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Article

Role of the Mad2 Dimerization Interface in the Spindle Assembly Checkpoint Independent of Kinetochores

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Summary

Background: The spindle assembly checkpoint (SAC) arrests cells when kinetochores are unattached to spindle microtubules. The signaling pathway is initiated at the kinetochores by one SAC component, Mad2, which catalyzes the initial steps of the cascade via the conformational dimerization of its open and closed conformers. Away from kinetochores, the dimerization surface of Mad2 has been proposed, based on data in vitro, to either interact with SAC activators or inactivators and thus to contribute to SAC activation or silencing. Here, we analyze its role in vivo.
Results: To analyze the putative pathway downstream of the kinetochores, we used two complementary approaches: we activated the SAC ectopically and independently from kinetochores, and we separated genetically the kinetochore-dependent and independent pools of Mad2. We found that the dimerization surface is required also downstream of kinetochores to mount a checkpoint response.
Conclusion: Our results show that away from kinetochores the dimerization surface is required for stabilizing the end-product of the pathway, the mitotic checkpoint complex. Surprisingly, downstream of kinetochores the surface does not mediate Mad2 dimerization. Instead, our results are consistent with a role of Mad3 as the main interactor of Mad2 via the dimerization surface.

Introduction

The spindle assembly checkpoint (SAC) is activated by the presence of kinetochores unattached to microtubules and by the ensuing lack of tension between sister chromatids [1, 2]. The target of the checkpoint is the anaphase promoting complex or cyclosome (APC/C), a ubiquitin ligase whose function is required for the degradation of mitotic cyclins and securin (primarily Clb2 and Pds1 in budding yeast, respectively) when cells enter anaphase [3]. The SAC does not directly inhibit APC/C, but it targets Cdc20, one of its coactivators [4]. SAC activation induces the formation of a complex between Cdc20 and three components of the pathway, Mad2, Mad3, and Bub3. The tetrameric complex Cdc20:Mad2:Mad3:Bub3, known as the mitotic checkpoint complex (MCC), binds the APC/C [5]. The resulting complex, APC/C^{MCC}, cannot target securin and mitotic cyclins for degradation.

A complex formed by the SAC components Mad1 and Mad2 (Mad1:Mad2) plays a crucial role in the generation of the MCC [6]. Mad1:Mad2 binds to unattached kinetochores and facilitates the binding of Mad2 to Cdc20, likely the first step in the formation of the MCC [6]. Depending on the conformation of the so-called safety belt, the C-terminal 50 residues, Mad2 is defined as either open or closed (O-Mad2 and C-Mad2) [7]. In cells, monomeric Mad2 is present in the open conformation, but O-Mad2 transits to the closed conformation when it binds its substrates, either Mad1 or Cdc20. Given the extensive structural rearrangement required for the conversion between O-Mad2 and C-Mad2, Mad2 binding to Cdc20 is very slow, as measured in vitro [8]. The Mad1:C-Mad2 complex catalyzes the structural conversion of O-Mad2 required for Cdc20/Mad2 binding. According to the "Mad2-template model," the catalytic reaction requires the transient binding of Mad1:C-Mad2 (the template C-Mad2) with O-Mad2, which occurs via dimerization of O-Mad2 with C-Mad2 in the Mad1:Mad2 complex [6]. The Mad1:C-Mad2:O-Mad2 intermediate is believed to release a molecule of "active" Mad2, not closed yet, but more prone to closing than O-Mad2 on encountering Cdc20. The binding reaction will give rise to the final conversion into C-Mad2 (the copy C-Mad2) (see Figure S1A available online).

Mad2 dimerization mutants have been instrumental for understanding the molecular mechanisms taking place during SAC signaling. In humans, Phe141 (Phe134 in budding yeast) lies at the core of the dimerization surface. Accordingly, Alanine point mutations of Phe141 impair the dimerization between C-Mad2^{F141A} and O-Mad2 [7]. Crucially, the effect of the mutation is asymmetric because O-Mad2^{F141A} can bind to wild-type (WT) C-Mad2 as efficiently as O-Mad2. In budding yeast, the *mad2^{F134A}* allele does not complement the deletion of the *MAD2* gene, showing that Mad2 dimerization is required for SAC signaling [9]. This result can be interpreted in light of the Mad2-template model if Mad2^{F134A} binding to Mad1 creates a Mad1:C-Mad2^{F134A} complex that cannot dimerize and thus cannot prime O-Mad2^{F134A} for binding to Cdc20 (Figure S1B, where we collectively refer to this part of the pathway as "kinetochore-dependent") [10].

In principle, the checkpoint deficiency of the Mad2^{F134A} mutant could also be due to a role of Mad2 downstream from kinetochores, independently from Mad1:C-Mad2 dimerization ("kinetochore-independent" in Figure S1B). Indeed, it has been pointed out that the Cdc20:C-Mad2 complex is structurally a copy of the Mad1:C-Mad2 template and is therefore predicted to contribute to the conversion of O-Mad2 into C-Mad2 via Mad2 dimerization. Evidence supporting this hypothesis comes from experiments in vitro [8]. However,

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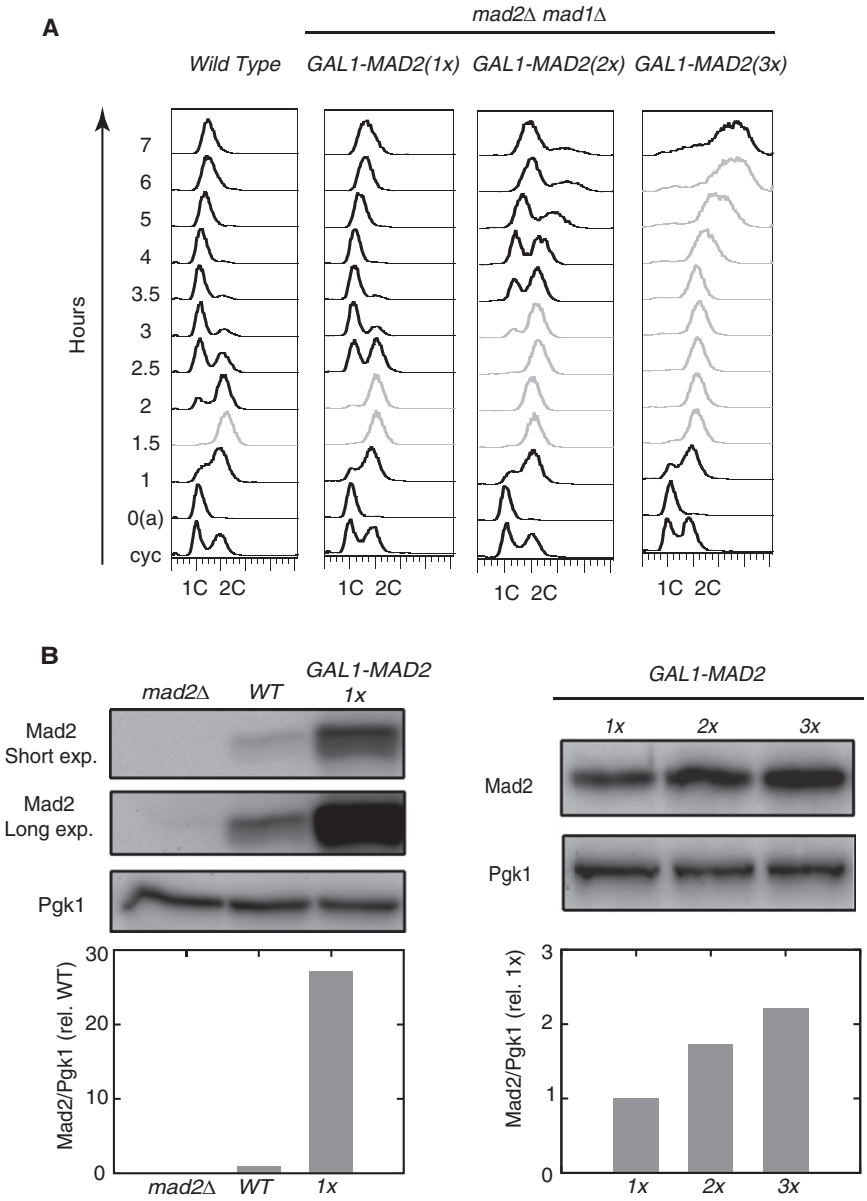

Figure 1. Mad2 Overexpression Can Induce an Arrest in Metaphase Independently from Mad1

(A) Cells were synchronized in G1 with α factor and released in galactose. α factor was re-added at hr 2. In parentheses, the number of copies of *GAL1-MAD2*: *1X* (yAC80), *2X* (yAC81), and *3X* (yAC82). WT cells (yAC1) were also analyzed. DNA content was analyzed by FACS; samples where more than 80% of cells have a 2C DNA content are shown in light gray.

(B) Cells were collected after 4 hr growth in galactose. Mad2 levels were analyzed by western blot with anti-Mad2 antibodies. Left shows a strain carrying one copy of *GAL1-MAD2* (yAC80), *MAD2* WT (*yAC1*), and *mad2Δ* (yAC5). Right shows strains carrying multiple copies of *GAL1-MAD2*: *1X* (yAC40), *2X* (yAC106), and *3X* (yAC41).

the discovery that the dimerization interface of C-Mad2 is implicated in the binding of other proteins, such as p31^{comet} (limitedly to metazoans) [9, 11] and BubR1/Mad3 [12, 13], suggests that this interface might be capped by other proteins away from kinetochores.

Here, we investigate SAC signaling downstream of kinetochores and show that the dimerization interface of Mad2 away from kinetochores is primarily required for stabilizing the MCC. Our results point to a possible role of Mad3 as the key interactor of Mad2 for helping MCC stabilization, whereas they do not support a role for Mad2 dimerization in amplifying the SAC signal away from kinetochores.

Results

Increasing Mad2 Overexpression Gradually Induces a Mitotic Arrest Independently from Mad1

To analyze the role of Mad2 dimerization surface independently from Mad1:C-Mad2, we aimed at activating the SAC ectopically in *mad1Δ* cells. In many systems, the overexpression of Mad2 causes an arrest in metaphase for several hours [14–16], followed by a process named adaptation in which arrested cells leave mitosis and reenter into G1. In fission yeast, the mitotic delay caused by overexpression of Mad2 is independent from Mad1 [15], likely because the amount of active Mad2 that forms spontaneously, negligible in physiological concentrations of Mad2, is enough to sequester Cdc20. We set out to verify whether this was the case in budding yeast as well (reactions in the "kinetochore-independent" box of Figure S1A).

To overexpress Mad2, we integrated its coding sequence under the galactose-inducible *GAL1* promoter in the yeast genome (*GAL1-MAD2*). We then produced strains carrying one, two, or three copies of *GAL1-MAD2* in a *mad1Δ mad2Δ* background. Cells were arrested in G1 with α factor and released in galactose-containing medium. α factor was re-added 2 hr after the release to arrest them before entering the next cell cycle. We referred to a population as "mitotic" when more than 80% of cells have a 2C DNA content as detected by FACS. We then identified as "arrested in mitosis" a cell population that remains mitotic after WT cells have entered G1. One copy of *GAL1-MAD2* was unable to induce a prolonged mitotic arrest, but with two copies, the cells were arrested in mitosis for 1.5 hr. Three copies induced an arrest that lasted for 5 hr (Figure 1A). The stabilization of Pds1, one of the substrates of APC/C^{Cdc20}, confirmed that mitotic cells were arrested in metaphase (Figure S1C). The time cells spent in mitosis increased with the number of copies of *GAL1-MAD2* and thus with the levels of Mad2, with one copy of *GAL1-MAD2* inducing approximately a 20-fold increase of Mad2 compared to the endogenous promoter (Figure 1B). Finally, we confirmed that the presence of Mad1 does not affect the outcome of Mad2 overexpression (Figure S1D).

We conclude that by overexpressing Mad2, it is possible to arrest cells in metaphase, independently from the presence of Mad1.

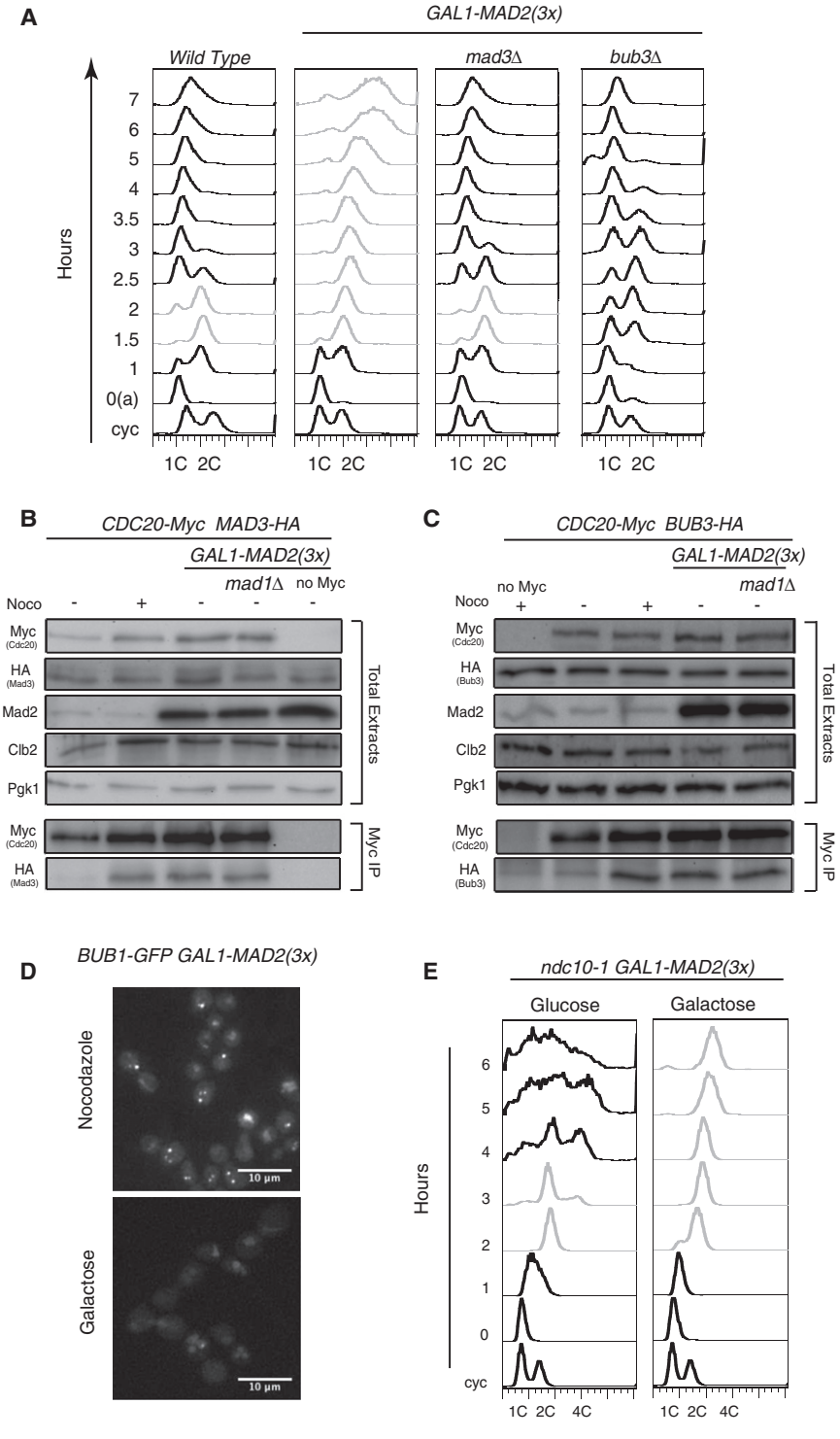

Figure 2. Mad2 Overexpression Induces a Genuine SAC Independently from Kinetochores

(A) WT (yAC1), *GAL1-MAD2* (*3X*) (yAC41), *GAL1-MAD2* (*3X*) *mad3Δ* (yAC436), and *GAL1-MAD2* (*3X*) *bub3Δ* (yAC454) cells were treated as in Figure 1A. Samples where more than 80% of cells have a 2C DNA content are shown in light gray.

(B and C) Cells were synchronized in G1 with α factor and released either in galactose (yAC396, yAC401, yAC394, yAC424, yAC383) or in nocodazole (yAC398, yAC363, yAC387). One hundred min after release, protein extracts were prepared and analyzed by immunoblotting with indicated antibodies either directly (total extracts) or after Cdc20-Myc immunoprecipitation with anti-Myc antibodies (Myc IP).

(D) *GAL1-MAD2* (*3X*) *BUB1-GFP* cells (yAC532) were synchronized in G1 and released in raffinose either with nocodazole or galactose. Figures were taken after 180 min.

(E) *GAL1-MAD2 (3X) ndc10-1* (yAC262) cells were arrested in G1 by α factor, and released at 37°C in nocodazole and either glucose or galactose.

Mad2 can induce a mitotic delay in the absence of Mad3 or Bub3. We synchronized *mad3Δ GAL1-MAD2* (3X) and *bub3Δ GAL1-MAD2* (3X) cells in G1 and released them in galactose-containing medium. In both cases, we did not observe any mitotic delay compared to the WT (Figure 2A). *GAL1-MAD2* (3X) *bub3Δ* cells divided more slowly than *GAL1-MAD2* (3X), likely because of the deletion of *BUB3*. Thus, Mad2 overexpression relieves a requirement for a component, Mad1, which is known to act upstream of Mad2 activation, but does not relieve a requirement for other SAC components that work with Mad2 in the checkpoint effector MCC.

To confirm that the arrest due to Mad2 overexpression was caused by MCC formation, we compared the amount of MCC present in WT cells arrested in metaphase by treatment with nocodazole or by Mad2 overexpression [*GAL1-MAD2* (3X)]. The metaphase arrest was evaluated by the stabilization of the mitotic cyclin Clb2 (Figures 2B and 2C) and was confirmed by DNA content (data not shown). As a read-out of MCC formation, we measured the amount of Mad3 or Bub3 bound to Cdc20 by immunoprecipitation. The levels of Cdc20-bound Mad3 and Bub3 were similar in the two settings (Figures 2B and 2C), further confirming that Mad2 overexpression induces a bona fide activation of the SAC independently from Mad1.

Mad2 Overexpression Induces a Bona Fide SAC Arrest

The Mad2 overexpression data are consistent with a scenario where high levels of Mad2 can spontaneously induce the formation of sufficient Cdc20:C-Mad2 seeds for the formation of a complete and active MCC through the recruitment of additional components (Mad3 and Bub3). In this case, the absence of additional components of the MCC should impair the ectopic activation of the checkpoint even in the presence of Mad2 overexpression. We thus tested whether high levels of

Ectopic Activation of the SAC Does Not Require Kinetochores

Our hypothesis is that Mad2 overexpression induces the SAC directly, by providing an initial seed for MCC formation. However, it is also possible that the overexpression of Mad2

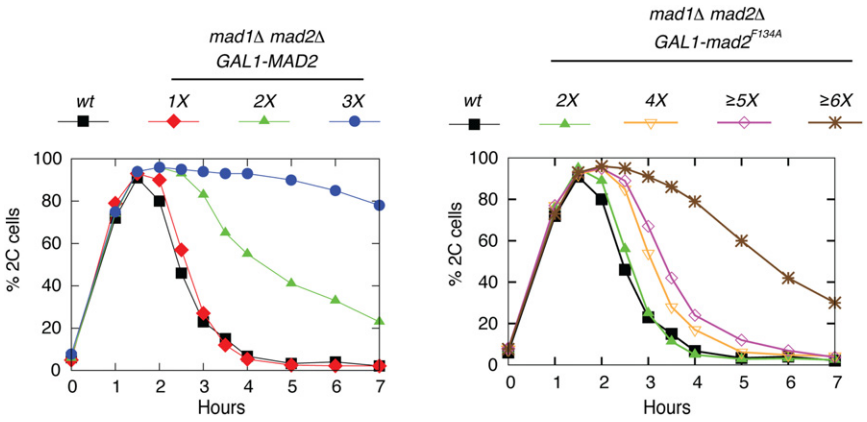

Figure 3. Ectopic SAC Activation Is Not Efficient in Dimerization Impaired Mad2 Mutants

The fraction of cells with 2C DNA content as measured by FACS. After α factor synchronization and release in galactose, α factor was re-added at hr 2. Strains had different number of copies of *GAL1-MAD2*: (left) *1X* (yAC80), *2X* (yAC81), and *3X* (yAC82); (right) *GAL1-mad2^{F134A}* *2X* (yAC157), *4X* (yAC234), *>5X* (yAC233), and *>6X* (yAC232). WT *MAD2* (yAC1) was used as a control.

impairs microtubule-kinetochore attachment, triggering a SAC response. To exclude this possibility, we used a fluorescently tagged version of Bub1 (Bub1-GFP) as an indicator of SAC signaling at the kinetochores [17]. Bub1, another element of the SAC pathway, localizes at kinetochores when the latter are not attached to microtubules. When cells are treated with nocodazole, Bub1-GFP localizes as expected at the kinetochores in metaphase-arrested cells (appearance of Bub1-GFP nuclear foci), whereas Mad2 overexpression induces a metaphase delay in the absence of Bub1 localization at the kinetochores (Figure 2D). To further confirm that Mad2 overexpression does not impact on microtubule-kinetochore attachment, we have analyzed the viability of cells overexpressing Mad2 and lacking Mad3. *GAL1-MAD2 (3X) mad3Δ* are not delayed in the cell cycle (Figure 2A) and thus, if overexpressed Mad2 affected the microtubule-kinetochore interaction, we would expect these cells to have reduced viability when grown in galactose. We have instead observed that both growth rate and viability of *GAL1-MAD3 (3X) mad3Δ* are very similar to WT cells (Figures S2A and S2B), further supporting the conclusion that Mad2 overexpression does not affect microtubule-kinetochore attachment.

Thus, Mad2 overexpression does not cause an overt defect in kinetochore-microtubule attachment, suggesting that the arrest induced by Mad2 overexpression develops independently of kinetochores. To test this hypothesis, we induced an ectopic SAC arrest in cells lacking kinetochores altogether. Conditional disassembly of kinetochores can be obtained by using a temperature-sensitive mutant of Ndc10, a fundamental structural component of the kinetochores [18, 19]. Indeed, *GAL1-MAD2* (3X) *ndc10-1* cells grown at 37°C in nocodazole and glucose (i.e., *GAL1* promoter repressed) are unable to activate the SAC and enter another round of S phase, as seen by 4C DNA content. However, cells grown at 37°C in galactose (i.e., *GAL1* promoter active) and nocodazole are arrested in mitosis regardless of the *ndc10-1* mutation, likely due to the ectopic activation of the SAC independently of kinetochores (Figure 2E).

These observations strongly support our contention that Mad2 overexpression induces a mitotic arrest by producing MCC independently from Mad1 and kinetochores.

A Dimerization-Impaired Mutant Fails in Ectopically Activating the SAC

Having established a system where SAC activation can be induced independently from kinetochores, we wanted to test whether the dimerization surface of Mad2 plays a role away from the kinetochores. We reasoned that if the dimerization defective Mad2^{F134A} was only impaired in relaying the Mad1:C-Mad2 signal at the kinetochores, its overexpression should produce the same ectopic activation of the SAC as the overexpression of Mad2 WT. If instead the dimerization surface of Mad2 is also required downstream from Mad1:C-Mad2, we would expect a defect in SAC activation. *mad1Δ mad2Δ GAL1-mad2^{F134A}* cells were synchronized in G1 and released in galactose containing medium. FACS analysis showed that to obtain merely a moderate delay in mitosis, more than five copies of *GAL1-mad2^{F134A}* were required (Figure 3), an effect weaker than what produced by three copies of *GAL1-MAD2*. This quantitative result is not due to different levels of Mad2 expression, because the same number of copies of *GAL1-mad2^{F134A}* and *GAL1-MAD2* expresses similar amounts of Mad2 (Figure S3).

In conclusion, the overexpression of Mad2^{F134A} shows that the dimerization surface plays a fundamental role in SAC signaling independently from kinetochores.

Engineering Strains Carrying Two Pools of Mad2 (2-Pool Strain)

We then set out to confirm that the C-Mad2 dimerization surface mutated in Mad2^{F134A} plays a fundamental role away from kinetochores also when Mad2 is expressed at endogenous levels and kinetochores signal normally. Testing this idea requires maintaining signaling from Mad1:C-Mad2 while at the same time interfering with the remaining Mad2 directed to Cdc20. To this aim, it is necessary to create two genetically distinct populations of Mad2: one unable to interact with Mad1 but capable to bind Cdc20 and a second one that signals to the first via Mad1 binding. An important precondition for the engineering of strains that meet this condition is that the Mad1:Mad2 complex is extraordinarily stable, so that its dissociation can be neglected within the timing of observation [20, 21].

We thus engineered yeast cells with two distinct pools of Mad2 (2-pool strain): WT Mad2, which interacts preferentially with Cdc20, and Mad2-kinetochore (Mad2$_{KT}$), capable to bind efficiently to Mad1 (Figure S4A). To ensure specificity between Mad2$_{KT}$ and Mad1, we took advantage of differences in the Mad2 binding motifs of Mad1 and Cdc20 in different species. Specifically, we were able to demonstrate in vitro that human Mad2 is unable to bind with high affinity to the Mad2-binding motifs of Cdc20 or Mad1 from *Saccharomyces cerevisiae*. Conversely, yeast Mad2 is unable to bind tightly to the Mad2-binding motifs of human Cdc20 or Mad1 (L.N. and A.M., unpublished data). To identify crucial residues responsible for species-specific specificity of the interaction of Mad2 with Mad1, we carried out a co-occurrence analysis [22]. This identified a set of Mad2 residues whose occurrence

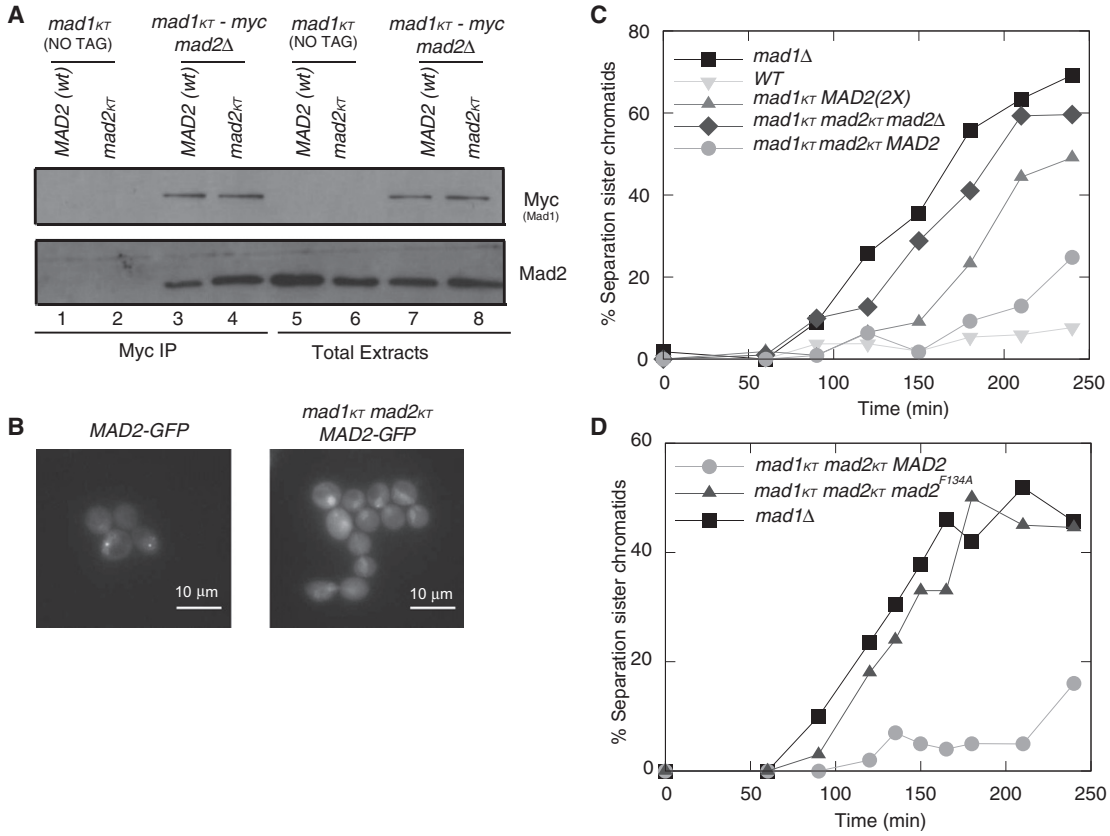

Figure 4. The 2-Pool Strain

(A) Protein extracts from cells of the indicated genotypes (yAC1217, yAC1208, yAC1276, yAC1282) were analyzed by immunoblotting with anti-myc or anti-Mad2 antibodies either directly (total extracts) or after $Mad1_{KT}$-13Myc immunoprecipitation with anti-Myc antibodies (Myc IP).

(B) Micrographs of cells of the indicated genotypes (yAC1513, yAC1544) taken 3 hr after release from α factor in nocodazole.

(C and D) Cells of the indicated genotypes (C: yAC1070, yAC1269, yAC1274, yAC1272, yAC1286; D: yAC1269, yAC1471, yAC1481) were arrested in G1 with α factor and released in nocodazole. Samples were collected at the indicated time points to score sister chromatids separation.

correlates strictly with the presence of mutations in the Mad2-binding motifs of Mad1 (Text S1).

Next, we introduced human specific mutations to "humanize" ScMad1 (defined as $Mad1_{KT}$) and ScMad2 ($Mad2_{KT}$). The mutations in Mad1 are located in the Mad2-binding region [21]. The latter was modified by replacing the yeast residues in positions 581–588 (RILQLRDG) with the equivalent mammalian residues (KVLHMSLN) (Figure S4B). We also modified ScMad2 in the Mad1-binding region (substitutions I62V and D164E). These substitutions do not affect the dimerization interface, which is very well conserved in evolution and which mediates, as previously shown, cross-species dimerization [10].

We tested the specificity of $Mad1_{KT}$/$Mad2_{KT}$ binding by comparing the amount of $Mad1_{KT}$-bound Mad2 in *mad1Δ* $mad1_{KT}$ *mad2Δ MAD2* and *mad1Δ* $mad1_{KT}$ *mad2Δ* $mad2_{KT}$ cells. Immunoprecipitation showed that $Mad2_{KT}$ binds $Mad1_{KT}$ ≈2 times better than Mad2 (Figure 4A, lanes 3 and 4), suggesting that we obtained a good, albeit not perfect, separation of the two pools of Mad2. As an additional test, we used Mad2 tagged with GFP (a gift from the Tanaka laboratory) to evaluate whether Mad2 binding to kinetochores is decreased by the presence of $Mad1_{KT}$ and $Mad2_{KT}$. In cells treated with nocodazole, we observed a clear reduction of kinetochore signal in *mad1Δ* $mad1_{KT}$ $mad2_{KT}$ *MAD2-GFP* cells as compared to *MAD2-GFP* cells (Figure 4B).

In conclusion, we took advantage of the species-specificity of Mad2 binding sites in Mad1 and Mad2 to produce "humanized" versions of Mad1 and Mad2 ($Mad1_{KT}$ and $Mad2_{KT}$) that interact specifically with each other.

Both $Mad2_{KT}$ and Mad2 WT Are Required for an Operational Checkpoint

Both catalysis of Mad1:C-Mad2 at the kinetochores and Mad2/Cdc20 binding away from kinetochores are required for a proficient checkpoint. Although Mad2 WT can bind Cdc20 efficiently, it is not proficient in $Mad1_{KT}$ binding. We thus expected the *mad1Δ* $mad1_{KT}$ *MAD2* strain to be checkpoint deficient and to require $Mad2_{KT}$ to restore the checkpoint.

To test checkpoint proficiency, we followed sister chromatids separation directly, by visualizing with GFP the pericentromeric region of chromosome V [23, 24]. Cells were synchronized by α factor arrest and released in nocodazole to activate the checkpoint. As indicated by the kinetics of sister separation, cells expressing only WT Mad2 are impaired in mounting a checkpoint response (50% of *mad1Δ* $mad1_{KT}$ *MAD2 (2X)* have entered anaphase after 4 hr, compared to 10% for WT cells, Figure 4C). The partial recovery of the SAC compared to *mad1Δ* can be related with the previous observation that Mad2 binding of $Mad1_{KT}$ is decreased compared to $Mad2_{KT}$ but not completely abolished (Figure 4A). Crucially, the functionality of the checkpoint increased greatly after we

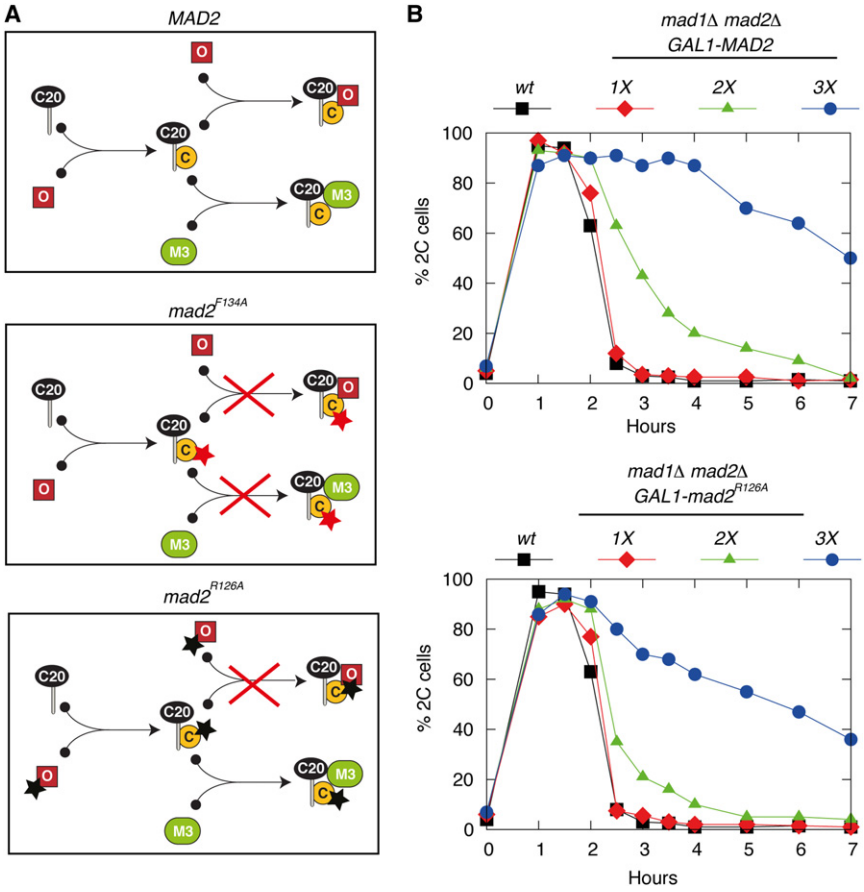

Figure 5. Overexpression of Mad2^{R126A} Is Similar to that of Mad2 WT

(A) Upper panel shows that in Mad2 WT, Cdc20:C-Mad2 can interact both with O-Mad2 and with Mad3. Middle panel shows that Cdc20:C-Mad2^{F134A} (red star) can interact with neither of the two. Lower panel shows that Mad2^{R126A} (black star) cannot dimerize but can bind Mad3.

(B) Cells were treated as in Figure 1A. Upper panel shows *GAL1-MAD2* (*1X* [yAC80], *2X* [yAC81], and *3X* [yAC82]), and lower panel shows *GAL1-mad2^{R126A}* (*1X* [yAC735], *2X* [yAC737], and *3X* [yAC741]). The plot shows the fraction of cells with 2C DNA content as measured by FACS. WT *MAD2* (yAC1) was used as control.

replaced one copy of *MAD2* with one copy of *mad2$_{KT}$* (which by itself is unable to recover the full checkpoint arrest). In 75% of *mad1Δ mad1$_{KT}$ mad2$_{KT}$ MAD2* cells, sister chromatids separation is arrested for at least 4 hr although cells do not express more Mad2 than *mad1Δ mad1$_{KT}$ MAD2 (2X)* (Figure S4C).

In summary, in the 2-pool strain the kinetochore-independent pool of Mad2 is unable to mount a full checkpoint response, which requires the contribution of the kinetochore-dependent pathway.

The Mad2 Dimerization Surface Is Needed Away from Kinetochores

We used the 2-pool strain to investigate the role of Mad2 dimerization downstream of kinetochores by replacing WT *MAD2* with *mad2^{F134A}*. The F134A mutation impairs the dimerization surface of Mad2 when the latter is in the closed conformation but O-Mad2^{F134A} binds C-Mad2 as efficiently as O-Mad2 [7]. For this reason, in the *mad1Δ mad1$_{KT}$ mad2$_{KT}$ mad2^{F134A}* strain, the signal emanating from the kinetochores is not expected to be silenced: O-Mad2^{F134A} can bind to Mad1:C-Mad2$_{KT}$ and thus can be primed for the binding with Cdc20. However, after Mad2^{F134A} binds Cdc20 and turns into the closed conformation, it might be unable to interact with additional molecules required for MCC formation (Mad2^{F134A} acts only in the kinetochore-independent box of Figure S4A). If this interaction were important for SAC signaling, we would expect to observe a checkpoint defect. Indeed, we observed that *mad1Δ mad1$_{KT}$ mad2$_{KT}$ mad2^{F134A}* is checkpoint deficient (similar to *mad1Δ*, Figure 4D). Our interpretation of the previous results only holds if Mad2^{F134A} does not significantly bind to Mad1$_{KT}$, because binding of Mad2^{F134A} to Mad1$_{KT}$ would silence SAC signaling at kinetochores. We thus tested Mad2^{F134A} binding to Mad1$_{KT}$ and found it to be negligible (Figure S4D, lanes 1 and 2).

In conclusion, these data confirm the results from Mad2 overexpression that the dimerization surface of C-Mad2 plays a role independently of Mad1, presumably when C-Mad2 is bound to Cdc20.

Mad2 Dimerization Does Not Play a Relevant Role Downstream of Kinetochores

Data obtained in vitro have shown that several proteins interact with the dimerization interface of Mad2. In metazoans, p31^{comet}, a negative regulator of the SAC whose homolog in yeast is unknown, interacts with Mad2 via the dimerization interface [9, 11]. Our results in two different experimental systems show that Mad2^{F134A} is impaired in SAC activation independently from kinetochores, ruling out the relevance at this stage of an interaction with a putative negative regulator of the SAC. The results could instead be explained by the inability of Cdc20:C-Mad2^{F134A} to interact with MCC components. The recently determined structure of the MCC [13] shows that the F134A mutation might impair the interaction between Cdc20:C-Mad2 and Mad3. By superimposing the structure of C-Mad2:Mad3 in the MCC with the C-Mad2:O-Mad2 dimer [7], it is also clear that the F134A mutation could impair the binding of Cdc20:C-Mad2 with O-Mad2. In summary, structural data show that the phenotype of the F134A mutation could be due to the impaired interaction with either Mad3 or O-Mad2.

To estimate the relevance of O-Mad2 binding in ectopic SAC activation, we need a separation of function mutant of Mad2 that cannot dimerize but preserves the capability to bind Mad3 (Figure 5A, lower panel). *mad2^{R126A}* fulfills all requirements: the structure of the MCC from fission yeast shows that Arg133 (the equivalent residue in fission yeast and in *Homo sapiens* of Arg126) does not take part in Mad3 binding to Cdc20:C-Mad2 [13]. However, although the product of *Hs-mad2^{R133A}* can acquire both open and closed conformations, the two mutant conformers do not form dimers as the R126A substitution affects the dimerization surface of both C-Mad2^{R126A} and O-Mad2^{R126A} [25]. Importantly, in budding

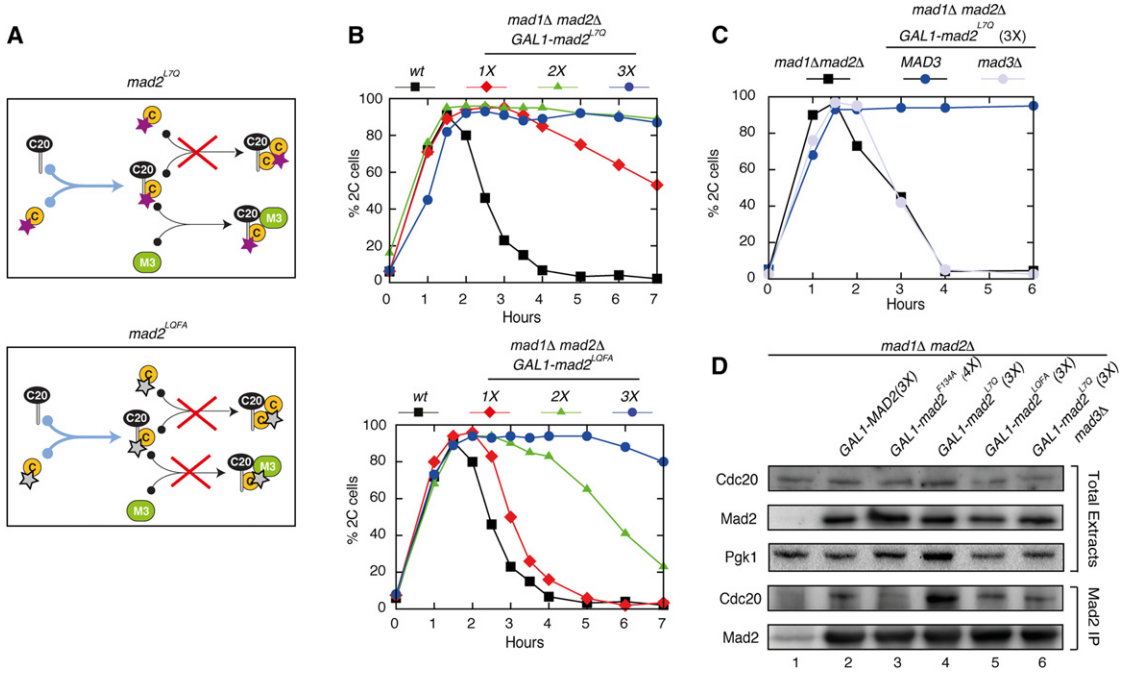

Figure 6. Overexpression of Mad2^{LQFA} Is Less Capable to Induce a Checkpoint Arrest than Mad2^{L7Q}

(A) Upper panel shows that the binding of Cdc20/Mad2 is favored (blue arrow) due to the L7Q mutation (purple star) and that Mad2 cannot dimerize. Lower panel shows that in the *mad2^{LQFA}* mutant (gray star) both dimerization and Mad3 binding are impaired.

(B) Cells were synchronized in G1 with α factor, and released in galactose to induce the expression of *GAL1-mad2^{L7Q}* (*1X* [yAC83], *2X* [yAC84], and *3X* [yAC86]) and *GAL1-mad2^{LQFA}* (*1X* [*yAC323*], *2X* [*yAC324*], and *3X* [yAC326]). α factor was re-added at hr 2. The plot shows the fraction of cells with 2C DNA content as measured by FACS.

(C) *mad1Δ mad2Δ* (yAC5) and *GAL1-mad2^{L7Q}* (*3X*) either with (yAC86) or without *MAD3* (yAC569) were treated and analyzed as in (B).

(D) Cells were arrested in G1 with α factor and released in galactose (*GAL1-MAD2* [*3X*] [yAC82], *GAL1-mad2^{F134A}* [*4X*] [yAC234], *GAL1-mad2^{L7Q}* [*3X*] [yAC86], *GAL1-mad2^{LQFA}* [*3X*] [yAC326], and *GAL1-mad2^{L7Q}* [*3X*] *mad3Δ* [yAC569]). At 80 min, protein extracts were collected and analyzed by immunoblotting with the indicated antibodies either directly (total extracts) or after Mad2 immunoprecipitation with anti-Mad2 antibodies (Mad2 IP).

yeast Mad2^{R126A} binds normally to Mad1 but is checkpoint deficient, likely as a result of the impaired dimerization [10].

We thus compared the effect of *MAD2* and *mad2^{R126A}* overexpression in *mad1Δ mad2Δ* cells to evaluate the role of Mad2 dimerization downstream of kinetochores. Our results show that overexpression of *mad2^{R126A}* and *MAD2* have a similar effect, which is much stronger than *mad2^{F134A}* overexpression (Figures 3 and 5B). We conclude that Mad2 dimerization does not contribute significantly to an ectopic checkpoint arrest induced by Mad2 overexpression. We also predict that the phenotype of *mad2^{F134A}* is mainly due to impairment in Mad3 binding.

Mad3 Binding Might Contribute to the Ectopic Activation of the SAC

We next wanted to confirm that, independently from Mad2 dimerization, the lack of Mad3 binding affects the kinetochore-independent pathway. To evaluate the relevance of C-Mad2/Mad3 binding, we proceeded in two steps. First, we created Mad2 mutants impaired in dimerization regardless of an intact F134 residue. Then, we used them to analyze the effect of the F134A mutation, which in this setting should impact on Mad3 binding only (Figure 6A). Glutamine point mutations of Leu7 destabilize O-Mad2 and thus Mad2^{L7Q} mutants accumulate in the closed-conformation and do not form dimers [7]. The fact that Mad2^{L7Q} acquires more easily the closed conformation is mirrored by its higher affinity for Cdc20 in vivo, because in mammalian cells the overexpression of an equivalent mutant of Mad2^{L7Q} (i.e., Mad2^{L13A}) induces a mitotic delay [26]. We confirmed this result in yeast, because when we overexpressed Mad2^{L7Q} in *mad2Δ mad1Δ* cells, we found that one copy of *GAL1-mad2^{L7Q}* was sufficient to induce a phenotype stronger than what we have observed with two copies of *GAL1-MAD2* (compare Figure 6B upper and Figure 3 left). Again, the presence of Mad3 is required for the arrest (Figure 6C), showing that Mad2^{L7Q} induces a genuine SAC response and that the L7Q substitution does not affect the capability of Mad2 to interact with Mad3.

We then combined the L7Q mutation with the F134A mutation in the same allele (*mad2^{LQFA}*). Phenotypic differences between *GAL1-mad2^{L7Q}* and *GAL1-mad2^{LQFA}* can be primarily attributed to the inability of Mad2^{LQFA} to interact with Mad3 because Mad2 dimerization is already impaired by the L7Q mutation. Whereas two copies of *GAL1-mad2^{L7Q}* were enough to induce a SAC arrest, three copies of *GAL1-mad2^{LQFA}* were required to achieve a comparable mitotic arrest (Figure 6B, lower). This quantitative difference suggests that the impaired binding between C-Mad2 and Mad3 contributes significantly to the reduced ability of Mad2^{LQFA} to induce an ectopic checkpoint.

Mad3 Binding Could Help Stabilize the Cdc20:C-Mad2 Complex

From a molecular viewpoint, one interpretation of our data is that the interaction between C-Mad2 and Mad3 via the F134A residue is relevant for creating a stable Cdc20:C-Mad2:Mad3

complex. This hypothesis comes with the prediction that Mad2 bound to Cdc20 decreases when the Mad2/Mad3 interaction is impaired. We thus aimed at testing whether $Mad2^{LQFA}$ binds Cdc20 less stably than $Mad2^{L7Q}$ due to the missing interaction with Mad3. Hence, we measured the amount of Cdc20 bound to Mad2 in *mad1Δ mad2Δ GAL1-mad2*^{L7Q} and *mad1Δ mad2Δ GAL1-mad2*^{LQFA} 80 min after release from α factor, when most cells were in metaphase as evaluated by spindle morphology (data not shown). As expected, we observed more Cdc20:C-$Mad2^{L7Q}$ than Cdc20:C-$Mad2^{LQFA}$ (Figure 6D, lanes 4 and 5) and WT Mad2 immunoprecipitated more Cdc20 than $Mad2^{F134A}$ (Figure 6D, lanes 2 and 3). A second prediction is that $Mad2^{L7Q}$ should not bind Cdc20 effectively in the absence of Mad3. Indeed, we noticed that without Mad3 the capability of $Mad2^{L7Q}$ to bind Cdc20 is decreased to the level of $Mad2^{LQFA}$ (Figure 6D, lanes 4, 5, and 6).

We conclude that in vivo the F134 residue of Mad2 is relevant for stabilizing the Cdc20:C-Mad2 complex likely via the interaction with Mad3.

Discussion

In this manuscript, we have investigated the SAC pathway away from kinetochores by analyzing the role of Mad2 dimerization interface. Our results showed that in vivo the F134 residue of Mad2, localized in the dimerization surface, is required for the formation of MCC independently from kinetochores.

What Role for Mad2 Dimerization Surface Downstream of Kinetochores

The recently determined structure of the mitotic checkpoint complex (MCC) shows that the dimerization surface of C-Mad2 in the Cdc20:C-Mad2 complex interacts with Mad3, with the F134 residue playing a pivotal role in the binding [13]. The very same residue is required for the dimerization between O-Mad2 and C-Mad2 [7]. Thus we have two potential interactions of Cdc20:C-Mad2—with O-Mad2 and Mad3—whose impairment may result in the phenotype of the F134A mutation.

What could be the rationale for Mad2 dimerization downstream of kinetochores? The O-Mad2:C-Mad2 dimer, in analogy with Mad1:C-Mad2, could be used by Cdc20:C-Mad2 to prime O-Mad2, help the formation of more Cdc20:C-Mad2 and amplify the SAC signal in an autocatalytic fashion (Figure S5). We have previously shown in vitro that indeed Cdc20:C-Mad2 can induce such a positive feedback loop [8]. Here, we show data that do not support the presence of such a circuit in vivo. Mad2 overexpression induces a gradual increase of cells arrested in mitosis and not the activation threshold that characterizes positive feedback loops [Figure 3, left, *GAL1-MAD2* (*2X*) is half-way between (*1X*) and (*3X*)]. Moreover, when we overexpress a separation of function mutant of Mad2 that can bind to Mad3 but cannot dimerize ($Mad2^{R126A}$), we do not see major differences from overexpressing Mad2 WT. The result is in agreement with what was reported by Lau and Murray, who could activate the checkpoint equally well by tethering Cdc20 with either Mad2 WT or a Mad2 mutant, which carries the R126E and Q127A mutations [27]. From the MCC structure, we infer that $Mad2^{RQEA}$ is mainly impaired in Mad2 dimerization, although the Q127A substitution should affect marginally also Mad3 binding. Notice that, in previous works, we have also used for in vitro assays the R126A mutation together with Q127A to create a more penetrant effect that eliminates the residual dimerization between $Mad2^{R126A}$ and dimerization competent forms of Mad2 (e.g., $Mad2^{wt}$) [10]. However, in the experiments we present here, cells express $Mad2^{R126A}$ only, and thus the R126A mutation is sufficient to generate mutants fully impaired in dimerization.

From the structural data [13] and the $Mad2^{R126A}$ experiments, we conclude that $Mad2^{F134A}$ is less capable to induce an ectopic SAC activation likely because Cdc20:C-Mad2 cannot interact with Mad3. Further supporting this prediction, we showed that, in a context where the dimerization of Mad2 is already minimal ($Mad2^{L7Q}$), the F134A mutation further destabilizes the MCC. We thus propose that Mad3 binding to the dimerization interface of C-Mad2 stabilizes the Cdc20:C-Mad2 complex downstream of kinetochores.

The Role of Mad3 in Cdc20/Mad2 Binding

Mad3 being required to stabilize the Cdc20:C-Mad2 complex implies that the complex by itself is unstable. This conclusion might be surprising when we consider the affinities of the binding reaction as measured in vitro. Using mammalian homologs of Mad2 and Cdc20, we observed a dissociation constant in the order of $\approx$100 nM [6, 8]. Given that concentrations of Cdc20 and Mad2 in metaphase are [Cdc20] $\approx$100 nM and [Mad2] $\approx$400 nM [28], the concentration of free Cdc20 is expected to be quite low. Even more so under Mad2 overexpression: because every copy of *GAL1-MAD2* leads to a $\approx\times$20 increase of [Mad2], we would expect only $\approx$1% of Cdc20 free in a *GAL1-MAD2* (*1X*). In such conditions, cells should be well arrested in metaphase, which is not the case. We thus conclude that a larger fraction than 1% of Cdc20 is free from Mad2 with one copy of *GAL1-MAD2*. The discrepancy between the measurement in vitro and the observed phenotype in vivo could be due to putative active mechanisms at place to destroy the Cdc20:C-Mad2 complexes, as observed in mammals [29] albeit not in yeast. The binding of Cdc20 requires a large conformational change of Mad2, and very few Cdc20:C-Mad2 complexes could form if the dissociation were faster than the closing time of Mad2 around Cdc20. The role of Mad3 could be to prevent such mechanisms to destabilize the Cdc20:C-Mad2 complex before the binding is complete. The lower efficiency of $Mad2^{F134A}$ in inducing a SAC arrest compared to Mad2 WT would thus be the result of the active dissociation processes not contrasted by the presence of Mad3. Even the few Cdc20:C-$Mad2^{F134A}$ formed would be quickly destabilized creating free Cdc20 and O-Mad2, which again would rebind only very slowly. We can speculate that in the extreme case when Cdc20 and Mad2 affinity is infinitely high, the mutation in the dimerization interface should bear no consequences because dissociation would be ineffective, and Mad2 would have enough time to bind Cdc20. This is indeed what Lau and Murray observe, using a different allele of Mad2 mutated in the dimerization interface [27].

Is then Mad3 role limited to stabilizing Cdc20:C-Mad2? In our system, Mad3 is not dispensable to generate an ectopic SAC, even in the presence of large overexpression of $Mad2^{L7Q}$. In Lau and Murray's system, where Cdc20 and Mad2 are tethered together, Mad3 is dispensable (although it is not so in a sizeable fraction of cells) [27]. This apparent discrepancy might be explained by the strength of the putative mechanisms that dissociate Cdc20:Mad2, so that even three copies of *GAL1-MAD2*^{L7Q} cannot cope with them as efficiently as tethering Cdc20 and Mad2 molecules together. Surely, additional work is needed to understand whether Mad3 plays

additional roles in the checkpoint besides stabilizing the MCC, in agreement with previous results [30, 31].

In conclusion, our results suggest that the very same dimerization interface of Mad2 is required for two different, yet fundamental, consecutive steps of SAC signaling: at the kinetochores for priming Cdc20/Mad2 binding, away from kinetochores for stabilizing this complex.

Experimental Procedures

Strains, Media, and Reagents

All yeast strains (Table S1) were derivatives of or were backcrossed at least three times to W303 (*ade2-1*, *trp1-1*, *leu2-3,112*, *his3-11,15*, *ura3*, *ssd1*). Cells were grown in YEP medium (1% yeast extract, 2% bactopeptone, 50 mg/l adenine) supplemented with 2% glucose (YEPD), 2% raffinose (YEPR), or 2% raffinose and 2% galactose (YEPRG). Unless otherwise stated, α factor and nocodazole were used at 3 μg/ml and 15 μg/ml, respectively. Re-addition of α factor was at 20 μg/ml. Synchronization experiments were carried out at 25°C, and galactose was added 1 hr before release from α factor. See Supplemental Experimental Procedure for plasmid construction, genetic manipulations, and other experimental details.

Supplemental Information

Supplemental Information includes five figures, one table, Supplemental Text, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2012.08.028.

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