A Mad2-Mediated Translational Regulatory Mechanism Promoting S-Phase Cyclin Synthesis Controls Origin Firing and Survival to Replication Stress

Highlights

- Mad2 promotes origin firing in rad53 defective cells upon replication stress
- Rad53 inhibits Clb5 degradation, while Mad2 promotes Clb5 synthesis
- Mad2 inhibits Caf20<sup>4E-BP</sup> by modulating its interaction with the translation machinery
- The role of Mad2 in translation does not depend on other SAC proteins

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In Brief

Here, Gay et al. show that, in addition to its well-established role in the spindle checkpoint, Mad2 promotes translation of S-phase cyclin mRNAs. This S-phase role of Mad2 is critical for cell survival following replication stress.
A Mad2-Mediated Translational Regulatory Mechanism Promoting S-Phase Cyclin Synthesis Controls Origin Firing and Survival to Replication Stress

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SUMMARY

Cell survival to replication stress depends on the activation of the Mec1ATR-Rad53 checkpoint response that protects the integrity of stalled forks and controls the origin firing program. Here we found that Mad2, a member of the spindle assembly checkpoint (SAC), contributes to efficient origin firing and to cell survival in response to replication stress. We show that Rad53 and Mad2 promote S-phase cyclin expression through different mechanisms: while Rad53 influences Clb5,6 degradation, Mad2 promotes their protein synthesis. We found that Mad2 co-sediments with polysomes and modulates the association of the translation inhibitor Caf204E-BP with the translation machinery and the initiation factor eIF4E. This Mad2-dependent translational regulatory process does not depend on other SAC proteins. Altogether our observations indicate that Mad2 has an additional function outside of mitosis to control DNA synthesis and collaborates with the Mec1-Rad53 regulatory axis to allow cell survival in response to replication stress.

INTRODUCTION

Inhibition of replication fork progression activates the Mec1ATR-mediated checkpoint that phosphorylates downstream effectors such as Rad53Chk1 (Branzei and Foiani, 2009). Once activated, the replication checkpoint stabilizes stalled replication forks (Sogo et al., 2002), stimulates dNTP production (Zhao et al., 1998; Zhao and Rothstein, 2002), influences origin firing (Marhineke and Hyrien, 2004; Santocanale and Diffley, 1998), regulates histone protein levels (Gunjan and Verreault, 2003), and releases topological tensions (Bermejo et al., 2012). Later in the cell cycle, the spindle assembly checkpoint (SAC) controls the bipolar attachment of chromosomes to microtubules (Foley and Kapoor, 2013; Musacchio, 2015). Abnormal physical tension between chromosomes and microtubules is sensed at the kinetochore by the Knl1 complex–Mis12 complex–Ndc80 complex (KMN network) (Godek et al., 2015) that recruits the mediator complex Mad1/Mad2 (Figure 1A). After activation, Mad2 forms a soluble effector complex with the proteins Bub3, Mad3BubR1, and Cdc20, which inhibits the anaphase-promoting complex (APC) (Fraschini et al., 2001; Sudakin et al., 2001). APC inhibition prevents the degradation of mitotic cyclins (Cib2CyclinB) and Pds1Securin, thus blocking the metaphase/anaphase transition (Shirayama et al., 1999; Wäsch and Cross, 2002). Deregression of SAC protein expression causes chromosome instability and cancer (Schuyler et al., 2012; Suijkerbuijk and Kops, 2008).

Despite the persistence of Mad1 and Mad2 proteins throughout the cell cycle, their role outside of mitosis is elusive. During interphase, Mad1 and Mad2 mainly localize to nuclear pores (Campbell et al., 2001; De Souza et al., 2009; Lee et al., 2008; Lussi et al., 2010). In the yeast Saccharomyces cerevisiae, Mad1, but not Mad2, favors protein nuclear transport and modulates nuclear import following SAC activation (Cairo et al., 2013). Mad2 has been shown to physically interact with proteins involved in transcription, mRNA processing, and translation (Batisse et al., 2009; Graumann et al., 2004; Uetz et al., 2000; Wong et al., 2007; Yu et al., 2008), although the involvement of Mad2 in these processes is still elusive.

We found that Mad2 contributes to cell survival in response to replication stress when the Rad53 checkpoint is defective. While Rad53 influences Clb5,6 degradation, Mad2 promotes Clb5,6 protein synthesis through its association with the inhibitor of translation Caf204E-BP. Altogether our observations indicate that Mad2, and not the other SAC proteins, counteracts the association of Caf20 with the translational machinery and stabilizes the complex formation between Caf20 and the initiation factor Cdc33eIF4E. Hence, Mad2 acts also during S-phase to promote origin firing and, together with the Rad53-mediated checkpoint, contributes to cell survival in response to replication stress.

RESULTS

Mad2 Contributes to Cell Survival in Response to Replication Stress when Rad53 Is Not Functional

We investigated whether spindle checkpoint proteins were required for survival to hydroxyurea (HU)-induced replication...
Figure 1. **MAD2 Deletion Increases the Sensitivity of rad53 Mutants to Replication Stress**

(A) Schematic representation of the SAC activation.

(B) Cells were spotted in 5-fold (A and C) or 2-fold (B) serial dilutions on YPDA/YP-Gal/HU/MMS or camptothecin plates, or on plates irradiated with UV. Relative growth was assayed after 2 days of culture at 28°C. The rad53-K227A allele is marked as rad53, and the GAL1-rad53-D339A allele is marked as GAL1-rad53.

(D) DNA damage induced by replication stress was monitored by western blot analysis using anti-phosphorylated H2AX antibodies. Cells were synchronized in G1 and released into YPD + 200 mM HU. Pgk1 was used as a loading control.

**Figure 1A.** Deletion of **MAD2** increases the sensitivity of rad53 mutants to replication stress. In the presence of HU, mad2Δ mutants were more sensitive to HU treatment than wild-type (wt) cells.

**Figure 1B.** Schematic representation of the SAC activation.

**Figure 1C.** Western blot analysis of γH2AX levels in rad53 mutants treated with HU.

**Figure 1D.** Western blot analysis of γH2AX levels in rad53 mutants treated with HU and CPT.

Molecular Cell 70, 628–638, May 17, 2018 629
Rad53 mediates cell survival under replication stress conditions but also under DNA damaging conditions. We tested the effect of MAD2 deletion in cells treated with the alkylating agent methyl methan-sulphonate (MMS); ultraviolet (UV) radiation, which leads to the formation of thymine dimers; or camptothecin (CPT), which forms a covalent complex between topoisomerase I and DNA. In all cases rad53-K227A mutants exhibited hypersensitivity compared to WT cells (Figure 1C); however, the combination of MAD2 deletion with rad53-K227A mutation did not cause synthetic growth defects upon DNA damage (Figure 1C). These results suggest that MAD2 deletion specifically affects the HU-induced replication stress response and not the DNA damage response of rad53-K227A mutants. Accordingly, the levels of H2AX phosphorylation, a readout of DNA damage, were comparable in WT and mad2Δ cells as well as in rad53-K227A and rad53-K227A mad2Δ mutants (Figure 1D).

**Mad2 Influences Origin Firing in Response to Replication Stress**

We then investigated whether the absence of Mad2 affected replication dynamics. WT, mad1Δ, mad2Δ, and mad1Δ mad2Δ cells were released from G1 into 200 mM HU for 1, 2, or 3 hr and analyzed by neutral-neutral 2D gels electrophoresis after in vivo electrophoresis (Lopes et al., 2001). However, the combination of MAD2 deletion with rad53-K227A mutation did not cause synthetic growth defects upon DNA damage (Figure 1C). These results suggest that MAD2 deletion specifically affects the HU-induced replication stress response and not the DNA damage response of rad53-K227A mutants. Accordingly, the levels of H2AX phosphorylation, a readout of DNA damage, were comparable in WT and mad2Δ cells as well as in rad53-K227A and rad53-K227A mad2Δ mutants (Figure 1D).

**Mad2 and the Intra-S Checkpoint promote the Expression of the S-Phase Cyclins Cib5,6**

Replication origin firing is a two-step process mediated by origin licensing and origin activation (Bell and Dutta, 2002; Diffley and Labib, 2002). During this later step, a subset of the potential origins bound by a pre-replicative complex are converted into active origins through a phosphorylation cascade which involves the DBF4-dependent kinase DBF4/Cdc7 and the cyclin-depend-ent kinases (CDKs) Cib5/Cdc28 and Cib6/Cdc28. cib5Δ cells exhibit a prolonged S-phase, a decrease in late origin firing in the absence of replication stress, and an increased sensitivity to HU (Donaldson et al., 1998; Epstein and Cross, 1992; Hsu et al., 2011). Moreover, CLB5 deletion is lethal in combination with a defective intra-S checkpoint, suggesting that Cib5 levels become particularly critical in the absence of a functional intra-S checkpoint (Gibson et al., 2004; Manfrini et al., 2012). We investigated whether Cib5 levels were affected by MAD2 deletion or RAD53 mutations. WT, mad2Δ, rad53-K227A, and rad53-K227A mad2Δ cells were synchronized in G1 and released into S-phase. Cib5 levels were analyzed throughout the cell cycle by western blotting. As previously described (Jackson et al., 2006), Cib5 accumulation started roughly 30 min after release from G1. Its levels peaked after 60 min from release and declined when cells went through mitosis (Figures 3A and 3B). Cib5 expression profile was similar in mad2Δ and WT cells, even though we observed a 40% loss in Cib5 levels at 75 min from the G1 release in mad2Δ cells compared to WT. We note that this loss was not due to a faster S-phase since FACS profiles were comparable in WT and mad2Δ cells (Figure 3B). In rad53Δ mutants, Cib5 accumulated with similar kinetics to WT cells. The contribution of Mad2 in the maintenance of Cib5 levels was far more evident when comparing rad53-K227A and rad53-K227A mad2Δ cells. Although the kinetics of Cib5 accumulation were similar between the two strains, Cib5 protein levels were between 30% and 60% less abundant in rad53-K227A mad2Δ cells compared to rad53-K227A cells (45–90 min after release from G1). Altogether, these results suggest that Mad2 influences the accumulation of Cib5 levels, even in the absence of replication stress.

We then studied the effect of replication stress on the Cib5 accumulation by releasing cells in complete medium in the presence of HU (Figure 3A). As previously described (Palou et al., 2010), in WT cells, Cib5 levels peaked 60 min after G1 release both in the presence and in the absence of replication stress. However, Cib5 levels were stabilized for a longer time in the presence of HU (Cib5 expression decline after 90 min of G1-release without HU versus 150 min in the presence of replication stress) (Figure 3A). Both MAD2 deletion and RAD53 mutations accelerated Cib5 decline. Indeed, a 60% reduction in Cib5 levels was observed 30 min before in mad2Δ and in rad53-K227A cells compared to WT. Cib5 reduction was even more pronounced in rad53-K227A mad2Δ cells in which Cib5 became hardly detectable. We conclude that both Mad2 and Rad53 contribute to maintain Cib5 levels in the presence of replication stress. Furthermore, their additive effect on Cib5 suggests that Rad53 and Mad2 maintain Cib5 levels through different mechanisms. Similar results were obtained for the other S-phase cyclin, Cib6 (Figure S3A). We note that Mad2 did not affect the general...
protein expression, since the abundance of the replication factor Orc2, of the cyclin Clb2, or of the housekeeping gene Pgk1, was not reduced by MAD2 deletion (Figure S3B).

**Rad53 Inhibits Clb5 Degradation under Replication Stress**

The decrease in cyclin expression that we have previously observed can result from a defect in transcription, translation, and/or protein degradation. To test the contribution of Rad53 or Mad2 in transcription, WT, mad2Δ, rad53-K227A, and rad53-K227A mad2Δ cells were synchronized in G1 and released into S-phase at 25°C in 200 mM HU. mRNA abundance was measured by reverse transcription (RT) and qPCR. As shown in Figure 3C, Clb5 transcription initiates at the end of the G1 phase, 30 min after α factor release. The levels of Clb5 transcripts were similar in all strains. We conclude that neither MAD2 deletion nor rad53 mutation influences Clb5 transcription. Similar results were observed for the other S-phase cyclin, Clb6 (Figures S3C and S3D).

We then investigated whether Mad2 or Rad53 could have an impact on Clb5 degradation. WT, rad53-K227A, and rad53-K227A mad2Δ cells were synchronized in G1 and released into S-phase in the presence of 200 mM HU and 100 μM MG132, a proteasome inhibitor, which specifically protects short-lived protein from degradation (Lee and Goldberg, 1996). Under MG132 and HU treatment, Clb5 protein levels remained almost constant in WT cells, even after 3 hr of release (Figure 3D). A similar result was observed in rad53-K227A cells. While the levels of Clb5 were reduced by 70% after 150 min of release without MG132, Clb5 levels decreased only by 15% during the same period in the presence of MG132 (Figures 3D and S3E). We ruled out a cell-cycle issue, since FACS profiles were comparable with or without MG132 (data not shown).

The same strategy was used to study the contribution of Mad2 to Clb5 degradation. Contrary to what we observed in rad53-K227A cells, the level of Clb5 gradually decreased under HU treatment in rad53-K227A mad2Δ cells, even in the presence of MG132 (Figure 3D). We conclude that Rad53 protects Clb5 from degradation during HU treatment, while Mad2 acts through a mechanism that is not affected by MG132 treatment.
Figure 3. Rad53 and Mad2 Regulate Clb5 Expression

(A) Expression of Clb5-HA was followed throughout the cell-cycle progression in WT, mad2Δ, rad53-K227A, and rad53-K227A mad2Δ cells by western blot analysis. Pgk1 and Ponceau S were used as loading controls. Cells were released from G1 into S-phase at 25°C in YPD ± 200 mM HU. In the absence of HU, α factor was re-added in the medium after 40 min of release in order to avoid cell re-entry into a new cell cycle. Clb5/Pgk1 signal ratio was plotted for each condition, using WT/rad53-K227A cells at 60 min as a reference. The error bars represent the SD of two independent experiments.

(B) WT, mad2Δ, rad53-K227A, and rad53-K227A mad2Δ cells were released from G1 into S-phase at 25°C in YPD ± 200 mM HU. The cellular DNA content was determined by FACS analysis at the indicated time points.

(C) Total RNAs were extracted from cells synchronized in G1 or released from G1 into S-phase at 25°C in YPD + 200 mM HU. After reverse transcription, the levels of Clb5 mRNA were determined by quantitative PCR. The ratio between the transcription level of CLB5 and the average expression of two reporter genes (TAF10 and ALG9) was plotted on a histogram. The error bars represent the SD of two independent experiments.

(legend continued on next page)
Mad2 Stimulates Clb5 Synthesis through 4E-BP Inhibition

High-throughput screenings performed in yeast, human, and plants have previously uncovered some intriguing physical interactions between Mad2 and translation factors (Table S1). These observations, added to our results, suggest that Mad2 could be implicated in Clb5 mRNA translation. To test this possibility, we quantified the translation rate of Clb5 mRNA in rad53-K227A and rad53-K227A mad2Δ cells (Figures 4A and S4). Cells were synchronized in G1 and released into S-phase for 45 min in the presence of HU. Cycloheximide was added to freeze the ribosomes onto the translating mRNAs. Cytoplasmic, monosome, and polysome fractions were separated on a sucrose gradient, and mRNA levels in polysomes (high translation) and monosomes (low translation) were measured by RT-qPCR. We observed that the deletion of MAD2 shifted Clb5 mRNA from heavy polysomes to smaller polysomes and monosomes, confirming that Mad2 may impact the translation process of Clb5 mRNA (Figures 4A and S4). We then investigated whether Mad2 co-sediments with polysomes by performing a western blot analysis on sucrose gradient fractions (Figure 4B). We observed that Mad2 co-fractionated with heavy polysomes in an EDTA- and RNase I-dependent manner (addition of EDTA leads to the separation of the 40S and 60S ribosomal subunits while RNase I is an endoribonuclease that preferentially degrades single strand RNA). This result supports the hypothesis that Mad2 interacts with the translational machinery. Interestingly, in S. cerevisiae, two independent screenings identified the translation factor Caf20 as a physical partner of Mad2 (Castelli et al., 2015; Graumann et al., 2004). Caf20 has been identified as a repressor of the cap-dependent translation and as a homolog of the eIF4E binding proteins (4E-BP). Consequently, we investigated whether Mad2 could control Clb5 expression through a caf20-dependent mechanism. rad53-K227A, rad53-K227A caf20Δ, rad53-K227A mad2Δ, and rad53-K227A mad2Δ caf20Δ cells were released from G1 into 200 mM HU. Clb5 expression was analyzed by western blot analysis (Figure 4C). The level of Clb5 protein remained similar in rad53-K227A and rad53-K227A caf20Δ cells, whereas Clb5 level increased in rad53-K227A mad2Δ caf20Δ cells in comparison of rad53-K227A mad2Δ cells. In addition, we found that the deletion of CAF20 suppressed the effect of MAD2 deletion on Clb5 mRNA translation (Figure 4A). Altogether, these results show that Mad2 impacts Clb5 mRNA translation in a Ca20-dependent manner.

Since CAF20 deletion restores the Clb5 expression in rad53-K227A mad2Δ cells, we investigated the effect of CAF20 deletion on replication origin firing and HU resistance. As expected, we observed a global increase in origin firing in rad53-K227A mad2Δ caf20Δ cells compared to rad53-K227A mad2Δ cells (Figure 4D). In agreement with this result, CAF20 deletion suppressed the HU sensitivity observed in rad53-K227A mad2Δ cells (Figure 4E). Hence, Mad2 inhibits the 4E-BP Caf20, which in turn counteracts Clb5 synthesis and efficient origin firing.

Mad2 Modifies the Interaction of Caf20 with the Translational Machinery

Mad2 could inhibit Caf20 by at least two different mechanisms: preventing its loading onto mRNA or counteracting its activity. To test the first possibility, rad53-K227A and rad53-K227A mad2Δ cells were released from G1 into S-phase for 45 min in the presence of HU. Protein complexes were fixed onto mRNA with formaldehyde. mRNAs bound to Caf20-HA were co-immunoprecipitated with an anti-HA antibody, reverse transcribed, and quantified using quantitative PCR. As it was previously described (Castelli et al., 2015; Costello et al., 2015), we found that Caf20 was strongly bound to its own mRNA (Figure S5). In addition, we observed that Clb5 mRNA was also bound by Caf20. We confirmed that this association was specific, since the attachment of Caf20 onto Clb5 mRNA was higher compared to the binding of Caf20 onto a negative control (Alg9 mRNA). However, we found that the deletion of MAD2 did not significantly modify the recruitment of Caf20 onto Clb5 mRNA. This result implies that Mad2 inhibits Caf20 by a mechanism that is independent of the recruitment of Caf20 onto mRNAs.

We then investigated whether Mad2 could counteract the activity of Caf20 by altering its interaction with the translational machinery. rad53-K227A and rad53-K227A mad2Δ cells were synchronized in G1 and released into S phase for 45 min in the presence of HU. We did not observe significant differences in Caf20 protein levels by western blotting in the two strains (data not shown). Caf20-HA and its interactors were co-immunoprecipitated. Protein partners were identified and quantified by mass spectrometry. As it was previously described (Costello et al., 2015), we found that Caf20 interacts with a large number of translation factors implicated in both initiation and elongation (Figures 5A and 5B; Table S2). In addition, we observed that, in the absence of Mad2, Caf20 globally increases its association with the whole translational machinery (translational initiation factors and ribosomal proteins). Conversely, only the translational initiation factor elf4E (Cdc33) reduced its affinity for Caf20 in the absence of Mad2 (Figure 5B). We conclude that Mad2 hinders the stable association between Caf20 and the translation machinery, while it specifically stabilizes the binding of Caf20 with elf4E.

DISCUSSION

Many oncogenes and tumor suppressors target cyclins and CDKs. For example, cMyc promotes Cyclin D/E/A transcription (Bretones et al., 2015), while p53 favors the transcription of p21, a CDK inhibitor (Georgakilas et al., 2017). In this study, we uncovered a Mad2-mediated regulatory circuit promoting S-phase cyclin expression. In particular, the levels of Clb5 and Clb6 are compromised when Mad2 is ablated in unperturbed conditions and in response to replication stress. Mad2 is a well-known effector of the spindle checkpoint, but its S-phase function is independent of the other SAC components like
Figure 4. Mad2 Controls Ctb5 mRNA Translation via a Caf20-Dependent Mechanism

(A) Translational rate of Ctb5 mRNA in rad53-K227A, rad53-K227A mad2Δ, and rad53-K227A mad2Δ caf20Δ cells. Cells were released from G1 into 200 mM HU for 45 min at 25°C. Polysome/monosome ratio (Poly/Mono) at the global level or on Ctb5 mRNA was plotted for each strain using rad53-K227A as a reference. Error bars represent the SD of two independent experiments.

(B) Mad2, Pgk1, and Rps6 protein distribution across the sucrose gradient was analyzed by western blotting in the absence or in the presence of EDTA/RNase I. Expression of Ctb5-HA was followed throughout the cell-cycle progression in rad53-K227A, rad53-K227A caf20Δ, rad53-K227A mad2Δ, and rad53-K227A mad2Δ caf20Δ cells by western blot analysis. Pgk1 and Ponceau S were used as loading controls. Cells were released from G1 into 200 mM HU at 25°C. Ctb5/ Pgk1 signal ratio was plotted for each condition, using rad53-K227A and rad53-K227A mad2Δ cells at 60 min as a reference. Error bars represent the SD of two independent experiments for rad53-K227A versus rad53-K227A caf20Δ, cells and of three independent experiments for rad53-K227A mad2Δ versus rad53-K227A mad2Δ caf20Δ cells. Cell-cycle progression of rad53-K227A mad2Δ and rad53-K227A mad2Δ caf20Δ cells was determined by FACS analysis.

(D) BrdU ImmunoPrecipitation and quantitative PCR on four early (ARS305, ARS306, ARS607, and ARS106) and one late (ARS106) origins in rad53-K227A mad2Δ and rad53-K227A mad2Δ caf20Δ cells. Cells were released from G1 into S-phase for 45 min at 25°C in the presence of 200 mM HU and 20 μg/ml BrdU. BrdU fold increase was calculated as a ratio: IP/input, normalized against BrdU signal of rad53-K227A mad2Δ. Error bars represent the SD of two independent experiments.

(E) Cells were spotted in 2-fold serial dilutions on YPDA/HU plates. Relative growth was assayed after 2 days of culture at 28°C. The rad53-K227A allele is marked as rad53.

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**Notes:**
- Section A: Description of translational rate of Ctb5 mRNA in different cell strains and the method of analysis using western blotting or FACS.
- Section B: Analysis of protein distribution across the sucrose gradient using western blotting and cell-cycle progression using western blot analysis.
- Section D: ImmunoPrecipitation and quantitative PCR on various origins in different cell strains.
- Section E: Relative growth assay of cells spotted in serial dilutions on YPDA/HU plates.
Mad1, Mad3, and Bub3. Moreover, since Mad1 also favors the proper localization of Mad2 at the kinetochore during mitosis and at the nuclear pore during interphase (Campbell et al., 2001; De Souza et al., 2009; Lee et al., 2008; Lussi et al., 2010), our results imply that the S-phase function of Mad2 influencing Cib5 expression does not depend on its localization at the kinetochore or at the nuclear pores. We ruled out a contribution of Mad2 in promoting Cib5 transcription or in regulating its degradation. Our results rather indicate that Mad2 promotes an optimal Cib5 protein synthesis.

Previous high-throughput studies have already identified some interactions between Mad2 and translational factors (Table S1), and to our knowledge, Mad2 is the only component of the spindle checkpoint which presents such interactions. Here we found that Mad2 co-sediments with translating ribosomes (Figure 4B), and that Mad2 directly promotes the translation of Cib5 mRNA via a Cib20-dependent mechanism (Figure 4A). We observed that Caf20 binds Cib5 mRNA, regardless of the presence or the absence of Mad2 (Figure S5). Recent studies demonstrated that Caf20 is recruited onto mRNAs via its interaction with the eIF4E factor, or via its fixation on the 3’UTR motif present on mRNAs. Interestingly, Cib5 and Cib6 mRNA share the properties of the 4E-independent recruitment. Indeed, both of them present a Caf20 consensus sequence in their 3’UTR (“AUAAUAUGUAUAUA” localized 100 nt after the stop codon to note that the same tendency has already been observed for a Caf20 mutant that has a defective eIF4E binding (Castelli et al., 2015).

We also found that, in response to replication stress, Rad53 controls Cib5 protein degradation (Figure 3). Interestingly, Cib6 protein degradation upon replication stress is also dependent on Rad53 (Palou et al., 2010). Although Cib5 stabilization occurs in response to both replication stress and intra-S DNA damage (MMS, treatment), the mechanisms seem different (Germain et al., 1997). In particular, the proteolysis inhibition observed in the presence of DNA damage is independent of the checkpoint proteins Mec1 and Rad53 (Germain et al., 1997). This observation could explain why rad53-K227A mad2 Δ cells are specifically sensitive to HU and not to other DNA damaging agents. Contrary to Rnr1, whose transcription level is maintained during the duration of S-phase upon replication stress through a Rad53 dependent mechanism (Figure S3D; Travesa et al., 2012), we note that we did not find evidence for a contribution of Rad53 in prolonging Cib5 or Cib6 transcription upon replication stress (Figures 3 and S3D).

Importantly, the Rad53–Mad2 synergistic and specific effect in response to replication stress seems to be at least partially conserved between different yeast species. Indeed, previous observations in S. pombe also underlined that (1) MAD2 and

**Figure 5. Mad2 Affects the Recruitment of the Translational Machinery**

(A and B) rad53-K227A CAF20-HA, rad53-K227A MAD2 Δ CAFA0-HA cells were released from G1 into S-phase at 25°C in 200 mM HU for 45 min. Proteins interacting with Caf20 (A, ribosomal proteins; B, initiator factors) were co-immunoprecipitated using an anti HA antibody and identified by mass spectrometry. Statistically significant differences between rad53-K227A and rad53-K227A MAD2 Δ cells were plotted on a logarithmic scale so that higher interaction in rad53-K227A MAD2 Δ cells is shown as a positive number. (Red) Interactors enriched in rad53-K227A MAD2 Δ cells; (green) interactor enriched in rad53-K227A Δ cells. (C) Model for the translational regulation of the S-phase cyclin mRNAs by Mad2. Mad2 regulates the affinity of Caf20 for the translational machinery. Mad2 decreases the association Caf20/ribosomes while it stabilizes the association Caf20/Cdc33.

![Figure 5](https://example.com/fig5.png)
CDS1 (RAD53 homolog) exhibit a negative genetic interaction in the presence of replication stress; (2) this interaction is specific for MAD2 over the other components of the spindle checkpoint (MAD1, MAD3, and BUB1); and (3) this interaction is specific to the cellular response to replication stress and not to DNA damage (Sugimoto et al., 2004). However, while in S. pombe the APC is hyperactivated in the absence of Mad2, we did not find any increase in APC activity in S. cerevisiae (no modification of Cib2 degradation). On the contrary, we proposed that Mad2 interferes with the translation process. It is particularly interesting to note that physical interactions between the spindle checkpoint protein Mad2 and the translational machinery have already been identified in different organisms including plants and humans (Table S1).

The Mad2 function described in our paper may have relevant implications for cancer cells. Expression of SAC proteins is often deregulated in tumor cells. However, Mad2 deregulation is particularly prominent over the other spindle checkpoints proteins. Oncomine database analysis revealed that only 2% of cancer cells show an upregulated expression of Mad1, whereas 18% of cancer cells exhibit Mad2 upregulation. This specific increase is particularly relevant in colorectal cancer, in which one-third of the tumor tissue analyzed exhibits an overexpression of Mad2. An additional role of Mad2 out of the spindle checkpoint could explain the particular prominence of Mad2 deregulation. It is also worth noting that many cancer cells exhibit a delocalization of Mad2 protein from the nucleus to the cytoplasm (Fung et al., 2007; Li et al., 2003), a cellular compartment in which Mad2 is not functional as a spindle checkpoint player (Kasai et al., 2002) but could be fully functional as a regulator of the translation process. Given that differences exist between yeast and mammals at the level of 4E-BP regulation, more work will be required to address whether Mad2 affects protein translation also in mammalian cells.

## STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **CONTACT FOR REAGENT AND RESOURCE SHARING**
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
- **METHODS DETAILS**
  - Drug sensitivity assay
  - Protein extraction and Western Blot analysis
  - 2D Gel Electrophoresis of replication intermediates
  - BrdU immunoprecipitation
  - Quantitative PCR
  - RNA extraction and reverse transcription
  - Fluorescence-activated cell sorter (FACS) analysis
  - Non-denaturing protein Co-immunoprecipitation
  - Mass-spectrometry (MS)
  - RNA co-immunoprecipitation
  - Polysome profiling
  - Quantification and statistical analysis
  - Data and software availability

## SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and four tables and can be found with this article at https://doi.org/10.1016/j.molcel.2018.04.020.

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## AUTHOR CONTRIBUTIONS

D.P. and S.G. executed the experiments. C.B., S.R., and S.B executed and analyzed the polysome profiling experiments. P.S. performed the mass spectrometry analysis. S.G. designed the experiments. W.C., D.P., and S.G. analyzed the data. M.F. and S.G. conceived the project and wrote the article. M.F. provided financial support.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Mouse monoclonal anti-phosphorylated Rad53 | In house (Fiorani et al., 2008) | Clone F9 |
| Mouse monoclonal anti-phosphorylated histone H2X (ser139) | Merck | Clone JBW301 Cat #05-636 |
| Mouse monoclonal anti-Pgk1 | ThermoFisher Scientific | Clone 22CSD8 Cat #459250 |
| Rabbit polyclonal anti-Cln2 | Santa-Cruz Biotechnology | Cat #sc9071 |
| Mouse monoclonal anti c-MYC (clone 9E10) | Santa Cruz Biotechnology | Cat #sc-40 |
| Mouse monoclonal anti HA | Biolegend | Clone 16B12 Cat #901503 |
| Mouse monoclonal anti BrdU | MBL | Clone 2B1 Cat #MI-11-3 |
| Rabbit polyclonal anti Mad2 | In house (Vernieri et al., 2013) | Clone IEO447S1353F |
| Rabbit polyclonal anti Rps6 | ABCam | Cat #ab40820 |
| Goat anti-Mouse IgG (H + L)-HRP Conjugate | Bio-Rad | Cat #1706516 |
| Goat anti-Rabbit IgG (H + L)-HRP Conjugate | Bio-Rad | Cat #1706515 |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| Hydroxyurea | Sigma-Aldrich | Cat #H8627 |
| Methyl methanesulfonate (MMS) | Sigma-Aldrich | Cat #129925 |
| MG132 | Sigma-Aldrich | Cat #M8699 |
| Cicloheximide | Sigma-Aldrich | Cat #C7698 |
| Camptothecin | Sigma-Aldrich | Cat #208925 |
| Anti-HA.11 Epitope Tag Affinity Matrix | Biolegend | Cat #900801 |
| Dynabeads Protein A | ThermoFisher Scientific | Cat #10001D |
| **Critical Commercial Assays** | | |
| Genomic DNA buffer genomic set | QIAGEN | Cat #19060 |
| Genomic tip 100/G | QIAGEN | Cat #10243 |
| Rneasy Mini Kit | QIAGEN | Cat #74104 |
| PCR purification kit | QIAGEN | Cat #28106 |
| QuantIFast SYBR green PCR kit | QIAGEN | Cat #204054 |
| **Deposited Data** | | |
| Proteomic raw data | Peptide Atlas repository | http://www.peptideatlas.org/PASS/PASS01156 |
| Raw Data | Mendeley Data | http://dx.doi.org/10.17632/wwcr942cl.1 |
| **Experimental Models: Organisms/Strains** | | |
| All Saccharomyces cerevisiae yeast strains used in this study were W303 derivatives; they are listed in Table S3 | This study | N/A |
| **Oligonucleotides** | | |
| See Table S4 for a list of oligonucleotides used in this study | Sigma-Aldrich | N/A |
| **Recombinant DNA** | | |
| pCH12 (GAL1-rad53-D339A) | Pellicoli et al., 1999 | N/A |

(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Marco Foiani (marco.foiani@ifom.eu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

The list of yeast strains used in this study is available in the Key Resources Table (Table S3). Yeast strains are derived from W303 background (S. cerevisiae). All strains, except cdc33 mutants, are corrected with the wild-type RAD5 locus. Gene deletions and carboxy-terminal tags were carried out using a standard PCR-based strategy to amplify resistance cassettes with appropriate flanking sequences, and replacing the target gene by homologous recombination. All strains, except rad53-D339A mutants, carry a repetition of the thymidine kinase (TK) at the URA3 locus in order to allow BrdU incorporation (Lengronne et al., 2001). GAL1-rad53-D339A plasmid was previously described in (Pellicioli et al., 1999). Strains were grown at 25°C in yeast extract/peptone with 2% glucose (YPD) or in synthetic complete medium lacking uracil. Exponentially growing cells (10^7 cells/ml) were arrested in G1 using α factor (5 μg/ml). Before release, cells were washed in YP. HU was added at 0.2 M, BrdU at 0.2 mg/ml and MG132 at 100 μM.

METHODS DETAILS

Drug sensitivity assay

Cells were grown in liquid culture overnight, counted, adjusted to 8X10^7 cells/ml and diluted before being spotted on YPD plates supplemented with adenine (YPDA) containing indicated concentrations of HU, MMS or Camptothecin. Plates were incubated for 2 days at 28°C. Note that serial dilutions of 1:2 were made for rad53 mutant cells plated on HU while serial dilutions of 1:5 were made in all other cases. rad53-D339A mutants were spotted on synthetic growth medium plates deprived of uracil to maintain the plasmid selection.

Protein extraction and Western Blot analysis

Protein extracts for western blotting were prepared following cell fixation using trichloroacetic acid and analyzed by SDS-polyacrylamide gel electrophoresis (gel 4/15% Criterion TGX, Biorad). Briefly, cells were quickly spun down and the pellet was resuspended in 20% TCA and lysed by bead beating. Lysate and precipitate/debris was mixed with 200 μL 5% TCA and pelleted. The pellet was resuspended in 100 μL Laemmli buffer 1X (β-mercaptoethanol as reducing agent) and 50 μL Tris base 1M, boiled for 5 min. After centrifugation, the supernatant was transferred in new tubes.

Antibodies used for detection are listed in the Key Resources Table. Detection was done through electrogenerated chemiluminescence (ECL, GE- Healthcare). Quantification was done using Image Lab software (Bio-Rad).

2D Gel Electrophoresis of replication intermediates

200m1 of exponentially growing cell culture was harvested in the presence of 1% sodium azide and cooled on ice for 15 minutes. Cells were then subjected to in vivo psoralen cross-linking. Briefly, cells were washed, resuspended in 5ml of cold water in small Petri dishes and kept on ice. 300 μL of 4,5’,8-tri-methyl-psoralen solution (0.2 mg/ml in EtOH 100%) was added prior to extensive resuspension by pipetting, followed by 5 min of incubation in the dark and then 10 min of UV irradiation at 365 nm (Stratagene UV Stratalinker). The procedure was repeated 3 times. Cells were centrifuged and resuspended in 5ml of nuclear isolation buffer (17% glycerol, 50mM MOPS, 2mM MgCl2, 150mM Kacetate, 0.5mM spermidine, 0.15mM spermine, pH 7.2) and mechanically lysed using an equal volume of glass beads (Sigma, G8772). After centrifugation (8000 rpm, 10 min, 4°C), the pellet was resuspended in 5ml
of G2 buffer (Genomic extraction kit, QIAGEN). Genomic DNA extraction was performed according to manufacturer recommendations. 4 µg of genomic DNA was digested overnight with 200 units of NcoI enzyme and then precipitated with 1/8 volume of Kacete and one volume of isopropanol. Signals were detected following 2D gel electrophoresis (Brewer and Fangman, 1987) and standard Southern blot procedures using a probe against ARS305 (Chr III 39002-40063). Quantification of bubble arc signal was performed using the Image Quant software (GE Healthcare).

**BrdU immuno-precipitation**

150 mL of exponentially cell culture was synchronized in G1, released into S-phase in the presence of 200mM HU and 200 µg/ml BrdU. At different time points, cells were harvested in the presence of 1% sodium azide and cooled on ice for 15 minutes. Cells were then washed in 20ml of TE and the genomic DNA was extracted using the Genomic extraction kit (QIAGEN) according to manufacturer recommendations. BrdU immuno-precipitations were performed as previously described (Bermejo et al., 2009; Katou et al., 2003). DNA was then resuspended overnight in 250 µL of TE and then shared using the Bandelin UW2070 sonicator (6 pulses, 20% power, 20 s/pulse). Immediately before immunoprecipitation, the genomic DNA was denatured at 100°C for 10 minutes, cooled immediately on ice and supplemented with 100 µL of ice cold PBS 2x and 200 µL of ice cold-PBS 1X-2% BSA-0,2% Tween20. BrdU immunoprecipitation was carried out overnight using 14 µg of genomic DNA, 20 µL of Protein A-magnetic Dynabeads (Thermo-fisher) and 4 µg of anti-BrdU antibody (clone 2B1, MBL, MI-11-3). 700ng of DNA was kept as an input. After immuno-precipitation, beads were washed 4 times with ice cold lysis buffer (50mM HEPES-KOH pH7.5, 140mM NaCl, 1mM EDTA, 1% Triton x100, 0.1% Na-deoxycholate), 2 times with ice-cold lysis buffer + 500mM NaCl, 2 times with ice-cold washing buffer (10mM Tris- HCl pH8.0, 250mM LiCl, 0.5% NP40, 0.5% Na-deoxycholate, 1mM EDTA) and once with TE. Beads were then resuspended in 50 µL of elution buffer (50mM Tris-HCl pH8, 10 mM EDTA, 1% SDS), incubated at 65°C for 10 minutes with shaking. After centrifugation and magnetic attachment, the supernatant was transferred into a new tube. 49 µL of TE and 1 µL of proteinase K 50 mg/ml was added to both IP and input tubes. Tubes were incubated at least 2 hours at 37°C. DNA was then purified using the PCR purification Kit (QIAGEN), eluted in 50 µL of the elution buffer of the kit and precipitated at −20°C overnight in the presence of 40mM Na-acetate, 1 µL of glycogen and 132.5 µL of 100% ethanol. After centrifugation, the pellet was washed in 70% ethanol, dried and resuspended in 50 µL of bi-distillated water. Input DNA was diluted 50 times and IP DNA 10 times before quantitative PCR.

**Quantitative PCR**

Primers used for quantitative PCR are listed in Table S4. Quantitative PCR was prepared using QuantiFast SYBR green PCR kit (QIAGEN) and run on the Lightcycler 480II (Roche) according to the manufacturer’s recommendations. Absolute DNA quantification was deduced from standard curve (100; 10; 1 and 0.1ng of input DNA).

**RNA extraction and reverse transcription**

Total miRNAs were extracted from 2.5x10^7 cells using the RNeasy mini kit (QIAGEN). Reverse transcription was performed using the SuperScript VILO cDNA synthesis kit (Invitrogen) with 2 µg of total RNA as starting material. 1/160 of the cDNA reaction was used for quantitative PCR. Two reporter genes (TAF10, ALG9) were chosen for their robustness (Teste et al., 2009) and their distance from replication origins (minimum 12 kb). Normalization was done by dividing the expression of the gene of interest by the average of the two reporter genes.

**Fluorescence-activated cell sorter (FACS) analysis**

1x10^7 cells were collected by centrifugation, and resuspended overnight in 70% ethanol/ 250mM Tris-HCl (pH 7.5) solution. Cells were then centrifuged and resuspended in 0.5M Tris-HCl (pH 7.5), resuspended in the same buffer containing 2 mg/ml of RNaseA and incubated at 37°C for at least 2h. Cells were then centrifuged in 500 µL of 200mM Tris-HCl (pH 7.5), 200mM NaCl, 80mM MgCl2, supplemented by 50 µg/ml of propidium-iodide (Sigma). Samples were then diluted 10-fold in 50mM Tris-HCl (pH 7.5) and analyzed using a Becton Dickinson FACSscan and the Cellquest software.

**Non-denaturing protein Co-immunoprecipitation**

2x10^8 cells were used for each immunoprecipitation. Cell pellet was washed two times in 50ml of TE, and resuspended in 1.6ml of JS buffer (35mM HEPES pH7.5, 105mM NaCl, 0.7% glycerol; 0.7% Triton, 1 mM MgCl2, 3.5mM EDTA, 1 tablet of Phostop-phosphatase inhibitor Roche, 1 tablet of Ultra-protease inhibitor-Roche). The same quantity of zirconium beads was added to the cell suspension and cells were mechanically disrupted at 4°C thanks to the use of a bead-beater. Cell lysate was collected using a gel tip (thin diameter to avoid beads) and proteins were quantified using the Bradford Protein Assay (Biorad). Proteins associated to Caf20-HA were immunoprecipitated overnight using 80 µL of Sepharose beads covalently coupled to an anti-HA antibody (Biolegend). After immunoprecipitation, beads were washed once with JS buffer, two times with JS buffer supplemented with 350 mM NaCl, and two times with JS buffer. At the last step, beads were resuspended in 40 µL of Laemmli buffer, boiled during 6 minutes at 98°C, and centrifuged. The supernatant was analyzed by SDS-polyacrylamide gel electrophoresis. Note that pre-lubricated tubes were used at all stages to avoid protein adherence.
Mass-spectrometry (MS)

**Protein digestion and peptide preparation**

Gel lanes were processed according to STAGE-digging protocol (Soffientini and Bachi, 2016). The entire protocol occurs in a p1000 tip (Sarstedt 70.762.100) filled at the orifice with a double C18 Empore Disk (3M, Minneapolis, MN) plug, named STAGE-digging tip. Briefly, after Coomassie staining, the entire lane was carefully cut into ~1 mm³ cubes and transferred into the STAGE-digging tip. These gel cubes were dehydrated with 100% acetonitrile (CAN, Carlo Erba) and rehydrated in 100 mM NH₄HCO₃ (Sigma) twice before being dehydrated by the addition of ACN. To ensure that the gel pieces do not create a sticky surface on the C18, all the solutions were added with a gel-loader tip. The removal of solutions was accomplished by centrifugation at 1800 rpm using the commercial tip box as holder. Reduction of protein disulfide bonds was carried out with 10 mM dithiothreitol (DTT) in 100 mM NH₄HCO₃ and subsequent alkylation was performed with 55 mM iodoacetamide (IAA) (Sigma), in complete darkness, in 100 mM NH₄HCO₃ at room temperature for 30 min. Both DTT and IAA were removed by centrifugation or by syringe as previously described. The gel pieces were rehydrated and dehydrated with 100 mM NH₄HCO₃ and ACN respectively prior to digestion. Gel pieces were rehydrated with 40 μL of Trypsin (12.5 ng/μL in 100 mM NH₄HCO₃), after few minutes 60 μL of NH₄HCO₃ were added and samples were incubated at 37°C O/n in a commercial tip box filled by water on the bottom to ensure that buffer will not evaporate. The digestion solution was then forced through the double plug with a syringe and the flow through was collected. Samples were acidified with 100 μL of 0.1% formic acid (FA, Fluka), forced with the syringe and collected as flow-through. In this way the desalting of peptides occurs. Peptides were eluted twice by adding 100 μL of a solution composed of 80% ACN, 0.1% FA, an extra step of extraction with 100% ACN was performed and then all the eluates were dried in a Speed-Vac and resuspended in 15 μL of solvent A (2% ACN, 0.1% formic acid), 3 μL were injected for each technical replicate on the Q-Exactive –HF mass spectrometer.

**MS analysis and proteins quantification**

Mass spectrometry analysis was carried out by LC–MS–MS on a quadrupole Orbitrap Q Exactive HF mass spectrometer (Thermo Scientific). Peptide separation was achieved on a linear gradient from 95% Solvent A to 50% Solvent B (80% acetonitrile, 0.1% formic acid) over 20 min and from 50% to 100% Solvent B in 2 min at a constant flow rate of 0.25 μl min⁻¹ on a UHPLC Easy-nLC 1000 (Thermo Scientific), where the LC system was connected to a 25 cm fused-silica emitter of 75 μm inner diameter (New Objective), packed in house with ReproSil–Pur C18–AQ 1.9 μm beads (Maisch) using a high-pressure bomb loader (Proxeon). MS data were acquired using a data-dependent top15 method for HCD fragmentation.

Survey full scan MS spectra (300–1750 Th) were acquired in the Orbitrap with 60,000 resolution, AGC target 1e6, IT 120 ms. For HCD spectra the resolution was set to 15,000, AGC target 1e5, IT 120 ms; normalized collision energy 28 and isolation width 3.0 m/z.

2 technical replicates of each sample were carried out. Raw data were processed with MaxQuant version 1.5.2.8. Peptides were identified from the MS–MS spectra searched against the uniprotKB_S.Cerevisiae database (3845 entries canonical + isoforms) using the Andromeda search engine, in which trypsin specificity was used with up to two missed cleavages allowed.

Cysteine carbamidomethylation was used as a fixed modification, methionine oxidation and protein amino-terminal acetylation as variable modifications. The mass deviation for MS and MS–MS peaks was set at 4.5 and 20 ppm respectively. The peptide and protein false discovery rates (FDRs) were set to 0.01; the minimal length required for a peptide was six amino acids; a minimum of two peptides and at least one unique peptide were required for high-confidence protein identification. The lists of identified proteins were filtered to eliminate reverse hits and known contaminants.

Label-free analysis was carried out, including a ‘match between runs’ option (time window of 5 min). A minimum ratio count of 2 was considered and the ‘LFQ intensities’, which are the intensity values normalized across the entire dataset, were used. 2 Biological and 2 technical replicates for every condition were submitted in a single MaxQuant run.

Statistical t test analyses were done using the Perseus program (Version 1.5.1.6) in the MaxQuant environment. For all the statistical analysis an FDR 0.05 was applied using a permutation test (500 randomizations) and S0 = 1.

Gene ontology annotation for biological process and molecular function was manually added by uniprot.org. Proteins belonging to translational process and ribosomal assembly (list of GO selected is reported in the Table S2, sheet Go_table) were filtered and only proteins with a valid ratio in both biological replicates were selected.

Ratios of the 2 biological replicates were averaged and plotted in logarithmic scale. Standard deviations were calculated and represented on the plot.

**RNA co-immunoprecipitation**

2x10⁶ cells were used for each immunoprecipitation. Proteins were fixed onto RNA with 1% formaldehyde at 4°C. In vivo crosslinking was stopped by addition of 0.1M glycine. Cells were washed four times with 20mL of water. After centrifugation, the cell pellet was resuspended in 0.8mL of Buffer A (20mM Tris-HCl pH8, 140mM NaCl, 1 mM MgCl₂, 0.5 mM DTT, 1 tablet of Phostop-phosphatase inhibitor Roche, 1 tablet of Ultra-protease inhibitor-Roche, and 80U/ml RNasin-Promega). The same quantity of zirconium oxide beads was added to the cell suspension and cells were mechanically lysed thanks to the use of a bead-beater. The lysate was recovered with a gel tip (to avoid beads) and centrifuged. Proteins of the supernatant were quantified using the Bradford Protein Assay (Biorad). 20mg of protein extract and 120 μL of Sepharose beads coupled with anti-HA antibody were used for overnight immunoprecipitation. 1mg of protein extract was kept as an input. After immunoprecipitation, beads were washed 3 times shortly with Buffer A, two times 15 minutes with Buffer A, and three times with the Rnase III buffer (10mM Tris-HCl pH8, 10mM MgCl₂, 60mM NaCl, 1mM DTT, 10U/ml RNasin-Promega). At the final wash, beads were resuspended in 100 μL of Rnase III buffer supplemented...
by 40 U RNasin, 1 U DNase I, 2 U RNase III, and incubate 10 minutes at 37°C. RNase III was deactivated by addition of 900 μL of Buffer E (20mM Tris-HCl pH8, 140mM NaCl, 0.5% NP40, 1mM EDTA, 0.5 mM DTT, 40U/ml RNasin-Promega). Beads were further washed three more times with buffer E. At the last wash, beads were resuspended in 270 μL of proteinase K buffer (10mM Tris-HCl pH8, 200mM NaCl, 1mM EDTA, 0.1% SDS, 10U/ml RNasin-Promega). Proteinase K buffer up to 270 μL was also added to the input samples. 50 μg of proteinase was added to IP and input and the samples were incubated for two hours at 40°C. After centrifugation, the supernatant was transferred into a new tube and incubated overnight at 65°C to reverse the crosslink. 750 μL of Trizol and 200 μL of Chloroform was added to each sample and the aqueous phase was collected. RNA was precipitated overnight at −20°C by addition of 500 μL of propanol and 1 μL of glycogen. After centrifugation, RNA pellet was washed in 70% ethanol and resuspended in 30 μL of bi-distilled water for IP samples and 20 μL of water for Input samples. Total mRNAs were then reverse-transcribed and amplified by quantitative PCR.

**Polysome profiling**

**Cell lysis.** 2*10^9 cells were used for each polysome profiling experiment. 100 μg/ml of cycloheximide was added for 5 minutes to each sample to “freeze” ribosomes onto RNAs. Cells were centrifuged, washed two times with 7 mL of pre-lysis buffer (20mM Tris-HCl pH8, 50mM KCl, 10mM MgCl2, 1mM DTT, 100 μg/mL cycloheximide, 200 μg/mL heparin) and then resuspended in a volume of lysis buffer (20mM Tris-HCl pH8, 50mM KCl, 10mM MgCl2, 1mM DTT, 100μg/mL cycloheximide, 200μg/mL heparin, Ultra-protease inhibitor 1X-Roche, and 40U/ml RNasin-Promega) equivalent to the cell pellet size. An equal volume of zirconium beads was added to the cell suspension and cells were mechanically lysed thanks to use of a bead-beater. Lysate was recovered and cleared by centrifugation.

**Polysomal profiles.** 15 and 50% sucrose buffers were prepared in 50mM NH4Cl, 50mM Tris-Acetate, 12mM MgCl2, 1mM DTT. To obtain a linear gradient, we used the Gradient Maker device (Hoefer SG) equipped with tubes adapted for a SW41 rotor. We used 11ml of each sucrose buffer in each gradient tube. Cytoplasmic extracts with equal amounts of RNA were loaded on the 15%–50% sucrose gradient and centrifuged at 4°C in a SW41Ti Beckman rotor for 3h30 min at 39,000 rpm Absorbance at 254 nm was recorded by BioLogic LP software (Bio-Rad) and fractions (0.5 ml each) were collected for subsequent RNA extraction.

To distinguish between active polysomes and co-sedimenting mRNPs, we treated our samples with 40 mM EDTA prior to gradient loading. Sucrose gradients were also supplemented with 30 mM EDTA and prepared without Mg2+.

**RNA purification.** The different fractions were pooled into 3 major fractions: cytoplasm, monosomes and polysomes. 100 μg/ml of proteinase K and 1% SDS was added to each major fraction. The fractions were then incubated for 1h30 at 37°C. 200mM NaCl and 1/4 volume of acidic phenol/chloroform/isoamyl alcohol (25:24:1) was added to the samples and the aqueous phase was transferred to a new tube. RNAs were precipitated overnight at −20°C after addition of 1 μL of glycogen and 1 volume of isopropanol. After centrifugation, RNA pellet was washed in 70% ethanol and resuspended in 40 μL of bi-distilled water. The RNA concentration was measured using nanodrop.

**Quantification and statistical analysis**

Number of replicates and statistical tests are specified in the corresponding figure legends. In all graphs, standard deviations are represented by error bars. Western blots were quantified using the Image lab software (Biorad). Raw files (.scn) were used to allow the quantification of linear and non-saturated signals. 2D gels were quantified using the Image Quant software (GE Healthcare). The mass spectrometry analysis is described in the corresponding paragraph of the STAR Methods.

**Data and software availability**

Proteomic data as raw files, total proteins and peptides identified with relative intensities and search parameters have been loaded on the Peptide Atlas repository (http://www.peptideatlas.org/PASS/PASS01156). The remaining raw data have been deposited to Mendel Data and are available at http://dx.doi.org/10.17632/wwcrg942cf.1.
Supplemental Information

A Mad2-Mediated Translational Regulatory Mechanism
Promoting S-Phase Cyclin Synthesis Controls
Origin Firing and Survival to Replication Stress

Sophie Gay, Daniele Piccini, Christopher Bruhn, Sara Ricciardi, Paolo Soffientini, Walter Carotenuto, Stefano Biffo, and Marco Foiani
SUPPLEMENTARY FIGURE:

Figure S1 (related to Figure 1): *Mad2, but not all HORMA domain proteins, affects cell survival in case of replication stress without alteration of the Rad53 activation.* (A) Twofold serial dilutions of *rad53-K227A* and *rad53-K227A rev7Δ* cells on YPDA/HU plates. Relative growth was assayed after 2 days of culture at 28°C. (B) Rad53 activation induced by replication stress was monitored by Western Blot analysis using anti-phosphorylated Rad53 antibodies. Cells were synchronized in G1 and released into YPD + 200mM HU. Pgk1 was used as loading control.
Figure S2 (related to Figure 2): MAD2 deletion does not affect origin firing in wt cells. (A) 2D gel analysis of replication intermediates in wt, mad1Δ, mad2Δ, mad1Δ mad2Δ cells at the early origin ARS305. Cells were released from G1 into S-phase at 25°C in 200mM HU. Quantification of replication intermediates was plotted on the histogram. Indeed, intensity of the bubble arc signal was divided by the intensity of the relative monomer spot, normalized against wt cells (arbitrary units). (B-D) BrdU Immunoprecipitation and quantitative PCR on early (B, D), centromeric (C) and inactive (D) origins in wt and mad2Δ cells. Cells were released from G1 into S-phase at 25°C in the presence of 200mM HU and 200μg/ml BrdU. BrdU fold increase was calculated as a ratio: IP/input, normalized against BrdU signal of wt cells after 45min of release. Error bars represent the standard deviation between two independent experiments.
Figure S3 (related to Figure 3): Rad53 and Mad2 specifically regulate Clb5/Clb6 protein expression. (A-B) Expression of Clb6-HA (A), Orc2-Myc and Clb2 (B) was followed throughout the cell cycle progression in wt, mad2Δ, rad53-K227A and rad53-K227A mad2Δ cells by Western Blot analysis. Pgk1 and Ponceau S were used as loading controls. Cells were released from G1 into S-phase at 25°C in YPD +/- 200 mM HU. In the absence of HU, alpha factor was re-added in the medium after 40 minutes of release in order to avoid cell re-entry into a new cell cycle. Clb6/Pgk1 signal ratio was plotted for each condition, using wt/rad53-K227A cells at 60 minutes as a reference. Error bars represent standard deviation of two independent experiments. (C) Clb6 mRNA relative expression in wt, mad2Δ, rad53-K227A and rad53-K227A mad2Δ cells. Total RNAs were extracted from cells synchronized in G1 or released from G1 into S-phase at 25°C in YPD + 200 mM HU. After reverse transcription, specific gene transcription was determined by quantitative PCR. The ratio between the transcription of a specific gene and the average expression of two reporter genes (TAF10, ALG9) was plotted on a histogram. Error bars represent the standard deviation of two independent experiments. (D) Rnr1, Clb5 and Clb6 mRNA relative expression in wt and rad53Δ sml1Δ cells. Experiments were performed as described in (C). (E) Expression of Clb5-HA in rad53-K227A cells upon HU treatment with or without a proteasome inhibitor (MG132). Pgk1 and Ponceau S were used as loading controls. Cells were released from G1 into S-phase at 25°C in YPD + 200 mM HU +/- 100 μM MG132. Clb5/Pgk1 signal ratio was plotted for each condition, using the 60 minute timepoint as a reference. The error bars represent the standard deviation of two independent experiments.
**Figure S4** (related to Figure 4): Association of Clb5 mRNA with monosomes or polysomes in *rad53-K227A* and *rad53-ΔK227* cells. Cells were released from G1 into 200 mM HU for 45 min at 25°C. mRNAs associated with monosomes and polysomes were separated on a 15–50% sucrose gradient. The abundance of Clb5 mRNA in the different fractions was determined by reverse transcription followed by quantitative PCR. Error bars represent the standard deviation of two independent experiments.
Figure S5 (related to Figure 5): Mad2 does not modify the recruitment of Caf20 onto Clb5 mRNA. *rad53-K227A CAF20-HA, rad53-K227A mad2Δ CAF20-HA* and wt (no tag) cells were released from G1 into S-phase at 25°C in 200mM HU for 45 minutes. mRNAs associated with Caf20 were co-immunoprecipitated using an anti-HA antibody, reverse transcribed and amplified by qPCR. Caf20 fold increase was calculated as a ratio: IP/input. The error bars represent the standard deviation of two independent experiments.
### SUPPLEMENTARY TABLES:

| Interaction | Protein | Detection | Reference | Protein function | Organism |
|-------------|---------|-----------|-----------|------------------|----------|
| Physical    | Caf20   | Affinity Capture & mass spectrometry | Graumann et al, 2004; Castelli et al, 2015 | Phosphoprotein of the mRNA cap-binding complex; involved in translational control | S.Cerevisiae |
| Physical    | Eap1    | Two hybrids | Wong et al, 2007 | eIF4E-associated protein | S.Cerevisiae |
| Physical    | Ebs1    | Two hybrids | Uetz et al, 2000 | Protein involved in translation inhibition and nonsense-mediated decay | S.Cerevisiae |
| Physical    | Rps3A   | Affinity Capture & mass spectrometry | Hein et al, 2015 | Ribosomal Protein S3A | H.Sapiens |
| Physical    | Fbr12   | Affinity Capture & mass spectrometry | Van Leene et al, 2010 | Eukaryotic initiation factor 5A-2 | A.Thaliana |

Table S1 (related to Figure 4): Previously published interactions between Mad2 and translation factors.
Table S3 (related to STAR Methods): Strains used in this study

| Name         | Called as | Genotype                                                                                                                                                                                                 | Source               |
|--------------|-----------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------|
| CY12512      | wt        | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, ura3::7x-TKs-URA3                                                                                                                               | Rossi et al, 2015    |
| CY12527      | rad53-K227A | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, rad53-K227A-KANMX4, ura3::7x-TKs-URA3                                                                                                                  | Rossi et al, 2015    |
| CY11935      | mad1Δ     | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, ura3::7x-TKs-URA3, mad1Δ::HIS3                                                                                                                   | This study           |
| CY11929      | mad2Δ     | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, ura3::7x-TKs-URA3, mad2Δ::HIS3                                                                                                                   | This study           |
| CY12006      | mad1Δ mad2Δ | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, ura3::7x-TKs-URA3, mad1Δ::HIS3, mad2Δ::HPHMX4                                                                                               | This study           |
| CY11942      | rad53 mad1Δ | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, rad53-K227A-KANMX4, ura3::7x-TKs-URA3, mad1Δ::HIS3                                                                                       | This study           |
| CY11933      | rad53 mad2Δ | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, rad53-K227A-KANMX4, ura3::7x-TKs-URA3, mad2Δ::HIS3                                                                                       | This study           |
| CY12009      | rad53 mad1Δ mad2Δ | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, rad53-K227A-KANMX4, ura3::7x-TKs-URA3, mad1Δ::HIS3, mad2Δ::HPHMX4                                                                  | This study           |
| CY13756      | rad53 mad3Δ | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, rad53-K227A-KANMX4, ura3::7x-TKs-URA3, mad3Δ::HIS3                                                                                       | This study           |
| CY13758      | rad53 bub3Δ | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, rad53-K227A-KANMX4, ura3::7x-TKs-URA3, bub3Δ::HIS3                                                                                           | This study           |
| CY14232      | rad53 pds1Δ | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, rad53-K227A-KANMX4, ura3::7x-TKs-URA3, pds1Δ::HIS3                                                                                           | This study           |
| CY11968      | GAL1-rad53-D339A | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, [cen3, URA+, Gal1-rad53-D339A]                                                                                                      | Pellicioli et al, 1999 |
| CY14562      | GAL1-rad53-D339A mad2Δ | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, [cen3, URA+, Gal1-rad53-D339A], mad2Δ::HIS3                                                                                   | This study           |
| CY15133      | rad53 GAL1-MAD2 | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, rad53-K227A-KANMX4, Mad2Δ::PromGAL1-MAD2-HIS3                                                                                       | This study           |
| CY14039      | rad53 rev7Δ | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, rad53-K227A-KANMX4, ura3::7x-TKs-URA3, rev7Δ::HIS3                                                                                         | This study           |
| CY14656      | wt Clb5-HA | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, ura3::7x-TKs-URA3, Clb5-3HA-TRP1                                                                                                               | This study           |
| CY14658 | rad53 Clb5-HA | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, rad53-K227A-KANMX4, ura3::7x-TkS-Ura3, Clb5-3HA-TRP1 | This study |
|---------|---------------|-------------------------------------------------------------------------------------------------|-----------|
| CY14660 | mad2Δ Clb5-HA| MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, ura3::7x-TkS-Ura3, mad2::His3, Clb5-3HA-TRP1 | This study |
| CY14652 | rad53 mad2Δ Clb5-HA | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, rad53-K227A-KANMX4, ura3::7x-TkS-Ura3, mad2::His3, Clb5-3HA-TRP1 | This study |
| CY15135 | wt Clb6-HA | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, ura3::7x-TkS-Ura3, Mad2::His3, Clb6-3HA-TRP1 | This study |
| CY15139 | rad53 Clb6-HA | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, rad53-K227A-KANMX4, ura3::7x-TkS-Ura3, Clb6-3HA-TRP1 | This study |
| CY15137 | mad2Δ Clb6-HA | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, ura3::7x-TkS-Ura3, mad2::His3, Clb6-3HA-TRP1 | This study |
| CY15141 | rad53 mad2Δ Clb6-HA | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, rad53-K227A-KANMX4, ura3::7x-TkS-Ura3, mad2::His3, Clb6-3HA-TRP1 | This study |
| CY15062 | caf20Δ Clb5-HA | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, ura3::7x-TkS-Ura3, caf20Δ::His3, Clb5-3HA-TRP1 | This study |
| CY15064 | rad53 caf20Δ Clb5-HA | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, rad53-K227A-KANMX4, ura3::7x-TkS-Ura3, caf20Δ::NatR, Clb5-3HA-TRP1 | This study |
| CY15078 | mad2Δ caf20Δ Clb5-HA | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, ura3::7x-TkS-Ura3, caf20Δ::NatR, Clb5-3HA-TRP1 | This study |
| CY15081 | rad53 mad2Δ caf20Δ Clb5-HA | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, rad53-K227A-KANMX4, ura3::7x-TkS-Ura3, caf20Δ::NatR, Clb5-3HA-TRP1 | This study |
| CY14046 | wt Orc2-3Hc | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, ura3::7x-TkS-Ura3, Orc2-13MYC-TRP1 | This study |
| CY14025 | rad53 Orc2- myc | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, rad53-K227A-KANMX4, ura3::7x-TkS-Ura3, Orc2-13MYC-TRP1 | This study |
| CY14047 | mad2Δ Orc2- myc | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, rad53-K227A-KANMX4, ura3::7x-TkS-Ura3, Orc2-13MYC-TRP1 | This study |
| CY14020 | rad53 mad2Δ Orc2- myc | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, rad53-K227A-KANMX4, ura3::7x-TkS-Ura3, Orc2-13MYC-TRP1 | This study |
| CY14646 | wt Clb5-myc | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, ura3::7x-TkS-Ura3, Clb5-13MYC-TRP1 | This study |
| CY14648 | rad53 Clb5-myc | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, rad53-K227A-KANMX4, ura3::7x-TkS-Ura3, Clb5-13MYC-TRP1 | This study |
| CY14663 | mad2Δ Clb5-myc | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, ura3::7x-TkS-Ura3, mad2::His3, Clb5-13MYC-TRP1 | This study |
| CY14651 | rad53 mad2Δ Cln2-myc | MAT a ade2-1, ura3-1, his3-11,15 leu2-3,112, trp1-1, CAN1, rad53-K227A-KANMX4, ura3::7x-TKs-URA3, mad2::HIS3, Cln2-13MYC-TRP1 | This study |
| CY12489 | rad53Δ sml1Δ | MAT a, ade2-1, ura3-1, his3-11,15, leu2-3,112, trp1-1, CAN1, sml1::TRP1, rad53::KANMX6, ura3::7x-TKs-URA3 | This study (Gift from Michele Giannattasio) |
Table S4 (related to STAR methods): Oligonucleotides used in this study

| Name     | Sequence                                      | Source               |
|----------|-----------------------------------------------|----------------------|
| ARS305 Fwd | TTTGGAGCTCAAGTGGATTGAG                      | Tittel-Elmer et al, 2012 |
| ARS305 Rv  | TGAAGACTGGACATATTTGAAGGAATTT                 | Tittel-Elmer et al, 2012 |
| ARS306 Fwd | CCCCCATCCCCAATAGTTCGA                       | Tittel-Elmer et al, 2012 |
| ARS306 Rv  | TGCAGCCGCTCATACGA                           | Tittel-Elmer et al, 2012 |
| ARS607 Fwd | CTTAGCTGGGTATGAGGAGGT                      | Tittel-Elmer et al, 2012 |
| ARS607 Rv  | TAAATGCACGAGGCGAAAACAA                     | Tittel-Elmer et al, 2012 |
| ARS106 Fwd | GGCCGACTTGCCATAATATCA                     | Tittel-Elmer et al, 2012 |
| ARS106 Rv  | TCAGACCGAGAAGGAGGTT                       | Tittel-Elmer et al, 2012 |
| Cen3 Fwd  (ARS308) | GATCAGCAGTCAAAACTATGGGAATTC            | Tittel-Elmer et al, 2012 |
| Cen3 Rv   (ARS308) | AACTTCCACCAGTAACGTTTCCATATCC         | Tittel-Elmer et al, 2012 |
| ARS440 Fwd (inactive ARS) | CGAAAGTGACGAAGTTCAATGC | Urulangodi et al, 2015 |
| ARS440 Rv (inactive ARS) | GCCATTGCTGATAAAGACGC | Urulangodi et al, 2015 |
| CLB5 Fwd  | AGGCAGCCAAGCTGCAGAGAGA                    | This Study           |
| CLB5 Rv   | TCTGCTGCTCGTACGTT                        | This Study           |
| CLB6 Fwd  | ACTCCCTCGGAAATTGCC                      | This Study           |
| CLB6 Rv   | TGGCTCTATAGGATCGGTT                      | This Study           |
| CLN2 Fwd  | TGTAGACGAGAGGCCCTACCA                    | This Study           |
| CLN2 Rv   | ACTGCCAGGCGGATAACATCA                    | This Study           |
| RNR1 Fwd  | CGCACTAGGCGATCCACCGTA                    | This Study           |
| RNR1 Rv | ACAACGTTGGAGAGGCCTGA | This Study |
|---------|------------------------|------------|
| **TAF10 Fwd** | ATATTCCAGGATCAGGTCTTCCGTAAGC | Teste et al, 2009 |
| **TAF10 Rv** | GTAGTCTTCTCATTCTGTGATGTTGTTGTTG | Teste et al, 2009 |
| **ALG9 Fwd** | CACGGATAGTGGCTTTTGGTGAACAATTAC | Teste et al, 2009 |
| **ALG9 Rv** | TATGATTATCTGCGAGCAGGAAAGAACTTGGG | Teste et al, 2009 |
| **CAF20 Fwd** | GCAGGACAAGCCAATCTTGGT | This Study |
| **CAF20 Rv** | TGCTTCGTCGTCTTCGCTTC | This Study |