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The Rad4<sup>TopBP1</sup> ATR-Activation Domain Functions in G1/S Phase in a Chromatin-Dependent Manner

Su-Jiun Lin¹,³, Christopher P. Wardlaw¹,³, Takashi Morishita¹, Izumi Miyabe¹, Charly Chahwan², Thomas Caspari³, Ulrike Schmidt³, Antony M. Carr¹*, Valerie Garcia¹

¹ Genome Damage and Stability Centre, University of Sussex, Brighton, Sussex, United Kingdom, ² Department of Molecular Genetics, University of Toronto, Toronto, Ontario, Canada, ³ School of Biological Sciences, Bangor University, Bangor, Gwynedd, United Kingdom

Abstract

DNA damage checkpoint activation can be subdivided into two steps: initial activation and signal amplification. The events distinguishing these two phases and their genetic determinants remain obscure. TopBP1, a mediator protein containing multiple BRCT domains, binds to and activates the ATR/ATRIP complex through its ATR-Activation Domain (AAD). We show that *Schizosaccharomyces pombe* Rad4<sup>TopBP1</sup> AAD–defective strains are DNA damage sensitive during G1/S-phase, but not during G2. Using lacO-LacI tethering, we developed a DNA damage–independent assay for checkpoint activation that is Rad4<sup>TopBP1</sup> AAD–dependent. In this assay, checkpoint activation requires histone H2A phosphorylation, the interaction between TopBP1 and the 9-1-1 complex, and is mediated by the phospho-binding activity of Crb2<sup>STBP1</sup>. Consistent with a model where Rad4<sup>TopBP1</sup> AAD–dependent checkpoint activation is ssDNA/RPA–independent and functions to amplify otherwise weak checkpoint signals, we demonstrate that the Rad4<sup>TopBP1</sup> AAD is important for Chk1 phosphorylation when resection is limited in G2 by ablation of the resecting nuclease, Exo1. We also show that the Rad4<sup>TopBP1</sup> AAD acts additively with a Rad9 AAD in G1/S phase but not G2. This manifests that AAD–dependent Rad3<sup>ATR</sup> checkpoint amplification is particularly important when DNA resection is limiting. In *S. pombe*, this manifests in G1/S phase and relies on protein–chromatin interactions.


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* E-mail: a.m.carr@sussex.ac.uk

These authors contributed equally to this work.

Introduction

The DNA damage checkpoint is an elaborate signal transduction pathway that monitors the integrity of the DNA, prevents cell cycle progression and promotes appropriate DNA metabolism [1] reviewed in [2]. The DNA damage sensors associated with checkpoint activation define two separate DNA structure-dependent signal transduction cascades. Each pathway engages a phospho-inositol-3 kinase-like protein kinase (PIKK); either the Ataxia Telangiectasia and Rad3 related (ATR) kinase [3]. ATM detects DNA damage–dependent DNA processing [4] or following the replication fork [5]. Once ssDNA needs to be formed — for example by DNA repair-dependent DNA processing [4] or following the replication machinery encountering the unrepaired lesion [5]. Once ssDNA is generated, it is immediately coated by replication protein A (RPA) (Reviewed in: [6]). Multiple ATR molecules are initially recruited to ssDNA regions via ATRs obligate binding partner, ATRIP, which binds directly to RPA [7,8]. ATR-ATRIP recruitment to ssDNA-RPA is necessary for “basal” ATR activation, but is insufficient for full checkpoint activation: co-recruitment of a second complex consisting of three PCNA-like proteins, Rad9, Hus1 and Rad1 (known as the 9-1-1 clamp) is also necessary. 9-1-1 is loaded in parallel to ATR recruitment at 5′ ssDNA/dsDNA junctions by the checkpoint clamp loader Rad17-RFC [2–5] [9,10,11]. (Figure 1A).

When ATR-ATRIP is first loaded at the site of ssDNA, its “basal” kinase activity promotes phosphorylation of its immediate neighbours, including ATRIP [12,13], an in trans phosphorylation of a residue within ATR itself, T1989 [14], and the subunits of the 9-1-1 clamp [13,16]. Dependent on the concomitant recruitment of 9-1-1, a further protein, TopBP1, is recruited [17]. TopBP1 is recruited via an interaction between its BRCT (1–4) domains and a constitutive phosphorylation on the C-terminus of Rad9 [18,19]. Similarly in both yeast systems, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, the TopBP1 homologs, Dpb11 and Rad4 respectively, are recruited by the phosphorylation of the C-terminus of Rad9<sup>Ntb1</sup> creating a binding site for a pair of BRCT domains (Figure 1A). In *S. pombe*, the C-terminal phosphorylations occur on Rad9 at residues T412 and S423 [20]. This subsequently recruits Rad4<sup>TopBP1</sup> via interaction with BRCT pair (3+4). However, unlike in mammalian cells, T412 and S423 in *S. pombe* are directly targeted by Rad3<sup>ATR</sup> in response to its ssDNA/RPA.
Author Summary

DNA structure-dependent checkpoint activation and the amplification of checkpoint signals are carefully modulated and allow the checkpoint kinases to delay mitosis and regulate DNA metabolism. While much work has gone into understanding how this checkpoint functions, the mechanism by which the checkpoint signal is amplified is less clear. We have characterised a conserved domain in the Schizosaccharomyces pombe TopBP1 homolog, Rad4

We have previously shown that S. pombe Rad4

binding and concomitant 9-1-1 loading [16,20,21]. Despite these differences, in both S. pombe [20] and mammalian cells [14], Rad4 recruitment promotes the formation of a Rad3/9-1-1/Rad4 complex (Figure 1A). However, the mode of interaction of Rad3, and Rad4 within this complex has not been defined in S. pombe.

Mammalian TopBP1 can directly activate ATR-ATRIP both in vitro in the absence of ssDNA/RPA and when over-expressed in cells. TopBP1-dependent ATR activation requires an ATR activation domain (AAD) situated between the 6th and 7th BRCT domains [17] and mutation of a conserved aromatic residue, W700 and Y735 were [22,23,24,25]. Interestingly, in the terminal tail of Ddc1, an AAD that can directly stimulate Mec1 activation domain (AAD) situated between the 6th and 7th th BRCT domains [17] and mutation of a conserved aromatic residue, W700 and Y735 [22,23,24,25].

In S. cerevisiae, two aromatic residues, W700 and Y735 were identified as critical for Dpb1 [28–648] AAD activity [24,25]. We similarly created an alignment of Rad4 with the C-terminal tails of a variety of TopBP1 homologs and identified a short sequence encompassing Y599 in S. pombe (Figure 1B, 1C), which we subsequently confirmed as defining the AAD activity of Rad4 [28–648] (see below). To characterise the function of the Rad4 AAD, we separately mutated the conserved aromatic amino acid to create strain rad4-Y599R and deleted the minimal conserved motif to create rad4Δ(535–601).

To establish if the Rad4 AAD interacts with Rad3 we expressed and purified recombinant Rad4 (288–648) (that includes BRCT (34)) and the unstructured C-terminal tail, as a fusion with GST. Constructs containing a mutation in either the AAD (Y599R), deletion of residues 643–645 encompassing a putative cyclin binding motif (RxL), or mutation of a CDK phosphorylation site (S641A) were similarly purified. Wild-type and all the mutated recombinant proteins, when incubated with yeast extracts, bound to and co-purified the control interacting partner of Rad4, Cdc13 (unpublished data). Wild-type Rad4-GST also co-purified Rad3-Y599R from extracts prepared from 3myc-rad3 cells (Figure 1D). In contrast, the amount of Rad3-Myc pulled-down with recombinant AAD-mutated Rad4-GST was reproducibly reduced (n = 4). Neither Rad3, nor Cdc13-S155/A were pulled-down with a GST-only control. We conclude that, in vitro, this residue of Rad4-GST is part of a Rad4 interaction domain for Rad3, consistent with it having the properties of an AAD. We also note that the interaction with Cdc13-K526 is not abolished by loss of the RxL motif and that mutation of S641 affects the Rad4 interaction in vitro (Figure 1D, lane 4).

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Function of *S. pombe* TopBP1-AAD
rad4-Y599R mutants show checkpoint defects associated with S phase

Both rad4-Y599R and rad4-A[595–601] displayed normal cell cycle progression (data not shown), indicating that, as is observed in *S. cerevisiae* [24], the Rad4ATR AAD is not required for unperturbed DNA replication. In response to UV, MMS and HU treatment, rad4-Y599R and rad4-A[595–601] showed intermediate sensitivity when compared to rad4+ and checkpoint defective rad3Δ (Figure 1E and Figure S1A). To establish if the sensitivity to DNA damage correlated with a defective G2 DNA damage checkpoint, we monitored cell cycle progression after cells were synchronised in G2 and UV-irradiated. Following exposure to 50 Jm−2 (Figure 2A), rad4-Y599R cells displayed premature release from cell cycle arrest (~20 min earlier than rad4+ after 50 Jm−2). We next monitored the sensitivity and checkpoint response to ionising radiation. rad4-Y599R mutant cells displayed only very mild sensitivity to IR (Figure 2B) and the checkpoint was mildly extended (Figure 2C). We do not know the reason for the slight extension of the G2 delay: no obvious increase in numbers or duration of Rad22Rad32 foci were observed, indicating no significant delay to DSB repair (Figure S1B).

We next examined Chk1 phosphorylation status in rad4+ and rad4-Y599R cells as a surrogate for checkpoint activation (Figure 2D). In response to 200 Jm−2 UV irradiation, asynchronously growing rad4-Y599R cells displayed reduced Chk1 phosphorylation when compared to rad4+ cells, consistent with the partial checkpoint defect observed. Conversely, in response to IR, no significant difference is seen between rad4-Y599R and rad4+. An upstream target of Rad3ATR is the C-terminus of histone H2A [31,32]. To establish if the UV-specific defect in Rad3ATR-dependent phosphorylation is specific to Chk1, we monitored γH2A formation following either UV or IR treatment (Figure 2E). As was seen for Chk1 phosphorylation, a significant decrease in γH2A is observed in rad4-Y599R cells following UV but not IR treatment when compared to rad4+ cells.

The pattern of DNA damage sensitivity seen for rad4-Y599R cells is consistent with a specific sensitivity within S phase. >70% of fission yeast cells in an asynchronous culture are in G2 and mitosis is followed rapidly by S phase: G1 is extremely short. In response to IR, the G2 DNA damage checkpoint is robustly activated and DNA repair completed before cells pass through mitosis and into S phase [33]. Thus, following IR, relatively few cells replicate damaged DNA. Conversely, the G2 checkpoint is not robustly activated following UV [34] and the majority of UV-irradiated cells pass through mitosis and enter S phase with damaged DNA. To monitor S phase-specific events, we thus examined γH2A induction in cells treated with either hydroxyurea (HU), an inhibitor of ribonucleotide reductase, or Camptothecin (CPT), an inhibitor of topoisomerase I (Figure 2F). Consistent with both agents manifesting cytotoxicity in S phase, γH2A levels were significantly reduced when comparing rad4-Y599R with rad4+ cells. Finally, since replication of UV damaged DNA induces Cds1Chk2 activity [35], we monitored the kinase activity of immuno-precipitated Cds1Chk2 following UV irradiation of rad4-Y599R and rad4+ cells (Figure 2G). Cds1Chk2 activation was reproducibly lower for rad4-Y599R, indicating an impaired S phase checkpoint activation (n = 3).

The rad4-Y599R mutants are sensitive to DNA damage in S-phase

If the rad4-Y599R mutant is deficient in activation of Rad3ATR in S phase, we would anticipate increased sensitivity to IR within S phase when compared to rad4+ cells. To test this possibility, rad4-Y599R mutant and rad4+ cells where either synchronised in G2 using cdc25-22 or in G1 using a cdc10-m17. Following the block, cells were released by reducing the temperature and cell cycle progression was monitored by FACS analysis (Figure 3A, 3B). Cells were irradiated with 50 Gy IR at the times indicated. rad4-Y599R cells showed significant increased sensitivity when compared to rad4+ when irradiated in S phase, but not when irradiated in G2, when S phase is complete (i.e. see Figure 3B). In an equivalent cdc25-22 block and release experiment, we monitored Chk1 phosphorylation and γH2A induction (Figure 3C). Unlike when asynchronous rad4-Y599R cells are irradiated (>70% of such cells are in G2), when rad4-Y599R cells were irradiated in early-mid S phase, Chk1 phosphorylation was moderately reduced for the first 40 minutes after irradiation and γH2A levels were similarly decreased when compared to rad4+ . Interestingly, following progression through S phase and into G2 (150 minute time point), Chk1 phosphorylation levels increased significantly in rad4-Y599R cells, although the same was not seen for γH2A levels.

To determine that the use of cdc25-22 synchronisation was not generating an artefact (Cdc25 is an activator of Cdc2-Cyclin B, which itself is required for normal DNA damage responses in G2 [36]), we used the alternative method of synchronisation where cells were arrested in G1 using cdc10-m17 and released directly into S phase (Figure 3D). Unlike IR treatment of asynchronous cultures where equivalent levels of γH2A were observed (Asyn IR), treatment of rad4-Y599R cells at 30, 60 or 90 minutes after release from arrest resulted in decreased γH2A levels and Chk1 phosphorylation when compared to rad4+ control cells.

LacO array-dependent checkpoint activation in *S. pombe* requires the Rad44TopBP1 AAD

In *S. cerevisiae*, co-localisation of two or more checkpoint proteins to arrays of lacO repeats bypasses the requirement for DNA damage in Mec1-mediated checkpoint activation [37]. To establish the role of the Rad44TopBP1 AAD in a what has previously been characterised as an RPA-ssDNA independent system, rad44TopBP1, rad9 and rad3ATR were each fused to a construct encoding GFP, the *E. coli* lac-repressor (LacI) and a nuclear localization signal (NLS); GFP/LN (Figure 4A). The resulting plasmids express the fusion construct under the control of a thiamine-repressible (mmtH4I) promoter. We established that each of the fusion constructs were functional by expressing them.
individually in the corresponding null mutants. Each was able to suppress the DNA damage sensitivity (and for rad4 TopBP1, the thermosensitivity) of the appropriate mutant, although for rad4- GFP/LN genotoxin resistance was not restored to wild-type levels (Figure S1C–S1E). When expressed in cells harbouring 256 repeats of the lac operator sequence (lacO) integrated at the ura4+ locus, each fusion protein formed a single nuclear focus. No foci were detected in cells devoid of lacO arrays (Figure 4B).

We used Chk1 phosphorylation as a readout for DNA damage checkpoint activation (Figure 4C). Following thiamine removal (induction takes between 12 and 16 hours [38]), Chk1 became phosphorylated in lacO containing cells, but not in lacO-negative control cells, when either Rad3XTR, Rad4TopBP1 or Rad9 LacI fusion proteins were expressed. Similar results were obtained when each pair-wise combinations of two fusion proteins were expressed (Figure 5B). In S. pombe, DNA damage checkpoint activation results in cell cycle arrest and cell elongation. Elongated cells were observed upon expression of single fusion proteins (data not shown), confirming checkpoint activation. From these data we conclude that, in S. pombe, DNA damage checkpoint activation results in cell cycle arrest and cell elongation. Elongated cells were observed upon expression of single fusion proteins (data not shown), confirming checkpoint activation. From these data we conclude that, in S. pombe, forced co-localisation of two checkpoint proteins is not required [37].
To establish if the Rad4TopBP1 AAD is involved in this damage-independent mode of checkpoint activation, we tested if Rad3 tethering could result in Chk1 phosphorylation in a rad4-Y599R mutant background (Figure 5A). While Rad3-GFP/LN expression resulted in induced Chk1 phosphorylation in rad4+ cells, Rad3-GFP/LN expression did not increase Chk1 phosphorylation in rad4-Y599R cells, demonstrating a role for the Rad4 TopBP1 AAD. Next we established if expression and tethering of the AAD-
was observed. Furthermore, while co-expression and tethering the checkpoint (Figure 5B). No induction of Chk1 phosphorylation visible foci (t = 24 hours).

lacO constructs were expressed in either a strain containing 256 integrated defective Rad4-Y599R protein to activate the checkpoint. It also supports the idea that the Rad4+TopBP1 AAD domain is required for the activation of Rad3TR and not simply recruiting it.

H2A phosphorylation is required for Rad4TopBP1 AAD-dependent checkpoint activation

While Rad3TR kinase activity is essential for Chk1 phosphorylation in response to DNA damage [40], it also depends on the 9-1-1 clamp, the Rad17 clamp loader and the Crb2/TopBP1 mediator. To characterise the dependencies for lacO-dependent checkpoint activation we examined which checkpoint genes were required for Chk1 phosphorylation during Rad3TR tethering (Figure 5C). Rad3TR+GFP/LN was expressed in lacO-positive strains deleted for rad1, rad9 (encoding 9-1-1 components), rad17 (clamp loader) and crb2. Each was required for Chk1 phosphorylation. Thus, Rad3TR tethering is not sufficient for checkpoint activation: the clamp loader, the 9-1-1 clamp complex and the Crb2 mediator are all required and this artificial checkpoint activation system does not bypass the usual requirements. However, Brc1, the proposed MDC1/PTIP ortholog is not required for Chk1 phosphorylation in this system (Figure S1F).

In both S. pombe and S. cerevisiae recruitment of the 53BP1 ortholog (Crb2 and Rad9 respectively) to chromatin in response to IR requires prior phosphorylation of histone H2A [32,41,42]. In addition to H2A phosphorylation, recruitment also requires the largely constitutive methylation of a further histone residue, H3K79 in S. cerevisiae or H4K20 in S. pombe. These modifications are effected by distinct methyltransferases in the two yeasts: Dot1 methylates H3K79 in S. cerevisiae [43] while Set9 methylates H3K20 in S. pombe [44,45]. In S. pombe it has been demonstrated that the C-terminal BRCT domains of Crb2TopBP1 and Rad9TopBP1 bind directly to H2A [42] while the Tudor domain binds directly to di-methylated H3K20 [46]. Both are required for Crb2TopBP1 chromatin association and show an epistatic relationship [45].

Using our Chk1 phosphorylation assay in response to Rad3TR tethering, we tested two strains harbouring charge reversal mutations of residues within the phospho-acceptor site of the C-terminal Crb2TopBP1 BRCT domains, crb2-K617E and crb2-K619E (Figure 5C) that disrupt the interaction with γH2A [42]. Chk1 phosphorylation was reduced in both mutants. We next established if checkpoint activation by Rad3TR tethering was affected in cells containing mutants in the two H2A genes that replace the phosphorylated residue with alanine, hta1-S129A hta2-S128A [32]. Chk1 phosphorylation was not observed in this background (Figure 5D), indicating that the Rad3TR tethering-dependent and Rad4TopBP1 AAD-dependent checkpoint activation acts in the context of chromatin modification.

Rad4TopBP1 tethering bypasses the requirement for Rad9 C-terminal phosphorylation

Upon activation of the DNA damage checkpoint in S. pombe, Rad3TRphosphorylates the Rad9 C-terminus on T412 and this is required to recruit Rad4TopBP1 [20]. Recruitment of Rad4TopBP1 allows subsequent recruitment of Crb2TopBP1 and consequent Chk1 activation [29]. A similar requirement for TopBP1 recruitment via Rad9 C-terminal phosphorylation is also evident in S. cerevisiae and
mammalian cells [19,47,48]. As expected, expression of Rad3\(^{ATR}\)-LacI in cells harbouring a rad9-T412A mutation did not result in Chk1 phosphorylation (Figure 5E) implying that lacO-recruited Rad3\(^{ATR}\) must phosphorylate endogenous Rad9 to promote Rad4\(^{ATR}\)-dependent recruitment to activate the checkpoint.

We reasoned that the requirement for Rad9-T412 phosphorylation during activation by Rad3\(^{ATR}\) tethering may solely be to bring the Rad4\(^{TopBP1}\)-AAD into proximity of Rad3\(^{ATR}\). In this case, we should be able to bypass the requirement for Rad9-T412 phosphorylation specifically for checkpoint activation by Rad3\(^{ATR}\) tethering by recruiting both Rad3\(^{ATR}\) and Rad4\(^{TopBP1}\)-LacI at the same time. Indeed, Chk1 phosphorylation was restored when we co-expressed Rad3\(^{ATR}\)-LacI and Rad4\(^{TopBP1}\)-LacI in a rad9-T412A mutant background (Figure 5E). Since checkpoint activation by co-expression of Rad3\(^{ATR}\)-LacI and Rad4\(^{TopBP1}\)-LacI remains lacO dependent (Figure S1G), these data suggest that Rad4\(^{TopBP1}\)-AAD can activate the Rad3\(^{ATR}\)-dependent checkpoint cascade in the absence of the recruitment activity of the Rad9 C-terminal tail.

The Rad4\(^{TopBP1}\) AAD is important when resection is limiting

We have shown that the Rad4\(^{TopBP1}\) AAD functions to protect cells from insult during S phase, but is not required for G2 checkpoint activation after IR. Further, we demonstrated that when rad4-Y599R (AAD-defective) mutant cells were synchronised in S phase, phosphorylation of both Chk1 and H2A in response to IR treatment was reduced when compared to rad4\(^{+}\) cells. The increase in Cdc2-Cdc13\(^{Ext} \text{Cdc13}\) activity as cells progress from S phase into G2 [49] is known to establish conditions conducive to HR by regulating factors required for DNA resection [36,50,51]. A consequence of this is that, in response to IR treatment but not in response to UV treatment, ssDNA RPA is predicted to be more prevalent in G2 cells when compared to G1/S phase cells. We thus predicted that reducing resection rates associated with IR treatment in G2 cells would create a dependency for full Chk1 phosphorylation on the Rad4\(^{TopBP1}\)-AAD and thus that Chk1 phosphorylation would be reduced in rad4-Y599R strains compared to rad4\(^{+}\) strains in response to an equal dose of IR.

To test this prediction we examined the induction of Chk1 phosphorylation in response to 100 Gy IR in exo1\(^{+}\) rad4\(^{+}\) and exo1\(^{+}\) rad4-Y599R strains (Figure 6). First we established that, when exo1 was deleted, Rad11\(^{RPA}\) foci were reduced in number, consistent with the expectation that resection is decreased in this background (Figure 6A). In contrast to rad4\(^{+}\) cells, where exo1 deletion did not reduce Chk1 phosphorylation levels, Chk1 phosphorylation was reduced to approximately 50\% when exo1 was deleted in AAD-defective cells. Previous work in both budding and fission yeasts has indicated that, in the absence of resection, Chk1 phosphorylation can occur through an alternative double strand break end-dependent Tel1\(^{ATM}\) pathway, as opposed to the canonical resection and ssDNA/RPA-dependent Rad3\(^{ATR}\) pathway [32]. Such a response could potentially mask some aspect of defects seen in the exo1\(^{+}\) background. Thus, we first confirmed that loss of tel1 alone does not influence Chk1 phosphorylation in our assay (Figure S1H) and then concomitantly deleted Tel1\(^{ATM}\) in both rad4\(^{+}\) and rad4-Y599R strains (Figure 6B, 6C). In the tel1\(^{−}\) exo1\(^{+}\) background, Chk1 phosphorylation was decreased by approximately 50\% for both rad4\(^{+}\) and rad4-Y599R when compared to the exo1\(^{+}\) alone background. These data are consistent with a general increase in Tel1-dependent checkpoint signalling when
resection is reduced by exo1 deletion. In the background of the rad4-Y599R mutation, this is superimposed on a decrease in Rad3 ATR-dependent signalling caused by the reduced resection.

The Rad4TopBP1 and Rad9 AADs co-operate in G1/S

A second ATR activation domain has recently been identified in the S. cerevisiae Ddc1Rad9 C-terminal tail [26]. Mutations in this domain define a function in Mec1ATR activation during G1, complementary to the function of the Dpb11TopBP1 AAD in promoting robust checkpoint activation in G2 in this organism [24]. Sequence alignments show that the two key Ddc1Rad9 AAD aromatic residues are conserved in S. pombe as Y271 (equating to W352Sc within the PCNA-like domain) and W348 (equating to W544Sc in the intrinsically disordered C-terminal tail) ([26] and Figure 7A, 7B). We thus created a rad9-AAD mutant by mutating both aromatic residues to alanine.

Figure 6. Chk1 phosphorylation is reduced in rad4-Y599R mutant cells when exo1 is deleted. A. exo1+ (WT) and exo1 deleted cells harbouring a rad11GFP allele were assayed for the number of Rad11GFP foci either with no treatment or after 100 Gy ionising radiation. B. Chk1 phosphorylation (*) was monitored using α-HA following 100 Gy IR in the indicated strains (immediately after irradiation). C. The phosphorylated (reduced mobility) bands were quantified relative to unphosphorylated Chk1 for each strain indicated. Error bars are the standard deviation from the mean (n = 3). doi:10.1371/journal.pgen.1002801.g006
Analysis of the resulting rad9-AAD strain demonstrates no clear sensitivity to DNA damaging agents that create problems during S phase, including CPT, MMS and UV (Figure 7C) or in G2 to IR. However, some increased sensitivity is evident to CPT and MMS when the Rad4 TopBP1 AAD mutant is present in the same strain.

We next assayed the ability of rad9-AAD mutants to activate Chk1 in response to either IR or UV treatment. Consistent with the lack of sensitivity, the level of Chk1 phosphorylation after IR was not reduced (Figure 7D), either in rad9-AAD mutant alone or in the rad9-AAD rad4-Y599R double mutant when compared to the rad4-Y599R single. In response to UV treatment, there was again no decrease observed for the single rad9-AAD mutant, but a further and reproducible decrease was seen for the rad9-AAD rad4-Y599R double mutant when compared to rad4-Y599R alone (Figure 7E).

Thus, the putative Rad9-AAD domain in S. pombe plays, at most, only a minor role in activating Rad3 ATR in response to DNA damage and this is only revealed in the absence of the Rad4 TopBP1 AAD.

Discussion

Understanding the mechanism of ATR activation is an important facet of gaining insight into how cells respond to unwanted DNA structures, itself a key aspect in maintaining genomic integrity. TopBP1 was initially implicated in the ATR-dependent checkpoint in fission yeast and later this was extended to higher eukaryotes [53]. TopBP1 is a multi-BRCT-domain containing protein that acts to scaffold proteins during both the...
Figure 8. Updated model for the checkpoint response in *S. pombe*. Rad3\(^{ATR}\) is initially activated via an ssDNA pathway which is independent of the Rad4\(^{TopBP1}\) AAD (right side). However, a secondary pathway of Rad3\(^{ATR}\) activation, which is dependent on \(\gamma\)H2A, its interaction with Crb2\(^{33BP1}\) and requires the Rad4\(^{TopBP1}\) AAD domain is important to amplify levels of activated Rad3\(^{ATR}\) and thus Chk1(left side). This chromatin Rad4-AAD pathway is of particular importance when the levels of ssDNA are low.

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We next used a genetic system to separate Rad3\(^{ATR}\) activation from the production of DNA damage and therefore ssDNA, thus allowing us to assess the pathway of Rad3\(^{ATR}\) activation dependent on the Rad4\(^{TopBP1}\) AAD. In this system, specific checkpoint proteins are recruited to a defined chromatin locus through dsDNA:protein binding [37,39]. Interestingly, recruitment of any one of the three checkpoint proteins (Rad3\(^{ATR}\), Rad4\(^{TopBP1}\) and Rad9) tested was sufficient to generate a checkpoint response and these responses followed the expected dependencies. This suggests that the recruitment of multiple copies of a single checkpoint protein results in the formation of active checkpoint complexes that utilise the endogenous proteins. Using this system, we observed that the ability of the Rad4\(^{TopBP1}\) AAD to activate Rad3\(^{ATR}\) is fully dependent on phosphorylation of H2A(\(\gamma\)H2A) and requires the ability of Crb2 to bind \(\gamma\)H2A. This leads us to conclude that, in the absence of ssDNA, the ATR activation domain of Rad4\(^{TopBP1}\) is particularly important for Rad3\(^{ATR}\) activation and acts in a chromatin:protein interaction dependent manner. Taking these data together with the requirement of the Rad4\(^{TopBP1}\) AAD to amplify checkpoint signals in either G1/S or G2 when resection was limited, we propose that Rad4\(^{TopBP1}\) acts to amplify the checkpoint in a chromatin-dependent manner when single-stranded DNA levels are limiting. We can therefore hypothesise that there is a threshold level for the amount of active Rad3\(^{ATR}\) required for a full checkpoint response. When ss-DNA is limited, such as in S-phase, the chromatin-dependent Rad4\(^{TopBP1}\) AAD-dependent pathway for Rad3\(^{ATR}\) activation becomes important to amplify the levels of activated Rad3\(^{ATR}\) to obtain a full checkpoint response (Figure 8).

In addition to analysing the Rad4\(^{TopBP1}\) AAD, we also created a mutant predicted to disable the Rad9 equivalent of the *S. cerevisiae* Ddc1\(^{Rad9}\) AAD and analysed the effect of this mutant in checkpoint activation. Unlike in *S. cerevisiae*, we observed no significant effect on DNA damage-induced checkpoint activation either in G1/S phase or G2. Although when combined with a Rad4\(^{TopBP1}\) AAD mutant, an additive effect to S-phase but not G2
DNA damage can be seen. This suggests that the Rad9 AAD acts in a separate but redundant pathway for Rad3\(^\text{ATR}\) activation in G1/S with the Rad1\(^\text{TopBP1}\) AAD. It appears that, during evolution, the mechanism of activating the ATR pathway has diverged significantly with the roles of different ATR activating domains being of more or less importance in different organisms. It will be interesting to establish if the ATR activating domain of TopBP1 in metazoan systems is particularly important in the context of low levels of ssDNA and whether its function is dependent on γH2AX, especially as a 53BP1/Chk2 and TopBP1 pathway for checkpoint activation in G1 has been previously reported in the mammalian system [54]. The differences in the dependencies of the specific ATR activators in different cell cycle phases between S. \textit{pombe} and S. \textit{cerevisiae} is not surprising as the checkpoint mechanism between these organisms has diverged. For example, in S. \textit{cerevisiae}, the S phase checkpoint is activated independently of the 9-1-1 complex, whereas in S. \textit{pombe} and mammalian cells ATR activation appears to be largely - if not entirely - dependent on 9-1-1 loading. Such distinctions are likely a result of evolutionary adaptation to the different cell cycle profiles of the two yeasts and it is interesting to note that significant evolutionary plasticity surrounds the interface between TopBP1 and the checkpoint apparatus. These distinctions will have to be considered when extrapolating mechanistic data from yeast to human systems. None the less, we believe that our findings shed light on the role of TopBP1 AAD in DNA damage responses and offer useful insights into metazoan mechanisms of DNA damage signalling.

Materials and Methods

\textit{S. pombe} strain construction and biological methods

Standard \textit{S. pombe} protocols were carried out as previously described [55]. \textit{rad} \(\text{i}\) and \textit{rad} \(\text{m}\) mutant strains were created using PCR site directed mutagenesis and integrated at their endogenous locus using \textit{Cre} recombinase-mediated cassette exchange [56] In brief, this system uses a “base strain” which is engineered so that the gene of interest is either replaced with the \textit{ura4} marker (i.e. \textit{radm}) or in the case of essential genes (i.e. \textit{radi}) has the marker integrated immediately after the stop codon. In both cases the gene/marker and loci’s promoter region are flanked by \textit{loxP} and \textit{loxM} sites. These two variant \textit{lox} sites are incompatible with each other. The marker (and, for essential genes, the actual gene also) is then replaced by transforming in either the wild type (as a control: \textit{radi}) or the various mutated copies on a plasmid. These are flanked by the equivalent \textit{loxP} and \textit{loxM} sites and the plasmid expresses \textit{Cre} recombinase, which results in \textit{loxP:loxP} and \textit{loxM:loxM} recombination. For \textit{cdc10-M17} synchronisation cells were grown to log phase at the permissive temperature (25°C) and shifted to the restrictive temperature of 36°C for 3.5 hours. Cells were then either irradiated with the indicated dose of gamma irradiation at 36°C and released at 25°C, or directly released at 25°C and irradiated at the given time points after release. \textit{cdc25-22} block and release [57] and lactose gradient synchronisation [12] were performed as described previously. For FACS analysis cells were resuspended in 50 mM tri-sodium citrate, 1 mg/ml final concentration RNaseA [Sigma], stained with 5 μg/ml Propidium iodide [Sigma] and analysed on FacsCalibur [Becton Dickinson].

Imaging

For live cell imaging concentrated culture was mounted onto a 2.5% agar patch in standard YE medim [Microworks] and imaged on a Deltavision Microscope. Sepation index was counted as previously described [12], \textit{lacO::NAT chkl-HA} strains were created by inserting the 10 Kb \textit{lacO} repeats into the PUC19 plasmid containing the NAT marker and homology to \textit{ura4}. This was integrated into the genomic \textit{ura4} locus. The appropriate strains were transformed [58] with pRep41-GFP-LacI-NLS (GFP/LN) into which either \textit{rad3} or \textit{rad9} had been cloned in frame for N-terminal tagging or \textit{rad4} cloned in frame for C-terminal tagging. Transformants were grown and expression of the fusion protein induced by the removal of thiamine. All \textit{lacO} repeats were checked by Southern hybridisation.

Biochemical assays

Protein extracts for western were prepared by TCA (trichloro-acetic acid) extraction from 1×10\(^{6}\) cells/ml and resuspended in SDS sample buffer [59]. Crude extracts for affinity analysis were prepared by mechanical disruption in liquid nitrogen. Antibodies used: α-HA [Santa cruz] 1:2500, α-Myc [Santa cruz] 1:2000, α-GFP [Roche] 1:2500, α-H2ApSi129 [Abcam] 1:2500 or 1:1000, α-Tubulin [Sigma] 1:5000, α-Cdc2 53-53 [Santa cruz] 1:2500, α-Cdc13 [Jacky Hayes] 1:500, α-Cds1 1:5000 [35]. The secondary antibodies used were HRP rabbit α mouse [Dako] 1:2500 or HRP swine α Rabbit [Dako] 1:2500. Chkl-HA phosphorylation was quantified as a percentage of total signal minus back ground on a ImageQuant LAS 4000 [GE Healthcare]. Cds1 kinase assay was carried out as described [35].

Supporting Information

Figure S1 A. Sensitivity of the indicated strains to HU. 10-fold serial dilutions of 1×10\(^{7}\) cells/ml were spotted onto YEA. \textit{rad4}\(^{\text{m}}\) indicates the RMCE control strain [56]. B. Rad22-GFP foci were visualised by fluorescence microscopy before and after 40 Gy ionising radiation: average of 2 experiments. C-E, pREP41-GFP/LN fused to wild-type \textit{rad3}, \textit{rad3} kinase dead (\textit{rad3K619}) \textit{rad9} or \textit{rad4} were used to transform wild type (WT) or appropriate mutant strains. Empty vector served as a control. Expression of the fusion proteins was induced (thiamine withdrawal for 16 hours) and 10-fold serial dilutions of 1×10\(^{7}\) cell/ml spotted onto selective media plates without thiamine either with or without HU or UV irradiation at the indicated doses. F. \textit{Rad3-GFP/LN (+R3)} or an empty vector control (+EV) was induced for 22 hours in either \textit{rad4}\(^{m}\), \textit{rad4-5399R} or \textit{loxM} cells harbouring the \textit{lacO} array. \textit{Rad3-GFP/LN} expression was monitored 0 and 22 hrs after induction. Chkl-HA phosphorylation was used as an indicator of Rad3-dependent checkpoint activation. G, \textit{Rad3\(^{\text{ATR}}\)} and \textit{Rad4\(^{\text{TopBP1}}\), when co-expressed as LacI fusions and co-recruited to a \textit{lacO} array, can bypass the requirement for Rad9. Top panel: \textit{rad9} is required when \textit{Rad3} is recruited alone. Bottom two panels: two independent experiments showing co-recruitment bypasses \textit{rad9} and remains \textit{lacO}-dependent. H. Deletion of \textit{tel1} alone does not affect Chkl1 phosphorylation. Quantification, relative to unphosphorylated Chkl1, of phosphorylated Chkl following 100 Gy IR in the indicated strains immediately after irradiation (α-HA). Error bars are the standard deviation from the mean (\(n=3\)).

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Author Contributions

Conceived and designed the experiments: AMC VG S-JL CPW. Performed the experiments: CPW S-JL VG TC US TM IM. Analyzed the data: AMC S-JL CPW CC VG IM. Contributed reagents/materials/analysis tools: TM. Wrote the paper: VG CPW AMC.
References

