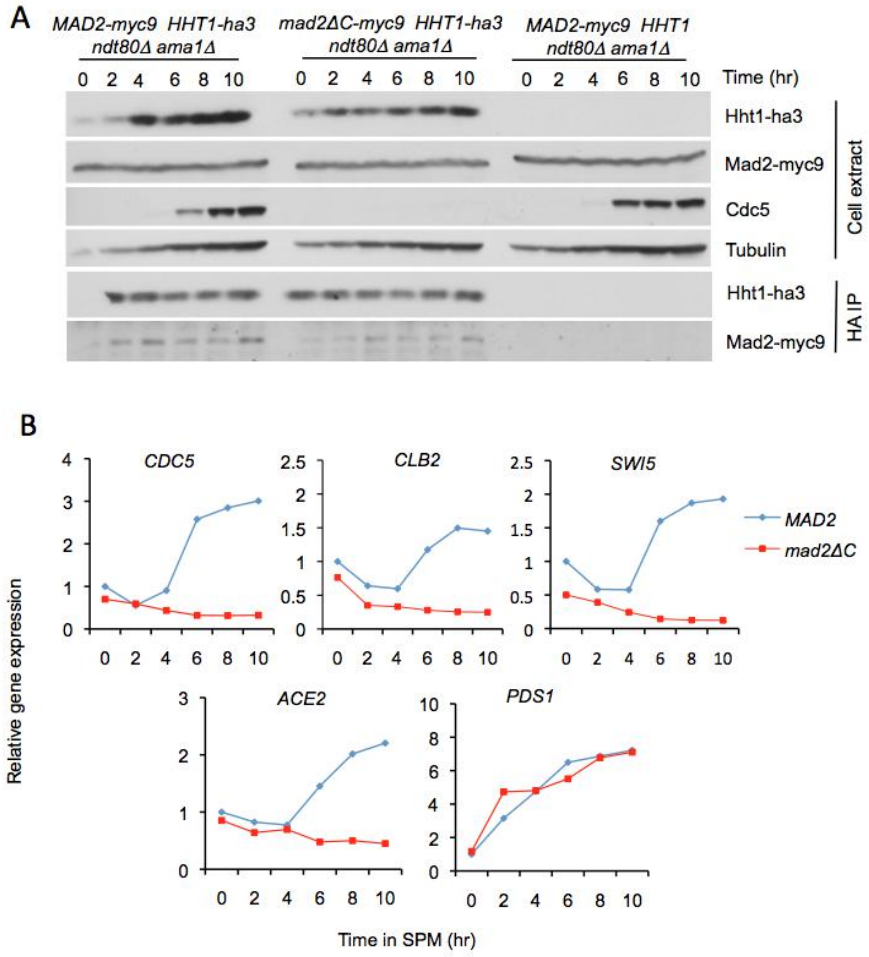

Figure 29. Deletion of Mad2 C-terminal tail reduces histone binding and transcription of *CLB2*-cluster genes. Meiosis was induced in synchronized cultures of *ndt80*Δ *ama1*Δ *HHT1-HA3 MAD2-myc9* (Z29955), *ndt80*Δ *ama1*Δ *HHT1-HA3 mad2-*Δ*C-myc9 (*Z30438*)* and *ndt80*Δ *ama1*Δ *MAD2-myc9* (Z29956) cells. After transfer to SPM, samples for immunoprecipitation and RNA extraction were collected every two hours. (A) Immunoblot detection of proteins from anti-HA immunoprecipites and whole cell extracts were shown. (B) Total RNA was converted to cDNA by reverse transcription. Transcription levels of selected genes were measured by real-time PCR.

3. Discussion

3.1. The SAC regulates transcription of key M phase genes

The *CLB2*-cluster is a group of important M phase genes including B-type cyclins (*CLB1* and *CLB2*), polo kinase *CDC5*, APC/C co-activator *CDC20*, and transcriptional factors *SWI5*, *ACE2* among others (Spellman et al., 1998). The transcription of *CLB2*-cluster is regulated by the transcriptional activator complex containing the MADS box protein Mcm1, the Forkhead box protein Fkh2 and the transcription coactivator Ndd1 (Haase and Wittenberg, 2014). The precise mechanism regulating the periodicity of *CLB2*-cluster genes in the cell cycle has been elusive. Recently, it has been shown that Cdk1-Clb- and Cdc5-dependent phosphorylation of Ndd1 is a critical step in the activation of the transcriptional activator complex during entry into M phase (Darieva et al., 2006; Darieva et al., 2003; Reynolds et al., 2003). The requirement for Ndd1 phosphorylation generates a positive feedback loop, wherein Clb2 and Cdc5, when expressed by Ndd1, feedback and activate their own gene expression by increasing Ndd1 phosphorylation. However, this poses a problem: if these two kinases are required to activate their own expression, it is unclear how this feedback loop is initiated. Epigenetic regulation might provide a clue. Sin3 transcriptional repressive complex and the chromatin remodeler Isw2 are shown to bind and repress *CLB2*-cluster promoters during interphase (Sherriff et al., 2007; Veis et al., 2007). S phase Cdk1 removes the Sin3 complex and Isw2 from *CLB2*-cluster genes during S phase, thus allowing basal transcription of *CLB2* and *CDC5* genes, which then might initiate the positive feedback loop. Our results provided another potential answer to this question. We showed that SAC proteins were required for the timely accumulation of *CLB2*-cluster proteins in M-phase. Deletion of individual SAC genes *MAD2, MAD1, MAD3, BUB1* or inhibiting the SAC kinase Mps1 reduced the accumulation of *CLB2*-cluster proteins at M-phase entry (Figures 6, 7, 8, 9, and 10). Surprisingly, however, the effect was

not due to elevated APC/C^{Cdc20} activity since inhibiting Cdc20 or APC/C activity did not allow re-accumulation of *CLB2*-cluster proteins in the absence of *MAD2* (Figures 11, 12, and 14). Our data showed that the SAC was required for transcription of *CLB2*-cluster genes, which has not been reported to date. Deletion of *MAD2* or *MAD1* completely prevented the accumulation of RNA transcripts of *CLB2*-cluster genes as determined by RT-qPCR (Figures 25, 26 and 27). Additionally, deleting the C-terminal tail of Mad2 (*mad2-ΔC*) also reduced *CLB2*-cluster gene transcription (Figures 29). Since C-terminal truncation locks Mad2 in its closed conformation (Luo et al., 2000; Luo et al., 2004; Sironi et al., 2001), this suggested that only the closed form of Mad2 was required for transcription. We also showed that Mad2 and Mad1 both interacted with histone H3 *in vivo* (Figures 28), consistent with previous *in vitro* studies (Schibler et al., 2016). We observed that the ability of Mad2 to interact with histone H3 was compromised by C-terminal truncation (Figure 29A), suggesting that the Mad2 conformational change was required for interaction with histone proteins. Expression of *CLB2*-cluster genes was reduced in the *mad-2ΔC* mutant, which suggested that the ability of Mad2 to regulate gene expression might depend on its association with histone H3. However, our data cannot yet answer how Mad2 specifically regulates *CLB2*-cluster genes given that histone proteins are ubiquitous on the genome. Without a DNA binding motif, Mad2 might need a sequence-specific DNA binding protein to execute its transcriptional function. It is known that the transcriptional factors Mcm1, Fkh2, and the transcriptional co-activator Ndd1 bind specifically to promoters of *CLB2*-cluster genes. Therefore, it is possible that Mad2 is recruited via interaction with one of these factors. However, so far we have not been able to detect interactions between Mad2 and Ndd1, Fkh2 or Mcm1 by immunoprecipitation (data not shown). It is possible that the immunoprecipitation method we have used is not optimal for capturing the transient interactions or another yet to be identified factor recruits Mad2 to *CLB2*-cluster gene promoters.

3.2. The SAC is a master regulator of M phase

The fidelity of chromosome segregation relies on stable chromosome-microtubule attachment, which allows all sister chromatids to align perfectly on the metaphase plate and coupling anaphase to kinetochore bipolar attachment, which ensures that chromosome segregation occurs only when all sister chromatids are attached to microtubules from the opposite spindle poles. Activated by unattached kinetochores, the spindle assembly checkpoint delays sister chromatids separation by inhibiting APC/C^{Cdc20} activity, which couples anaphase initiation to kinetochore attachment. Therefore, it is thought that SAC protects genomic integrity mainly by delaying chromosome segregation in the presence of unattached kinetochores. However, it is increasingly clear that SAC proteins also actively regulate microtubule-kinetochore attachment. For example, Mad2 regulates kinetochore-microtubule attachment by regulating Aurora B kinase localization (Kabeche and Compton, 2012; Shandilya et al., 2016). Mad2 depletion reduces kinetochore-microtubule attachment, conversely *MAD2* overexpression hyperstabilizes it independent of checkpoint function in human cells. Spindle formation is triggered by Clb-Cdk1 activity. Our results show that SAC is required for transcriptional upregulation of *CLB1* and *CLB2,* which encode M-phase cyclins that activate M-Cdk1 activity leading to spindle formation. Since SAC is known to stabilize Clb1 and Clb2 proteins by inhibiting APC/C -dependent degradation, our data and others show that the SAC regulates microtubule-kinetochore attachment by modulating expression and protein stability of M-phase cyclins.

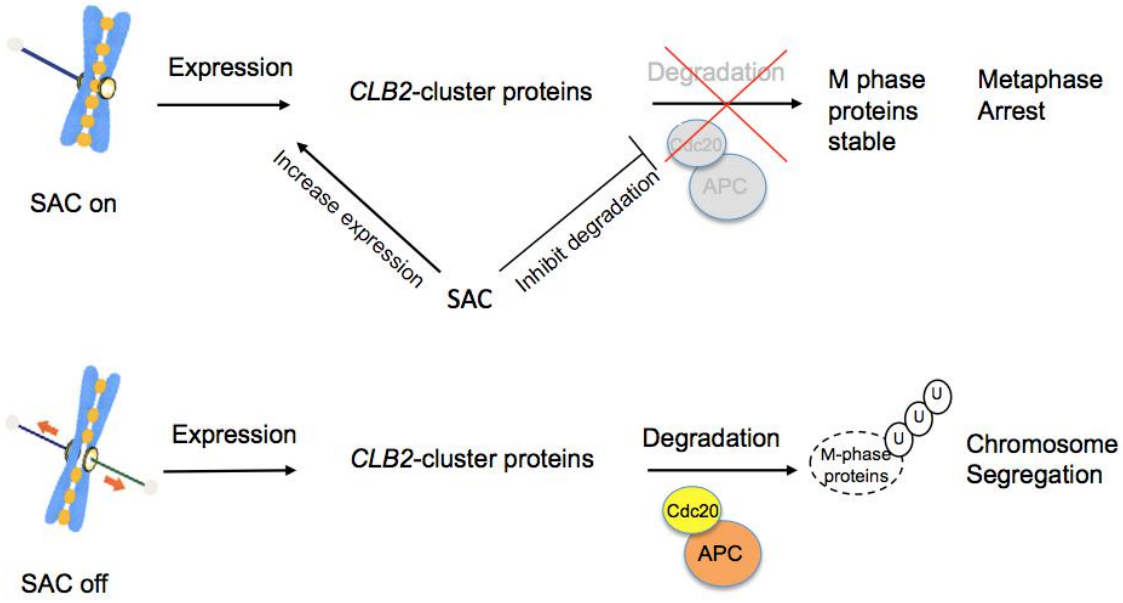

Figure 30. The SAC regulates metaphase to anaphase transition. When the SAC is on, SAC promotes accumulation of *CLB2*-cluster proteins by augmenting their transcription and shutting down the APC/C dependent protein degradation. Therefore, high level of B-type cyclins Clb1 and Clb2 induce cells in high kinase state, in which anaphase is delayed until all sister chromatids acquire bi-polar attachment. When the SAC is off, low level of *CLB2*-cluster proteins are produced to allow cells to enter metaphase. APC/C is subsequently activated to degrade *CLB2*-cluster proteins, which drives faithful chromosome segregation.

3.3. Cross-talk between the SAC and Cdk1

In response to chromosome-microtubule attachment errors, SAC stabilizes M phase cyclins by inhibiting APC/C dependent proteolysis. Consequently, cells are arrested in metaphase by reaching high kinase state. Now, our results provide another mechanism on how high kinase state is maintained during SAC activation. SAC activity is required for optimal gene expression of *CLB2*-cluster including M phase cyclins *CLB2* and *CLB1*. SAC silencing by deleting individual SAC genes or inhibiting the Mps1 kinase reduces the transcription levels of *CLB2*-cluster genes and spindle formation, which depends on Cdk1 activity. Therefore, the SAC maintains high kinase state not only by inhibiting protein degradation of M phase cyclins but also by promoting transcriptional activation of these genes. Recently, it is shown that the SAC and the Cdk1 are interdependent (Kamenz and Hauf, 2014; Rattani et al., 2014; Vazquez-Novelle et al., 2014). The dependence of the SAC on Cdk1 activity is required to solve the “anaphase problem”, which suggests when sister chromatids are splitted during anaphase, why the SAC is not re-activated despite the lack of chromosome-microtubule attachment (Kops, 2014). These studies show that inactivation of the Cdk1 is a critical step in taming the mitotic checkpoint in anaphase (Kamenz and Hauf, 2014; Rattani et al., 2014; Vazquez-Novelle et al., 2014). By using a non-degradable variant of the cyclin B in human cell, mouse oocyte and the fission yeast, they show that persistant Cdk1 activity results in unstable kinetochore-microtubule attachment and SAC re-engagement, as evidenced by the production of the SAC effector MCC complexes and APC/C inhibition. In summary, our data and others show that the SAC and Cdk1 are interdependent.

3.4. The HORMA domain acts as a versatile protein-protein interaction module.

The HORMA domain was identified through sequence comparison of three yeast proteins: **Ho**p1, **R**ev7, and **Ma**d2 (Aravind and Koonin, 1998). Hop1 is a protein involved in meiotic synaptonemal complex assembly (Hollingsworth et al., 1990). Rev7 is a subunit of DNA polymerase zeta (Nelson et al., 1996), which is involved in double-strand break repair. Mad2 is a SAC protein capable of inhibiting APC/C^{Cdc20} activity in the presence of unattached kinetochores. All of these three HORMA domain-containing proteins are involved in protein-protein interactions. Hop1 forms a complex with Red1 and Mek1, which is the component of the axial element protein cores in synaptonemal complexes. Rev7 interacts with Rev1 and Rev3 as a part of the repair-polymerase-zeta complex. Mad2 interacts with Mad1 and Cdc20 when SAC is activated. Moreover, Mad2 binds the insulin receptor to regulate metabolism homeostasis. Recently, it is reported that Mad2 interacts with histone H3 in an *in vitro* binding assay. Consistently, we observed that Mad2 indeed interacts with histone H3 *in vivo* (Figure 27A). Since Mad2 protein is almost entirely made up of HORMA domain, it is likely that HORMA domain mediates Mad2-histone interaction. Recently, several chromatin-associated proteins that contain HORMA domain in mammals including HORMAD1, HORMAD2, and Rev7 have been discovered (Rosenberg and Corbett, 2015). Therefore, the HORMA domain represents a new class of histone-binding domain. It not only mediates histone interaction, but also mediates other protein-protein interactions. For example, two autophagy-signaling proteins Atg13 and Atg101 also possess HORMA domains, which mediate interaction with PI3K kinase and Atg13, respectively (Jao et al., 2013; Suzuki et al., 2015). In all of these signaling pathways, the function of HORMA domain is highly conserved, which acts as a signal responsive adaptor protein mediating protein-protein interactions.

Genes	Species	Essential Functions	Refs
ScHop1	*S. cerevisiae*	Meiotic recombination	(Hollingsworth et al., 1990)
ScMad2	*S. cerevisiae*	SAC	(Li and Murray, 1991)
ScRev7	*S. cerevisiae*	DNA damage repair	(Nelson et al., 1996)
hMad2A	*Homo sapiens*	SAC	(Luo et al., 2000)
hMad2B	*Homo sapiens*	SAC	(Chen and Fang, 2001; Pfleger et al., 2001)
ScAtg13	*S. cerevisiae*	Autophagy kinase	(Reggiori et al., 2004)
hHORMAD1	*Homo sapiens*	Homologue alignment and synaptonemal-complex formation	(Daniel et al., 2011)
hHORMAD2	*Homo sapiens*	Meiotic DSBs repair	(Wojtasz et al., 2012)

Table 3. HORMA-domain containing proteins

3.5. The function of the SAC beyond inhibiting APC/C^{Cdc20} activity

It's becoming increasingly clear that the SAC regulates a variety of cell functions in addition to inhibiting APC/C^{Cdc20} activity. It has been shwon that the SAC can regulate nuclear transport and DNA damage response (DDR). For example, the SAC and the DDR have intensive cross talks. It was shown that the important DDR regulators Chk1 kinase and the Fanconi anemia protein FANCA are required for optimal SAC signalling (Eliezer et al., 2014; Nalepa et al., 2013; Zachos et al., 2007). DNA damaging agents even induce SAC activation in a ATM and ATR kinases-dependent machanism (Kim and Burke, 2008). SAC proteins are also required for proper DDR. A single double strand break (DSB) not only trigers DDR but also SAC signalling, and Mad2 is required for the prolonged arrest induced by DSB (Dotiwala et al., 2010). In mammalian oocytes, DNA damage also induces meiotic arrest by activating the SAC (Collins et al., 2015; Marangos et al., 2015). Therefore, the DDR and the SAC function in synergy to protect genomic integrity. Nuclear pore complexes (NPC) provide selective barriers for the trafficking of macromolecules between the nucleus and the cytoplasm (Wente and Rout, 2010). It is thought that SAC proteins only localize to kinetochore during SAC activation. However, the SAC proteins Mad1 and Mad2 were shown to associate with NPC during interphase (Campbell et al., 2001; Iouk et al., 2002). Additionally, NPCs in interphase cells also function as scaffolds for generating APC/C inhibiting Mad1/Mad2 complexes (Lee et al., 2008; Rodriguez-Bravo et al., 2014). Therefore, both kinetochores and NPC emit "wait anaphase" signals that preserve genomic integrity. Interestingly, the SAC protein Mad1 can also regulate nuclear transport (Cairo et al., 2013b). Cairo et al. showed that kinetochore-microtubule detachment arrests nuclear import mediated by the transport factor Kap121 through a mechanism that requires Mad1 cycling between unattched metaphase kinetochores and binding sites at the NPC (Cairo et al., 2013a). Results described in this thesis have discovered, for the first time, that the SAC also regulates transcription of a group of key mitotic genes, the *CLB2*-cluster. In summary, the SAC regulates diverse cell

functions, ranging from transcriptional regulation to DNA damage response and nuclear transport, in addition to its APC/C inhibiting function.

3.6. The SAC, aneuploidy, and tumorigenesis

Aneuploidy, a condition characterized by gain or loss of chromosomes, is a hallmark of human cancer (Fang and Zhang, 2011). Aneuploidy can arise from missegregation of sister chromatids during mitosis. In normal cells, the SAC prevents chromosomal instability. Complete abrogation of the SAC function is lethal to normal cells due to massive chromosome mis-segregations. It is postulated that a weakened SAC function may underlie many human cancers (Silva et al., 2011). Indeed, mutations of certain SAC genes have been identified in many types of cancer (Cahill et al., 1998; Ohshima et al., 2000; Ru et al., 2002). Consistently, haploinsufficiency of the SAC genes *MAD2, MAD1, BUBR1, or BUB1* leads to a high incidence of aneuploidy and tumors in mice (Baker et al., 2009; Dai et al., 2004; Iwanaga et al., 2007; Michel et al., 2001). Interestingly, not only reduced expression of SAC genes leads to cancer, but also elevated level of *MAD2* gene expression promotes tumorigenesis in a p53 mutant mouse model (Schvartzman et al., 2011; Sotillo et al., 2007), highlighting the intricate requirement of SAC activity in genetic stability and carcinogenesis. Constitutive activation and deregulation of CDK activity has been associated with human cancer (Malumbres and Barbacid, 2009). Our finding that the SAC positively regulate transcription of M phase cyclin genes *CLB1* and *CLB2* thus provides a potential explanation for tumorigenesis induced by SAC deregulation. When expression of SAC genes increases, Cdk1 is more active than normal due to high expression of M phase cyclins, which leads to unrestrained growth of tumor cells. To the contrary, when SAC is not functional, CDK activity is low due to low expression of M phase cyclins, which leads to premature chromosome segregation, aneuploidy and cancer.

3.7. Why do meiotic cells lacking *AMA1* and *NDT80* fail to initiate anaphase?

Although entry into M phase is universally driven by cyclin-dependent kinase 1 bound to cyclin B (Cdk1-Clb), the timing of this event differs dramatically in mitosis and meiosis. Mitotic cells activate Cdk1-Clb shortly after S phase, whereas meiotic cells activate Cdk1-Clb after a long prophase during which homologous chromosomes undergo recombination. Actually, mitotic and meiotic M phase in yeast are initiated by different transcription factors. Mitotic cells enter M phase by activating the transcriptional factor Ndd1 (Loy et al., 1999), whereas meiotic cells enter M phase by activating Ndt80, a meiosis specific transcriptional factor that triggers spindle formation by promoting the accumulation of M phase cyclins Clb1 and Clb4 and the polo kinase Cdc5 (Chu and Herskowitz, 1998; Sourirajan and Lichten, 2008). Abrupt accumulation of Ndt80 at exit from prophase I is regulated by two positive feedback loops; the *NDT80* gene is activated by itself and is repressed by Sum1, which is inhibited, in turn, by the Cdk1 activity that results from Ndt80's appearance (Pak and Segall, 2002; Shin et al., 2010). In the presence of double strand breaks, however, accumulation of Ndt80 is blocked by the meiotic recombination checkpoint (RC) (Tung et al., 2000). Since mitotic and meiotic factor can both trigger entry into M phase, how activation of mitotic factor Ndd1 is prevented in meiosis has been elusive. Recently, Okaz at al., show that APC/C^{Ama1} suppresses mitotic cell-cycle controls during prophase I by triggering the degradation of Ndd1, M phase cyclins, and Cdc5 (Okaz et al., 2012). This is essential for an extended prophase I that is controlled by the RC and for proper homolog segregation at meiosis I. Mathematical modeling also supports a crucial role for APC/C^{Ama1} in the irreversible transition from prophase I to metaphase I (Okaz et al., 2012). Interestingly, meiotic cells lacking both *AMA1* and *NDT80* enter meiosis I by triggering the mitotic factor Ndd1. *ndt80Δ ama1Δ* cells produce Clb4, Clb1 and Clb2 in two waves, which resembles mitotic cells (Fitch et al., 1992; Okaz et al., 2012; Richardson et al., 1992). Interestingly, however, although meiotic *ndt80*Δ

*ama1*Δ cells enter high kinase state, these cells fail to disassemble spindles, degrade Pds1 and M phase proteins and fail to undergo nuclear division (Okaz et al., 2012). We show that the failure to initiate anaphase is not because of the SAC. Inactivation of the SAC by deleting the individual SAC genes or inhibiting the SAC kinase Mps1 does not allow *ndt80*Δ *ama1*Δ cells to enter anaphase (Figures 6, 7, 8, 9, and 10). It has been shown that the kinase Swe1 regulates metaphase-anaphase transition by inhibiting Cdk1 dependent phosphorylation and activation of APC/C^{Cdc20} (Lianga et al., 2013). Future work should address whether deleting *SWE1* allows anaphase in *ndt80*Δ *ama1*Δ cells.

4. Material and methods

4.1. Yeast strains

All experiments were performed with diploid cells of the fast-sporulating SK1 background of *Saccharomyces cerevisiae* strains (*ho::LYS2 lys2 ade2Δ::hisG trp1::hisG leu2::hisG his3Δ::hisG ura3*)(Kane and Roth, 1974). Table 1 describes in detail the genotypes of strains used in this work. Diploid strains were made by mating of the corresponding haploid cells. Mutations in diploid strains are homozygous unless stated otherwise. The following mutations have been described previously: *CDC20* under the control of the mitotic *SCC1* promoter, *ndt80Δ::HIS3* (Okaz et al., 2012), the mutations *PDS1myc18::KlTRP1* and *ama1Δ::NatMX4* (Oelschlaegel et al., 2005), *cdc14-3* (Visintin et al., 1998), *cdc20-3* (Shirayama et al. 1998), *mps1-as1* (Jones et al., 2005), *tor1-1 fpr1Δ RPL13A-FKBP12* (Haruki et al., 2008).

4.2. Construction of yeast strains

Strains that contain C-terminal HA3, HA6, MYC9, MYC18 or FRB tagged proteins were produced by one-step PCR mediated epitope tagging (Haruki et al., 2008; Knop et al., 1999). The deletions of genes *MAD2, MAD1, MAD3, and BUB1* were obtained by one-step gene replacement by amplifying by PCR the appropriate antibiotic resistance cassette conferring resistance to the kanamycin derivative G418, nourseothricin, or hygromycin B (Goldstein and McCusker, 1999).

4.3. Time course experiment of synchronous meiosis

Meiotic time courses were prepared and carried out at 30 °C unless stated otherwise (Oelschlaegel et al., 2005). Healthy zygotes obtained with the appropriate haploid strains were streaked to single colonies on glycerol plates (YPG). Single colonies were picked after 30 hours and transferred to yeast

extract peptone dextrose (YPD) plates, making a patch of 2 cm^2. After a period of no longer than 23 hours, the resulting patch was plated to an approximately one-cell thick homogeneous lawn on YPD plates with a dry, smooth surface. Simultaneously, a loop-full of the patch was transferred to solid sporulation medium (SPM, 2% K-acetate). After a period of no longer than 23 hours, the meiotic proficiency of the diploids on the sporulation plate was evaluated by looking at the cells under a phase-contrast microscope. The best diploids were then inoculated into 250 ml of YEPA medium (YP plus 2% K-acetate) in 2.8 L flasks to an OD_{600} ~0.3. The cultures were shaken at 200 rpm for 11-12 hours at 30 °C in an orbital shaker. At the end of this period, the OD_{600} reached 1.5-1.7 and cells arrested in G1, with less than 15% of budded cells. The cultures were then concentrated by centrifugation at 3500 rpm for 3 min, washed once with 150 ml of sporulation medium, centrifugated one more time, and finally resuspended in 100 ml of SPM, resulting in a final OD_{600} of 3.0 - 3.5.

In time courses, including *mps1-as1* strains, the inhibitor 1NM-PP1 (Jones et al., 2005), Cayman Chemicals) was added to a final concentration of 10 μM from a stock solution of 5 mM in DMSO, stored at -20°C until use. For measuring the half-life of proteins, cycloheximide was added to meiotic cultures to a final concentration of 500 μg/ml from a stock solution of 10 mg/ml in DMSO.

4.4. Gene expression analysis by real-time PCR

1ml of meiotic cell culture was used for isolation of total RNA with Qiagen RNeasy kit according to the protocol of the manufacturer. RNA was eluted from column with 70 μl of RNase-free water. The typical yield was 5-20 μg of total RNA. RNA concentration was measured on a ThermoFisher nanodrop instrument. RNA quality was determined on Agilent Bioanalyzer 2100 with RNA 6000 Nano kit. 1 μg of total RNA was reverse transcribed into cDNA (SuperScript III first strand synthesis SuperMix) as described by the protocol. cDNA product (20 μl) was diluted 5 fold with PCR grade water to be compatible with real-time PCR. Real-time PCR was carried out with 4 μl of diluted cDNA in 20 μl reaction

on Applied Biosystem StepOne instrument using SYBR green dye as detection method (Applied Biosystem Power SYBR green master mix). For amplification of *TFC1* and *ACT1*, primers were used at 0.2 µM, whereas for other genes, primers were used at 0.5 µM. Negative control (NC) reactions lacking cDNA template were included to monitor non-specific amplification by primers. Melting curve analysis was always included to monitor amplicon size. Gene expression was quantified by comparative $2^{-\Delta C_T}$ method using *TFC1* or *ACT1* as internal reference gene. Table 2 lists all primer sequences used for RT-qPCR.

4.5. Preparation and separation of protein sample by SDS-PAGE

9 ml of meiotic culture were centrifugated at 4000 rpm for 2 min, resuspended in 1 ml of 10% TCA, and transferred to a 1.5 ml safe-lock Eppendorf tube and centrifuged again at 8000 rpm for 2 min at 4 °C. The pellets were snap-frozen in liquid nitrogen and then stored at -80 °C. For breakage, pellets were thawed on ice, inside a 4 °C cold room. 200 µl of glass beads (diameter = 0.5 mm) and 400 µl of 10 % TCA were added, and the samples were mechanically disrupted by shaking them on a bead beater set at maximal speed for 30 min. The resulting supernatant was transferred to a fresh safelock Eppendorf tube and spun at 3000 rpm at 4 °C for 10 min. The acidic pellets were thoroughly resuspended in 200 µl of 2X concentrated Laemmli buffer with freshly added β–mercaptoethanol (62.5 mM Tris-HCl pH 6.8, 10 % glycerol, 2 % SDS, 0.01 % bromophenol blue, 0.4 M β–mercaptoethanol) and then neutralized with 100 µl of 1M Tris Base. Samples were mixed thoroughly, boiled for 10 min at 95 °C, and finally spun for 10 min at 13000 rpm. Protein concentration in the extracts was measured with the Bradford protein assay (BioRad) and 60 µg of total protein were loaded on SDS-8% polyacrylamide gels.

4.6. Western blotting and immunodetection of protein

Semidry western blotting (0.45 mA/cm^2 for 1 hr) was used to transfer proteins to a PVDF membrane (Immobilon P, Millipore). Membranes were then blocked for 1

hour in PBS buffer containing 0.1% Tween 20 (PBS-T) and 4% non-fat milk powder. Primary antibodies were incubated for 1 hour at room temperature. After three washes with PBS-T, the membrane was incubated for 1 hour, or overnight at 4°C, with secondary antibodies conjugated to horseradish peroxidase. After three washes with PBS-T, the membranes were incubated for 20 s with ECL solution (ECL detection system, GE Healthcare) and were developed on a Kodak X-omat machine. Mouse monoclonal antibodies 12CA5 (1:500, Zachariae lab) and 9E10 (1:200, Zachariae lab) were used for the detection of HA and Myc tagged proteins,

respectively. Rabbit polyclonal antibodies were used for the detection of: Ama1 1:5000 (Oelschlaegel et al., 2005), Cdh1 (1:5000, Zachariae lab), Cdc5 1:5000 (Matos et al., 2008), Cdc20 1:2000 (Camasses et al., 2003), Clb2 1:2000 (Okaz et al., 2012), Ndt80 1:10000 (Pierce et al., 2003), Rec8 1:5000 (Petronczki et al., 2006), Tub2 1:20000 (a gift from Wolfgang Seufert, University of Regensburg, Germany), Mad3 1:1000 (a gift from Kevin Hardwick), Sic1 1:600 (Santa Cruz sc-50441), Cdc28 1:5000 (Zachariae lab), phospho-cdc2 Thr161 1:1000 (Novus NB100-81837), phospho-cdc2 Tyr15 1:1000 (Cell signalling 4539), Spo13 1:5000 (Matos et al., 2008), mTOR (human FRB domain) 1:2000 (Enzo life sciences ALX-215-065-1), Cin8 1:1000 (a gift from Tony Hyman), Kip1 1:2000 (a gift from Tony Hyman), Mad1 1:200 (ACRIS BP4531) and Dbf4 1:5000 (Matos et al., 2008), Goat polyclonal antibodies were used for the detection of: Clb1 1:300 (Santa Cruz sc-7647), Clb4 1:400 (Santa Cruz sc-6702), Clb5 1:100 (Santa Cruz sc-6704), Ime2 1:100 (Santa Cruz sc-26444), Cdc14 1:1000 (sc-12045 Santa Cruz), Mad2 1:200 (Santa Cruz sc-6331), Fkbp12 1:200 (Santa Cruz sc-6174), Swi5 1:500 (Santa Cruz sc-15545), Swe1 1:500 (Santa Cruz sc-7171).

4.7. Analysis of protein interaction by immunoprecipitation

30 ml of meiotic cell culture were used for immunoprecipitation. Sample were collected and washed with 20 ml cold water plus 2 mM PMSF/DMSO. Cell pellets were resuspended with 0.4 ml of cold breakage buffer (50mM HEPES/KOH

pH7.4, 70 mM KOAc, 5 mM MgOAC, 0.1% Triton X100, 10% glycerol, freshly added 1mM DTT, 20 mM beta-glycerophosphate, Roche EDTA-free protease inhibitor and PhoSTOP phosphatase inhibitor cocktail) and 0.4 ml of cold glass beads. To break cells, samples were vibraxed for 4 min for 4 times on a bead-beater with 4 min cooling on water-ice between vibrax runs. After lysis, whole cell extracts were cleared by centrifugation for 30 min at maximum speed (14000 rpm). After centrifugation, cell extracts were incubated with 150 µl protein-A agarose beads on a rotator for 30 min to eliminate non-specific interaction. Then, equal amount of protein extracts (10 mg for example) from each samples were incubated with 20 µl of concentrated 9E10 anti-myc or 12CA5 anti-HA on a rotator for 1 hour. 60 µl of BSA-blocked protein-A agarose beads were then added to capture the immune complex for 1 hour. Beads were washed sequentially with 1 ml of the following buffer for 5 min: twice of B70-BSA, once of B150, once of B200, twice of B70. Immune complexes were separated from the beads by boiling at 95 °C for 5 min. Immunoprecipitates were then seperated on a 1 mm acrylamide gel.

4.8. Histone H1 kinase assay

Cdc28-Clb4-ha3 complexes were prepared by anti-HA immunoprecipitation from meiotic cell culture as described in previous method. Kinase assay were carried out in kinase reaction mix containing histone H1, “cold” ATP, γ-^{32}P-ATP (Perkin Elmer) and purified Cdk1 complexes for 10 mins at 30 °C. Reactions were terminated by adding 25 µl of 3 X Laemmli sample buffer and heating for 5 min. Samples were seperated on an acrylamide gel. After staining, fixation and drying, protein gels were exposed to x-ray films, which were then developed on Kodak film developer instrument. Kinase activity was quantified digitally by imageJ software.

4.9. Indirect immunofluorescence

Indirect immunofluorescence was performed with cells fixed overnight at 4 °C in

3.7% formaldehyde. Samples were then washed twice with 1 ml of 0.1 M potassium phosphate buffer pH 6.4, one time with 1 ml spheroplasting buffer (0.1 M potassium phosphate buffer pH 7.4, 1.2 M sorbitol, 0.5 mM magnesium chloride) and finally resuspended in 200 µl of spheroplasting buffer. 6 µl of a freshly prepared 10 % solution of β–mercaptoethanol were added to each sample. After incubation at 30 °C for 15 min, samples were incubated with 10 µl of zymolyase solution (Zymolyase 100T from Amsbio, 1 mg/ml in spheroplasting buffer) for around 10 min, and then, the refractivity of the cells was assessed at the phase contrast microscope. When about 75% of the fixed cells looked as a dark rounded mesh with fuzzy edges, adding 1 ml of cold spheroplasting buffer stopped digestion. After gentle centrifugation, the spheroplasts were resuspended in 200 µl of spheroplasting buffer. 5 µl of spheroplasts per time point were loaded on a polylysine-covered 15-well slide. Spheroblasts were allowed to adhere to the surface for 5 min, the excess volume was aspirated and the cells were dehydrated by incubating the slides 3 min in methanol and 10 s in acetone, both at -20 °C. The slides were rehydrated by incubating with 5 µl of PBS per well, and then blocked with PBS containing 1 % bovine serum albumin (PBS-BSA). Slides were incubated with primary antibodies for 1 hour. Slides were washed four times with PBS-BSA for 5 min. Secondary antibodies were incubated for one hour and after four washes with PBS-BSA, the wells were covered with 4 µl of 4',6-diamidino-2-phenylindole (DAPI) to stain DNA, and the slides were sealed with coverslip.

The following primary antibodies were used: monoclonal mouse anti-Myc 9E10 (1:5, Zachariae lab), monoclonal rat anti-tubulin YOL 1/34 (1:300, Serotec), polyclonal rabbit anti-Myc (1:300, Gramsch CM-100). Secondary fluorophore-labeled antibodies were goat anti-mouse CY3 (1:400, Jackson ImmunoResearch), goat anti-rat Alexa 488 (1:300, Jackson ImmunoResearch), goat anti-rat CY3 (1:400, Jackson ImmunoResearch), goat anti-Rabbit Alexa 488 (1:200, Chemicon).

Cells were scored as Pds1myc18-positive when clear, bright nuclear staining

was observed. The first nuclear division was counted when cells produced two distinguishable masses of DNA. The second nuclear division was scored when cells presented 4 masses of DNA. Cell counting was done on an Zeiss Axioskop 2 epifluorescence microscope. A 100x α-Plan-Fluar 1.40 NA oil immersion was used as objective lens (Carl Zeiss). 100 cells per time point were counted. A SPOT RT210 CCD camera (Diagnostic Instruments) controlled by Quick Capture software was used to take the pictures and Adobe Photoshop was used to process them into images.

4.10. Abbreviations

as - analog-sensitive

APC/C - anaphase-promoting complex/cyclosome

BSA - bovine serum albumin

CDK1 - cyclin-dependent kinase 1

CHX - cycloheximide

DAPI - 4’,6-diamidino-2-phenylindole

DMSO - dimethyl sulfoxide

DNA - deoxyribonucleic acid

DSB - double-strand break

M – molar

MDa – Megadalton

NA - numerical aperture

NCO - non-crossover

OD - optical density

PAGE - polyacrylamide gel electrophoresis

PCR - polymerase chain reaction

MeiRC - meiotic recombination checkpoint

S – Svedberg

SAC - spindle assembly checkpoint

SC - synaptonemal complex

SCF - Skp1-cullin-F-box protein family of ubiquitin ligases

SDS - sodium dodecylsulfate

SPM - sporulation medium

TCA - trichloroacetic acid

YEPA - yeast peptone medium plus 2% K-acetate

YPD - yeast peptone dextrose medium

Table 4. List of *Saccharomyces cerevisiae* SK1 strains used in this work

Strain[1]	Genotype[2]
Z19545	*MATa/MATalpha ndt80Δ::HIS3 ama1Δ:: NatMX4*
Z20094	*MATa/MATalpha ndt80Δ::NatMX4 ama1Δ::KanMX4 PDS1myc18::KlTRP1*
Z20225	*MATa/MATalpha mad2Δ::KlURA3 ndt80Δ::NatMX4 ama1Δ::KanMX4 PDS1myc18::KlTRP1*
Z20488	*MATa/MATalpha mad1Δ::KlURA3 ndt80Δ::NatMX4 ama1Δ::KanMX4 PDS1myc18::KlTRP1*
Z20818	*MATa/MATalpha mad3Δ::KlTRP1 ndt80Δ::NatMX4 ama1Δ::KanMX4 PDS1myc18::KlTRP1*
Z20948	*MATa/MATalpha ndt80Δ::HIS3 ama1Δ::NatMX4 mps1::KanMX4::mps1-as1-myc10-TRP1*
Z21076	*MATa/MATalpha cdc20::hsl1p-CDC20-HphMX4 mad2Δ::KlURA3 ndt80Δ::NatMX4 ama1Δ::KanMX4 PDS1myc18::KlTRP1*
Z21099	*MATa/MATalpha leu2::DMC1p-CLB1ha6-LEU2 ndt80Δ::NatMX4 ama1Δ::KanMX4 PDS1myc18::KlTRP1*
Z21100	*MATa/MATalpha mad2Δ::KlURA3 leu2::DMC1p-CLB1ha6-LEU2 ndt80Δ::NatMX4 ama1Δ::KanMX4 PDS1myc18::KlTRP1*
Z21101	*MATa/MATalpha ura3::DMC1p-CLB2ha3-URA3 ndt80Δ::HIS3 ama1Δ::NatMX4 PDS1myc18::KlTRP1*
Z21102	*MATa/MATalpha mad2Δ::KlURA3 ura3::DMC1p-CLB2ha3-URA3 ndt80Δ::HIS3 ama1Δ::NatMX4 PDS1myc18::KlTRP1*
Z21330	*MATa/MATalpha mad2Δ::KlURA3 ura3::DMC1p-clb2-dkbm-ha3-URA3 ndt80Δ::HIS3 ama1Δ::NatMX4 PDS1myc18::KlTRP1*
Z21331	*MATa/MATalpha ura3::DMC1p-clb2-dkbm-ha3-URA3 ndt80Δ::HIS3 ama1Δ::NatMX4 PDS1myc18::KlTRP1*

Z21470	*MATa/MATalpha ndt80Δ::NatMX4 ama1Δ::KanMX4 FKH2myc9::KlTRP1 NDD1-HA3::KlTRP1*
Z21471	*MATa/MATalpha mad2Δ::KlURA3 ndt80Δ::NatMX4 ama1Δ::KanMX4 FKH2myc9::KlTRP1 NDD1-HA3::KlTRP1*
Z21546	*MATa/MATalpha bub1Δ::KlURA3 ndt80Δ::NatMX4 ama1Δ::KanMX4 PDS1myc18::KlTRP1*
Z21548	*MATa/MATalpha mad2Δ::KlURA3 leu2::DMC1p-clb1mdb/mkb-ha6-LEU2 ndt80Δ::HIS3 ama1Δ::NatMX4 PDS1myc18::KlTRP1*
Z21549	*MATa/MATalpha leu2::DMC1p-clb1mdb/mkb-ha6-LEU2 ndt80Δ::HIS3 ama1Δ::NatMX4 PDS1myc18::KlTRP1*
Z21872	*MATa/MATalpha CLB4ha3-TRP1::clb4::KanMX4 ndt80Δ::NatMX4 ama1Δ::KanMX4 PDS1myc18::KlTRP1*
Z21873	*MATa/MATalpha mad2Δ::KlURA3 CLB4ha3-TRP1::clb4::KanMX4 ndt80Δ::NatMX4 ama1Δ::KanMX4 PDS1myc18::KlTRP1*
Z21979	*MATa/MATalpha cdc20-3 mad2Δ::KlURA3 ndt80Δ::NatMX4 ama1Δ::KanMX4 PDS1myc18::KlTRP1*
Z22414	*MATa/MATalpha SIC1-ha3::HIS3MX6 ndt80Δ::NatMX4 ama1Δ::KanMX4 PDS1myc18::KlTRP1*
Z22416	*MATa/MATalpha mad2Δ::KlURA3 SIC1-ha3::HIS3MX6 ndt80Δ::NatMX4 ama1Δ::KanMX4 PDS1myc18::KlTRP1*
Z23829	*MATa/MATalpha RPL13A-2xFKBP12::TRP1 APC2-FRB::NatMX6 tor1-1::HIS3 fpr1::KanMX4 PDS1myc18::KlTRP1*
Z23831	*MATa/MATalpha tor1-1::HIS3 fpr1::KanMX4 PDS1myc18::KlTRP1*
Z23832	*MATa/MATalpha APC2-FRB::NatMX6 tor1-1::HIS3 fpr1::KanMX4 PDS1myc18::KlTRP1*
Z25473	*MATa/MATalpha APC2-FRB::NatMX6 tor1-1::HIS3 fpr1::KanMX4 ndt80::NatMX4 PDS1myc18::KlTRP1*

Z25474	*MATa/MATalpha mad2Δ::KIURA3 APC2-FRB::NatMX6 tor1-1::HIS3 fpr1::KanMX4 ndt80::NatMX4 PDS1myc18::KITRP1*
Z25475	*MATa/MATalpha RPL13A-2xFKBP12::TRP1 APC2-FRB::NatMX6 tor1-1::HIS3 fpr1::KanMX4 ndt80::NatMX4 PDS1myc18::KITRP1*
Z25476	*MATa/MATalpha mad2Δ::KIURA3 RPL13A-2xFKBP12::TRP1 APC2-FRB::NatMX6 tor1-1::HIS3 fpr1::KanMX4 ndt80::NatMX4 PDS1myc18::KITRP1*
Z26627	*MATa/MATalpha CKS1-HA3::HIS3MX6 ndt80Δ::NatMX4 ama1Δ::KanMX4 PDS1myc18::KITRP1*
Z26628	*MATa/MATalpha mad2Δ::KIURA3 CKS1-HA3::HIS3MX6 ndt80Δ::NatMX4 ama1Δ::KanMX4 PDS1myc18::KITRP1*
Z27605	*MATa/MATalpha trp1::DMC1p-CDC5-TRP1 cdc20::hsl1p-CDC20-HphMX4 ndt80Δ::NatMX4 ama1Δ::KanMX4 PDS1myc18::KITRP1*
Z27608	*MATa/MATalpha mad2Δ::KIURA3 trp1::DMC1p-CDC5-TRP1 cdc20::hsl1p-CDC20-HphMX4 ndt80Δ::NatMX4 ama1Δ::KanMX4 PDS1myc18::KITRP1*
Z27793	*MATa/MATalpha cdc20::SCC1p-CDC20-KanMX4 ama1Δ::MatMX4 PDS1myc18::KITRP1 NDD1-ha3::KITRP1*
Z29955	*MATa/MATalpha HHT1-HA3::KIURA3 MAD2-myc9::HIS3MX6 ndt80Δ::NatMX4 ama1Δ::KanMX4*
Z29956	*MATa/MATalpha MAD2-myc9::HIS3MX6 ndt80Δ::NatMX4 ama1Δ::KanMX4*
Z30438	*MATa/MATalpha mad2ΔC-myc9::HIS3MX6 HHT1-HA3::KIURA3 ndt80Δ::NatMX4 ama1Δ::KanMX4*

1. The genetic background of *S. cerevisiae* SK1 is: *ho::LYS2 ura3 leu2::hisG trp1::hisG his3::hisG ura3 leu2::hisG trp1::hisG his3::hisG*

2. Each mutation is homozygous unless stated otherwise.

Table 5. List of qPCR primers

Gene	Forward primer	Reverse primer
ACT1	ATTATATGTTTAGAGGTTGCTGCTTTGG	CAATTCGTTGTAGAAGGTATGATGCC
TFC1	GCTGGCACTCATATCTTATCGTTTCACAATGG	GAACCTGCTGTCAATACCGCCTGGAG
PDS1	TGATATCGAAATAGCACCACAGA	TGGGGAATAGCCTTCTGGTA
CLB4	TGCTGCCAAGTTTGAAGAGA	TCCAGCATGTAAACTAGATCATCC
CLB2	GCTGAGCTGCCTGCAAATA	CATGCTGGATTATCTCCTTCG
CDC5	AACAAAGAGACTAGATCCGAATAATGA	AAGCTGATAACTTTTCCCTTTTCTT
SWI5	GGAAGATCTCTCCTGCTTCAGA	GGGAAATCATTGGTGAAAGG

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6. Curriculum vitae

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Publications

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2. Sun L*, **Li H***, Chen J*, et al. (2013). PIASy mediates hypoxia-induced SIRT1 transcriptional repression and epithelial-to-mesenchymal transition in ovarian cancer cells. *Journal of Cell Science*. 126: 3939-3947
3. Sun L, **Li H**, Chen J, et al. (2013) A SUMOylation-Dependent Pathway regulates Sirt1 transcription and lung cancer Metastasis. *Journal of the National Cancer Institute*. 105: 887–898
4. Wu X, Kong X, Chen D, **Li H**, et al. (2001) SIRT1 links CIITA deacetylation to MHC II activation. *Nucleic Acids Research*. 39: 9549-58

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