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The Cdk1 interacting protein Cip1 is regulated by the S phase checkpoint in response to genotoxic stress

Ze Zhang[1], **Ping Ren**[2], **Ajay A. Vashisht**[3], **James A. Wohlschlegel**[3], **David G. Quintana**[2,#], and **Fanli Zeng**[1,2,#]

[1]College of Life Sciences, Hebei Agricultural University, Baoding, Hebei; Key Laboratory of Hebei Province for Plant Physiology and Molecular Pathology, Hebei, 071001, China

[2]Department of Biochemistry and Molecular Biology, Biophysics Unit, School of Medicine, Universitat Autonoma de Barcelona, Bellaterra, Catalonia, 08193, Spain

[3]Department of Biological Chemistry, University of California, Los Angeles, Los Angeles, California, 90095, United States of America

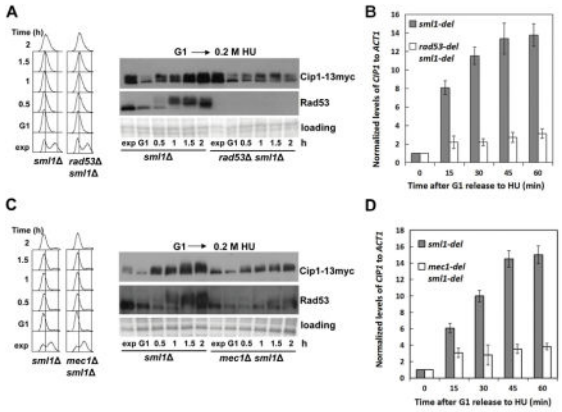
Abstract

In eukaryotic cells a surveillance mechanism, the S phase checkpoint, detects and responds to insults that challenge chromosomal replication, arresting cell cycle progression and triggering appropriate events to prevent genomic instability. In the budding yeast *Saccharomyces cerevisiae*, Mec1/ATM/ATR, and its downstream kinase Rad53/Chk2, mediate the response to genotoxic stress. In this study, we place Cip1, a recently identified Cdk1 inhibitor (CKI), under the regulation of Mec1 and Rad53 in response to genotoxic stress. Cip1 accumulates dramatically in a Mec1 and Rad53 dependent manner upon replication stress. This increase requires the activity of MBF, but not the transcriptional activator kinase Dun1. At the protein level, stabilization of replication stress induced Cip1 requires continued *de novo* protein synthesis. In addition, Cip1 is phosphorylated at an S/TQ motif in a Mec1 dependent manner. Deletion of Cip1 affects proliferation in hydroxyurea containing plates. Significantly, the sensitivity is increased when the dosage of the G1 cyclin CLN2 is increased, compatible to a role of Cip1 as a G1-CDK inhibitor. In all, our results place Cip1 under the S phase checkpoint response to genotoxic stress. Furthermore, Cip1 plays a significant role to preserve viability in response to insults that threaten chromosome replication.

Graphical Abstract

We place Cip1, a recently identified Cdk1 inhibitor (CKI), under the regulation of Mec1 and Rad53 in response to genotoxic stress. Cip1 accumulates dramatically in a Mec1 and Rad53 dependent manner upon replication stress. Cip1 is phosphorylated at an S/TQ motif in a Mec1 dependent manner.

[#]Correspondence to Fanli.Zeng@pku.edu.cn; DavidG.Quintana@uab.cat.
DR. FANLI ZENG (Orcid ID : 0000-0003-2241-1890)



Keywords

YPL014W; Cip1; Cdk1; Cln2; S phase checkpoint; Rad53; MBF; Mec1

Introduction

Cells are under constant pressure from endogenous and exogenous agents that cause DNA damage or interfere with DNA replication, globally termed as genotoxic stress. In response to genotoxic stress, cells activate a crucial surveillance mechanism, S phase checkpoint, which protects ongoing DNA replication and arrests cell cycle progression in order to repair DNA damage.

In budding yeast, replication forks stall upon replication stress, leading to the activation of the central transducer protein kinases Mec1 and Tel1 (Sanchez *et al.* 1996), the orthologs of human ATM and ATR. Mec1, in turn, activates the effector protein kinases Chk1 and Rad53 (Sanchez *et al.* 1999), the orthologs of human Chk1 and Chk2, respectively. The checkpoint response includes the stabilization of stalled replisomes, suppression of recombination at arrested replication forks, block of late firing origins of replication (Santocanale & Diffley 1998; Tercero & Diffley 2001), prevents cells entry into mitosis and the segregation of sister chromatids (Cohen-Fix & Koshland 1997), and also activates a transcriptional response that includes the induction of ribonucleotide reductase genes to counteract the stress (Zhou & Elledge 1993).

In human cells, the S phase checkpoint is now regarded as an anti-cancer barrier in early tumorigenesis (Bartkova, Bakkenist, *et al.* 2005; Bartkova, Horejsí, *et al.* 2005). Cyclin Dependent Kinase (CDK) activity is under strict checkpoint regulation upon different genotoxic stresses (Hartwell & Weinert 1989; Furnari *et al.* 1997; Palou *et al.* 2015). Overexpression of G1 cyclin E or cyclin D causes genomic instability such as increased aneuploidy and gene amplification (Zhou *et al.* 1996; Spruck *et al.* 1999). In budding yeast, deregulated G1 cyclin expression also induces genomic instability (Tanaka & Diffley 2002).

We previously reported that budding yeast Cip1 (YPL014W) specifically interacts with G1 cyclins as an inhibitor of cyclin dependent kinase Cdk1 (Ren *et al.* 2016). Our results now

place Cip1 under the S phase checkpoint. Cip1 accumulates in response to genotoxic replication stress in a Mec1 and Rad53 dependent manner. In addition, in response to replication stress Cip1 is phosphorylated at an S/TQ consensus motif in a Mec1 dependent manner. Deletion of Cip1 affects proliferation in hydroxyurea containing plates. Significantly, the sensitivity is increased when the dosage of the G1 cyclin CLN2 is increased, suggesting that Cip1 may play a role as a G1-CDK inhibitor as part of the S phase checkpoint response.

Results

Cip1 accumulates upon genotoxic stress

In a proteomic study to identify proteins differentially associated with Cdk1in response to Rad53 activation, we identified 1,078 proteins (Figure S1). 90 proteins were found specifically associated to Cdk1 in wild type cells but not in *rad53* mutant cells upon hydroxyurea (HU) treatment (Figure S1, Table S3). Validating the findings, 40 out of 90 proteins were previously known to be directly connected to Cdk1 and/or to replication stress. For example, the second hit, Srl3 is a Cdk1 interacting protein previously identified as a suppressor of the lethality of a *rad53* null mutation when overexpressed (Desany *et al.* 1998). More recently, Srl3 has been shown to be part of the negative control of Start (Yahya et al. 2014).

We recently characterized the most intense, Rad53 dependent Cdk1 interactor, Cip1, as a G1-Cdk1 inhibitor (Ren *et al.* 2016). Because we found Cip1 associated to Cdk1 in cells under replication stress and in a Rad53 dependent manner, we subsequently wished to explore the role of Cip1 in response to genotoxic stress. We first explored the abundance of Cip1 protein during an unperturbed cell cycle and in cells under replication stress. Cells synchronized in pre-Start G1 were synchronously released into S phase, either in the presence or in the absence of hydroxyurea, a reagent that generates replication stress by depleting the pool of dNTPs. As shown in Figure 1A, when cells enter S phase in the presence of HU, Cip1 accumulates dramatically and the electrophoretic mobility decreases, in a manner compatible with phosphorylation.

Significantly, the accumulation of Cip1 occurs not only in response to replication stress, but also in response to DNA damage generated with different standard reagents (Figure 1B), such as methyl methane-sulphonate (MMS), a reagent that generates DNA methylation damage, and camptothecin (CPT), a Topoisomerase I poison that generates single strand breaks on the DNA. In contrast, Cip1 accumulation is not significant when cells were arrested in G2/M in the presence of the spindle depolymerizing drug nocodazole (Noc), and are similar to those seen in an unperturbed cell cycle in cells in mitosis (Figure 1A, YPD, 90 min time point).

The increase of Cip1 abundance under replication stress depends on the S phase checkpoint

The three different types of genotoxic stress shown above to result in an increase of Cip1 levels are known to activate the checkpoint kinase Rad53. We therefore next explored

whether the increase in Cip1 abundance depends on Rad53. The experiment described in Figure 1A was repeated, now comparing wild type cells and *rad53* null mutant cells. As shown in Figure 2A, the *rad53* mutant fails to increase the Cip1 protein levels, even though DNA replication is effectively blocked in both cases (see left panel).

The accumulation of Cip1 protein might be achieved through increased transcription or through protein stabilization. The checkpoint kinase Rad53 mediates the transcriptional response to genotoxic stress (Zhou & Elledge 1993; Bastos de Oliveira et al. 2012; Jaehnig et al. 2013). Analysis of the *CIP1* mRNA levels shows that *CIP1* transcript accumulates in the response to replication stress (Figure 2B). In addition, also the observed protein accumulation in response to replication stress, but not the slower electrophoretic mobility, are dependent on the checkpoint kinase Rad53.

Cip1 upregulation in response to genotoxic stress also depends on the checkpoint central transducer kinase Mec1. We repeated the experiment described in Figure 2A and 2B now comparing a *mec1* null mutant with its isogenic MEC1 control. As shown in Figure 2C and 2D, the accumulation of Cip1 mRNA and protein in response to replication stress is also dependent on Mec1. Interestingly, the slower mobility of Cip1 is also affected in *mec1* null mutant cells, which suggests a Mec1 dependent modification of Cip1 upon replication stress.

In all, these results indicate that the upregulation of Cip1 upon replication stress depends is part of the S phase checkpoint response mediated by the conserved Mec1 and Rad53 kinases.

Cip1 expression is induced via Nrm1 upon DNA replication stress

Accumulation of *CIP1* transcript might result either from mRNA stabilization or from induction of transcription. Two major pathways mediate the transcriptional response triggered by Rad53 in response to genotoxic stress. In one of the pathways, Rad53 activates the downstream kinase Dun1, which subsequently phosphorylates and inactivates the transcriptional repressor Crt1, thereby upregulating the expression of a cluster of genes including those encoding the ribonucleotide reductase subunits *RNR2,3,4* (Zhou & Elledge 1993; Huang *et al.* 1998). Another branch of the transcriptional response mediated by Rad53 depends on the selective reactivation of MBF transcription. When S phase is challenged by genotoxic stress, Rad53 inactivates the Nrm1 repressor that suppresses MBF transcription as cells enter S phase (de Bruin *et al.* 2008; Travesa *et al.* 2012).

As shown in Figure 3A, a *dun1*Δ mutant strain remains proficient to increase the levels of Cip1 in response to replication stress to the same extent as wild type cells. Therefore, increase of Cip1 expression in the presence of HU does not require the Dun1 mediated transcriptional program under Rad53.

We then checked whether *CIP1* is one of the MBF genes reactivated by Rad53 by inactivation of the MBF repressor Nrm1. We took advantage of a dominant allele of Nrm1, Nrm1ΔN, that supersedes Rad53 when overexpressed and keeps MBF expression off (Palou *et al.* 2010; Bastos de Oliveira *et al.* 2012). As shown in Figure 3B and 3C, whereas Cip1 expression at both mRNA and protein levels increases in the presence of HU in control cells,

the response is abrogated in cells overexpressing Nrm1ΔN, in the continued presence of HU and active Rad53. These observations indicate that MBF transcription is required to maintain the presence of Cip1 in a compromised S phase.

Stabilization of replication stress induced Cip1 requires continued *de novo* protein synthesis

As mentioned above, Cip1 accumulation might also result from reduced protein turnover. We therefore explored whether Cip1 protein is stabilized in response to replication stress. We analyzed the evolution of Cip1 protein levels when protein synthesis is inhibited with of cycloheximide (CHX) in cells undergoing replication stress in S phase. Wild type cells were synchronized in pre-Start G1 with α-factor, and released into the S phase in the presence of HU. After 1 h the culture was split in two, and CHX was added to one of the cultures.

As shown in Figure 4A, ells are completely unable to accumulate Cip1 when protein synthesis is blocked. Interestingly, the population of Cip1 with lower mobility shows a much more rapid elimination upon inhibition of protein synthesis. Therefore, the excess Cip1 protein accumulated upon replication stress remains highly unstable, indicating that Cip1 abundance is regulated through *de novo* protein synthesis.

Mec1 dependent phosphorylation of Cip1 in response to DNA replication stress

We showed above that in addition to the increase in Cip1 protein levels, the protein displays a slower electrophoretic mobility in response to DNA replication stress (Figure 1A). Despite the increase of Cip1 expression is dependent on the downstream kinase Rad53, the slower mobility appears to depend on Mec1 rather than on Rad53 (Figure 2). A recent high-throughput proteomic study to profile DNA damage-induced phosphorylation in budding yeast suggested that Cip1 is phosphorylated at an SQ motif (Zhou *et al.* 2016). SQ/TQ is the consensus phosphorylation sequence for the DNA damage response ATM/ATR kinases and their yeast homologs, Tel1/Mec1 (Abraham 2001). Taking these observations into consideration, we asked whether Cip1 is phosphorylated by Mec1 in response to DNA replication stress.

As shown in Figure 5A, Cip1 has three putative Mec1 phosphorylation sites, amino acid residues S18, T42 and S288. We used antibodies that specifically detect phosphorylated S/TQ motifs (anti-pSQ/pTQ antibodies), and analyzed Cip1 from wild type cells and *mec1* null cells exposed or not to treatment with HU. Cip1 was immunopurified from native whole cell extracts and western-blotted with anti-pSQ/pTQ antibodies. As seen in Figure 5A, at least one SQ/TQ site of Cip1 is phosphorylated in wild type cells treated with HU (Lane 1), but not in untreated wild type cells (Lane 2). Interestingly, the phosphorylation signal is absent in *mec1* null mutants exposed to replication stress (lane 3). These results indicate that Cip1 is phosphorylated at S/TQ motif in a Mec1 dependent upon DNA replication stress, robustly placing Cip1 as part of the S phase checkpoint response. In addition, the Mec1 paralog Tel1 is unable to replace Mec1 in such role.

We previously showed that cells over-expressing Cip1 upon release from the α-factor arrest significantly delay cell budding, an event triggered by Cln1/2–Cdk1 (Ren *et al.* 2016). To explore whether Cip1 phosphorylation by Mec1 affects Cdk1 activity, we generated a strain

that over-expressing non-phosphorylatable mutant of Cip1 (Gal-Cip1-3AQ) and checked budding index in the indicated strains released from the α-factor arrest. As shown in Figure 5B, budding defects in cells over-expressing Cip1 is robustly rescued when Cip1 S/TQ sites were mutated to non-phosphor AQ. The arrested cell cycle progression of cells overexpressing Cip1 is significantly rescued by nearly 10-fold in cells with phosphor-mutated Cip1, as quantified by 10-fold serial dilution assay shown in Figure 5C. The overexpression extends of both wild type and non-phosphorylatable Cip1-3AQ is identical as controlled in Figure 5D.

CIP1 mutants are sensitive to replication stress when the dosage of the G1 cyclin CLN2 is increased

Our results above placed Cip1 under the regulation of Mec1 and Rad53 kinases in response to replication stress. We therefore examined the result of Cip1 ablation in cells exposed to replication stress. As shown in Figure 6, proliferation of the *cip1* null mutant is decreased in the presence of hydroxyurea compared to wild type cells. The effect is more evident at 0.2 M HU and when the two circles seeded with the highest cell densities are compared between the two strains.

Because we previously identified Cip1 as a putative G1-CKI (Ren *et al.* 2016), we asked whether the role of Cip1 in the response to replication stress had to do with control of the G1 cyclin-Cdk1 activity present in S phase (Wittenberg *et al.* 1990). We took advantage of a 2-micron viral origin driven plasmid (pRS425-CLN2) to create strains carrying multiple copies of the *CLN2* gene under its own promoter. As shown in Figure 6, wild type cells carrying the pRS425-CLN2 gene survive normally to replication stress. In deep contrast, upregulation of the G1 cyclin in cells lacking Cip1 results in a dramatic sensitivity to DNA replication stress.

Discussion

In this study, we showed that Cip1 is upregulated by the S phase checkpoint in response to genotoxic stress. CIP1 expression is induced in response in a Rad53 dependent manner. Rad53/Chk2 is the checkpoint effector kinase responsible for the transcriptional response to genotoxic stress (Zhou & Elledge 1993; Bastos de Oliveira *et al.* 2012; Jaehnig *et al.* 2013). As a result, Cip1 protein levels accumulate, despite the lower mobility pool of the protein remains highly unstable. In addition, Cip1 is phosphorylated at an SQ/TQ motif in a Mec1 dependent manner. Mec1/ATM/ATR is the central transducer kinase of the S phase and DNA damage checkpoint (Sanchez et al. 1996; McGowan & Russell 2004).

The fact that Cip1 is upregulated by the S phase checkpoint prompted us to explore the function of Cip1 in response to DNA replication stress. Whereas Cip1 null mutants are as viable as wild type cells under normal conditions, *cip1* mutant cells are clearly sensitive to replication stress. Because we previously identified Cip1 as a putative G1-CKI (Ren *et al.* 2016), the simplest explanation is that checkpoint mediated accumulation of Cip1 is required to downregulate G1 cyclin-Cdk1 activity. A prediction derived from such hypothesis is that increased G1 cyclin-Cdk1 activity should enhance the hydroxyurea sensitivity phenotype in *cip1* null cells. Indeed, *cip1* mutants carrying multiple copies of the *CLN2* gene are unable

to cope with replication stress, whereas $CIP1^{+}$ cells carrying the same multicopy plasmid respond normally to the stress.

Replication stress is sensed in S phase. Despite G1 cyclins start declining by the G1-S transition, their presence lingers well into S phase (Wittenberg *et al*. 1990), where G1 cyclin-Cdk1 activity has been proposed to be continuously required for proper polar growth (McCusker *et al*. 2007). We showed that Cln2 levels in cells under replication stress in S phase parallel those of an unperturbed S phase. Based on our results, residual G1 cyclin-Cdk1 activity during genotoxic stress may be deleterious.

Several possibilities may account for the observed loss of viability in *cip1* mutants. One, deregulated G1 cyclin-Cdk1 may result in inadequate polarization of the cell. Persistent G1 cyclin-Cdk1 activity has been shown to result in excessive polar growth (Lew & Reed 1993; Barral *et al*. 1995). Because the S phase checkpoint keeps mitotic cyclin-Cdk1 inactive in response to genotoxic stress (Palou *et al*. 2015), the polar-to-isotropic growth switch is expectably delayed. Under those conditions Cip1 may be required to fine-tune the Cln1,2-Cdk1 activity to maintain the adequate polarity during the cell cycle arrest.

A second possibility is that unrestrained G1 cyclin-Cdk1 activity during genotoxic stress results in genomic instability. G1 cyclin overexpression has been reported to cause genomic instability in human cells (Zhou *et al*. 1996; Spruck *et al*. 1999) and in budding yeast (Tanaka & Diffley 2002). The observed sensitivity of *cip1* mutants to replication stress might be the result of cells being unable to preserve genomic integrity under such conditions. The dramatic loss of viability observed in *cip1* null cells may indeed underscore a significant degree of genomic instability.

Finally, the strong and lasting accumulation of Cip1 protein, past the presence of Cln2, is also compatible with Cip1 playing a role other than as a G1-CKI. Work is currently going on in our lab to distinguish between the different possible possibilities, and determine what is the role of Cip1 to prevent loss of viability in the presence of genotoxic stress.

In all, our results place Cip1 under the DNA damage response. Significantly, Cip1 is required for survival in the presence of replication stress. The loss of viability is aggravated when the dosage of Cln2 cyclin is increased, which strengthens the proposed role of Cip1 as a G1 cyclin-Cdk1 inhibitor (G1-CKI).

Experimental procedures

Strains, Constructs, Culture Media, Cell Synchronization, DNA Content Analysis and Inhibition of Protein Synthesis

The strains used in this study are listed in Table S1. All strains were derived from *Saccharomyces cerevisiae* W303-1a (Thomas & Rothstein 1989). Yeast cells used in this study were grown at 30°C in either YPD medium or Synthetic Dextrose (SD) medium supplemented with the required essential nutrients. For the hydroxyurea sensitivity tests, SD medium plates were chosen and incubated at 30°C, to minimize the effect of the Trp auxotrophy reported for the limiting tryptophan concentration in rich medium plates and at

lower temperatures (Godin *et al.* 2016). Cell cycle synchronization with the pheromone α-factor, and DNA content analysis by propidium iodide Fluorescent Activated Cell Sorting (FACS) were done as previously described (Palou *et al.* 2015; Ren *et al.* 2016). Where indicated, protein synthesis was inhibited by adding 100 μg/ml cycloheximide (CHX) to the medium as previously described (Palou *et al.* 2010).

Site-directed DNA mutagenesis

The site-directed DNA mutagenesis for generation of CIP1-3AQ mutants was carried out using a modified method based on Single-Primer Reactions IN Parallel (SPRINP) method (Edelheit *et al.* 2009; Zeng *et al.* 2017). Cip1-3AQ refers to the mutation of the three S/TQ sites of Cip1 to Alanine (S18A, T42Q, S228A).

Western Blotting and Antibodies

Whole cell extracts for western blotting were prepared by glass beads beating in trichloroacetic acid (TCA), then resolved by SDS-PAGE as previously described (Palou *et al.* 2015; Ren *et al.* 2016). The primary antibodies used in this study were anti-Rad53 (Santa cruz sc-6749), anti-Myc (9E10, monoclonal mouse hybridoma supernatant), and anti-pS/TQ (Cell Signaling Technologies 2851).

RNA Extraction and qRT-PCR

Around 10^9 cells were harvested for each sample and washed once with pre-chilled diethy pyrocarbonate (DEPC)-treated water. Cell pellets were snap-frozen in liquid nitrogen and stored at −80°C until RNA extraction. Total RNA was obtained using Trizol reagent (Invitrogen) as recommended by the manufacturer. RNA was then reverse transcribed using PrimeScript™ RT kit (Takara, RR014A). The cDNA library was used as template in real-time PCR using Takara SYBR Premix Ex-Taq (Tli RNaseH Plus) kit (Takara, RR420A). The primers for *CIP1* and the control *ACT1* used in this study are listed in Table S2.

Immunoprecipitation

For immunoprecipitation, around 10^9 cells treated with 0.2 M HU for 2 hours were collected and extracted by glass beads beating in lysis buffer (50 mM Tris-HCl pH 7.8, 175 mM NaCl, 1 mM EDTA, 0.1% (v/v) Nonidet-P40) supplemented with protease inhibitors (1 mM AEBSF, 0.15 mM aprotinin, 1 mM leupeptin, 1 mM pepstatin) and phosphatase inhibitors (0.5 mM sodium pyrophosphate, 2 mM NaF, 2 mM β-glycerophosphate). 20 μl of anti-myc HA-7 agarose matrix (Sigma A2095, mouse monoclonal purified IgG) were added to extracts to immunopurify Cip1-13myc. The samples were gently rotated for 1 h at 4°C. The beads were then washed four times with cold lysis buffer. Finally, the proteins were released by boiling the beads in Laemmli sample buffer. Enriched proteins were resolved by SDS-PAGE and checked by western blotting. For mass spectroscopy analysis of Cdk1 interacting proteins, proteins from the immunoprecipitate were released from the beads by mixing with 50 μl of room temperature 8M urea in 100 mM Tris pH 8.0 for 5 min.

Serial dilution assay

Yeast cells were grown overnight in YPD at 30°C to mid-log phase. Serial 10-fold or 5-fold dilutions from the cultures were spotted on indicated plates and incubated at 30°C for 3 days. For cells containing 2-micron based plasmids pRS425, leucine minus SD medium was used instead of YPD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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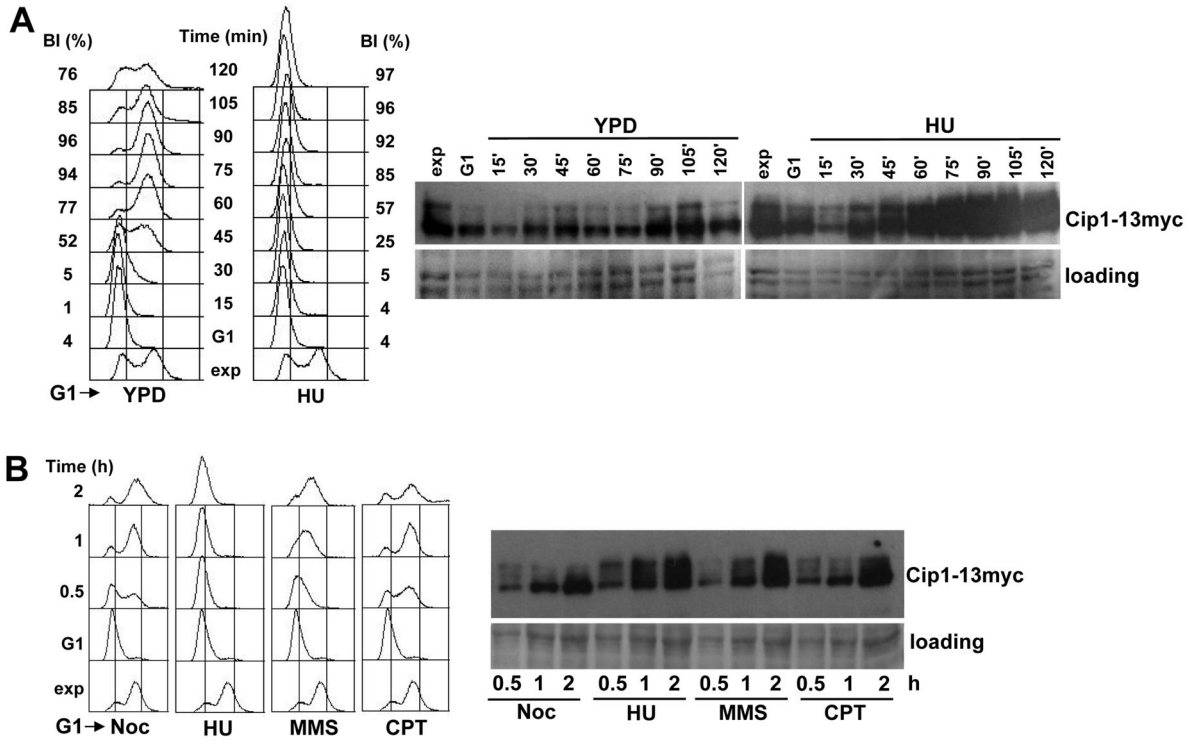

Figure 1. Cip1 accumulates upon genotoxic stress

(A) Cip1-13myc cells (YFL117 strain) were synchronized with α-factor and released into S phase in the presence of HU. The samples were taken at the indicated time points (min). Left panel, fluorescence-activated cell sorting analysis of DNA content. The presence of HU abolishes DNA replication, but not cell cycle entry, as assessed by the progression of the budding indexes. Right panel, TCA whole cell extracts were analyzed by immunoblotting with anti-Myc (Cip1) antibodies. A Ponceau S-stained region of the same membrane used for immunoblotting is shown as a loading control. (B) Wild type cells (YFL110) were released from the G1 arrest, and allowed to progress into S phase in the presence of Noc, or in the presence of HU, MMS, and CPT respectively. The samples were taken at the indicated time points. Left panel, fluorescence-activated cell sorting analysis of DNA content. Right panel, whole cell extracts were analyzed by immunoblotting with anti-Myc (Cip1). A Ponceau S-stained region of the same membrane used for immunoblotting is shown as a loading control.

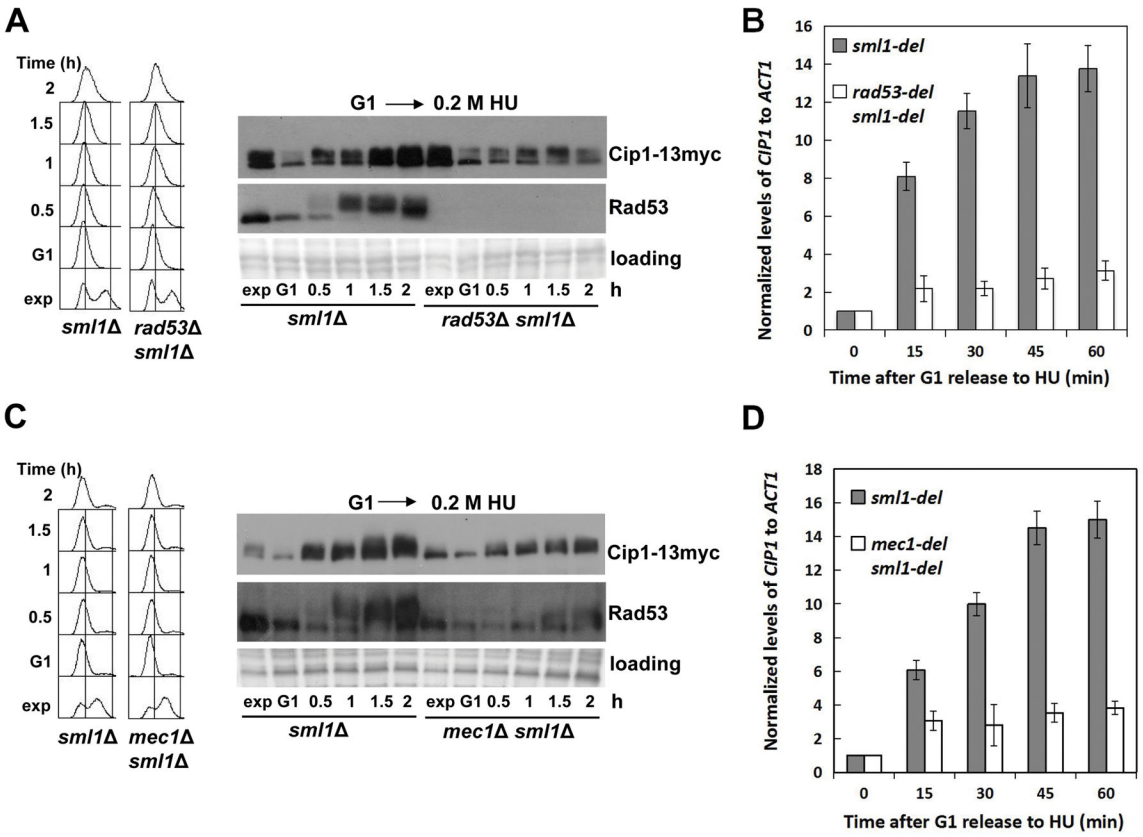

Figure 2. Increase of Cip1 abundance upon replication stress depends on the S phase checkpoint kinases Mec1 and Rad53

(A) Accumulation of Cip1 protein upon replication stress is dependent on Rad53. Rad53 positive cells (*sml1*Δ, YFL101 strain) and *rad53*Δ null mutant cells (*rad53*Δ *sml1*Δ, strain YFL102) were synchronized in pre-Start G1 (α) and released in the presence of 200 mM HU. Samples were taken at the indicated times (min). Left panel, fluorescence-activated cell sorting analysis of DNA content. Right panel, TCA whole cell extracts were analyzed by immunoblotting with anti-Myc (Cip1) or anti-Rad53 (Rad53) antibodies. (B) Accumulation of CIP1 mRNA is dependent on Rad53. The normalized relative amount of CIP1 mRNA to ACT1 control in different strains in the presence of 200 mM HU is shown. (C) Accumulation of Cip1 protein upon replication stress is dependent on Mec1. Mec1 positive cells (*sml1*Δ, YFL101 strain) and *mec1*Δ null mutant cells (*mec1*Δ *sml1*Δ, strain YZZ2) were synchronized in pre-Start G1 (α) and released in the presence of 200 mM HU. Samples were taken at the indicated times (min). Left panel, fluorescence-activated cell sorting analysis of DNA content. Right panel, TCA whole cell extracts were analyzed by immunoblotting with anti-Myc (Cip1) or anti-Rad53 (Rad53) antibodies. (D) Accumulation of CIP1 mRNA is dependent on Mec1. The normalized relative amount of CIP1 mRNA to ACT1 control in different strains in the presence of 200 mM HU is shown.

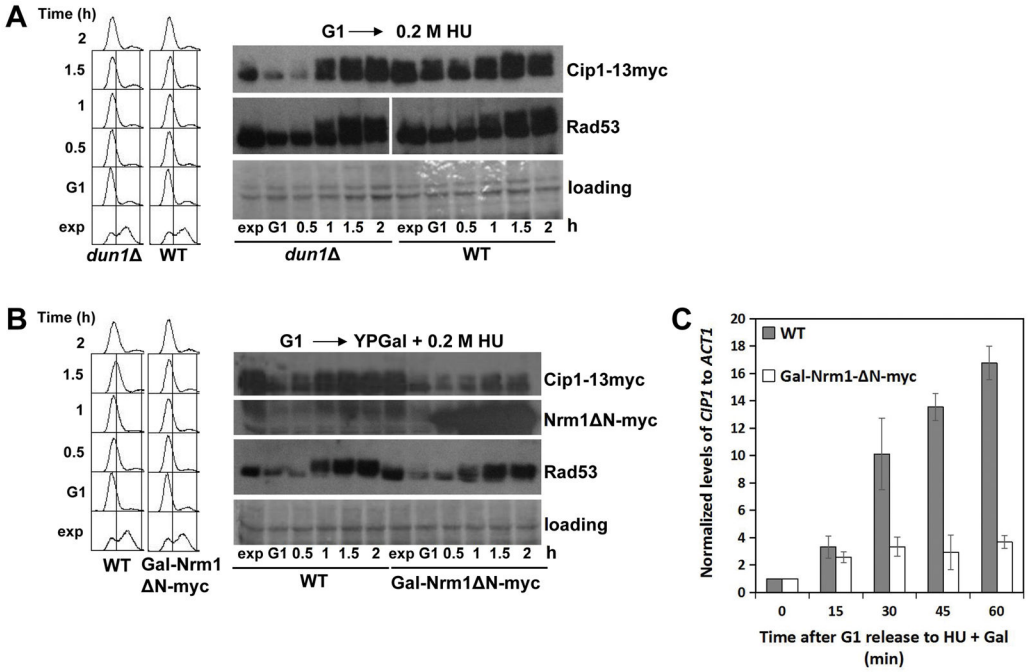

Figure 3. Increase of Cip1 abundance upon replication stress depends on MBF transcription

(A) Mutant *dun1*Δ cells (YFL122 strain) and wild type cells (YFL100 strain) were synchronized in pre-Start G1 (α) and then released in the presence of 200 mM HU. Samples were taken at the indicated times (min). TCA whole cell extracts were analyzed by immunoblotting with anti-Myc (Cip1) or anti-Rad53 antibodies. (B) Gal-Nrm1ΔN-myc cells (YFL120 strain) and wild type cells (YFL100 strain) were synchronized in pre-Start G1 (α) in YPRaff. The cells were then released from the α-factor arrest into YPGal supplemented with 200 mM HU. Samples were taken at the indicated times (min). TCA whole cell extracts were analyzed by immunoblotting with anti-Myc (Cip1, Nrm1ΔN) or anti-Rad53 (Rad53) antibodies. (C) The normalized relative amount of CIP1 mRNA to ACT1 control in the indicated conditions is shown.

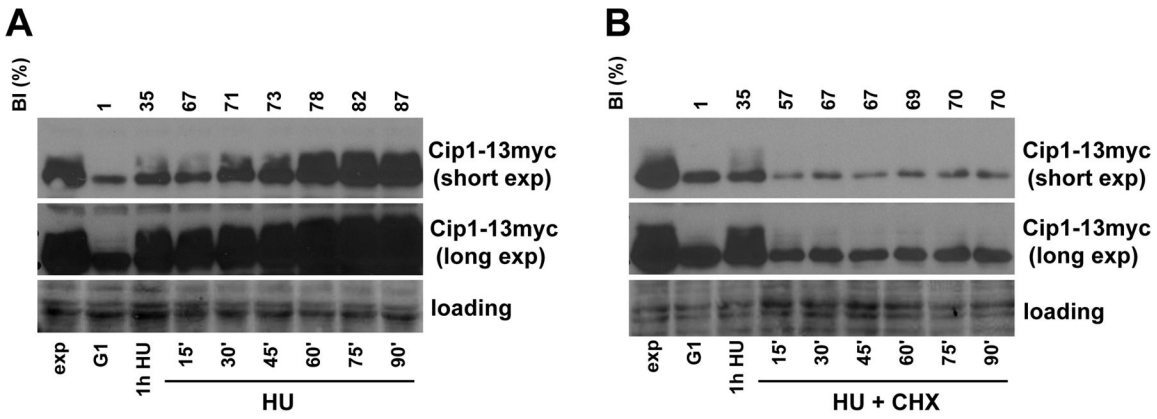

Figure 4. Increase of Cip1 abundance upon replication stress requires continued *de novo* protein synthesis

Cip1-13myc cells (YFL100 strain) were synchronized in pre-Start G1 (α) and released in the presence of 200 mM HU for 1h. The culture was then split in two. One half was further incubated under the same conditions (HU) (A), whereas cycloheximide was added to the other half (B), keeping the presence of hydroxyurea (HU + CHX). The samples were collected every half hour, and whole cell extracts were analyzed by immunoblot with anti-Myc antibodies (Cip1). Budding indexes (BI) are shown on top as measures of synchronicity and cell cycle progression to confirm that cells progress into the cell cycle despite the absence of DNA replication or protein synthesis.

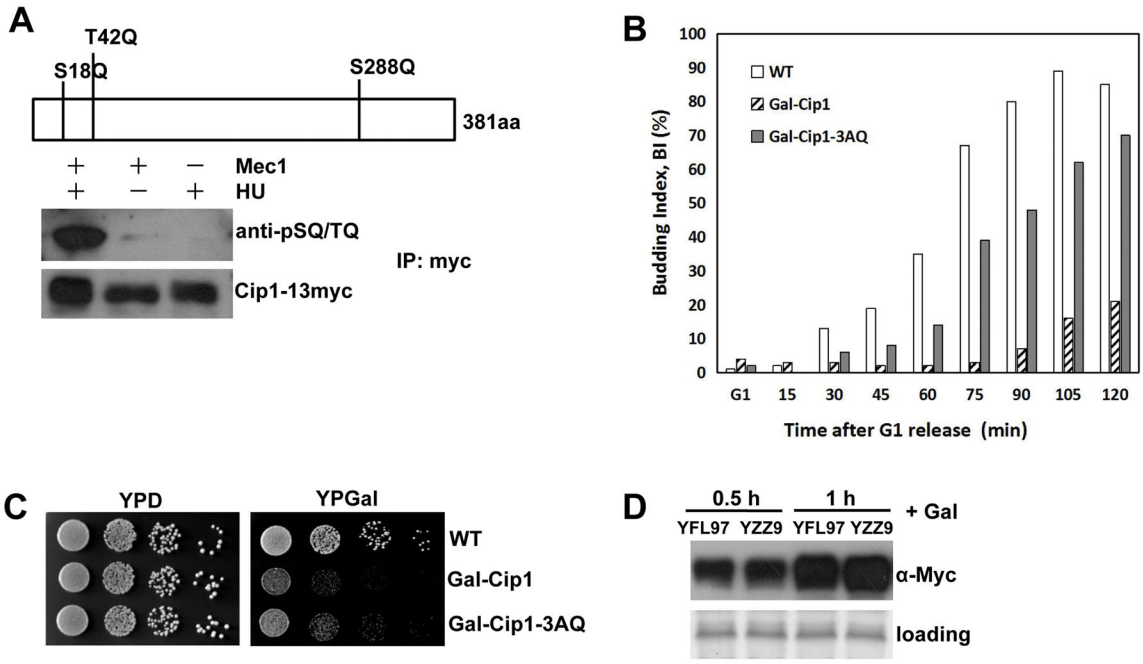

Figure 5. Mec1 dependent phosphorylation of Cip1 in response to replication stress

(A) Upper panel, a schematic diagram of Cip1 showing the three SQ/TQ motif sites present. Lower panel, Mec1 positive cells (*sml1*Δ, YFL101 strain) and *mec1*Δ null mutant cells (*mec1*Δ *sml1*Δ, strain YZZ2) were treated with or without 0.2 M HU and subjected to Cip1-13myc immunopurification followed by immunoblotting with anti-Myc (Cip1) or anti-pS/TQ antibodies. (B) GAL-Cip1 (strain YFL97), GAL-Cip1-3AQ (strain YZZ9) or wild-type control cells (strain YFL3) were synchronized in pre-Start G1 (α). Galactose was added while keeping the presence of α-factor for 30 min. Cells were then released from the α-factor while keeping the presence of galactose in the medium. Samples were taken at the indicated points (min) and the budding indexes (BI) were measured. (C) Cultures of GAL-Cip1 (strain YFL97), GAL-Cip1-3AQ (strain YZZ9) or wild-type control cells (strain YFL3) were 10-fold serial spotted on YPD or YPGal plates. (D) Whole cell extracts analyzed by immunoblot with anti-Myc (Cip1) antibodies show that the proteins are being overexpressed equally in the indicated strains. A coomassie blue-stained region of the same membrane used for immunoblot is shown as a loading control.

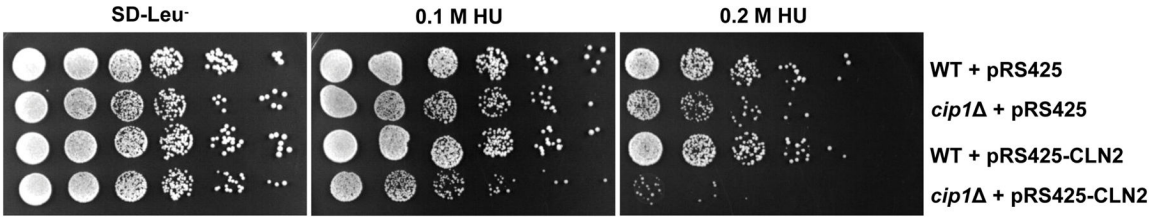

Figure 6. *CIP1* deletion results in sensitivity to replication stress when the dosage of the G1 cyclin CLN2 is increased

WT cells and *cip1*Δ mutants transformed with pRS425 or pRS425-CLN2 vectors were 5-fold serial spotted on SD-Leu$^-$ with or without the HU at the indicated concentrations.