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Prolonged Cell Cycle Arrest in Response to DNA damage in Yeast Requires the Maintenance of DNA Damage Signaling and the Spindle Assembly Checkpoint

Felix Y Zhou, David P Waterman, Marissa Ashton, Suhaily Caban-Penix, Gonen Memisoglu, Vinay V Eapen, James E Haber

Department of Biology and Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, USA • Department of Molecular Genetics & Cell Biology, University of Chicago, Chicago, USA

Abstract

Cells evoke the DNA damage checkpoint (DDC) to inhibit mitosis in the presence of DNA double-strand breaks (DSBs) to allow more time for DNA repair. In budding yeast, a single irreparable DSB is sufficient to activate the DDC and induce cell cycle arrest prior to anaphase for about 12 to 15 hours, after which cells "adapt" to the damage by extinguishing the DDC and resuming the cell cycle. While activation of the DNA damage-dependent cell cycle arrest is well-understood, how it is maintained remains unclear. To address this, we conditionally depleted key DDC proteins after the DDC was fully activated and monitored changes in the maintenance of cell cycle arrest. Degradation of $Ddc2^{ATRIP}$, Rad9, Rad24, or $Rad53^{CHK2}$ results in premature resumption of the cell cycle, indicating that these DDC factors are required both to establish and to maintain the arrest. Dun1 is required for establishment, but not maintenance of arrest, whereas Chk1 is required for prolonged maintenance but not for initial establishment of the mitotic arrest. When the cells are challenged with 2 persistent DSBs, they remain permanently arrested. This permanent arrest is initially dependent on the continuous presence of Ddc2, Rad9, and Rad53; however, after 15 hours these proteins become dispensable. Instead, the continued mitotic arrest is sustained by spindle-assembly checkpoint (SAC) proteins Mad1, Mad2, and Bub2 but not by Bub2's binding partner Bfa1. These data suggest that prolonged cell cycle arrest in response to 2 DSBs is achieved by a handoff from the DDC to specific components of the SAC. Furthermore, the establishment and maintenance of DNA damage-induced cell cycle arrest requires overlapping but different sets of factors.

eLife Assessment

This is an **important** study on the damage-induced checkpoint maintenance and termination in budding yeast that provides novel and **convincing** evidence for a role of the spindle assembly checkpoint and mitotic exit network in halting the cell cycle after prolonged arrest in response to irreparable DNA double strand breaks (DSBs). The study identifies particular components from these checkpoints that are specifically required for the establishment and/or the maintenance of a cell cycle block triggered by such DSBs. The authors propose an interesting model for how these different checkpoints intersect and crosstalk for timely resumption of cell cycling even without repairing DNA damage that has been revised by addressing the bulk of the reviewers' comments to the first version of the manuscript.

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Introduction

DNA double-strand breaks (DSBs) are one of the most deleterious forms of DNA damage (Mehta and Haber 2014). In response to DSBs, cells evoke the DNA damage checkpoint (DDC) to halt the metaphase to anaphase transition (known as the G_2/M checkpoint). Activation of DDC gives cells an extended opportunity to repair DSBs and therefore, prevents the inheritance of broken chromosomes, which can lead to aneuploidy, chromosome aberrations and genome instability (Waterman, Haber, and Smolka 2020).

In budding yeast, a single irreparable DSB is sufficient to trigger the DDC through the activation of Mec1, a PI3K-like kinase and homolog of the mammalian ATR (Mantiero et al. 2007; Pellicioli et al. 2001). Mec1 activation depends on 5' to 3' resection of the DSB ends that exposes single-stranded DNA (ssDNA) which is rapidly coated by the ssDNA binding protein, RPA (reviewed by (Marechal and Zou 2015)). As resection proceeds, the PCNA-related 9-1-1 clamp, made up of Ddc1, Rad17, and Mec3, is loaded at the resected ss/dsDNA junction by the Rad24-Rfc2-5 clamp loader (Ellison and Stillman 2003; Majka et al. 2006). Mec1 is recruited to DSB sites via its obligate binding partner, $Ddc2^{ATRIP}$ interacting with RPA-bound ssDNA (Dubrana et al. 2007; Zou and Elledge 2003). Following its localization to DSBs, Mec1's kinase activity is stimulated by Dbp11, Dna2 and the Ddc1 subunit of the 9-1-1 clamp (Navadgi-Patil and Burgers 2009; Melo, Cohen, and Toczyski 2001). Impairing Mec1's kinase activity by the PI3K-like kinase inhibitor caffeine, by using temperature-sensitive Mec1 mutants or by degradation of Mec1's binding partner Ddc2, rapidly extinguishes checkpoint signaling (Pellicioli et al. 2001; Tsabar et al. 2015; Vaze et al. 2002), illustrating that continual Mec1 activity is needed to activate and sustain DDC. In contrast to Mec1, yeast's other PI3K-like kinase, Tel1, the homolog of mammalian ATM, is dispensable for DDC activation and maintenance, as *TEL1* deletion only shortens damage-induced cell cycle arrest by a few hours (Dubrana et al. 2007).

Following the induction of a DSB, numerous proteins are phosphorylated either directly by Mec1 or by the downstream effector kinases Rad53 and Chk1 (human CHK2 and CHK1), which are themselves Mec1 substrates (Lanz, Dibitetto, and Smolka 2019; Smolka et al. 2007). Mec1 and Tel1 substrates also include histone H2A-S129, called γ-H2AX, which spreads on both sides of the DSB via two distinct mechanisms (Rogakou et al. 1998; Shroff et al. 2004). γ-H2AX then recruits the scaffold protein Rad9, which brings the effector kinase Rad53 in close proximity to Mec1 for activation (Durocher et al. 1999; Emili 1998; Schwartz et al. 2002). Activated Rad53 then amplifies the DDC signal through autophosphorylation in *trans*, also stimulating the

transcription regulator Dun1 kinase, while restraining the degradation of Pds1 (securin) to inhibit mitosis (Chen, Smolka, and Zhou 2007; Fiorani et al. 2008; Pellicioli et al. 1999; Usui and Petrini 2007; Yam et al. 2020).

In addition to the DDC, unattached kinetochores can induce cell cycle arrest through the activation of spindle assembly checkpoint (SAC) (reviewed by (Musacchio 2015)). Several studies have suggested a crosstalk between the SAC and the DDC. For example, deletion of SAC components *MAD1* or *MAD2* shorten the cell cycle arrest in response to DNA damaging agents in the absence of DDC genes *RAD9* and *RAD24* (Garber and Rine 2002; Kim and Burke 2008). Furthermore, *MAD1*, *MAD2* or *BUB1* mutants arrest for less time than wild-type cells following the induction of a single persistent DSB (Dotiwala et al. 2010). In mouse oocytes, inhibition of the SAC overrides the activation of DDC-mediated metaphase arrest during the first meiotic division (Marangos et al. 2015). The mitotic exit network (MEN) is another signaling cascade activated during anaphase to promote cell cycle re-entry (Matellan and Monje-Casas 2020); therefore, defects in MEN lead to mitotic arrest in late anaphase (Geymonat et al. 2002; Bardin, Visintin, and Amon 2000; Shirayama, Matsui, and Toh 1994). In addition to SAC, MEN also communicates with the DDC in response to DNA damage. For instance, a key regulator of MEN, the heterodimer Bub2/Bfa1 complex, is modified in a Rad53 and Dun1-dependent manner following damage (Hu et al. 2001). Supporting the idea of crosstalk between MEN and DDC, our lab had shown that deletion of *BUB2* shortened the duration of the arrest in response to a single unrepaired DSB (Dotiwala et al. 2010).

Here, we present new mechanistic insights into the *maintenance* of the cell cycle arrest following DNA damage by employing the auxin-inducible degron (AID) strategy to conditionally deplete DDC and SAC proteins. An advantage of the AID system, compared to null or temperature-sensitive mutants, is that AID-tagged proteins retain wild-type function until the addition of the plant hormone auxin (indole-3-acetic acid, IAA), which triggers rapid degradation of AID-tagged proteins in the presence of the TIR1 E3 ubiquitin ligase (Morawska and Ulrich 2013; Nishimura et al. 2009). To investigate how conditional depletion of DDC or SAC proteins alter the maintenance of cell cycle arrest, we engineered a yeast strain that permanently arrests due to the presence of 2 persistent DNA breaks. We find that the maintenance of the cell cycle arrest requires constant presence of some, but not all, checkpoint activation proteins. Surprisingly, we find that the DDC proteins which are essential to induce the cell cycle arrest and sustain it at the early stages of the arrest become dispensable nearly 15 hours after DNA damage induction. Conversely, SAC proteins are dispensable for the establishment and the initial steps of the cell cycle arrest but become essential at later stages of the DNA damage dependent cell cycle arrest. Based on these findings, we posit that prolonged cell cycle arrest in response to DNA damage is sustained by both SAC and DDC; however, each checkpoint sustains the arrest at different stages.

Results

Measuring DNA Damage Checkpoint Arrest and Maintenance

To study the role of DDC initiation proteins in the *maintenance* of the cell cycle arrest, we utilized the well-characterized strain JKM179 (Lee et al. 1998), in which the site-specific HO endonuclease is expressed from a *GAL1-10* promoter (*GAL-HO*) to induce a single DSB within the *MAT* locus on chromosome III (referred to as the 1-DSB strain). In this 1-DSB strain, we inserted an additional HO cleavage site 52 kb from the centromere on chromosome IV to induce another DSB (referred to as the 2-DSB strain) (Kim et al. 2007; Lee et al. 1998). At both loci, HO-mediated cleavage after galactose induction is nearly complete within 30-45 minutes (Lee et al. 2014). In both strains, we also deleted the *HML* and *HMR* donors to prevent repair by homologous recombination. With continuous *HO* expression, nonhomologous end-joining occurs in only 0.2% of these cells (Moore and Haber 1996) thus both DSBs are essentially irreparable.

Following the induction of two DSBs, we monitored cell cycle arrest in 4 ways: (1) with an adaptation time-course assay where we micromanipulated individual G_1 cells on agar plates and scored the percentage of cells that are able to re-enter mitosis (Lee et al. 1998), (2) by monitoring the percentage of G_2/M-arrested cells in liquid culture based on cell morphology (**Figure 1A**), (3) by monitoring nuclear division by DAPI staining of nuclei, and (4) by assaying Rad53 phosphorylation by western blot (Pellicioli et al. 2001).

In both 1-DSB and 2-DSB strains, 4 h after induction of DNA damage, >90% of cells arrested at G_2/M as determined by an adaptation assay (**Figure 1B, 1E**) and DAPI staining (**Figure 1D** and **1G**). In agreement, western blot analysis showed that Rad53 was hyperphosphorylated (**Figure 1C** and **1F**), demonstrating that DDC was fully activated in both these strains after DNA damage. By 12 to 15 h after the induction of a single persistent DSB, most 1-DSB cells adapted; that is, they escaped the G_2/M arrest and re-entered mitosis (**Figure 1B**). The timing of Rad53 dephosphorylation following the induction of a single irreparable DSB correlated with the timing of adaptation and escape from the G_2/M arrest (**Figure 1C**), as previously shown (Pellicioli et al. 2001) In contrast, in the 2-DSB strain, over 90% of cells remained permanently arrested in G_2/M with persistently hyper-phosphorylated Rad53 throughout the 24 h time-course (**Figure 1E-G**). We leveraged this permanent cell cycle arrest observed in the 2-DSB strain to study how the DNA damage-induced cell cycle arrest is maintained once it had been established.

Analysis of Checkpoint Factors Required for Checkpoint Maintenance

We used the auxin-inducible degron (AID) system (Nishimura et al. 2009) to conditionally deplete DDC proteins after G_2/M arrest had been established to study the maintenance of cell cycle arrest. To this end, we appended an AID tag with 9 copies of the c-MYC epitope tag to the C-terminus of Ddc2, Rad9, Rad24, and Rad53, which are all components of the Mec1 signaling cascade (Memisoglu et al. 2019; Sweeney et al. 2005; de la Torre-Ruiz, Green, and Lowndes 1998). Hereafter, all AID-tagged proteins will be designated simply as -AID, e.g., Ddc2-9xMyc-AID as Ddc2-AID.

AID-tagging of DDC proteins did not alter the establishment of G_2/M arrest; however, *RAD9-AID*, *RAD24-AID*, and *RAD53-AID* strains are hypomorphic and adapted 24 h after DNA damage in the absence of IAA, while a 2-DSB wild-type counterpart cells remained fully arrested (Figure S1A). This premature escape from the cell cycle arrest was dependent on the presence of TIR1 (Figure S1B) and is likely due to low levels of IAA as a natural intermediate in amino acid biosynthesis (Rao et al. 2010). IAA treatment prior to the induction of 2 DSBs in a control strain, which does not contain any AID-tagged proteins, did not alter the prolonged cell cycle arrest following DNA damage (Figure S2A), illustrating that IAA treatment by itself does not alter response to DNA damage. However, rapid degradation of Ddc2-AID, Rad9-AID, Rad24-AID and Rad53-AID by IAA treatment 2 h prior to the induction of DSBs largely prevented cell cycle arrest as well as DDC signaling, evident from the absence of detectible Rad53 hyperphosphorylation (Figure S2B-E). These results underlie the importance of Ddc2, Rad9, Rad24 and Rad53 in initiating DDC signaling and cell cycle arrest in response to DNA damage, agreeing with previous reports (Pellicioli et al. 2001; Paciotti et al. 2000; Emili 1998; de la Torre-Ruiz, Green, and Lowndes 1998).

To test whether the DDC proteins are required for *maintenance* of the cell cycle arrest following DNA damage, we employed the AID tagged strains with 2 DSBs and depleted the AID proteins 4 h *after* inducing DSBs. In the absence of IAA, *DDC2-AID*, *RAD9-AID*, or *RAD24-AID* strains all activated the DDC signaling 4 h after DSB induction, with 89% - 99% of cells arrested in G_2/M (**Figure 2A-C**), demonstrating that AID tagging of these proteins did not impair their function. Within 1 h after IAA treatment, Ddc2-AID, Rad9-AID, or Rad24-AID were all rapidly depleted, which caused a gradual Rad53 dephosphorylation, as detected by western blotting (**Figure 2A-C**). Moreover, agreeing with the loss of Rad53 phosphorylation, IAA treatment of DDC-AID

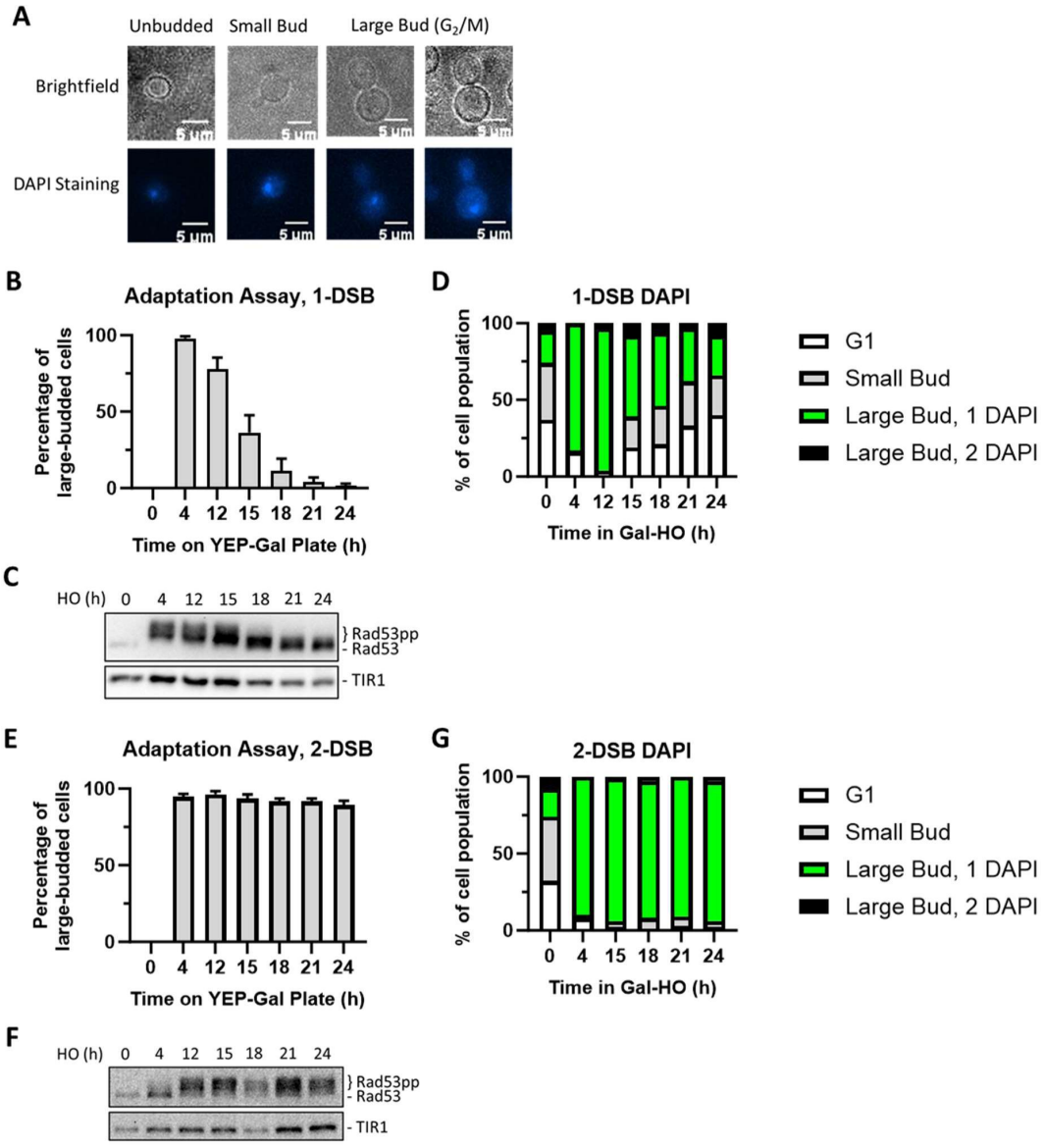

Figure 1.

Measuring checkpoint arrest in a 1-DSB and 2-DSB strains

(A) Morphological categories of budding yeast cells using brightfield microscopy and DAPI staining were used to determine G_2/M arrest. Cells that arrest at G2/M shift towards a large bud state. G_2/M arrested cells that progress into anaphase. (B) Adaptation assay with 1 DSB strain on a YEP-Gal plate. G_2/M arrest was determined based on cell morphology as shown in **Fig. 1A**. Data are shown from 3 independent experiments, error bars represent standard error of the mean (SEM). (C) Profile of DAPI stained cells in a 1-DSB strain after DNA damage induction in liquid culture. Cells were grouped based on cell morphology and DAPI staining profiles, as explained below the graphs. (D) Rad53 phosphorylation kinetics in 1 DSB strain by western blotting. Samples collected after the induction of DNA damage during the time course experiment and blotted with α-Rad53 to monitor DDC signaling. α-Rad53 can both detect unphosphorylated and hyperphosphorylated Rad53 species. TIR1-Myc was detected with α-Myc and serves as a loading control. (E) Same as (B) for a 2-DSB strain. (F) Same as (C) with a 2-DSB strain. (G) Same as (D) with a 2-DSB strain.

strains triggered release from G_2/M arrest, while the untreated control cells remained fully arrested. DAPI staining of *DDC2-AID* cells after IAA treatment revealed the accumulation of large-budded cells with 2 distinct DAPI signals, indicating that cells started to progress into anaphase following Ddc2 depletion (Figure S3A). These findings illustrate that the upstream DDC factors Ddc2, Rad9 and Rad24 are essential for initiating and sustaining the cell cycle arrest in response to DNA damage.

Compared to *DDC2-AID*, *RAD9-AID* or *RAD24-AID*, we found that *RAD53-AID* cells maintained G_2/M arrest for an additional 4 h after complete depletion of Rad53 (**Figure 2D**). In contrast to Ddc2-AID depletion, cell cycle analysis by DAPI staining showed that Rad53 depletion led to a more gradual transition into late anaphase (Figure S3B). This delay after the conditional depletion of Rad53 could be due to continued signaling from downstream targets activated by Rad53 kinase, such as Dun1, or from other targets of the Mec1 kinase, downstream of Ddc2, Rad9 and Rad24.

Chk1 Sustains Checkpoint Signaling in the Absence of Ddc2, Rad9, Rad24, or Rad53

Rad53 and Chk1 kinases both contribute to the maintenance of the cell cycle arrest after DNA damage (Dotiwala et al. 2007; Pellicioli et al. 2001). Agreeing with previously published reports (Sanchez et al. 1999), we find that Chk1 is involved in maintaining the permanent arrest following the induction of 2 DSBs. Deletion of *CHK1* did not impair the induction of cell cycle arrest (**Figure 3A**); however, it inhibited the permanent cell cycle arrest, as >95% of *chk1*Δ cells adapted by 24 h (**Figure 3B**). We then asked whether the delay in cell cycle re-entry observed when Rad53 was degraded was due to Chk1's independent role in maintaining arrest. To test this, we induced 2 DSBs in *RAD53-AID chk1Δ* cells for 4 h and then added IAA to deplete Rad53. Compared to the depletion of Rad53-AID alone (**Figure 2D**), the depletion of Rad53-AID in the absence of *CHK1* led to a significant decrease in the number of G_2/M-arrested cells within 1 h of IAA treatment (**Figure 3C**). These results suggest that Chk1 functions in conjunction with Rad53 to sustain cell cycle arrest in response to DNA damage.

To explore further how Chk1 signaling contributes to the maintenance of DDC-dependent cell cycle arrest, we depleted DDC factors Ddc2-AID, Rad9-AID, or Rad24-AID in *chk1Δ* cells 4 h after the induction of DSBs. Depletion of these upstream factors in the absence of *CHK1* led to a more rapid release from the cell cycle arrest (**Figure 3D-F**) compared to the depletion of DDC factors alone (**Figure 2A-C**). Collectively, these findings demonstrate that Chk1 plays a key role in maintaining cell cycle arrest in response to DNA damage.

Tel1 is thought to play a minor role in response to DSBs, as the establishment of DSB-induced cell cycle arrest normally depends entirely on Mec1 (Dotiwala et al. 2010). However, previous studies have shown that, in addition to Mec1, Tel1 can also target Chk1 for phosphorylation (Limbo et al. 2011; Sanchez et al. 1999). Additionally, when the initial 5' to 3' end resection of DSB ends is impaired, Tel1 alone can activate the DDC (Usui and Petrini 2007). To study how Tel1 contributes to the maintenance of the permanent cell cycle arrest, we deleted *TEL1* in 2-DSB strain. Unlike *chk1*Δ with 2 DSBs, a *TEL1* deletion did not affect either the establishment of the DDC nor the maintenance of checkpoint arrest up to 24 h (Figure S4), agreeing with previously published results (Dubrana et al. 2007). Taken together, these data illustrate that DNA damage-dependent cell cycle arrest is initiated by Mec1 branch of the DDC via Ddc2, Rad9 and Rad24, and the cell cycle arrest is largely sustained by the downstream kinases Rad53 and Chk1, with minor contributions from other downstream targets of DDC.

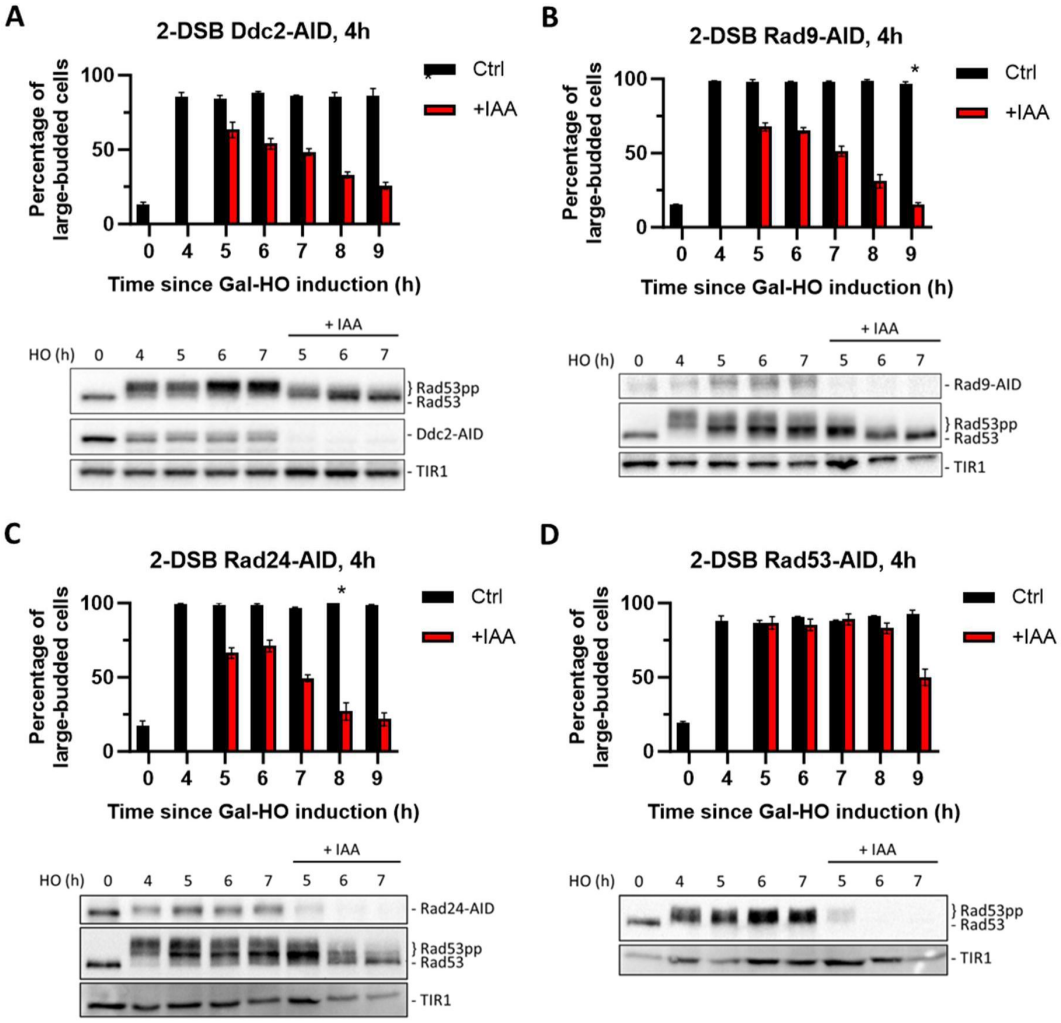

Figure 2.

Checkpoint maintenance requires Ddc2, Rad9, Rad24, and Rad53 activity.

(A) Above, percentage of G_2/M arrested cells in a 2-DSB *DDC2-AID* strain after DNA damage induction in a liquid culture. Cultures were split 4 h after galactose treatment to induce DNA damage by GAL::HO and treated either with auxin (+IAA) (1mM) or with ethanol (Ctrl). Data are shown from 3 independent experiments with error bars representing standard error of the mean (SEM). The asterisk marks the time point when the percentage of large-budded G_2/M cells returned to pre-damage levels. Below, western blots ran with samples collected at various time points during the same time course, probed with α-Rad53, to determine DDC status, and α-Myc, to determine Ddc2-AID-Myc protein abundance and TIR1-Myc as a loading control. (B) Same as (A) for 2-DSB *RAD9-AID*. (C) Same as (A) for 2-DSB *RAD24-AID*. (D) Same as (A) for 2-DSB *RAD53-AID*.

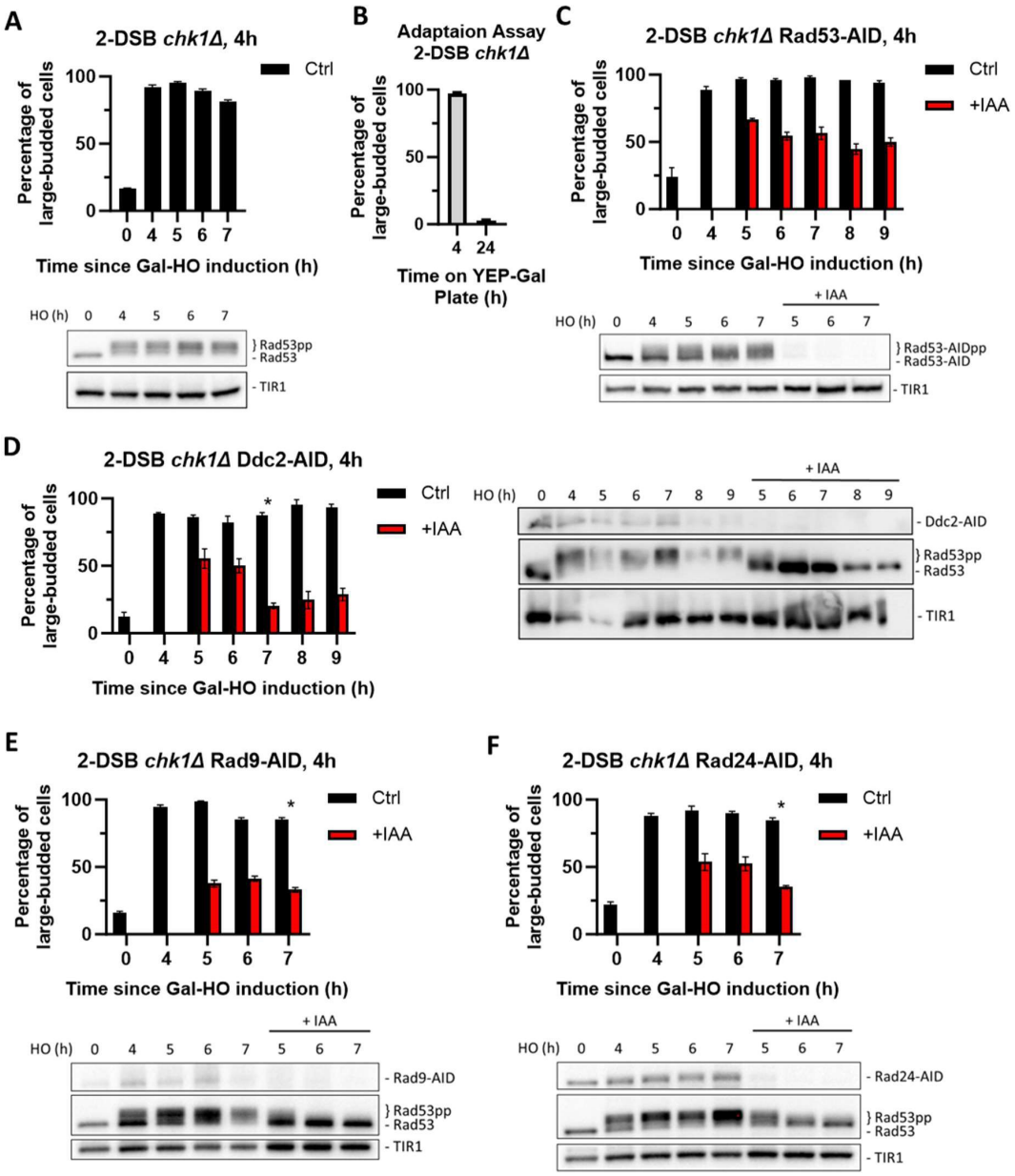

Figure 3.

Chk1 is dispensable for activation of the cell cycle arrest, but essential for its maintenance.

(A) Percent G_2/M cells in a 2-DSB *chk1Δ* strain following DNA damage. Data are shown from 3 independent experiments with error bars representing the standard error of the mean (SEM). Western blot probed with α-Rad53 to determine the status of DDC and α-Myc to determine TIR1-Myc protein abundance. (B) Adaptation assay with 2-DSB *chk1Δ* strain. (C) Percentage of G_2/M arrested cells a 2-DSB *chk1Δ RAD53-AID* strain after DNA damage. Cultures were split 4 h after DSB induction and treated with 1 mM auxin (+IAA) or with ethanol (Ctrl). Data are shown from 3 independent experiments with error bars representing the standard error of the mean (SEM). Western blot probed with α-Myc for Rad53-AID and TIR1-Myc as a loading control. (D) Same as (C) for 2-DSB *chk1Δ DDC2-AID*. Western blot probed with α-Rad53 and α-Myc. α-Rad53 shows both an unphosphorylated protein and multiple phosphorylated species. α-Myc shows Ddc2-AID degradation and TIR1-Myc as a loading control. The asterisk shows when the percentage of large-budded cells returned to pre-damage levels. (E) Same as (D) for 2-DSB *chk1Δ RAD9-AID*. (F) Same as (D) for 2-DSB *chk1Δ RAD24-AID*.

Dun1 is Required for the Initiation but not for the Maintenance of Cell Cycle Arrest

Our findings show that a small number of cells remain arrested in the absence of *CHK1* when Rad53 is depleted. We posited that Rad53 could modulate the expression of other DDC factors which, in turn, sustain the cell cycle arrest in the absence of Rad53 and Chk1. One candidate protein is Dun1, a Rad53-activated protein kinase that regulates transcription in response to DNA damage (Chen, Smolka, and Zhou 2007; Yam et al. 2020; Zhou and Elledge 1993). Deleting *DUN1* significantly impaired checkpoint activation: compared to the wild-type control strain, only 60% of *DUN1* cells arrested in G_2/M 4 h after the DSB induction, and only 25% remained in G_2/M arrest at 7 h (**Figure 4A**). Additionally, depletion of Dun1-AID 4 h after damage induction did not cause a significant change in the proportion of G_2/M arrested in these otherwise wild type cells, nor did it affect Rad53 phosphorylation (**Figure 4B**); however, deleting *CHK1* triggered an exit from checkpoint arrest following the depletion of Dun1-AID 4 h after DSB induction (**Figure 4C**) further demonstrating the role of Chk1 in checkpoint maintenance. These results suggest that Dun1 is required for the initiation of DDC and concomitant cell cycle arrest but is dispensable for maintenance.

Ddc2 and Rad53's Role in Maintaining Arrest Become Dispensable in Extended G_2/M Arrest

Previously, we reported that Ddc2 protein abundance initially increases over time as after the induction of a single DNA break, which is followed by near-complete depletion of Ddc2 around the time that cells adapt (Memisoglu et al. 2019). Given that Ddc2 overexpression leads to permanent cell cycle arrest following DNA damage (Clerici et al. 2001), we concluded that Ddc2 abundance is intimately tied to the duration of the arrest. Here, we asked whether the presence of 2 DSBs instead of a single DSB would lead to an increase in Ddc2 protein abundance and therefore, the permanent cell cycle arrest. We examined the levels of Ddc2 in both 1 and 2-DSB strains following DNA damage but did not detect a difference in the changes of abundance of Ddc2 protein (Figure S5A-C), even though cells adapt to 1 DSBs and remained terminally arrested after 2 DSBs (**Figure 1A-1D**).

If Ddc2 activity is essential to maintain the cell cycle arrest in the 2-DSB strain, then degradation of Ddc2-AID around the time wild-type cells adapt to a single DNA break should interrupt the permanent cell cycle arrest and trigger cell cycle re-entry. We find that complete depletion of Ddc2-AID 15 h after the induction of 2 DSBs leads to diminished Rad53 hyperphosphorylation (**Figure 5A**). However, surprisingly, Ddc2-AID degradation did not alter the percentage of G_2/M arrested cells even 9 h after Ddc2 depletion (**Figure 5A**). Furthermore, despite the diminished Rad53 phosphorylation, Ddc2-AID cells mostly remained arrested in G_2/M after the depletion of Ddc2-AID at 15 h as illustrated by DAPI staining (**Figure 5B**), in contrast to Ddc2-AID depletion soon after induction of 2 DSBs which causes cells to rapidly resume mitosis (**Figure 2A**). These results hint that the maintenance of the permanent cell cycle arrest in response to 2 DSBs at later stages could be independent of the DDC signaling.

We then asked whether other DDC factors such as Rad9, Rad24 and Rad53 are dispensable for the prolonged arrest following the induction of 2 DSBs. However as noted above, AID-tagged DDC activation proteins were unable to maintain this prolonged arrest in a 2-DSB strain even in the absence of IAA, which precludes their use in this analysis. To be able to study the contribution of these DDC factors in prolonged cell cycle arrest, we turned to the AID version 2 (AID2) system (Yesbolatova et al. 2020). To this end, we integrated a TIR1-F74G point mutation, and used 5-phenyl-IAA (5-Ph-IAA) instead of IAA to lower the basal degradation of AID-tagged proteins. Switching to the AID2 system did not fully restore function to *RAD9-AID2* and *RAD24-AID2* strains,

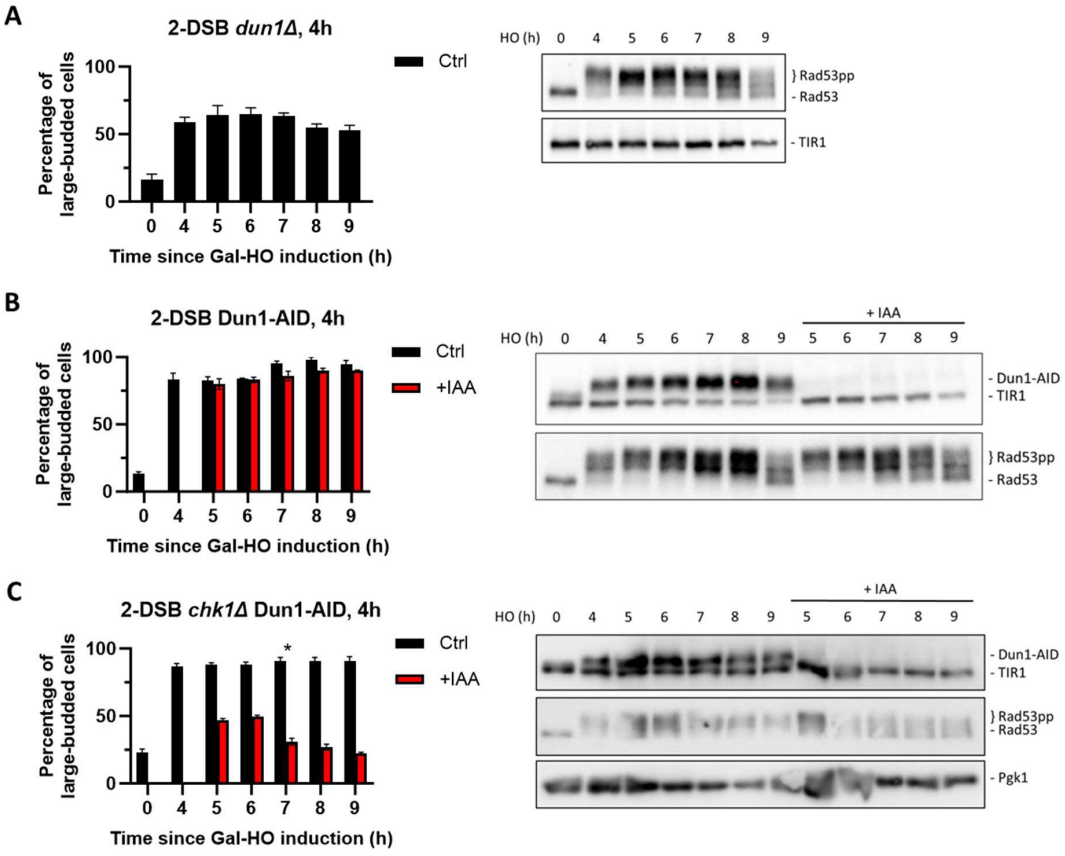

Figure 4.

Dun1 is not required for checkpoint maintenance

(A) Adaptation assay of 50 G_1 cells on a YEP-Gal plate with 2-DSB *dun1Δ*. G_2/M arrest was determined based on cell morphology as shown in **Figure 1A**. Data is shown from 3 trials with standard error of the mean (SEM). Western blot probed with α-Rad53 and α-Myc for TIR1-Myc as a loading control. (B) Percent G_2/M arrested cells for 2-DSB *DUN1-AID* after HO induction. Data are shown from 3 trials with standard error of the mean (SEM). Cultures were split 4 h after DSB induction; with auxin (1 mM) (+IAA). Western blot probed with α-Rad53 and α-Myc. α-Rad53 shows both an unphosphorylated protein and multiple phosphorylated species. α-Myc shows Dun1-AID degradation and TIR1-Myc as a loading control. (C) Same as (B) for 2-DSB *chk1Δ DUN1-AID*. The asterisk marks when the percentage of large-budded cells returned to pre-damage levels.

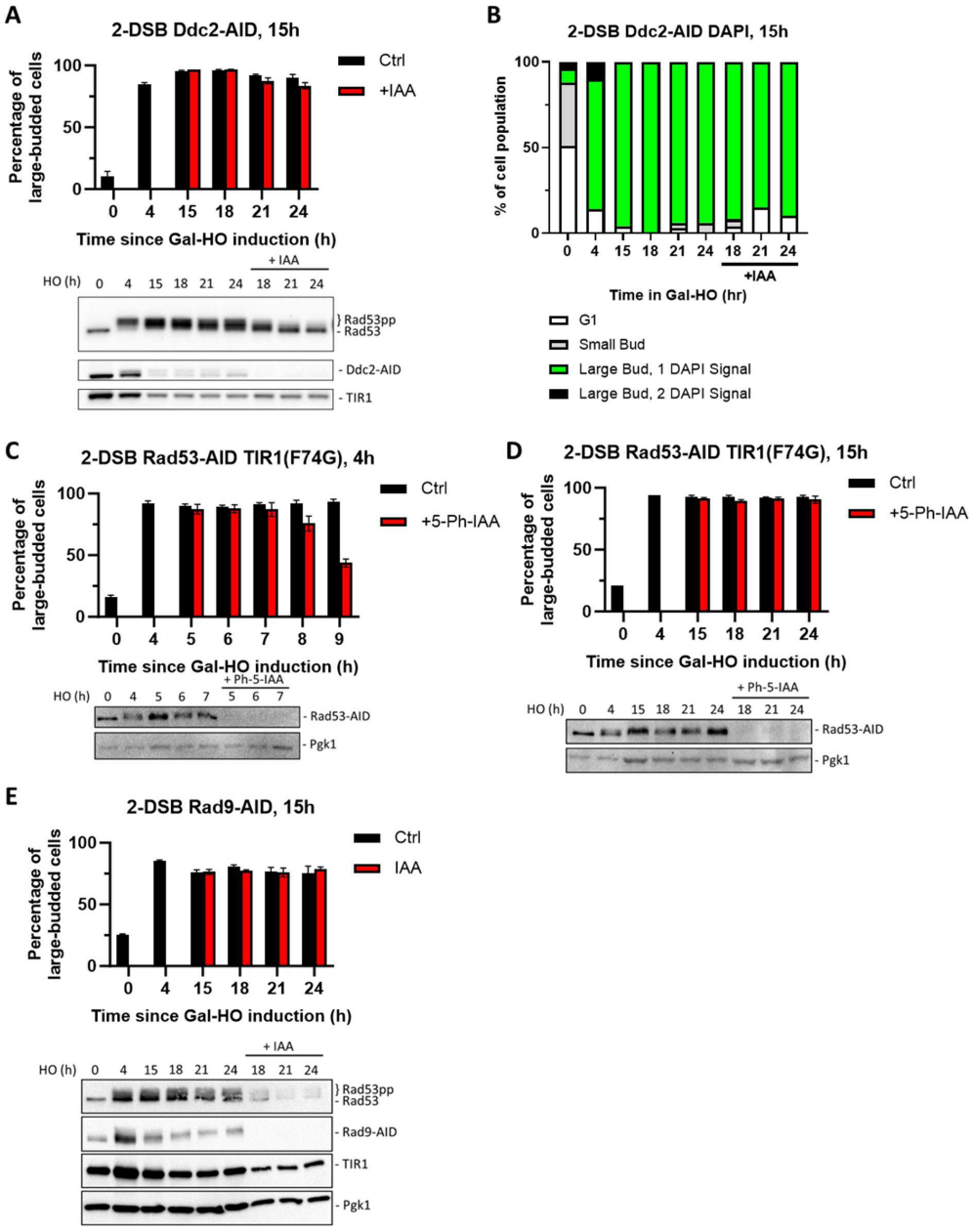

Figure 5.

Ddc2 and Rad53 are dispensable for >24 h checkpoint arrest

(A) Percent G_2/M arrested cells for 2-DSB *DDC2-AID* after HO induction. Data is shown from 3 trials with standard error of the mean (SEM). Western blot probed with α-Rad53 and α-Myc. α-Rad53 shows both an unphosphorylated protein and multiple phosphorylated species. α-Myc shows Ddc2-AID degradation and TIR1-Myc as a loading control. (B) Profile of DAPI stained cells in a 2-DSB *DDC2-AID* strain after HO induction. Cells were categorized based on cell morphology and number of DAPI signals. (C) Percent G_2/M arrested cells for 2-DSB *RAD53-AID TIR1(F74G)* after HO induction. 5-Ph-IAA was added 4 h after HO induction. Data is shown from 3 trials with standard error of the mean (SEM). Western blot probed with α-Rad53, α-Myc, and α-Pgk1. α-Rad53 shows both an unphosphorylated protein and multiple phosphorylated species. α-Myc shows Rad53-AID degradation. α-Pgk1 probed as a loading control. (D) Same as (C) where 5-Ph-IAA was added 15 h after HO induction. (E) Percentage G_2/M arrested cells for 2-DSB *RAD9-AID* plus pRad9-AID after HO induction. Data shown from 3 trials with standard error of the mean (SEM). Western blot probed with α-Rad53 and α-Myc. α-Rad53 shows both an unphosphorylated protein and multiple phosphorylated species. α-Myc shows Rad9-AID degradation and TIR1-Myc as a loading control. α-Pgk1 probed as a loading control.

as they mostly adapted after 24 h after the exposure to DNA damage, but 87% of *RAD53-AID2* cells remained in G_2/M arrest at 24 h (Figure S1C). Using Rad53-AID2, we then asked whether Rad53 is required for extended G_2/M arrest in response to 2 DSBs. Rapid depletion of Rad53-AID2 with 5-Ph-IAA 4 h after DSB induction led cells to escape G_2/M arrest, but as with the *RAD53-AID* strain, we detected a 4 h delay in cell cycle re-entry, confirming our previous results (**Figure 5C** and **Figure 2D**). However, degradation of Rad53-AID2 15 h after DSB induction did not prompt cell cycle re-entry even 9 h after complete depletion of Rad53 (**Figure 5D**, S. Table 1), akin to what we observe following the depletion of Ddc2-AID (**Figure 5A-5B**).

Because TIR1-mediated degradation of Rad9-AID even without auxin caused most cells to adapt 24 h after inducing DNA damage, we asked whether overexpression of *RAD9-AID* could overcome this effect. We added a *TRP1* centromere-containing plasmid copy of *RAD9-AID* with its endogenous promoter (pRAD9-AID) to our 2-DSB *RAD9-AID* strain. Degradation of Rad9-AID by IAA 15 h after DSB induction did not trigger release of cells from G_2/M arrest (**Figure 5E**, S. Table 1). However, unlike degradation of Ddc2-AID or Rad53-AID2 in this same situation, Rad53 remained hyperphosphorylated up to 9 h after adding IAA (**Figure 5E**). Therefore, while the DDC proteins Ddc2, Rad9, and Rad53 are required for the maintenance of checkpoint arrest at early stages, surprisingly, they are dispensable for prolonged arrest following induction of 2 DSBs. These results suggest that prolonged cell cycle arrest is maintained by signaling proteins other than the Mec1 branch of the DDC.

Spindle Assembly Checkpoint Proteins Mad1 and Mad2 are Required for Prolonged Arrest

In addition to the DDC, the spindle assembly checkpoint (SAC) maintains genomic integrity by halting mitosis at the metaphase/anaphase transition in response to unattached kinetochores, to ensure accurate chromosome segregation (reviewed by (McAinsh and Kops 2023)). We have previously shown that inactivation of the SAC by a *MAD1*, *MAD2* or *MAD3* deletion shortened the duration of the cell cycle arrest induced by a single DSB (Dotiwala et al. 2010). To test whether SAC is involved in enforcing and sustaining permanent cell cycle arrest in response to 2 DSBs, we deleted *MAD2* in the 2-DSB strain. Adaptation assay results illustrate that nearly all *mad2Δ* cells arrested at 4 h but began to adapt between 12-15 h after DNA damage (Figure S6A). To explore whether deletion of *MAD2* can antagonize the permanent cell cycle arrest due to hyperactive DDC signaling, we overexpressed Ddc2 in *mad2Δ* 2-DSB cells and assayed mitotic progression. We find that both in 1-DSB and 2-DSB strains, *MAD2* deletion leads to cell cycle re-entry even when Ddc2 is overexpressed (Figure S6B). Based on these data, we concluded that mitotic inhibition is enforced by the SAC proteins as DDC factors become dispensable 12 to 15 h after the induction of damage.

If SAC proteins are only required at later stages of cell cycle arrest when DDC proteins are dispensable, then the depletion of SAC protein Mad2 or its binding partner Mad1 soon after the induction of DNA damage should not affect DDC or cell cycle arrest up to 12 to 15 hours. In agreement with this idea, after Mad1-AID or Mad2-AID depletion at 4 h, cells remained arrested up to 15 h following DSB induction, with persistent Rad53 hyperphosphorylation (Figure S7A-C). Strikingly, these cells eventually re-entered the cell cycle by 24 h (20 h after depletion of Mad1 and Mad2). It is notable that cells resumed cell cycle progression despite persistent Rad53 hyperphosphorylation. This result reinforces our conclusion that Mad1 and Mad2 are not required for the activation and initial maintenance of arrest but are essential for prolonged cell cycle arrest in response to 2 DSBs. These data also suggest that in the absence of Mad1 or Mad2, cells become insensitive to the arrest normally imposed by DDC.

To monitor the effect of SAC proteins Mad1 and Mad2 at late stages of prolonged cell cycle arrest when DDC signaling becomes dispensable, we depleted Mad1-AID or Mad2-AID 15 h after DSB induction. Following the depletion of Mad1-AID and Mad2-AID, we observed an immediate reduction in the percentage of G_2/M-arrested cells (**Figure 6A-C**). DAPI staining confirmed that

Mad1-AID or Mad2-AID deplete cells re-entered the cell cycle, evident from an increase in the G1 cell population as well as an increase in percentage of large-budded cells with 2 DAPI foci (**Figure 6B, D**). Similar to what we observe following Mad1-AID and Mad2-AID depletion 4 h after damage, this cell cycle re-entry occurred despite persistent Rad53 hyperphosphorylation (**Figure 6A, C**).

To show that the late stages of the permanent cell cycle arrest in response to 2 DSBs is independent of DDC and dependent on SAC, we inactivated DDC by depleting Ddc2-AID together with Mad2-AID. Although the simultaneous depletion of Ddc2-AID and Mad2-AID led to Rad53 dephosphorylation as detected by western blotting, we found no statistically significant difference in the percentage of cells escaping G_2/M arrest in response to *DDC2-AID MAD2-AID* double depletion strain compared to Mad2-AID alone (Figure S8A-B, and S. Table 1). Collectively, our findings indicate that DDC initiates and sustains the cell cycle arrest approximately for 15 h following DNA damage, but after that DDC becomes dispensable and the permanent arrest is sustained by SAC.

Mitotic Exit Network Proteins Bfa1 and Bub2 have Different Roles in the DDR

To investigate the possible contribution of the mitotic exit network (MEN) to the maintenance of the extended cell cycle arrest in response to 2 DSBs, we appended AID tags to upstream MEN proteins Bub2 and Bfa1. Degradation of Bub2-AID both 4 h and 15 h after induction of 2 DSBs suppressed G_2/M arrest (**Figure 7A**, S9A-B), akin to what we observe following Mad1-AID or Mad2-AID depletion (**Figure 6A-B**). In contrast to Bub2-AID, we see that Bfa1-AID degradation did not trigger a significant release from G_2/M arrest and did not alter the phosphorylation of Rad53 (**Figure 7B**, S9C-E). Analysis of cell cycle distribution with DAPI staining showed that neither the inactivation of Bub2 nor Bfa1 led to the accumulation of a significant number of cells with two separate DAPI-staining nuclei, which is indicative of mitotic exit defects (**Figure 7B, D** and S9B, D, F). These results imply that although Bub2 and Bfa1 have interdependent functions for MEN signaling, they carry out independent roles in response to DNA damage.

The Location of the Second DSB Site Relative to the Centromere Affects Prolonged Arrest

In contrast to the permanent cell cycle arrest we observe in response to 2 DSBs, a recent study using two HO-mediated persistent DSBs showed that cells in fact adapt (Sadeghi et al. 2022). One difference between these two 2-DSB systems is the relative position of the DSBs, which might affect how SAC components become engaged, and thus might determine the extent of mitotic arrest. Supporting this, we previously showed that deleting *CEN3* in a strain with a DSB at *MAT* on chromosome III eliminated the Mad2-dependent delay in adaptation, but deleting *CEN3* when the DSB was on chromosome VI had no effect (Dotiwala et al. 2010). In our adaptation-defective 2-DSB strain, the DSBs are located at *MAT* (86 kb from *CEN3*) and near *FAB1* (42 kb from *CEN6*). Sadeghi et al. employed 2 strains, both of which escape prolonged G_2/M arrest, with at least one DSB site far from its centromere; at *URA3* (36 kb from its centromere) and *ADH1* (170 kb) or at *MIC2* (32 kb) and *DLD2* (316 kb).

To investigate whether the distance between the second DSB site and the centromere would affect whether cells will remain permanently arrested, we created several strains which contain a second cut site at various distances from the centromere, in addition to the cut site at *MAT* on chromosome III (Figure S10). Strain YSL53, which has a second DSB at chromosome V, 86 kb away from *CEN5* (Lee et al. 1998), and strain DW417, which has a second DSB 52 kb away from *CEN6* (Lee et al. 2014) mostly remained in G_2/M arrest 24 h after the induction of DNA breaks. However, in GEM188, which had a second cut site 230 kb away from the *CEN2*, only 37% of cells

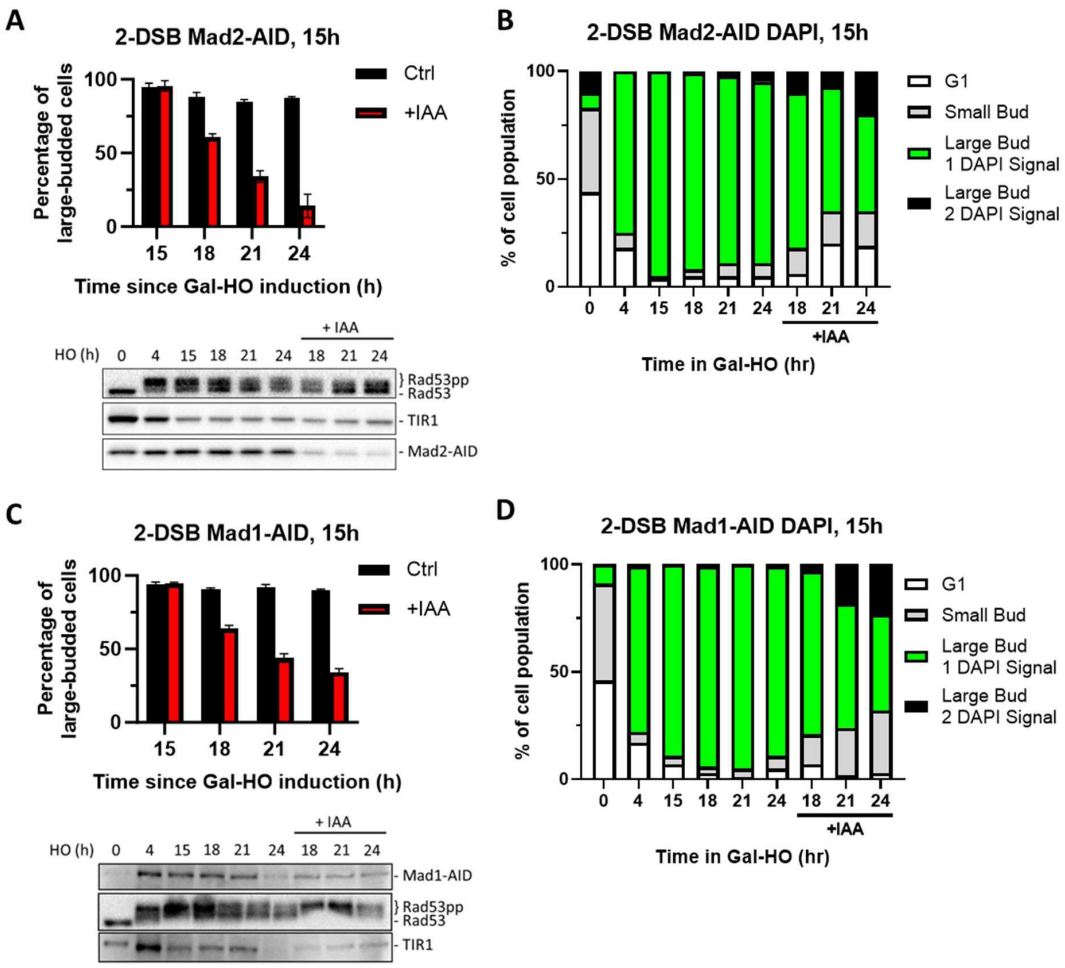

Figure 6.

Degradation of Mad2 or Mad1 at 15 h releases cells from checkpoint arrest

(A) Percent G_2/M arrested cells for 2-DSB *MAD2-AID* after HO induction. Data is shown from 3 trials with standard error of the mean (SEM). Western blot probed with α-Rad53 and α-Myc. α-Rad53 shows both an unphosphorylated protein and multiple phosphorylated species. α-Myc shows Mad2-AID degradation and TIR1-Myc as a loading control. (B) Profile of DAPI stained cells in a 2-DSB *MAD2-AID* strain after HO induction. Liquid cultures were split 15 h after HO induction and treated with either IAA or ethanol. Cells were scored based on cell morphology and number of DAPI signals. (C) Same as (A) for 2-DSB *MAD1-AID*. (D) Same as (B) for 2-DSB *MAD1-AID*.

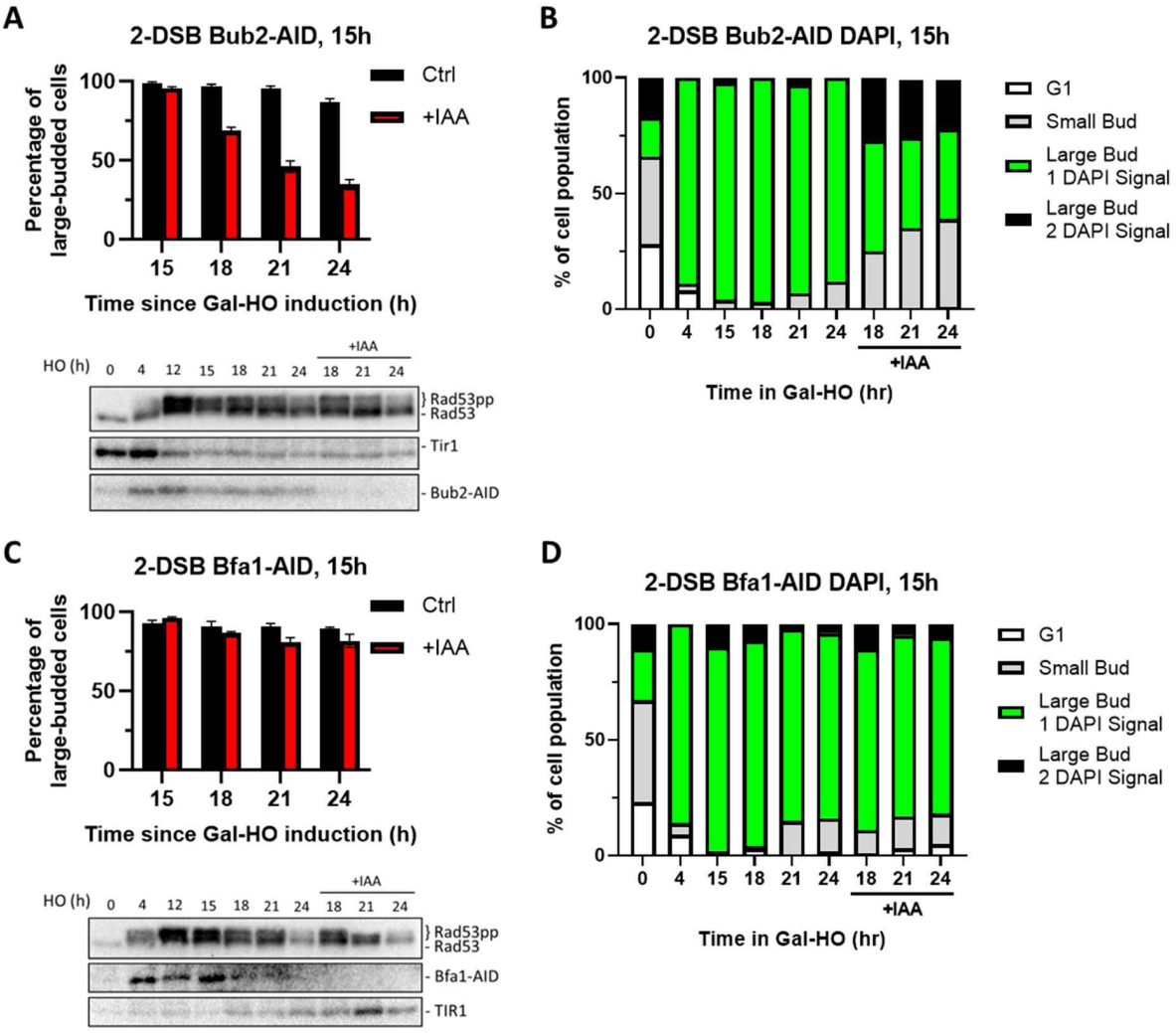

Figure 7.

Degradation of Bub2 but not Bfa1 at 15 h releases cells from checkpoint arrest

(A) Percent G_2/M arrested cells for 2-DSB *BUB2-AID* after HO induction. Data is shown from 3 trials with standard error of the mean (SEM). Western blot probed with α-Rad53 and α-Myc. α-Rad53 shows both an unphosphorylated protein and multiple phosphorylated species. α-Myc shows Bub2-AID degradation and TIR1-Myc as a loading control. (B) Profile of DAPI stained cells in a 2-DSB *BUB2-AID* strain after HO induction. Liquid cultures were split 15 h after HO induction and treated with either IAA or ethanol. Cells were scored based on cell morphology and number of DAPI signals. (C) Same as (A) for 2-DSB *BFA1-AID*. (D) Same as (B) for 2-DSB *BFA1-AID*.

remained in G_2/M arrest by 24 h. Thus, the increased distance of the second DSB site to the centromere in GEM188 appears to have led to a less robust triggering of the SAC compared with YSL53 and DW417.

Our previous data have suggested that the involvement of the SAC in prolonging DSB-induced arrest was dependent on centromere sequences on the broken chromosome and involved post-translational modification of chromatin by the Mec1- and Tel1-dependent phosphorylation of the histone H2A (Dotiwala et al. 2010). In budding yeast, histone H2B is also targeted by DDC kinases upon DNA damage (Lee et al. 2014). To test whether the presence of these chromatin modifications around centromeres would be sufficient to elicit a SAC response, we examined cell cycle progression in strains in which both histone H2A and/or histone H2B genes were mutated to their putative phosphomimetic forms (H2A-S129E and H2B-T129E). We note that although histone H2A-S129E is recognized by an antibody specific for the phosphorylation of histone H2A-S129 (Eapen et al. 2012), the mutation to S129E may not be fully phosphomimetic. As shown in Figure S11, there was no effect on the growth rate of either the single or the double mutants, suggesting that cells did not experience a SAC-dependent delay in entering mitosis because of these modifications.

Discussion

Here, we studied how cells maintain cell cycle arrest following DNA damage, by using a yeast strain that permanently halts cell cycle with two persistent DNA breaks. We find that most of the DDC signaling proteins must remain active to maintain G_2/M arrest, highlighting the importance of continuous checkpoint signaling in preventing premature mitotic entry and therefore, genome instability (**Figure 8**).

Phosphorylation of Rad53 by Mec1 is tightly linked to cell cycle arrest following DNA damage (Pellicioli et al. 2001). While Rad53 is also shown to be targeted by Tel1, deleting *TEL1* did not affect the prolonged checkpoint arrest in a 2-DSB strain. This finding supports the previous reports showing that cell cycle arrest in response to enzymatic DNA breaks is largely orchestrated by Mec1, with a minor contribution from Tel1 (Pellicioli et al. 2001; Vaze et al. 2002). Here, we add that Mec1 inactivation via Ddc2 depletion, 4 h after DSB induction, results in rapid resumption of the cell cycle, most likely through Ptc2, Ptc3, Pph3 and Glc7-dependent dephosphorylation and inactivation of the DDC signaling protein Rad53 (Bazzi et al. 2010; Leroy et al. 2003; O'Neill et al. 2007). However, the regulation and consequences of Rad53 phosphorylation are apparently more complex, given that cells can re-enter mitosis in the presence of persistent Rad53 phosphorylation when SAC proteins Mad1 and Mad2 are depleted (see below).

Mec1 activation in the S and G_2 cell cycle phases is achieved by at least two converging mechanisms; first, through Ddc2 binding to RPA-coated ssDNA created the 5' to 3' resection of the DSB ends, and second, through binding of the 9-1-1 clamp subunit Ddc1 (Dubrana et al. 2007; Navadgi-Patil and Burgers 2009; Zou and Elledge 2003; Melo, Cohen, and Toczyski 2001). Before Ddc1 can activate Mec1, the 9-1-1 checkpoint clamp must be loaded by the clamp loader, which consists of Rad24-Rfc2-5 (Melo, Cohen, and Toczyski 2001). DDC activation largely depends on a functional clamp loader, as cells lacking *RAD24* proceed directly into mitosis in response to a single DSB, with only a brief delay (Aylon and Kupiec 2003). If the clamp loader acts just once to load the 9-1-1 clamp and the clamp then slides away from the DSB as DNA is resected, then removal of Rad24 after the checkpoint had been robustly activated should not perturb arrest. However, we find that Rad24 depletion leads to rapid cell cycle resumption in response to two DSBs, suggesting that multiple 9-1-1 clamp loading events are required to sustain extended cell cycle arrest. We posit that 9-1-1 clamp may require continuous reloading, as the 5' end is being continuously resected by Exo1 and Sgs1-Rmi1-Top3-Dna2 exonucleases (Zhu et al. 2008).

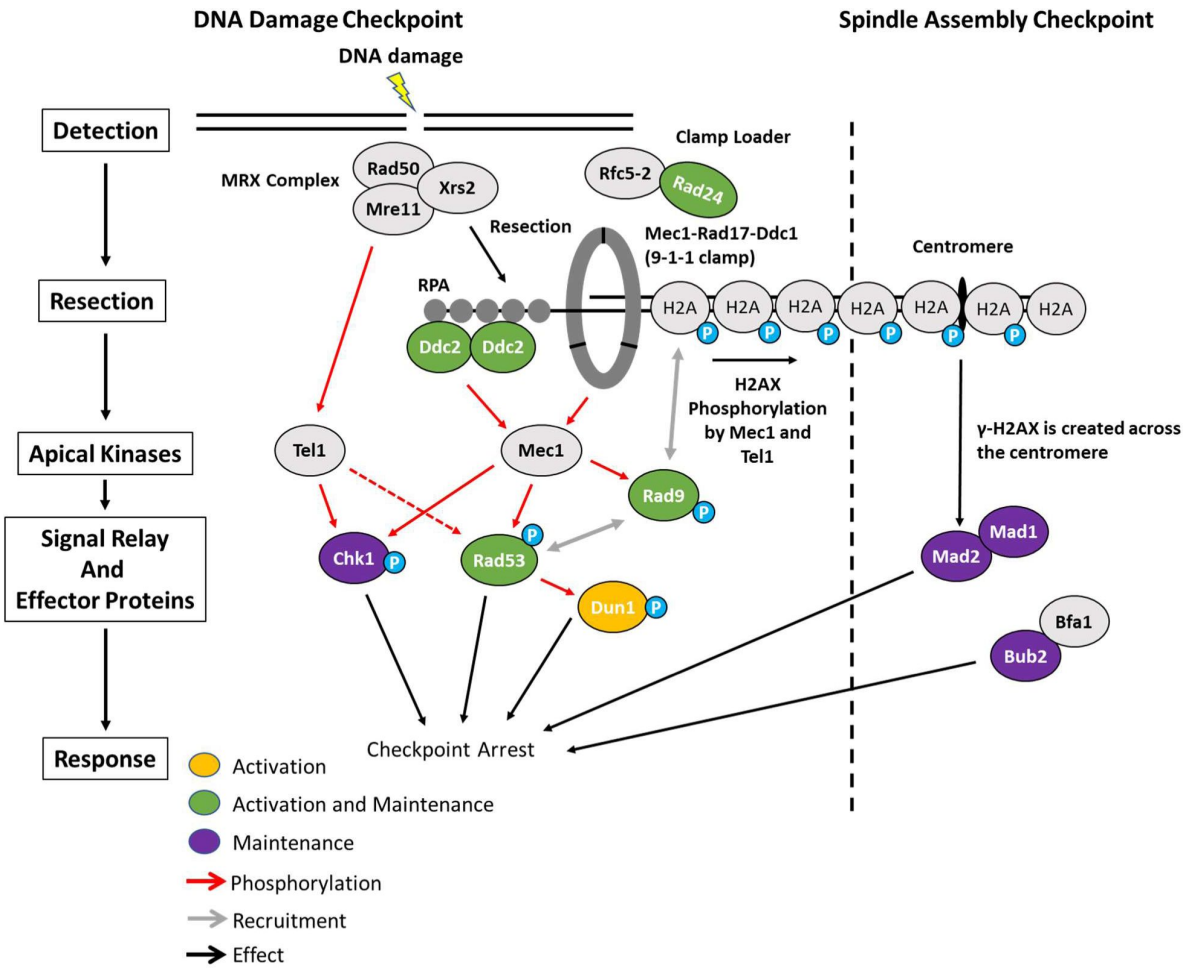

Figure 8.

Activation and maintenance of checkpoint arrest in response to a DSB

The Mre11-Rad50-Xrs2 (MRX) complex is one of the first complexes recruited to DSBs and initiates the resection of dsDNA to ssDNA. ssDNA is then coated with RPA which recruits Ddc2. Mec1 is the primary kinase responsible for checkpoint arrest in budding yeast and is activated by Ddc2 and Ddc1 from the 9-1-1 clamp. Proteins in green (Ddc2, Rad9, Rad24, and Rad53) were required for the activation and maintenance of checkpoint arrest. While Chk1 was not required for establishment of G_2/M arrest, it contributed to the maintenance of arrest. In contrast, Dun1 was required for checkpoint activation but was dispensable 4 h after DSB induction. Prolonged arrest >24 h in a 2-DSB strain was dependent on the SAC proteins Mad2, Mad1, and Bub2 as well as the distance between the 2nd HO-cut site and the centromere.

The adaptor kinase Rad9, downstream of Mec1 and Tel1, is responsible for scaffolding and activating the effector DDC kinases Rad53 and Chk1 (Emili 1998; Schwartz et al. 2002; Sweeney et al. 2005). Here, we show that conditional depletion of Rad9 shortly after the induction of 2 DSBs prompts mitotic re-entry and terminates the DDC, evident from rapid Rad53 dephosphorylation. Thus, Rad9 is required for continued maintenance of Rad53 phosphorylation.

Depletion of Rad53-AID 4 h after inducing 2 DSBs triggers release of cells from G_2/M arrest, albeit resumption of mitosis is delayed by 4 h compared to resumption of mitosis seen when upstream DDC factors Ddc2, Rad9, or Rad24 are depleted. We discovered that this residual delay in mitotic re-entry is dependent on Chk1 signaling. We postulate that Chk1 may delay cell cycle re-entry in the absence of Rad53 by phosphorylating and stabilizing Pds1 (Agarwal et al. 2003).

Agreeing with previously published work (Yam et al. 2020), we find that deleting *DUN1* resulted in a less robust DDC activation and a shortened G_2/M arrest. However, unlike the depletion of other DDC proteins examined in this study, depletion of Dun1-AID 4 h after DSB induction was not sufficient to promote cell cycle re-entry. It is possible that the transcripts upregulated by Dun1 after DNA damage (Zhao and Rothstein 2002; Zhou and Elledge 1993) are stable for several hours and are sufficient to sustain the arrest even in the absence of Dun1. We also find that cell cycle arrest upon depletion of Dun1 is Chk1-dependent. These results are in consistent with Dun1's role in stabilizing Pds1 through a Chk1-independent mechanism (Yam et al. 2020).

Here, we show that prolonged cell cycle arrest following induction of 2 DSBs becomes independent of DDC proteins Ddc2, Rad9, and Rad53, but dependent on SAC proteins Mad1 and Mad2. Depletion of Mad1 or Mad2 4 h after DSB induction did not immediately result in cell cycle re-entry, but around 15 h cells began to resume cell cycle. The timing of cell cycle re-entry for Mad1/2-depleted cells following the induction of 2 DSB is about the same as the timing of cell cycle re-entry in response to a single DNA break in wild-type cells. Even when DDC signaling is artificially upregulated via Ddc2 overexpression, cells re-enter mitosis if Mad2 is depleted. Surprisingly, in the absence of Mad1 or Mad2 cells escaped arrest despite persistent Rad53 hyperphosphorylation, suggesting that cells become "deaf" to the DDC signal once SAC takes over.

Previous work indicates that SAC proteins contribute to the DNA damage response (Dotiwala et al. 2010; Garber and Rine 2002; Kim and Burke 2008). Work from our lab has suggested that γ-H2AX spreading from a DSB to the centromere of the same chromosome might impair kinetochore attachment and thus trigger a SAC response (Dotiwala et al. 2010). SAC signaling is not sufficient to elicit a permanent cell cycle arrest in response to a single DSB; however, we find that inducing 2 DSBs, each within 100 kb of its centromere, elicits a SAC-dependent permanent arrest. As strength of the SAC has a direct relation to the number of unattached kinetochores (Dick and Gerlich 2013), the addition of a second DSB on another chromosome might trigger a stronger SAC response and result in a permanent cell cycle arrest.

The fact that not all combinations of two DSBs produce permanent arrest (Sadeghi et al. 2022) can be explained by the idea that the distance between the DSB site and its corresponding centromere is an important determinant of the extent of cell cycle arrest. We previously observed that a strain with a single DSB 200 kb away from the centromere had shorter cell cycle arrest compared to a strain with a DSB 86 kb away from the centromere (Dotiwala et al. 2010). Here we provide further evidence suggesting that the distances between DSBs and their corresponding centromeres determine whether SAC will be fully activated to prolong the arrest. Our previous results showed that when *MAD2* was deleted, the length of cell cycle arrest was the same in strains with a single DSB, irrespective of the DSB's distance to its centromere (Dotiwala et al. 2010). We suggest that the strains used by (Sadeghi et al. 2022) do not remain permanently arrested because one of the two DSBs in their strains is sufficiently far from its centromere to fully trigger SAC.

In addition to blocking the metaphase to anaphase transition, components of the SAC also block mitotic exit (reviewed by (Matellan and Monje-Casas 2020)). We examined the role of Bub2/Bfa1 heterodimer, the most upstream components of the MEN pathway (Matellan and Monje-Casas 2020). Much like Mad1 and Mad2, we found that neither Bub2 nor Bfa1 was required for the establishment of cell cycle arrest in response to DNA damage; but our study revealed a surprising result: Bub2, but not its partner Bfa1, is essential to prolong cell cycle arrest, indicating that Bub2 has a Bfa1-independent role.

By using conditional depletion of various proteins that contribute to cell cycle arrest, we show that the establishment, maintenance, and inactivation stages of DNA damage-provoked cell cycle arrest involve different sets of factors following DNA damage. After the DNA damage checkpoint is established, its maintenance proves to be divided into two distinct phases. Arrest up to about 15 h requires the constant presence of most of the identified DDC proteins, including Ddc2, Rad9, Rad24, and Rad53, with Dun1 playing an important but nonessential role. Although Chk1 was not required either to establish or initially to maintain cell cycle arrest, its absence shortened arrest, most notably when Rad53 was depleted. Surprisingly, neither Ddc2, Rad9, nor Rad53 (and we suggest likely Rad24) are necessary for the prolongation of cell cycle arrest lasting longer than 15-24 h. Instead, this prolonged arrest is enforced by SAC proteins Mad1, Mad2, and Bub2.

Methods and materials

Yeast Strain and Plasmid Construction

All AID-tagged mutant strains were derived from a modified version of strains JKM179 (S. Table 2). To create the strain with two HO cleavage sites (DW417), an HO cut site, designated HOcse6, with an adjacent HPH marker was integrated into chromosome VI, 52 kb from the centromere. To create auxin-inducible degron strains, we first integrated osTIR1 at *URA3* after digesting plasmid pNHK53 (Nishimura et al. 2009) with *Stu*I. To integrate osTIR1-F74G at *URA3*, the plasmid pMK420 (Yesbolatova et al. 2020) was digested with *Stu*I. For degron-tagging of DDC proteins, AID-9xMyc (AID) PCR products were generated with mixed oligos with homology to the C-terminal end of the corresponding open reading frames by using plasmids pKan–AID–9xMyc (pJH2892) or pNat–AID–9xMyc (pJH2899) as templates (Morawska and Ulrich 2013). Deletion of ORFs and insertion of AID tags were introduced with the one-step PCR homology cassette amplification and the standard yeast transformation method (Wach et al. 1994). Cas9 editing was done by inserting a gRNA into plasmid bRA90 (Anand et al. 2017) and co-transformed into our strain of interest with a donor sequence. Transformants were verified by PCR, western blotting, and sequencing. To create strain GEM188 with 2-DSBs, we inserted a second HO-cut site into JKM179 at *LYS2* locus by CRISPR/Cas9 (Anand et al Protocol Exchange, 2017) using a synthetic DNA template with 117 bp consensus HO recognition site. Non-phosphorylatable and phosphomimetic mutants of H2A were generated in a JKM179 background using CRISPR/Cas9 to target HTA1 and HTA2 genes at serine 129 and 80nt templates to mutate serine to either alanine (non-phosphorylatable) or glutamic acid (phosphomimetic). H2B mutants were generated in a JKM179 background using CRISPR/Cas9 to target *HTB1* and *HTB2* genes at threonine 129 with 80nt repair templates to mutate threonine to either alanine or glutamic acid.

The *CEN/ARS* plasmid pFZ052-*pRAD9-AID-*9Myc::Trp1* (pRAD9-AID) was obtained by digesting the plasmid pFL36.1 (Lazzaro et al. 2008) with *SmaI* and *AscI* to excise the *3-HA* tag on the C-terminal end of Rad9. A *9xMyc-AID* PCR product generated from the plasmid pJH2892 (pKan-9xMyc-AID) was cut with *AscI* and sticky/blunt end cloned into the *SmaI-AscI*-digested pFL36.1 to add the *9xMyc-AID* tag to the C-terminal end of Rad9. pRad9-AID was retained by growing cells in a Trp-media with 2% raffinose. Primers used for strain and plasmid creation are listed in S. Table 3. Plasmids are listed in S. Table 4.

Culturing Conditions, HO expression, and Auxin Treatment

Strains containing degron fusions and galactose-inducible HO were cultured using standard procedures. Briefly, a single colony grown on a YEPD plate (1 % yeast extract, 2 % peptone, 2 % dextrose, 2.5 % agar) was inoculated in 5 ml YEP-lactate (YEP containing 3 % lactic acid) and was grown for ~15 hours at 30° C with agitation. Next day, the overnight culture was used to inoculate a 500-100 ml of YEP-lactate culture such that the cell density reached an OD_{600} of 0.5 the following day. After harvesting 15 ml liquid culture before treatment, HO expression was induced by galactose treatment with a 2% final concentration. Then, cultures were split either at 4 h or 15 h following induction with galactose. The split cultures were treated either with IAA or 5-Ph-IAA or an equivalent volume of 200 proof ethanol. Indole-3-acetic acid (IAA, Sigma-Aldrich, I3750) was resuspended in ethanol for a 500 mM stock and used at a 1 mM final concentration both for liquid media and for agar plates. 5-Ph-IAA was dissolved in ethanol for a 1 mM stock and used at a final concentration of 1µM. 15 ml of liquid culture were harvested at various time points and prepared for microscopy or western blot analysis as described below.

Culturing Conditions, HO expression, and Auxin Treatment

To measure growth rate, strains were grown in 5 ml of YEPD with 2% dextrose at 30 °C with an initial OD_{600} of 0.1. The OD was measured 3, 5, 7, and 10 h after the initial OD measurement using a Thermo Scientific NanoDrop 2000c Spectrophotometer. To measure the OD, 50µl of culture was added to 950µl of fresh YPD in a cuvette at a dilution of 1:20.

TCA Protein Extraction

Protein extracts were prepared for western blot analysis by TCA extraction protocol as previously explained (Miller-Fleming et al. 2014). Briefly, 15 ml of liquid culture was spun down and the media was discarded. Harvested cells were incubated on ice in 1.5 ml microcentrifuge tubes with 20% TCA for 20 minutes. Cells were washed with acetone and the pellet was air dried. 200 µl of MURBs buffer (50 mM sodium phosphate, 25 mM MES, 3M urea, 0.5% 2-mercaptoethanol, 1 mM sodium azide and 1% SDS) was added to each sample with acid washed glass beads. Cells were lysed by mechanical shearing with glass beads for 2 min. Crude cell lysates were harvested by poking a hole in the bottom of the 1.5 ml microcentrifuge tube and spinning the tubes on a 15 ml conical tube. Samples were boiled at 95 ^{0}C for 10 min prior to loading on SDS-PAGE.

Western Blotting

8 – 20 µl of denatured protein samples prepared by TCA extraction were loaded onto a 10 % or 8 % SDS-PAGE gels. Proteins were separated by applying constant voltage at 90 V until the 37 kDa protein standard band reached the bottom of the gel. Gels were transferred to an Immun-Blot PVDF membrane (BioRad) using a wet transfer apparatus set to 100 V constant voltage for 1 h at 4 °C. Membranes were then blocked with OneBlock blocking buffer (Genesee Scientific Cat #: 20-313) for 1 h at room temperature, After three 10 minute washes with 1x TBS-T, blots were incubated with either anti-Myc [9E11] (Abcam, ab56) to detect TIR1 and AID fusions, anti-Rad53 [EL7.E1] (Abcam, ab166859), anti-Pgk1 (Abcam, ab30359), or anti-Rad9 (Usui, Foster, and Petrini 2009) for 1 h at room temperature or at 4 ^{O}C overnight. Blots were washed 3 times with 1x TBS-T and incubated with anti-mouse HRP (GE Healthcare Cat # NXA931) or anti-rabbit HRP secondary antibody (Sigma-Aldrich, Cat # A6154) for 1 h at room temperature. After washing the membranes 3 times with 1x TBS-T, Amersham ECL Prime Western Blotting Detection Reagent was added to fully coat the blots and left to incubate for 5 min at room temperature with gentle agitation. Blots were imaged using a BioRad ChemiDoc^{TM} XR+ imager and prepared for publication using ImageLab 6.1 software (BioRad) and Adobe Photoshop CC 2017. Reagents used are listed in S. Table 5.

Microscopy, DAPI staining, and Cell Morphology Determination

Aliquots from YEP-Lac cultures were taken either 4 h or 15 h after adding galactose, diluted 20-fold in sterile water and plated on a YEP-Agar with 2 % galactose with or without 1 mM IAA or 1 µM 5-Ph-IAA. Cells were counted on a light microscope with a 10x objective, examined and binned into three categories: unbudded, small buds, and G_2/M arrested cells with large buds. For each time-point, >250 cells were analyzed. For DAPI staining, 450 µl of culture was added to 50 µl of 37% formaldehyde and incubated in the chemical hood at room temperature for 20 min. Samples were spun down at 8000 rpm for 5 min and washed with 1X PBS 3 times. Cells were resuspended in 50 µl of DAPI mounting media (VECTASHIELD® Antifade Mounting Medium with DAPI H-1200-10) and incubated at room temperature for 10 min, away from direct light. The samples were imaged by using a Nikon Ni-E upright microscope equipped with a Yokogawa CSU-W1 spinning-disk head, an Andor iXon 897U EMCCD camera, Nikon Elements AR software, a 60x oil immersion objective, and a 358 nm laser. 15 z-stacks with a thickness of 0.3 µm were collected per image.

Adaptation and Auxin Plating Assays

We performed adaptation assays as previously described (Eapen et al. 2012; Lee et al. 1998). Cells grown in YEP-Lac overnight were diluted 20-fold in sterile water and plated on a YEP-agar plate containing 2 % galactose. Using micromanipulation, 50 G_1 cells were isolated and positioned in a grid followed by incubation at 30° C. To quantify the percentage of adapted cells, the number of cells that re-entered cell cycle, grew to a microcolony (3+ cells) after 24 h was divided by the total number of cells. For an auxin plating assays, damage was induced in a YEP-Lac liquid culture by adding galactose at a final concentration of 2 %, as described above. Cells were then transferred onto YEP-agar plates containing 2 % galactose and 1mM IAA or 1 µM 5-Ph-IAA 4 h or 15 h after adding galactose. For each timepoint, >250 cells were scored and categorized as described above for the adaptation assay.

Quantification and Data Analysis

Graphs were prepared using GraphPad Prism 10 (Dotmatics). Statistical analysis for differences in the percentage of large budded (G_2/M arrested) cells at different timepoints listed in S. Table 1 was done using a one-way Anova test in GraphPad Prism 10. Protein quantification of Ddc2-myc blots was done using ImageLab 6.1 (BioRad). To categorize DAPI stained cells based on their morphology and number of DAPI signals, images were captured as described above and viewed using ImageJ with the Fiji addon.

Competing Interest Statement

The authors declare no competing interests.

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Author Contributions

Conceptualization and initial data collection was conducted by David P. Waterman Vinay V. Eapen and James E. Haber. Data collection, strain making, imaging, primer design, and data analysis were performed by Felix Zhou, David P. Waterman, Marissa Ashton, Suhaily Caban-Penix, Gonen Memisoglu, and Vinay Eapen. The manuscript was authored and edited by Felix Zhou, David P. Waterman, Gonen Memisoglu, and James E. Haber.

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Editors

Reviewing Editor
Andrés Aguilera
CABIMER, Universidad de Sevilla, Seville, Spain

Senior Editor
Jonathan Cooper
Fred Hutchinson Cancer Research Center, Seattle, United States of America

Reviewer #1 (Public review):

Summary:

In their manuscript, Zhou et al. analyze the factors controlling the activation and maintenance of a sustained cell cycle block in response to persistent DNA DSBs. By conditionally depleting components of the DDC using auxin-inducible degrons, the authors verified that some of them are only required for the activation (e.g., Dun1) or the maintenance (e.g., Chk1) of the DSB-dependent cell cycle arrest, while others such as Ddc2, Rad24, Rad9 or Rad53 are required for both processes. Notably, they further show that after a prolonged arrest (>24 h) in a strain carrying two DSBs, the DDC becomes dispensable and the mitotic block is then maintained by SAC proteins such as Mad1, Mad2 or the mitotic exit network (MEN) component Bub2.

Strengths:

The manuscript dissects the specific role of different components of the DDC and the SAC during the induction of a cell cycle arrest induced by DNA damage, as well as their

contribution for the short-term and long-term maintenance of a DNA DSB-induced mitotic block. Overall, the experiments are well described and properly executed, and the data in the manuscript are clearly presented. The conclusions drawn are generally well supported by the experimental data. Their observations contribute to drawing a clearer picture of the relative contribution of these factors to the maintenance of genome stability in cells exposed to permanent DNA damage.

Weaknesses:

The main weakness of the study is that it is fundamentally based on the use of the auxin-inducible degron (AID) strategy to deplete proteins. This widely used method allows an efficient depletion of proteins in the cell. However, the drawback is that a tag is added to the protein, which can affect the functionality of the targeted protein or modify its capacity to interact with others. In fact, three of the proteins that are depleted using the AID systems are shown to be clearly hypomorphic, and hence their capacity to induce a strong checkpoint response might be compromised. A corroboration of at least some of the results using an alternative manner to eliminate the proteins would help to strengthen the conclusions of the manuscript.

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Reviewer #2 (Public review):

Summary:

The manuscript analyzes and attempts to discriminate genetic requirements for DNA damage-induced cell cycle checkpoint induction, maintenance, and adaptation in budding yeast bearing one or two unrepairable DNA double strand breaks using auxin-induced degradation (AID) of key DNA damage response (DDR) factors. The study paid particular attention to solving a puzzle regarding how yeasts bearing two unrepaired DNA breaks fail to engage in "adaptation" whereas those with a single unrepairable break eventually resume cell cycling after a prolonged (up to 12 h) G2 arrest.

The key findings are: 1. Genetic requirements for the entry and the maintenance of DDC are separable. For instance, Dun1 is partially required for the entry but not the DDC maintenance whereas Chk1 is only required for maintenance. 2. Cells with two unrepairable breaks respond to DDR only up to a certain time (~12-15 h post damage) and beyond this point, depend on spindle assembly checkpoint (SAC) and mitotic exit network (MEN) to halt cell cycling. 3. The authors also propose an interesting concept that the location of DNA breaks and their distance to centromeres are important factors dictating the effect of SAC/MEN on the duration of cell cycle arrest after prolonged arrest (and cells become "deaf" to persistent arrest signals) and yeast's adaptability following DNA damage. The results provide most compelling evidence to date on the role of SAC/MEN in DNA damage response and cell cycle arrest albeit its impact might be limited to the handful of model systems due to the vastly different centromeric elements and far larger chromosome sizes in metazoan cells. The study albeit briefly discussed the basis of transitions from entry, maintenance, and adaptation (ex. changes in centromeric architectures), it does not offer detailed explanations or a testable hypothesis to this topic.

Overall, the conclusion of the study is well supported by the elegant set of genetic experimental data and employed multiple readouts on DDC factor depletion on checkpoint integrity and cell cycle status. Although the study simply measures Rad53 phosphorylation as the primary metric to assess checkpoint status, it successfully demonstrated how the signaling is modified through the different stages and that eventually cells become recalcitrant to DDC signaling after a prolonged arrest. The results are clear, and rigorously

tested and carefully interpreted with good discussion on the possible limitations. The revision provided detailed responses to the reviewers' comments and addressed a few key concerns, one of which is universally raised by the reviewers on the full functionality of AID tagged DDC factors, by simply expressing excess Rad9-AID to restore more normal looking checkpoint response. It will be interesting if the excess expression of other DDC factors could overcome suboptimal checkpoints in cells after 24 h post damage.

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Reviewer #3 (Public review):

Summary:

The DNA damage checkpoint (DDC) inhibits the metaphase-anaphase transition to repair various types of DNA damage, including DNA double strand breaks (DSBs). One irreparable DSB can maintain the DDC for 12-15 hours in yeast, after which the cells resume the cell cycle. If there are two DSBs, the DDC is maintained for at least 24 hours. In this study, the authors take advantage of this tighter DDC to investigate whether the best-known proteins involved in establishing the DDC are also responsible for its long-term maintenance during irreparable DSBs. They do this by cleverly degrading such proteins after DSB formation. They show that most, but not all, DDC proteins maintain the cell cycle block. Interestingly, DDC proteins become dispensable after 15 hours and the block is then maintained by spindle assembly checkpoint (SAC) proteins.

Strengths:

The authors have engineered a tight yeast system to study DDC shutdown after irreparable DSBs and used it to address whether checkpoint proteins (DDC and SAC) contribute to the long-term maintenance of DSB-mediated G2/M block. The different roles of Ddc2, Chk1 and Dun1 are interesting, while the fact that SAC overtakes DDC after 15 hours is intriguing and highlights how DSBs near and far from centromeres can have a profound impact on cell adaptation to DSBs. In their revision, the authors have now improved the Rad9-AID methodology to place Rad9 in the context of DDC adaptation, as well as widening the association between adaptation and proximity to centromeres.

Weaknesses:

Some of the results they present essentially confirm their own previous findings, albeit with a tighter strain design for long-term arrest. Conclusions about the maintenance of G2/M in several mutant combinations could have been strengthened by adding simple microscopy experiments with DAPI staining. No clear mechanism for how depletion of Bub2, but not Bfa1, can relieve the G2/M (metaphase) block is given.

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Author response:

The following is the authors' response to the original reviews.

> ***Reviewer #1 (Recommendations For The Authors):***
>
> *To hopefully contribute to more strongly support the conclusions drawn by the authors, I am including a series of concerns regarding the manuscript, as well as some suggestions that could be useful to address these issues:*

> *(1) The main results of this study derive from the use of auxin-inducible degron (AID)-tagged proteins. Despite the great advantages of the AID strategy to conditionally deplete proteins, the AID tag can affect the normal function of a protein. In fact, some of the AID-labeled DDC components generated in this work are shown to be hypomorphic. Hence, the manuscript would have benefited from the additional confirmation of some of the observations using a different way to eliminate the proteins (e.g., temperature-sensitive mutants).*

Most ts mutants are also hypomorphic; hence we don't see there is much advantage to their use. The addition of the AID to these proteins alone does not interfere with the ability to sustain checkpoint arrest as demonstrated in Figure S1. Instead we found that by overexpressing Rad9-AID we could demonstrate that inactivating Rad9 after 15 h behaved the same way as the inactivation of Ddc2, significantly strengthening our finding that the DDC checkpoint becomes dispensable while the SAC takes over.

> *(2) In cells depleted of Rad53-AID, the deletion of CHK1 stimulates an earlier release from a mitotic arrest induced by two DSBs (Figures 2D and 3C). Likewise, the authors claim that a faster escape from the cell cycle block can also be observed when upstream factors such as Ddc2, Rad9, or Rad24 are depleted in the absence of CHK1 (Figures 2A-C and Figures 3D-F). However, this earlier release from the cell cycle arrest, if at all, is only slightly noticeable in a Rad9-AID background (Figures 2B and 3E). In this sense, it is also worth pointing out that Rad9-AID chk1Δ (Figure 3E) and Rad24-AID chk1Δ (Figure 3F) cells were only evaluated up to 7 h, while in all other instances, cells were followed for 9 h, which hinders a fair assessment of the differences in the release from the cell cycle arrest.*

As noted above, we have now been able to examine Rad9 over the long-time frame.

> *(3) Although only 25% of the cells depleted for Dun1 remained in G2/M arrest 7 h following the induction of two DSBs, it is shocking that Rad53 was nonetheless still phosphorylated after the cells had escaped the cell cycle blockage (Figure 4A).*

This persistence of Rad53 phosphorylation is also seen with the inactivation of Mad2, allowing escape in spite of continued Rad53 phosphorylation.

> *(4) Generation of Rad9-AID2 and Rad24-AID2 strains did not fully restore the function of these proteins, since most cells had adapted 24 h after induction of two DSBs (Figure S1C). Nonetheless, Rad9-AID2 and Rad24-AID2 are still likely more stable than their AID counterparts, and hence the authors could have instead used the AID2 proteins for the experiments in Figure 2 to better evaluate the role of Rad9 and Rad24 in the maintenance of the DDC-dependent arrest.*

We note again that we have found a way to study Rad9 up to 24 h.

> *(5) Deletion of BFA1 has been shown to promote the escape from a cell cycle arrest triggered by telomere uncapping (Wang et al. 2000, Hu et al. 2001, Valerio-Santiago et al. 2013). Likewise, while cells carrying the cdc5-T238A allele cannot adapt to a checkpoint arrest induced by one irreparable DSB, BFA1 deletion rescues the adaptation defect of this mutant CDC5 allele (Rawal et al., 2016). The authors show how, using AID-degrons of Bfa1 and Bub2, that only Bub2, but not Bfa1, is required to maintain a prolonged cell cycle arrest after the induction of two DSBs. To reinforce this point, and as shown for mad2Δ cells (Figure S6A), the authors could perform a complete time course*

> *using both the Bfa1-AID and a bfa1Δ mutant to demonstrate that they do indeed show the same behavior in terms of the adaptation to a two DSB-induced cell cycle arrest.*

We thank the reviewer for noting these other instances where bfa1D promoted an escape from arrest. We tested a 2-DSB *bfa1* deletion, data has been added to Figure S9E-F. We did not observe a difference in the percentage of cells escaping arrest between the 2-DSB *bfa1* deletion and the 2-DSB *BFA1-AID* strains.

> *(6) Bypass or adaptation of a checkpoint-induced cell cycle arrest in S. cerevisiae often leads to cells entering a new cell cycle without doing cytokinesis and, hence, to the accumulation of rebudded cells. However, the experiments shown in the manuscript only account for G1 or budded cells with either one or two nuclei. Do any of the mutants show cytokinesis problems and subsequent rebudding of the cells? If so, this should have been also noted and quantified in the corresponding assays.*

In the cases we have studied we have not seen instances where the cells re-bud without completing mitosis (at least as assessed by the formation of budded cells with two distinct DAPI staining masses). In the morphological assays we have done, we score the continuation of the cell cycle by the appearance of multiple buds, G1, and small budded cells. In our adaptation assays when cells escaped G2/M arrest they formed microcolonies indicating no short-term deficiency in cell division.

> *(7) The location of the DSB relative to the centromere of a chromosome seems to be a factor that determines the capacity of the SAC to sustain a prolonged cell cycle arrest. The authors discuss the possibility that the DSB could somehow affect the structure of the kinetochore. Did they evaluate whether Mad1 or Mad2 were more actively recruited to kinetochores in those strains that more strongly trigger the SAC after induction of the DSBs?*

We have not attempted to follow Mad1/2 recruitment. ChIP-seq could be used to monitor Mad1/2 localization at the 16 centromeres in response to DSBs and the spread of g-H2AX across the centromere. Our previous data showed that g-H2AX could spread across the centromere region and could create a change that would be detected by Mad1/2. This change does not, however, affect the mitotic behavior of a strain in which the H2A genes have been modified to the possibly phosphomimetic H2A-S129E allele.

> *(8) The authors could speculate in the discussion about the reasons that could explain why the DDC is required for the maintenance of checkpoint arrest at early stages but then becomes dispensable for the preservation of a prolonged cell DNA DSB-induced cycle arrest, which is instead sustained at later stages by the SAC.*

Our suggestion is that cells would have adapted, but modification of the centromere region engages SAC.

> *Finally, some minor issues are:*
>
> *(1) The lines in the graphs that display the results from adaptation assays (e.g., Figures 1B and 1E) or cell and nuclear morphology (e.g., Figures 1D and 1G) are too thick. This makes it sometimes difficult to distinguish the actual percentages of cells in each category, particularly in the experiments monitoring nuclear division.*

Fixed

> *(2) While both the adaptation assay and the analysis of nuclear division in Figures 1E and 1G, respectively, show a complete DDC-dependent arrest at 4h, the Western blot in Figure 1F suggests that Rad53 is not phosphorylated at that time point. Do these figures represent independent experiments? Ideally, the analysis of cell budding and nuclear division, which is performed in liquid cultures, and the Western blot displaying Rad53 phosphorylation should correspond to the same experiment.*

Cell budding in liquid cultures and adaptation assays were performed in triplicate with 3 biological replicates and the collective results are shown in each graph showing the percentage of large-budded cells. Western blot samples were collected in each liquid culture experiment. The western blot in 1G is a representative western blot.

> *(3) It is somewhat confusing that the blots for the proteins are not displayed in the same order in Figures 2A (Rad53 at the top) and 2B or 2C (Rad53 in the middle).*

Fixed. We place Rad53 – the relevant protein - at the top.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *(1) Yeast with the two breaks responds to DNA damage checkpoint (DDC) until sometimes 4-15 h post DNA damage. Since the auxin-induced degradation does not completely deplete all the tagged proteins in cells, the results should be more carefully considered and not to interpret if the checkpoint entry or maintenance depends on each target protein's ability to induce Rad53 phosphorylation. It should be theoretically possible if checkpoint maintenance requires only a modest amount of checkpoint factors especially because the experiments involve the induction of one or two DSBs. The low levels of DDC factors may be insufficient for Rad53 activation but could still be effective for cell cycle arrest. Indeed, the Haber group showed that the mating type switch did not induce Rad53 phosphorylation but still invoked detectable DNA damage response. To test such possibilities, the authors might consider employing yet another marker for DDC such as H2A or Chk1 phosphorylation besides Rad53 autophosphorylation. Alternatively, the authors might check if auxin-induced depletion also disrupts break-induced foci formation for checkpoint maintenance or their enrichment at DNA breaks using ChIP assays at various points post-damage.*

DAPI staining of Ddc2-AID cells show that when IAA is added 4 h after DSB induction (Figure S3A), cells escape G2/M arrest as evidenced by the increase in large-budded cells with 2 DAPI signals, small budded cells, and G1 cells. Overexpression of Ddc2 can sustain the checkpoint past 24 h, but without SAC proteins like Mad2 they will eventually adapt (Figure S6B).

That Rad9-AID or Rad24-AID in the absence of added auxin (but in the presence of TIR1) is unable to sustain arrest suggests to us that low levels of Rad9 or Rad24 are not sufficient to maintain arrest. As the reviewer notes, normal MAT switching doesn't cause Rad53 phosphorylation or arrest, though early damage-induced events such as H2A phosphorylation do occur. But our point is that Rad9 or Ddc2 is needed to maintain arrest only up to a certain point, after which they become superfluous and a different checkpoint arrest is imposed. At that point apparently a low level of these proteins plays no obvious role.

> *(2) It is interesting that DDC no longer responds to the damage signaling after 15 h of DSB-induced prolonged checkpoint arrest after two DNA double-strand breaks. Is this also applicable to other adaptation mutants? The results might improve the broad impact of the current conclusions. It is also possible that the transition from DDC to SPC depends on simply the changes in signaling or in part due to the molecular changes in the status of DNA breaks or its flanking regions. Indeed, the proposed model suggests*

> *that the spreading of H2A phosphorylation to centromeric regions induces SAC and thus mitotic arrest. The authors could measure H2A phosphorylation near the centromere using ChIP assays at various intervals post-DNA damage. It is particularly interesting if depletion of Ddc2 at 15 h post DNA damage does not alter the level of H2A phosphorylation at or near centromere.*

Our previous data have suggested that the involvement of the SAC in prolonging DSB-induced arrest involved post-translational modification of centromeric chromatin such as the Mec1- and Tel1-dependent phosphorylation of the histone H2A (Dotiwala). In budding yeast there is also a similar DSB-induced modification of histone H2B (Lee et al.). To ask if there is an intrinsic activation of the SAC if the regions around centromeres were modified by checkpoint kinase phosphorylation, we examined cell cycle progression in strains in which histone H2A or histone H2B was mutated to their putative phosphomimetic forms (H2A-S129E and H2B-T129E). As shown in Figure S11, there was no effect on the growth rate of these strains, or of the double mutant, suggesting that cells did not experience a delay in entering mitosis because of these modifications. We note that although histone H2A-S129E is recognized by an antibody specific for the phosphorylation of histone H2A-S129, the mutation to S129E may not be fully phosphomimetic.

> *(3) It is puzzling why Rad9-AID or Rad24-AID are proficient for DDC establishment but cannot sustain permanent arrest in the two break cells. It appears Rad53 phosphorylation for DDC is weaker in cells expressing Rad9-AID or Rad24-AID according to Fig.2B and C even though their protein level before IAA treatment is still robust. This might also explain why the results of depleting Rad53 and Rad9 are very different. It also raises concern if the effect of Rad24 depletion on checkpoint maintenance is in part due to the weaker checkpoint establishment. It might be necessary to use the AID2 system to redo Rad24 depletion to exclude such a possibility.*

We believe that the AID mutants are very sensitive to the low level of IAA present in yeast. The instability of the protein is entirely dependent on the TIR1 SCF factor, so the proteins themselves are not intrinsically defective; they are just subject to degradation. Overexpressing Rad9 allowed us to evaluate its role at late time points.

> *(4) It is intriguing that the switch from DDC to SAC might take place at around 12 h when yeasts with a single unrepairable break ignore DDC and resume cell cycling (so-called "adaptation"). Since 4h and 15h are far apart and the transition point from DDC to SAC likely takes place between these two points, it will be very helpful to analyze and compare cell cycle exit after 24 h by treating IAA at multiple points between 4-15h.*

When we add IAA to Mad2-AID and Mad1-AID 4 h after DSB induction, cells remain arrested for up to 12 h after DSB induction. At 15 h cells begin to exit checkpoint arrest indicating that the handoff of checkpoint arrest must occur between 12 to 15 h after DSB induction. If we degraded DNA damage checkpoint proteins at any point before Mad2, Mad1, and Bub2 begin to contribute to checkpoint arrest, then arrested cells will likely adapt in a similar manner to when IAA was added 4 h after DSB induction.

> *(5) Some of the Western blot quality is poor. For instance, in Figure 6C, Mad1-AID level after IAA addition is not compelling especially because the TIR level (the loading control) is also very low.*

In Figure 6C, while the relative levels of TIR1 are similar in the IAA treated and untreated samples, there is no detectable amount of Mad1-AID in the IAA treated samples indicating that Mad1-AID was successful degraded with the AID system.

> *(6) Fig. 8 is complex. It might be helpful to define the different types of arrows in the figure. The legend also has a spelling error, Rad23 should be Rad24.*

We've defined what each arrow means in the legend and corrected the spelling error in the figure legend.

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *Major concerns:*
>
> *Much of the manuscript states that two unrepairable DSBs lead to a long and severe G2/M arrest. Two main cytological approaches are used to make this statement: bud size and number on plates after micromanipulation (microcolony assay), and cell and nuclear morphology in liquid cultures. While the latter gives a clear pattern that can be assigned to a G2/M block as expected by DDC, i.e. metaphase-like mononucleated cells with large buds, the former can only tell whether cells eventually reach a second S phase (large budded cells on the plate can be in a proper G2/M arrest, but can also be in an anaphase block or even in the ensuing G1). The authors always performed the microcolony assay, but there are several cases where the much more informative budding/DAPI assay is missing. These include Dun1-aid and others, but more importantly chk1D and its combinations with DDC proteins. Incidentally, for the microcolony assay, it is more accurate to label the y-axis of the corresponding graphs (and in the figure legends and main text) with something like "large budded cells"; "G2/M arrested cells" is misleading.*

Figures have been updated to more accurately reflect what we are measuring.

> *The results obtained with the Bfa1/Bub2 partner are intriguing. These two proteins form a complex whose canonical function is to prevent exit from mitosis until the spindle is properly aligned, acting in a distinct subpathway within the SAC that blocks MEN rather than anaphase onset. The data presented by the authors suggest that, on the one hand, both SAC subpathways work together to block the cell cycle. However, why does canonical SAC (Mad1/Mad2) inactivation not lead to a transition from G2/M (metaphase-like) arrested cells to anaphase-like arrest maintained by Bfa1-Bub2? Since Bfa1-Bub2 is a target of DDC, is it possible that DDC knockdown also inactivates this checkpoint, allowing adaptation? On the other hand, can the authors provide more data to confirm and strengthen their claim of a Bfa1-independent Bub2 role in prolonged arrest? Perhaps long-term protein localization and PTM changes. Bub2-independent roles for Bfa1 have been reported, but not vice versa, to the best of my knowledge.*

In the mitotic exit network Bfa1/Bub2 prime activation of the pathway by bringing Tem1 to spindle pole bodies. Phosphorylation of Bfa1 causes Tem1 to be released and phosphorylate Cdc5 to trigger exit by MEN. It has been shown that DNA damage, in a *cdc13-1* ts mutant, phosphorylates Bfa1 in a Rad53 and Dun1 dependent manner. This phosphorylation of Bfa1 could release Tem1 and prime cells to exit checkpoint arrest when cells pass through anaphase. Looking at Tem1 localization to spindle pole bodies and interactions with Bfa1/Bub2 in response to DNA damage might give insight into why cells don't experience an anaphase-like arrest when they are released by either deactivation of the DNA damage checkpoint or SAC.

We have previously shown that a deletion of *bub2* in a 1-DSB background shortens DSB-induced checkpoint arrest. Deletion of *bfa1* in a 2-DSB background showed ~80-70% of cells stuck in a large-budded state as measured through an adaptation assay tracking the morphology of G1 cells on a YP-Gal plate and DAPI staining. Deletion or degradation of *bfa1* might not release cells from arrest because the Mad2/Mad1 prevent cells from transitioning

into anaphase. Our DAPI data for Bub2-AID shows an increase in cells with 2 DAPI signals (transition into anaphase) and small budded cells indicating that degradation of Bub2 is releasing cells into anaphase and allowing cells to complete mitosis.

> *Further suggestions:*
>
> *It would be richer if authors could provide more than one experimental replicate in some panels (e.g., S1A,B; S4A; and S6B).*

S1C confirms that Rad9-AID and Rad24-AID will adapt by 24 h even with the point mutant TIR1(F74G) which has lower basal degradation than TIR1. S4A has been updated with additional experimental replicates. The 48 h timepoint after DSB induction was to show the importance of Mad2 even when Ddc2 is overexpressed.

> *Figure 1: Rearrange figure panels when they are first mentioned in the text. For example, it makes more sense to have the plate adaptation assay as panel B for both 1-DSB and 2-DSB strains, budding plus DAPI as panel C, and Rad53 as panel D.*

These figures have been rearranged in the order that they are mentioned in the paper.

> *Figure 5: Correct Ph-5-IAA in the Rad53 WBs (it should be 5-Ph-IAA).*

This has been corrected.

> *Figure S2: The straight line under the "+IAA" text box is misleading. I think it should also cover the "-2" time point, right? Also, check the figure legend. Information is missing and does not correspond to the figure layout.*

This has been corrected.

> *Figure S3: Perhaps "Cell cycle profile as determined by budding and DAPI staining" is a better and more accurate legend title.*

The legend title has been updated to “Cell cycle profile as determined by budding and DAPI staining in Ddc2-AID and Rad53-AID mutants ± IAA 4 h after galactose.”

> *Figure S5: Detection of both Rad53 and Ddc2 in the same blot could lead to misinterpretation as hyperphosphorylated Rad53 appears to coincide with Ddc2 migration.*

Figure S5A-B are representative western blots where Rad53 was probed to show activation of the DNA damage checkpoint by Rad53 phosphorylation. When measuring the relative abundance of Ddc2 we did not probe all blots for Rad53.

> *Table S1: Include the post-hoc test used for comparisons after ANOVA.*

A Sidak post-hoc test was used in PRISM for the one-way ANOVA test. PRISM listed the Sidak post-hoc test as the recommended test to correct for multiple comparisons. A column has been added to S. Table 1 to show which post-hoc test was used.

> *Page 10, line 4: The putative additive effect of chk1 knockout with Dun1 depletion should also be compared to chk1 alone (in Figure 3A).*

We address the additive effect of *chk1* knockout with Dun1-AID depletion in a later section on Page 11, line 6. Since we had not explored possible effects from downstream targets of Rad53 for prolonging checkpoint arrest when Rad53 was depleted, we did not mention the effect of the *chk1* knockout on Dun1 depletion.

> *Page 14, second paragraph, line 4: "Figure 6A-D", is it not?*

Figure S6A is measuring checkpoint arrest in a deletion of *mad2* in a 2-DSB strain. Figure 6A-D shows how degradation of Mad2-AID and Mad1-AID after the handoff of arrest causes cells to exit the checkpoint in a Rad53 independent manner.

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