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Phosphorylation of the DNA damage mediator Rad9 by cyclin-dependent kinases (CDKs) regulates activation of Checkpoint kinase 1 (Chk1)

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Abstract

Extensive cell cycle-dependent phosphorylation is characteristic of DNA damage response mediators but is of unknown function. Here, we show that cell cycle phosphorylation of the Rad9 DNA damage mediator depends on B-type cyclin (Clbs) forms of the major cyclin-dependent kinase Cdc28 (Cdk1) in budding yeast. This phosphorylation does not inhibit Rad9 checkpoint activity in response to normal replication structures. Instead, we propose that Cdk1 regulates Rad9 DNA damage functions. In particular, we have found that the integrity of nine putative Cdk1 phosphorylation sites located in the N-terminal region of Rad9 is required for Chk1 activation, specifically in the G2/M phase of the cell cycle. Phosphorylation of Rad9 N-terminus by Clb-forms of Cdk1 regulates Rad9 interaction with Chk1, independently of the recently reported Rad9-Dpb11 interaction. Our data suggests a model where Rad9 and Chk1 interact constitutively, with remodeling of this complex in response to DNA damage requiring Mec1-dependent phosphorylation.

Introduction

Eukaryotic cells have developed highly conserved surveillance pathways, collectively termed the DNA damage response (DDR), to preserve genome integrity after genotoxic insult. These pathways inhibit replication and segregation of damaged DNA by activating checkpoints and regulating transcription, replication and repair (Harper and Elledge 2007). Defects in the DDR contribute to human cancer, primarily due to defective induction of apoptosis and senescence (Halazonetis et al. 2008).

Central to the DDR are protein kinases that are activated by DNA lesions. The human phosphatidylinositol 3-kinase-like kinases (PIKKs), ATM, ATR and DNA-PK, and their yeast homologues, are the apical kinases at the heart of DNA damage-induced signaling pathways (Durocher and Jackson 2001; Harper and Elledge 2007). DNA-PK does not exist in the budding and fission yeast model systems, ATM corresponds to Tel1 (in both yeasts), whereas ATR corresponds to Mec1 in *S. cerevisiae* and Rad3 in *S. pombe* (Durocher and Jackson 2001). Once activated by DNA-protein structures generated in response to lesions, PIKKs regulate numerous DDR proteins, including the downstream 'checkpoint' or CHK kinases, Chk1 (in all three species) and Chk2 (Rad53 or Cds1, in budding and fission yeast respectively). These two 'effector' kinases in turn phosphorylate target proteins leading to the downstream biological consequences of DDR activation (Stracker et al. 2009).

DDR induction after DNA double-strand breaks (DSBs) involves not only recognition and processing of the breaks, but also cyclin-dependent kinases (CDK) (Wohlbold and Fisher 2009). CDK activity allows DSB processing leading to the amplification of the checkpoint signal by stimulating the switch from ATM to ATR activity, as well as impacting upon the choice of the DSB repair mechanisms (Stracker et al. 2009; Wohlbold and Fisher 2009).

Evidence also suggests that CDK activity might regulate other DDR proteins not necessarily involved at the DSB processing step. For example, CDK activity regulates multiple steps of the homologous recombination process (Wohlbold and Fisher 2009). Our growing understanding of the role of CDKs in the early events of the DDR suggests that other non-inducible protein kinases (e.g. DDK or casein kinase 2) may also be regulated after DNA damage to become key components of the DDR.

DNA damage mediators, molecular adaptors that facilitate protein-protein interactions at sites of DNA damage, contribute to the PIKK-dependent activation of CHK kinases (FitzGerald et al. 2009; Jungmichel and Stucki 2010; O'Donovan and Livingston 2010). *Saccharomyces cerevisiae* Rad9, the first checkpoint protein identified (Weinert and Hartwell 1988), is the prototypical DDR mediator. Rad9 is a 148 kDa protein required for cell survival in response to DNA damage. It is homologous to *S. pombe* Crb2 (Saka et al. 1997; Willson et al. 1997) and shares functional and structural similarities to three human mediators 53BP1, MDC1 and BRCA1 (FitzGerald et al. 2009; Jungmichel and Stucki 2010; O'Donovan and Livingston 2010). It is required throughout the cell cycle for checkpoint delays (O'Shaughnessy et al. 2006) but also has other functions in the DDR, including roles in DNA repair (de la Torre-Ruiz and Lowndes 2000; Barbour et al. 2006; Toh et al. 2006; Murakami-Sekimata et al. 2010). The best understood biological function for Rad9 is its role as an adaptor-catalyst for the activation of the Rad53 kinase (Pellicioli and Foiani 2005). Rad9 is required as a scaffold to bring Rad53 in close proximity to Mec1 for its pre-activation and it is also required to increase the local concentration of Rad53 allowing *in trans* autophosphorylation to occur (Pellicioli and Foiani 2005). In contrast, Chk1 seems to autophosphorylate *in cis* upon DNA damage (Chen et al. 2009) Activation of Chk1 appears well conserved from yeast to human, requiring phosphorylation by the ATR kinase that in

turn depends upon various DNA damage mediators, which bring Chk1 into close proximity to ATR (Stracker et al. 2009). Among the mediators, Claspin-dependent activation of CHK1 is best characterised and involves PIK kinase-dependent phosphorylation of Claspin itself (Chen and Sanchez 2004; Smits et al. 2010). Mrc1 is the budding and fission yeast Claspin orthologue. However, budding yeast Mrc1 seems to mostly control Rad53 in the DNA replication checkpoint (Tanaka 2010) and a role in Chk1 activation remains to be reported. In both budding yeast and fission yeast the prototypical mediator, Rad9/Crb2, has also been implicated in Chk1 activation after DNA damage through unknown mechanisms (Blankley and Lydall 2004). In higher cells, Rad9-like mediator proteins, including BRCA1 and MDC1, play a complex role, which require further mechanistic characterisation (Stracker et al. 2009; Smits et al. 2010).

Mediators are typically phosphoproteins, phosphorylated by multiple kinases, especially PIKKs upon DNA damage (Saka et al. 1997, Lou, 2003 #545; Emili 1998; Vialard et al. 1998; Cortez et al. 1999; Rappold et al. 2001; Xia et al. 2001; Goldberg et al. 2003, Xia, 2001 #160; Lou et al. 2003; Xu and Stern 2003). DNA damage-induced and PIKK-dependent phosphorylation of budding yeast Rad9 is required for its activation and correlates with the remodeling of a large ≥850 kDa Rad9 complex into a smaller 560 kDa complex, containing the DNA damage induced hyperphosphorylated form of Rad9, which mediates the activation of Rad53 (Gilbert et al. 2001; Gilbert et al. 2003) and Rad9 oligomerisation (Usui et al. 2009).

DNA damage mediators are frequently phosphorylated during cell cycle progression in the absence of exogenous DNA damage (Ruffner and Verma 1997; Vialard et al. 1998; Esashi and Yanagida 1999; Ruffner et al. 1999; Rappold et al. 2001; Jullien et al. 2002). The 'Rad9-like' mediators share a high number of consensus motifs for phosphorylation by CDKs (S/T-

P-x-K/R, where x can be any amino acids, or minimal S/T-P sites) but their functions remain unknown. Rad9 contains an exceptionally high density of such motifs, twenty S/T-P sites of which nine conform to the full CDK consensus phosphorylation site, compared to the yeast proteome (Ubersax et al. 2003; Moses et al. 2007). The mobility of Rad9 during electrophoresis suggests that it is a heavily modified protein and phosphatase-sensitive isoforms of slower mobility have been observed in the S and M phases (Vialard et al. 1998). Not surprisingly, Rad9 has been identified as an *in vitro* substrate of Cdk1 (Ubersax et al. 2003; Loog and Morgan 2005). Mass spectrometric studies have supported the *in vivo* phosphorylation of 15 of these CDK consensus sites (Smolka et al. 2005; Albuquerque et al. 2008; Holt et al. 2009). However, the biological role of CDK-dependent phosphorylation of Rad9 remains to be characterised.

Here, we have demonstrated that phosphorylation of Rad9 during the S, G2 and M phases of the cell cycle, is dependent upon Cdc28/Clb complexes (Cdc28, also called Cdk1, the *S. cerevisiae* equivalent to CDK1, can bind six cyclin B equivalents, Clb1 to 6). We propose that CDK-dependent phosphorylation of Rad9 modulates the activity of multiple Rad9 functions during the DDR. As proof-of-principle we have focused on one of these functions, the regulation of Chk1 activation. We show here that Cdc28-dependent *in vivo* phosphorylation of consensus CDK phosphorylation sites located in the N-terminal 231 amino acids of Rad9 is required for activation and maintenance of Chk1-dependent, but Rad53-independent, signaling after DNA damage. Our results are consistent with a model in which prior CDK-dependent phosphorylation of the Rad9 N-terminus during S, G2 and M phases, is required for the interaction between Chk1 and Rad9. PIK kinase-dependent phosphorylation of both Chk1 and Rad9 at sites of DNA damage is then followed by release of Rad9 in association with activated Chk1, which can then phosphorylate its substrates.

Results

DNA damage-independent phosphorylation of Rad9 during S, G2 and M requires Cdc28/Clb activity

Rad9 migrates with an SDS-page mobility ranging from 180 to 220 kDa (Vialard et al. 1998). This mobility shift results from phosphorylation of Rad9 ((Emili 1998; Vialard et al. 1998; O'Shaughnessy et al. 2006) and Figure S1). The slowest migrating forms, previously termed hyper-phosphorylated Rad9 (Grenon et al. 2001), are dependent upon Mec1/Tel1 and DNA damage (Emili 1998; Vialard et al. 1998; Grenon et al. 2001; O'Shaughnessy et al. 2006). Rad9 is not detectably phosphorylated in G1 arrested cells, but is phosphorylated during arrest in either S or M phase without DNA damaging treatments. These Rad9 phospho-forms were previously termed hypo-phosphorylated Rad9 as they result in a smaller mobility shift (Grenon et al. 2001). To more clearly distinguish between the DNA damage and cell cycle regulated forms, here we term the former D-Rad9 and the latter, C-Rad9.

The fastest migrating C-Rad9 forms were detected in cells progressing through G1, with slower migrating phospho-forms appearing as cells entered S phase and persisting with ever decreasing mobility as cells moved through S, G2 and into mitosis (Figure 1A). These phospho-forms disappeared towards the end of mitosis, with the fastest migrating form of Rad9 reappearing in G1 cells. This pattern was repeated over approximately 2.5 cell cycles that were analysed. We conclude that Rad9 is increasingly phosphorylated during passage through S, G2 and M phases of the cell cycle.

The cyclic pattern of Rad9 phosphorylation strikingly reflects the cell cycle activity of budding yeast Cdc28 (also termed Cdk1) that regulates cell cycle progression (Enserink and

Kolodner 2010). Cdc28 can associate with 9 different cyclins. Cdc28/Cln1-3 complexes are active in G1 phase; whereas the Cdc28/Clb1-6 complexes function throughout S, G2 and mitosis (Enserink and Kolodner 2010). *In vitro*, Rad9 is one of 360 proteins that can be phosphorylated by Cdc28-Clb2 and Cdc28-Clb5 complexes (Ubersax et al. 2003; Loog and Morgan 2005).

To test the hypothesis that the *in vivo* phosphorylated forms of Rad9 observed during cell cycle progression require Cdc28 activity we used cells expressing Cdc28-as1, an analogue sensitive version of Cdc28 (Bishop et al. 2000). In the presence of the bulky ATP analogue, 1-NMPP1, Cdc28-as1 is inactivated within 5-15 minutes. Dephosphorylation of substrates is then dependent upon counteracting phosphatases (Ubersax et al. 2003). Dephosphorylation of Orc6, a known Cdc28 target (Liang and Stillman, 1997) was used to follow inactivation of Cdc28-as1 (Ubersax et al. 2003). Rad9 slower migrating forms detected in asynchronous and nocodazole-arrested cells (i.e. G2/M cells) were completely abolished as a consequence of Cdc28 inactivation (Figure 1B). The migration of Rad9 in G1 arrested cells was not affected by the inactivation of Cdc28. Thus the increasing phosphorylation of Rad9 as cell progress through S, G2 and M phase is dependent upon Cdc28.

Clb1 to 6 are obvious candidates for the cyclin partners of Cdc28 required for Rad9 cell cycle phosphorylation. We therefore examined Rad9 migration in conditional CDC mutants with different levels of Cdc28/Clb activity at non-permissive temperature (Figure 1C). Cdc4 (a protein from the SCF complex) is required for destruction of Sic1, a potent and specific inhibitor of B-type cyclin CDK complexes (Enserink and Kolodner 2010). G1 cells with high levels of the Cdc28-Clb inhibitor, Sic1, can be generated by arresting *cdc4-1* cells with a factor and shifting to the restrictive temperature (Schwob et al. 1994; Piatti et al. 1996; Nash et al. 2001). Upon release from the a factor block, cell cycle-dependent

phosphorylation of Rad9 was severely abrogated in *cdc4-1* cells, whereas this did not occur with *cdc7-1* cells, which also block cell cycle progression prior to S phase but with high CDK activity (Figure 1C). This abrogation of Rad9 cell cycle phosphorylation is dependent upon Sic1, as cell cycle phosphorylation of Rad9 is restored in similarly treated *cdc4-1 sic1Δ* cells (Figure 1C). In fact, in the absence of Sic1, Rad9 is phosphorylated even during a factor arrest as a consequence of residual Cdc28-Clb activity. Additionally, by manipulation of the levels of the Cdc6 protein we could establish that Rad9 can be normally phosphorylated irrespective of whether DNA synthesis takes place or not (Figure S1B). Together, our data indicate that Rad9 cell cycle phosphorylation is dependent upon B-type cyclin forms of the major CDK of budding yeast and independent of the generation of S phase structures.

Abrogation of Rad9 cell cycle phosphorylation does not affect cell proliferation

To determine whether consensus CDK sites in Rad9 contribute to its cell cycle-specific phosphorylation *in vivo*, we generated a strain in which Rad9 cell cycle phosphorylation is almost completely abrogated. Twelve of the twenty putative CDK phosphorylation sites present in Rad9, including all nine conforming to the strict S/T-P-X-K/R consensus, were converted from serine/threonine to alanine (Figure 1D). Phosphorylation of Rad9 and Rad9^{12A} was analyzed during cell cycle progression, which was followed by western analysis (Figure 1E, note that Sic1 and Clb2 are expressed in G1 and G2/M, respectively (Enserink and Kolodner 2010)) and by analysis of budding and DNA content (Figure S1C). Rad9 cell cycle phosphorylation was dramatically abrogated in the *rad9^{12A}* mutant indicating that the consensus CDK motifs mutated represent the majority of the sites in Rad9 that are phosphorylated *in vivo*.

Given the known roles of Rad9 in DNA structure-specific checkpoint responses, one possibility is that CDK-dependent phosphorylation of Rad9 is required for its inactivation upon entry into S phase in order to prevent inappropriate signaling from normal replication structures. This is consistent with proposals for an S phase-specific threshold that allows cells to tolerate damage-like DNA structures present at normal replication forks, e.g. naturally occurring ssDNA (Shimada et al. 2002; Cobb et al. 2004). However, cell cycle progression of the *rad9*^{12A} mutant was not detectably different from wild type cells. In particular, major cell cycle transitions and the extent of S phase, as judged by FACS analysis and budding index (Figure S1C), as well as the Sic1 and Clb2 cell cycle markers (Figure 1E), were not detectably perturbed. Consistent with this data, in proliferating *rad9*^{12A} cells without exogenous damaging treatments, neither Rad53 nor Rad9 were activated (Figure 1E) and no increased number of large budded cells was observed in these undamaged mutant cells (Figure S1C and D). Importantly, after ionizing radiation, *rad9*^{12A} cells largely retain normal checkpoint regulation and Rad9^{12A} can be hyper-phosphorylated with normal kinetics (Figure S1D and E). Thus, Cdc28-dependent phosphorylation of Rad9 is unlikely to be required to prevent inappropriate sensing of structures generated in S phase.

CDK phosphorylation sites in the CAD region of Rad9 are required for Chk1 activation

Half of the consensus CDK phosphorylation sites of Rad9 are located in its amino terminus (Figure 2A). Deletion of this region, termed the Chk1 Activating Domain or CAD (Blankley and Lydall 2004)), results in defective damage-dependent Chk1 phosphorylation through an unknown mechanism. We hypothesized that phosphorylation of the 9 CDK sites present in the CAD is important for Chk1 activation. Therefore, we generated a mutant, termed *rad9*$^{CDK1\text{-}9A}$ in which the four serines and the five threonines corresponding to CDK sites 1-9 were mutated to alanines. This mutant was also compared to *rad9*$^{CAD\Delta}$ cells expressing a

truncated Rad9 protein lacking the CAD region. The survival of *rad9*$^{CDK1\text{-}9A}$ and *rad9*$^{CAD\Delta}$ cells, unlike *rad9Δ* cells, was similar to WT cells in response to IR, bleocin (which primarily causes DNA strand breaks) and 4-NQO (4-nitroquinoline 1-oxide, which causes single strand breaks and lesions repaired by nucleotide excision repair) treatments indicating that these mutants are largely functional (Figure 2B). Interestingly, *chk1Δ* cells displayed pronounced sensitivity, equivalent to *rad9Δ*, when plated on bleocin, which was rescued when the plates also contained nocodazole. The bleocin sensitivity of proliferating *chk1Δ* cells could indicate a role for *CHK1* in surviving bleocin-induced lesions during S phase, which can be rescued by extended arrest in G2/M. Interestingly, *rad9*$^{CDK1\text{-}9A}$ and *rad9*$^{CAD\Delta}$ cells were not bleocin sensitive indicating that this phenotype is independent of the CAD of Rad9.

We used western analysis to determine the extent of Rad9 cell cycle phosphorylation in *rad9*$^{CDK1\text{-}9A}$ cells (Figure 2C). Rad9$^{CDK1\text{-}9A}$ migrated faster than Rad9 (but to a reproducibly lesser extent than Rad9^{12A}, consistent with mutation of additional CDK sites in Rad9^{12A}) in blots using extracts prepared from both asynchronous and G2/M arrested cells. In both cases the faster migrating forms of Rad9 were lost in *cdc28-as1* cells in the presence of 1-NMPP1. Thus, at least some of the CDK sites mutated in Rad9$^{CDK1\text{-}9A}$ and Rad9^{12A} are phosphorylated *in vivo*.

CDK sites in the Rad9 CAD are specifically required for Chk1 activation in G2 phase

The ability of CDK1-9 to mediate Chk1 activation was assessed by analysing the DNA damage-induced phospho-shift of tagged Chk1-3HA by western blotting as before (Sanchez et al. 1999). In response to 4-NQO treatment of asynchronously growing wild type cells, a higher mobility Chk1 phospho-form rapidly appears (Figure 2D). Chk1 activation in this

assay was fully defective in *rad9Δ* and *rad9*$^{CAD\Delta}$ cells, as previously reported (Sanchez et al. 1999; Blankley and Lydall 2004). Interestingly, the damage induced Chk1 phospho-shift was also mostly dependent on the integrity of the CDK1-9 sites. Similar results were obtained in response to IR, bleocin, UV and 4-NQO (Figure S2A and Figure 3 below). Our results suggest that the role of CDK sites in the Rad9 CAD is to regulate DNA damage induced-Chk1 phosphorylation.

Comparison of G1 and G2/M arrested cells reveals that Chk1 activation after bleocin treatment in *rad9*$^{CDK1\text{-}9A}$ cells was severely defective in G2/M cells, although it retained residual activation in G1 arrested cells (Figure 3A and B). Similar results were obtained with IR, 4-NQO and UV (Figure S2A). Notably, the defect in activating Chk1 in G2/M of the cell cycle cannot be explained by an inability to form D-Rad9$^{CDK1\text{-}9A}$ or D-Rad9$^{CAD\Delta}$ after DNA damage (Figure 3C and Figure S2B). Thus, Chk1 activation correlates well with elevated CDK activity and Rad9 cell cycle phosphorylation in G2/M.

The CDK1-9 sites of Rad9 specifically control the Chk1 branch

An important aspect of the role of the Rad9 CAD region is its specificity in regulating Chk1 and not Rad53 (Blankley and Lydall, 2004). We observed strong activation of Rad53 in G2/M arrested *rad9*$^{CDK1\text{-}9A}$ cells after DNA damage (Figure 3D and Figure S2C). Relative to *rad9D* cells, Rad53 could also be significantly activated after DNA damage in *rad9*$^{CAD\Delta}$ cells. Together, our data suggest that the CDK1-9 sites themselves are specifically regulating Chk1 activation.

To confirm that the CDK1-9 sites function exclusively in the Chk1 activation pathway, we performed an epistasis analysis using the G2 cell cycle checkpoint assay (O'Shaughnessy et al. 2006). The G2/M delay in this assay requires Rad9-dependent regulation of two additive

branches involving both checkpoint kinases, Chk1 and Rad53/Chk2 (Gardner et al. 1999; Sanchez et al. 1999). Similarly to single deletion of *chk1Δ* or *rad53Δ*, *rad9*$^{CDK1\text{-}9A}$ cells displayed a partially defective G2/M checkpoint (Figure 3E). A similar partial defect was observed in *rad9*$^{CDK1\text{-}9A}$ *chk1Δ* cells, whereas *rad9*$^{CDK1\text{-}9A}$ *rad53Δ* cells were completely defective in this G2 checkpoint assay. The epistatic relationship between *rad9*$^{CDK1\text{-}9A}$ and *chk1Δ*, as well as the additive relationship between *rad9*$^{CDK1\text{-}9A}$ and *rad53Δ*, strongly indicate that the CDK1-9 sites of Rad9 function specifically to regulate Chk1 activation in response to DNA damage.

While generating *rad9*$^{CDK1\text{-}9A}$ cells, we collected multiple CDK site mutants, containing different combinations of mutated CDK sites and covering the CAD region, in an attempt to determine whether any specific site or sites out of the nine CDK sites in the CAD is absolutely necessary for Chk1 activation (seven such mutants are shown in Figure S2E). These mutants ranged from single site mutants to mutants with up to five mutated sites, however, none of these mutants was fully defective for the DNA damage-induced phosphorylation of Chk1. Thus, it is unlikely that any single CDK site plays a critical role, rather a subset of the nine CDK sites, that will require extended investigation to decipher, must work together to effect efficient activation of Chk1.

CDK-dependent activation of Chk1 is not specific to DSB-inducing agents

It has been well established that Rad53 activation in response to an inducible HO DSB relative to single strand breaks and DNA adducts repaired by NER is cell cycle regulated and dependent on Cdc28 activity. This is due to a key role for Cdc28 in controlling DSB resection (Ira et al. 2004; Enserink and Kolodner 2010). However, Chk1 activation is less characterized. Therefore, we used G2/M arrested cells harbouring the *cdc28-as1* allele,

which can be rapidly inhibited, to investigate Chk1 activation after bleocin and 4-NQO treatments (Figure 4), as well as IR (Figure S3). In the absence of Cdc28 activity, both Rad53 and Rad9 were not activated after bleocin treatment, whereas their activation was delayed after treatment with 4-NQO (Figure 4A). In contrast, Cdc28 activity was essential for Chk1 activation in response to either DSB inducing agents, or 4-NQO. These results suggest that Chk1 activation upon DNA damage is absolutely dependent upon Cdc28 activity, rather than a downstream consequence of the Cdc28-dependent resection that occurs at DSBs.

Inactivation of Cdc28 activity after Chk1 had been activated by bleocin treatment resulted in rapid loss of the active phospho-form of Chk1 (Figure 4B, note that while C-Rad9 also requires continuous Cdc28 activity, D-Rad9 does not), indicating a continuous requirement for Cdc28 activity for maintenance of Chk1 signaling. Maintenance of Rad53 signaling also requires continuous Cdc28 activity and has also been observed by others (Ira et al. 2004).

Our data indicate that Cdc28 activity is absolutely required for initiation and maintenance of Chk1 activation with all DNA damaging agents tested. This is consistent with a role for Cdc28 in controlling Chk1 activation, regardless of the type of DNA damage, by regulating cell cycle-dependent phosphorylation of CDK sites in the N-terminus of Rad9.

Both CDK1-9 sites and Cdk1 activity are required for physical interaction between Rad9 and Chk1

A mechanistic clue as to how Chk1 is activated comes from the reported interaction between Rad9 and Chk1 in yeast 2-hybrid (Y2H) assays (Sanchez et al. 1999). We have independently confirmed this Y2H interaction using full length Rad9 (Figure 5A and Figure S4A and B for protein expression in the Y2H strains). Furthermore, the N-terminal Rad9

CAD alone is sufficient for this interaction. Importantly, the interaction between full length Rad9 and Chk1 is absolutely dependent on intact CDK1-9 sites. This is consistent with CDK-dependent phosphorylation of these sites being required to regulate the interaction between Rad9 and Chk1. In agreement with this hypothesis, the Y2H interaction between Rad9 and Chk1 is dependent upon Cdc28 activity since it is mostly abolished in G2/M arrested *cdc28-as1* cells treated with 1-NMPP1 (Figure 5B and Figure S4C). Interestingly, the Y2H interaction between Rad9 and Chk1 still occurred in G1 arrested cells (Figure 5B), in agreement with the Rad9 dependency of Chk1 activation after DNA damage in this phase of the cell cycle (Figure 3A). However, the Rad9-Chk1 interaction observed in G1 arrested cells, unlike G2/M arrested cells, clearly was Cdc28-independent, suggesting that a Cdc28 independent mechanism of regulation of Rad9 and Chk1 interaction must exist in G1 phase. Note that the interaction is still dependent on the integrity of the CDK1-9 sites in G1 phase (Figure S4D) and these results are in agreement with the decreased Chk1 activation observed in G1 arrested $rad9^{CDK1\text{-}9A}$ cells (Figure 3A). Thus, yeast two-hybrid analyses demonstrate that interaction between Rad9 and Chk1 is fully dependent on the Rad9 CAD, the nine CDK sites located in this region and the activity of Cdc28 during the G2/M phase of the cell cycle.

We used co-immunoprecipitation to confirm our Y2H Rad9-Chk1 interaction data (Figure 5C). Rad9 can be detected in Chk1-3FLAG immunoprecipitates with extracts prepared from G2/M arrested wild type cells that had been either mock or bleocin treated. Notably, Chk1 preferentially associated with Mec1/Tel1-phosphorylated D-Rad9 after DNA damage, even though C-Rad9, the phosphoform dependent upon Cdc28, was the major form detected in the whole cell extracts. The Rad9-Chk1 interaction can also be observed in reciprocal co-immunoprecipitation experiments in which Rad9-9MYC was immunoprecipitated from G2/M arrested cells treated with bleocin (Figure 5E). In parallel experiments using $rad9^{CDK1\text{-}}$

^{9A} mutant cells we were able to demonstrate that the Chk1-Rad9 interaction detected in Chk1 immunoprecipitates from extracts prepared from either mock or bleocin treated cells was lost (Figure 5D).

We have recently reported an interaction between Dpb11 and Rad9 (Granata et al. 2010). We have addressed the relationship between this interaction and the Rad9-Chk1 interaction reported here. Interestingly, and unlike the case with Chk1-Rad9 interaction, the Dpb11 interaction with C-Rad9 was not completely lost in extracts from bleocin treated cells (Figure 5C). Additionally, we observed that the extent of Dpb11 interactions with Rad9, in the presence or in the absence of DNA damage, appeared much less than we observed for the Chk1-Rad9 interaction under similar conditions. Importantly, Dpb11 was never observed in the Chk1 immunoprecipitates and vice versa, Chk1 was absent from Dpb11 immunoprecipitates, suggesting the presence of two distinct Rad9 sub-complexes in our whole cell extracts that either contain Chk1 or Dpb11.

Interestingly, although the Dpb11-Rad9 interaction detected in Dpb11 immunoprecipitates from *rad9*$^{CDK1\text{-}9A}$ cells after DNA damage was lost, the Dpb11-Rad9 interaction could still be detected in extracts from *rad9*$^{CDK1\text{-}9A}$ cells that had not been subject to bleocin treatment. Taken together our results indicate that CDK-dependent phosphorylation of the nine sites located in the N-terminus of Rad9 regulate the interaction between Chk1 and Rad9 both in the presence and in the absence of DNA damage.

In vitro ATP-dependent release of Rad53 but not Chk1 from Rad9 complexes

We previously reported a Rad9-Rad53 interaction and demonstrated that Rad53 could be released from this complex *in vitro* by addition of ATP (Gilbert et al. 2001; Gilbert et al. 2003; O'Shaughnessy et al. 2006). We therefore tested whether Chk1 could also be released

from Rad9 immunoprecipitates in an ATP dependent manner. Rad9 complexes were washed, incubated with either ATP, its non-hydrolyzable analog, ATP-γS, or simply mock treated. As previously observed (Gilbert et al. 2001), Rad53 became further phosphorylated and was released into the supernatant in a manner dependent upon ATP hydrolysis (Figure 5F). In contrast, Chk1 phosphorylation was not further modified by ATP addition and no release from the beads was observed. This *in vitro* data is consistent with distinct molecular mechanisms regulating the activity of the two distinct checkpoint kinases, Chk1 and Rad53.

Discussion

Complex cell cycle phosphorylation of Rad9 by Cdc28-Clb complexes

We show here that the budding yeast DNA damage response (DDR) mediator, Rad9, follows a complex pattern of phosphorylation during the cell cycle. Both the pattern of modification and genetic evidence implicate B-type cyclin (Clb1 to 6) forms of Cdc28 (also termed Cdk1) in this regulation. Our *in vivo* evidence demonstrating Cdc28/Clb-dependent phosphorylation of Rad9 is supported by published *in vitro* studies demonstrating phosphorylation of Rad9 by purified Cdc28/Clb5 and Cdc28/Clb2 complexes (Loog and Morgan 2005). It is likely that all forms of Cdc28/Clb complexes contribute to Rad9 cell cycle phosphorylation, switching from Clb5 and 6, followed by Clb3 and 4 and finally Clb1 and 2 as cells traverse the S, G2 and M phases.

DDR mediators from higher cells, such as 53BP1, BRCA1, MDC1, are also rich in consensus CDK sites, some of which are known to be phosphorylated *in vivo*, suggesting that modulation of the DDR by CDK-dependent phosphorylation of mediators is evolutionarily widespread. For example, CDK-dependent phosphorylation of S379 of mouse

53Bp1 has been shown to be required for binding to the mitotic kinase, Plk1 (van Vugt et al. 2010) and S1497 is needed for BRCA1 subcellular localization (Ruffner et al. 1999).

Rad9 functions in the DDR are regulated by CDK-dependent phosphorylation

The Rad9^{12A} mutant, containing mutation of 12 consensus CDK sites, retains most Rad9 functions but is severely defective for its cell cycle phosphorylation, demonstrating that phosphorylation of some of these sites occurs *in vivo*. In addition, nine of these twelve sites have also been identified as phospho-sites *in vivo* by mass spectrometric analyses (Smolka et al. 2005; Albuquerque et al. 2008; Holt et al. 2009). Importantly, lack of Rad9 cell cycle phosphorylation in *rad9^{12A}* cells did not perturb cell cycle proliferation or checkpoint activation. Thus it is unlikely that Rad9 is cell cycle phosphorylated in order to prevent inappropriate checkpoint activation in response to DNA structures generated during a normal S phase. In fact, we have shown that S phase structures are not needed to drive Rad9 cell cycle modifications under conditions in which the cell cycle proceeds to mitosis without initiating DNA replication.

An alternative hypothesis is that CDK-dependent phosphorylation of Rad9 may modulate its multiple functions in the DDR. The mutants presented here only abrogate some Rad9 functions in the DNA damage response, whereas a *rad9-18A* mutant (eighteen of the 20 consensus CDK sites have been mutated) displays greater defects (Bonilla et al. 2008) suggesting that individual CDK sites, or subsets of these sites, might regulate specific Rad9 functions. Consistent with this we have observed specific phenotypes, including checkpoint recovery defects and specific sensitivity to DNA damaging agents (our unpublished results) with specific CDK site mutants. In this report, we have investigated the role of nine CDK sites in the N-terminal region of Rad9 in the regulation of the checkpoint kinase, Chk1.

Chk1 activation is dependent on Cdc28 phosphorylation of Rad9 in G2/M cells

The first 250 amino acids of Rad9, out of a total of 1309, contain both half the consensus CDK sites and the previously identified CAD, or Chk1 activation domain (Blankley and Lydall, 2004). The molecular mechanisms by which the Rad9 CAD activates Chk1 were previously unknown. Here we establish that the nine CDK sites located in the first 231 amino acids of Rad9 are required for Chk1 activation in response to DNA damage. These sites are not involved in the activation of Rad53, nor do they significantly impact on the regulation of Rad9 by PIK kinases. These nine sites are necessary for normal cell cycle phosphorylation of Rad9 in vivo, and six (S11, S26, S56, S83, T155 and T218) have been mapped as sites of *in vivo* phosphorylation by mass spectrometry (Smolka et al. 2005; Albuquerque et al. 2008; Holt et al. 2009). Consistent with a role for Cdc28-dependent phospho-sites in the Rad9 CAD, we show that Cdc28 controls Chk1 activation in response to several DNA damaging agents that result in either DSBs, SSBs or bulky lesions repairable by nucleotide excision repair. Thus, Chk1 regulation, unlike DSB specific activation of Rad53, is not simply a downstream consequence of the involvement of CDK1 in regulating DSB specific responses including DSBs resection (Limbo et al. 2007; Huertas et al. 2008) or the reported Cdc28-dependent role for Rad9 in the inhibition of ssDNA generation at DNA ends (Lazzaro et al. 2008). Taken together, our results establish that CDK phosphorylation of up to 9 sites in the Rad9 N-terminal region regulate the activation of Chk1 via its interaction with Rad9 during periods of the cell cycle when Cdc28/Clb kinases are active. Interestingly, our results suggest that DNA damage-dependent activation of Chk1 in G1 phase also requires interaction with Rad9 via the CDK1-9 sites. However, it does not depend on CDK activity suggesting that this interaction is regulated by phosphorylation of CDK

sites by another kinase. Alternatively, these sites may be required in a G1 specific role that is independent of their phosphorylation.

Novel Molecular mechanism regulating Chk1 activation

Our results are consistent with a novel model for Chk1 activation illustrated in Figure 5G and based on the constitutive interaction between Rad9 and Chk1 controlled by Cdc28-dependent modification of Rad9 in S/G2/M. The Rad9 and Chk1 proteins, both of which are believed to dynamically associate with chromatin even in the absence of DNA damage (Smits 2006; Grenon et al. 2007; Granata et al. 2010), interact constitutively in the absence of DNA damage [Figure 5G, step 1]. While associated with Chk1, Rad9 undergoes enhanced chromatin recruitment in the vicinity of damaged DNA by exposure to or generation of specific histone marks (H3K79me and gH2A (Grenon et al. 2007; Hammet et al. 2007), as well as by binding to Dpb11 (Granata et al. 2010), recruited in a 9-1-1 complex-dependent manner (Puddu et al. 2008) [Figure 5G, step 2]. The 9-1-1/Dpb11 complex-dependent activation of Mec1 bound to RPA-coated ssDNA, conserved from yeast to human (Navadgi-Patil and Burgers 2009) results in DNA damage dependent phosphorylation and remodeling of both Rad9 (Gilbert et al. 2003) and Chk1 (Tapia-Alveal et al. 2009). This would lead to *in cis* autophosphorylation of Chk1 (Chen et al. 2009) and the release of the D-Rad9/Chk1 complex [Figure 5G, step 3]. This contrasts with Rad53 full activation by *in trans* auto-phosphorylation after its Mec1 (ATR)-dependent priming (Pellicioli and Foiani 2005). In this respect Chk1 activation is distinct from Rad53 activation, since the Rad53 kinase interacts specifically with Mec1/Tel1 phosphorylated Rad9 (D-Rad9) generated in response to DNA. Additionally, *in vitro* data suggest that activated Rad53 is released from Rad9 in an ATP-dependent mechanism (Gilbert et al. 2001), which we could not observe with Chk1. It is possible that activated Chk1 could be released from Rad9 by an ATP-independent

mechanism allowing the targeting of substrates throughout the nucleus in agreement with studies of budding yeast *chk1* mutants that can be activated in a Rad9 independent manner (Chen et al. 2009). Alternatively, or perhaps in addition, a proportion of activated Chk1 could be kept in the proximity of damaged chromatin via its interaction with Rad9 to phosphorylate local targets.

Interestingly, Dpb11 (termed Cut5 in fission yeast and TOPBP1 in higher cells), like Chk1, also interacts with Rad9 both in the absence or presence of DNA damage (Granata et al. 2010). However, and in contrast with the Chk1-Rad9 interaction, only the DNA damage-induced interaction between Dpb11 and Rad9 is dependent on the integrity of CDK sites in the Rad9 CAD (Granata et al. 2010). In addition, a far smaller fraction of Rad9 is associated with Dpb11 compared to Chk1 in our assays. Perhaps Rad9 interaction with Chk1 is a prerequisite for Rad9 binding with Dpb11. In our assays simultaneous binding of Rad9, Chk1 and Dpb11 was not detected, whereas in fission yeast a Crb2-Chk1-Cut5 interaction has been reported (Mochida et al. 2004). We cannot exclude the possibility that a Rad9-Chk1-Dpb11 interaction can occur *in vivo* but is lost in our extracts. Nevertheless, our work reveals a novel mode of Chk1 activation that contrasts with the previously reported DNA damage-induced recruitment of Rad53 to the DNA damage activated (PIK kinase phosphorylated) Rad9 mediator protein.

In higher cells, the role played by DDR mediator proteins is more complex than in yeasts as multiple mediators are known and whose molecular mechanisms in checkpoint activation remain largely uncharacterised (Stracker et al. 2009; Smits et al. 2010). Among the mediators, Claspin-dependent activation of CHK1 is best characterized, however, BRCA1 and MDC1 also play an undefined role in this activation. Our work suggests that activation of human CHK1 by mediator proteins, possibly those related to budding yeast Rad9, may

also be integrated into cell cycle stage by their prior CDK-dependent phosphorylation and dynamic interaction with CHK1. Additionally, the extensive cell cycle phosphorylation of these proteins is likely to fine-tune their DNA damage functions, as we have observed with their budding yeast homologue, Rad9.

Material and Methods

Strains

All strains used in this work are listed in Table S1 and in the W303 background (*MATa ade2-1 trp1-1 can1-100 leu2-3,12 his3-11,15 ura3*) with the exception of CG378 used in Figure 1A. All mutant and tagged alleles used were integrated on the chromosome apart from the yeast two-hybrid experiments. Yeast strain and plasmid constructions are described in supplementary material. Plasmids and oligonucleotides used in this study are listed in Tables S2 and S3.

Cell cycle arrest and Checkpoint experiments

These experiments were performed as described earlier (O'Shaughnessy et al. 2006) and are described in detail in supplemental information.

Antibodies

Western blotting was performed as previously described (Vialard et al. 1998; O'Shaughnessy et al. 2006). Rad9 phosphoforms, Rad53, Swi6, Dpb11-13MYC, RAD9-9MYC and Chk1-3FLAG were probed with NLO5 and NLO16 (Vialard et al. 1998; O'Shaughnessy et al. 2006), NLO2 (D. Lee & N. Lowndes), anti-MYC (9E11, Abcam) and anti-FLAG (M2, Sigma) antibodies respectively. Sic1, Clb2, Chk1-3HA and Orc6 were with anti-Sic1 (JD156 from J. Diffley), anti-Clb2 (sc9071 from Santa Crutz), anti-HA (12CA5) and anti-Orc6

(SB49 from B. Stillman) antibodies respectively.

Yeast two-hybrid

Interactions were assessed by using the Clontech Matchmaker TM Gold Yeast Two Hybrid System (Catalog no 630489) according to the manufacturer's instructions. A triple-plasmids based assay was used to study the Cdc28-dependent interaction between Rad9 and Chk1 protein as described earlier (Granata et al. 2010).

Yeast native extracts and immuno-precipitations

Clarified crude extracts (CCE) of wild type or *rad9*$^{CDK1\text{-}9A}$ cells, expressing either both Chk1-3FLAG and Rad9-9MYC or both Chk1-3FLAG and Dpb11-13MYC, arrested in G2/M phase and either mock treated or treated with 20μg/ml of bleocin (Calbiochem) for 45 min were generated as previously described (Granata et al. 2010). One ml of CCE at a concentration of 10mg/ml was used in immunoprecipitation experiments as previously described (Granata et al. 2010) with anti-FLAG, anti-MYC or IgG antibodies. Beads were finally resuspended in 40μl of 3x Laemmli buffer, boiled for 5 min and released proteins were separated on SDS-PAGE gels for western blot analysis. Note that all tagged strains were confirmed for functionality (Figure S4E).

ATP-dependent release assay

ATP-dependent release assays were performed as previously described (Gilbert et al. 2001) using Rad9-9MYC immunoprecipitates from nocodazole-arrested wild-type cells expressing both Rad9-9MYC and Chk1-3FLAG, and treated with 20μg/ml of bleocin for 45 min. The amount of Rad9-9MYC, Chk1-3FLAG and Rad53 remaining bound to anti-MYC protein G beads (Beads) or released in to the supernatant (Elut) after incubation with ATP (+), an ATP

non-hydrolysable analogue (γS-ATP) or after mock treatment without any nucleotide (-) was determined by western blotting.

Additional details and methods are described in the Supplemental Information.

Supplemental Information

Supplemental Information includes 4 figures, 3 tables and supplemental experimental procedures.

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Author contributions

Conceived and designed the experiments: MG NFL with contribution from RK, CMA. Performed the experiments: RK CMA KF KC NFL MG. Analyzed the data: MG NFL with

contribution from RK CMA KF KC Contributed reagents/materials/analysis tools: RK CMA KF KC NL MG. Wrote the paper: MG NFL with contributions from RK, CMA, KF, KC.

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Figures Legend

Figure 1: Rad9 is phosphorylated by Cdk1 in the absence of DNA damage.

(A) Cell cycle regulation of Rad9 phosphoforms. An a-factor block and release experiment with yeast strain CG378. Rad9 phosphoforms were identified in protein extracts by western blotting. The phospho-protein Swi6 serves as a loading control. The cell cycle phases of synchronously cycling cells determined by budding analysis are indicated above the blots. The insert in the graph shows Rad9 from asynchronous cells or cells arrested with a-factor, HU or nocodazole. (B) Rad9 phosphorylation is dependent on Cdc28 activity. Rad9 and Orc6 western blots shown are prepared from asynchronously growing and a-factor or nocodazole arrested *cdc28-as1* cells either mock or 1-NMPP1 treated. (C) Rad9 phosphorylation is dependent on Cdc28/Clb activity. The indicated cells were arrested with a-factor, shifted to 30°C and samples were taken at the indicated times for Rad9 western and FACS analysis. (D) Schematic representation of Rad9 showing the 20 consensus sites for phosphorylation by Cdc28 and those mutated to alanine in *rad9*^{12A} cells. (E) Rad9^{12A} is severely defective in cell cycle-dependent phosphorylation and *rad9*^{12A} cells cycle normally without detectable checkpoint activation. Western blot analysis of the indicated proteins from WT and isogenic rad9^{12A} cells.

Also see Figure S1.

Figure 2: The nine N-terminal CDK consensus sites contribute to Cdc28-dependent phosphorylation of Rad9 *in vivo* and are required for activation of Chk1. (A) Schematic representation of the Rad9$^{CDK1\text{-}9A}$ mutant protein. (B) Cells expressing Rad9$^{CDK1\text{-}9A}$ as their only Rad9 protein are not sensitive to the indicated DNA damaging treatments. Drop test were performed in the indicated strains. (C) Rad9$^{CDK1\text{-}9A}$ displays defective cell cycle and

Cdc28-dependent phosphorylation *in vivo*. Rad9 western blot prepared from the indicated strains. 1-NMPP1 treatment of *cdc28-as1* cells was used to indicate Cdc28-dependent phosphorylation. (D) DNA damage induced Chk1 phosphorylation is defective in $rad9^{CDK1\text{-}9A}$, $rad9^{CAD\Delta}$ and *rad9 Δ* cells. Asynchronously growing cells were treated with 4-NQO for the indicated times and Chk1 phosphorylation analysed by western blotting.

Figure 3: The CDK1-9 sites specifically act in the Chk1 branch of the G2/M checkpoint. Cells were grown and arrested in the cell cycle as indicated with either a-factor or nocodazole and treated with bleocin for the indicated times. Extracts prepared for western blotting of Chk1-3HA (A & B), Rad9 (C) or Rad53 (D). (E) The CDK1-9 sites of Rad9 function specifically in the Chk1 branch and not the Rad53 branch of the G2/M checkpoint. The indicated strains were examined for epistatic relationships using the G2/M checkpoint assay. All strains contained the *sml1Δ* mutation necessary for the viability of *rad53Δ* cells.
Also see Figure S2.

Figure 4: CDK is required for the activation and maintenance of Chk1-dependent signaling. Cdc28 activity was regulated using the 1-NMPP1 inhibitor in G2/M arrested *cdc28-as1* cells treated with bleocin or 4-NQO to examine the activation of Chk1 signaling (A) and the maintenance of Chk1 signaling (B). Rad9 and Rad53 were followed as markers of checkpoint activation, while Orc6 phosphorylation serves as a marker for Cdk1 inactivation.
Also see Figure S3.

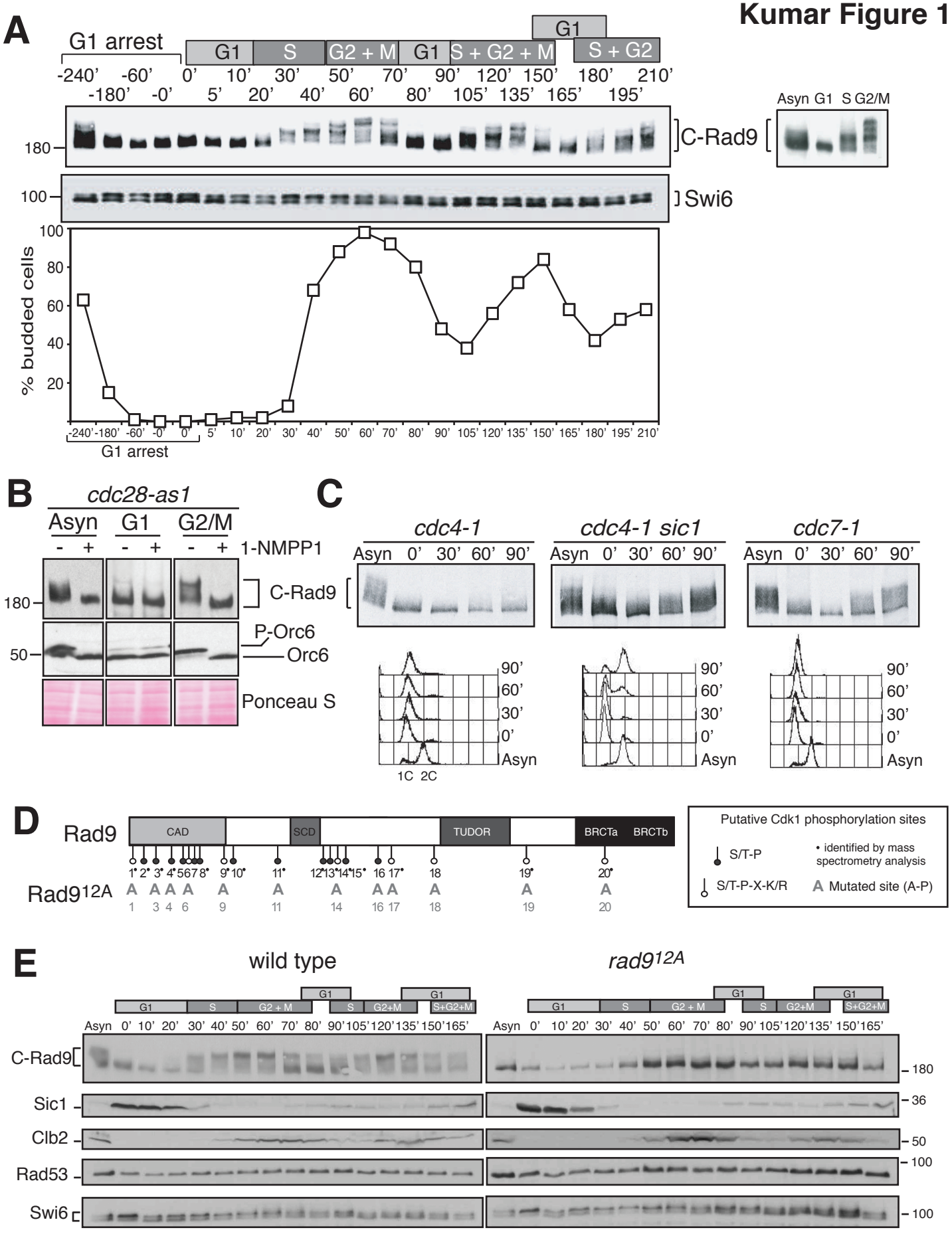
Figure 5: CDK-dependent phosphorylation of the nine N-terminal CDK sites in Rad9 regulates the Rad9-Chk1 interaction. (A) Rad9 and Chk1 interaction measured *in vivo* using a yeast two-hybrid (Y2H) assay is dependent on the CDK1-9 sites. Y2H interaction of

specific bait and prey plasmids shown on the left is indicated by the white color of the otherwise red cells, their resistance to Aureobasidin A and their blue color on media containing the X-alpha-gal substrate, as for the p53/T antigen interaction. (B) The Y2H interaction between Rad9 and Chk1 is dependent on CDK activity in G2/M cells. The indicated bait and prey plasmids were introduced into *cdc28-as1* cells treated or not with 1-NMPP1 1h after synchronization of cells with either nocodazole or alpha factor and prior to induction of bait expression. CDK-dependent Rad9-Dpb11 interaction was used as a control. (C) The Rad9 and Chk1 interaction measured using immunoprecipitation (IP) occurs both in the absence and presence of DNA damage. IPs were performed as indicated on extracts prepared from nocodazole-arrested cells, expressing both Chk1-3FLAG and Dpb11-13MYC, and either mock treated or treated with 20μg/ml of bleocin for 45 min. Mock (IgG) or MYC IPs were performed as controls. Rad9, Chk1-3FLAG and Dpb11-13MYC specific bands were detected in western blots. Lower exposures of the extracts are shown to facilitate their visualization. (D) Chk1 interaction with Rad9 is dependent on the CDK1-9 sites. As in panel C, except *rad9*$^{CDK1\text{-}9A}$ cells were used. (E) Western analysis of the indicated proteins in a reciprocal IP using Rad9-9MYC and Chk1-3FLAG expressing cells confirms the Rad9 and Chk1 interaction. Anti-MYC antibodies were used with extracts from nocodazole-arrested cells, treated with 20μg/ml of bleocin for 45 min and a mock (IgG) control was performed. Rad9 binding to Rad53 was used as a further control. Different exposures of the crude extracts and the IPs lanes are shown to allow visualization of Rad9-9MYC, Chk1-3FLAG and Rad53 specific bands. (F) ATP-dependent release of Rad53, but not Chk1 from Rad9 IPs. Assays were performed as described (Gilbert et al 2001) except Rad9-9MYC was immunoprecipitated using an anti-MYC monoclonal antibody. The extract was prepared from nocodazole-arrested cells, treated with 20μg/ml of bleocin for 45 min that expressed

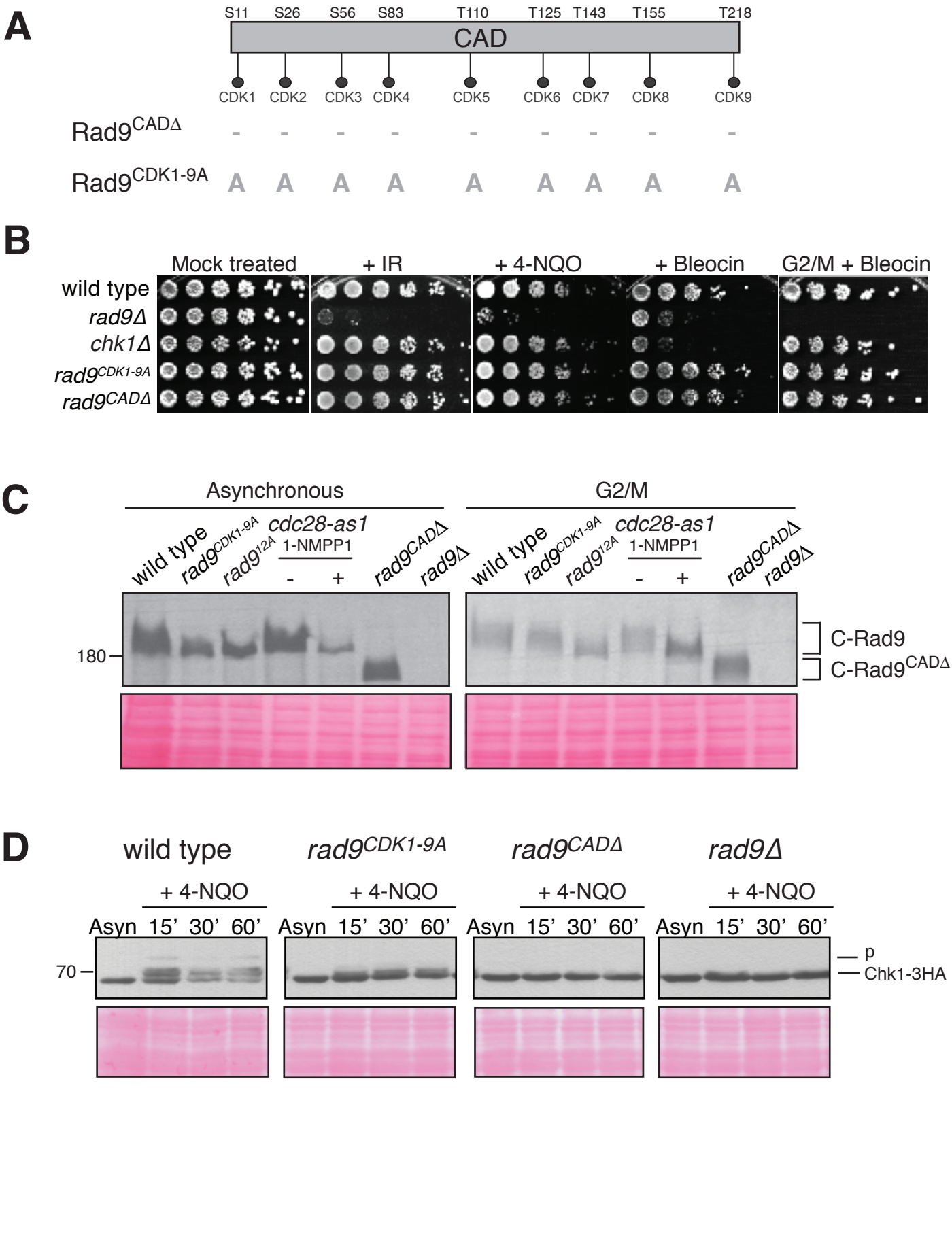
both Rad9-9MYC and Chk1-3FLAG. The amount of Rad9-9MYC, Chk1-3FLAG or Rad53 remaining on the beads (Beads) or released (Elution) after incubation with ATP (+), γS-ATP (γS), a non-hydrolysable analogue, or mock treatment without any nucleotide (-) was determined by western blotting. Different exposures of the crude extracts, elutions and beads lanes are shown to allow visualization of Rad9, Chk1 and Rad53 specific bands. (G) Model of Chk1 activation in response to DNA damage. See text for details.

Also see Figure S4.

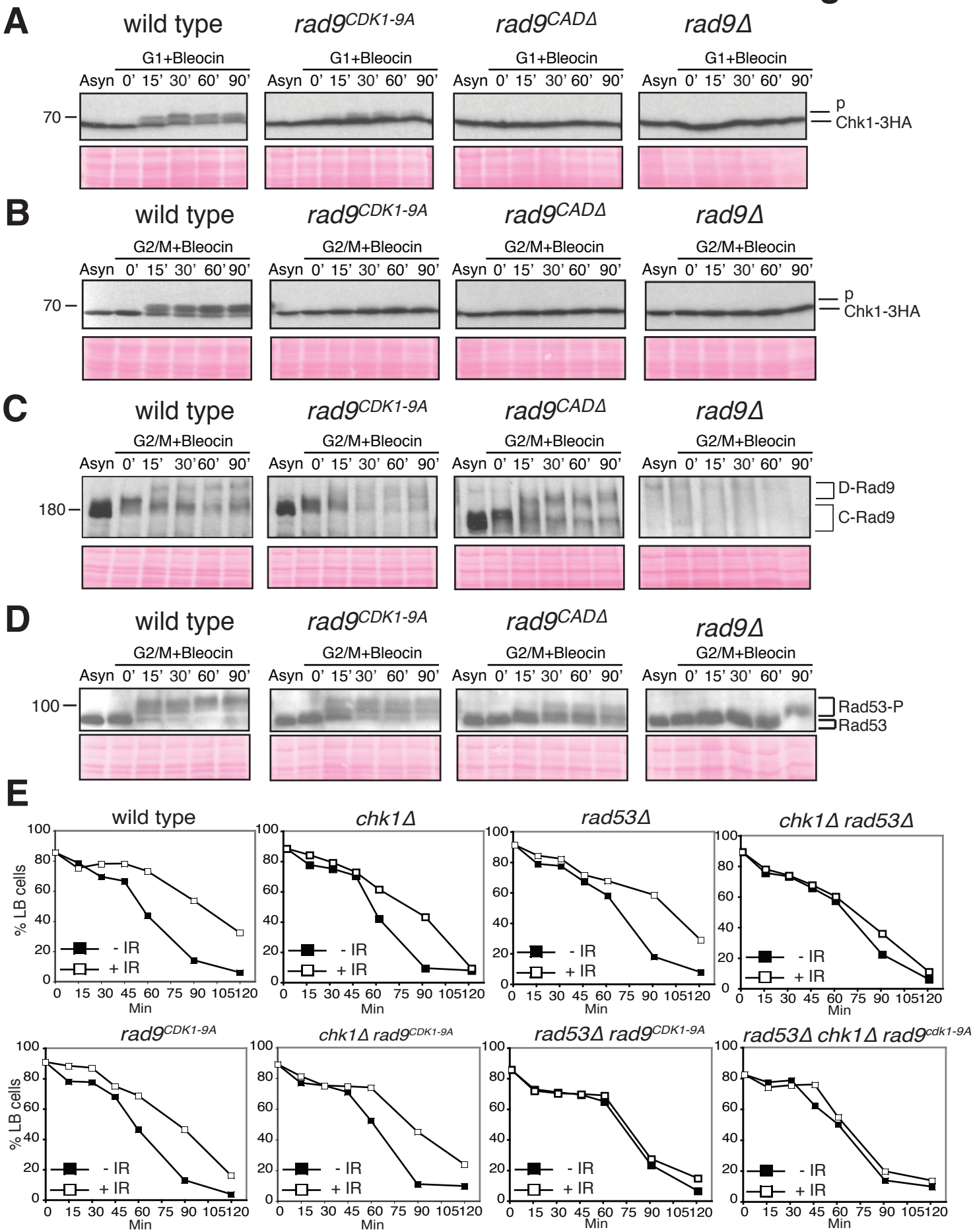
Kumar Figure 1



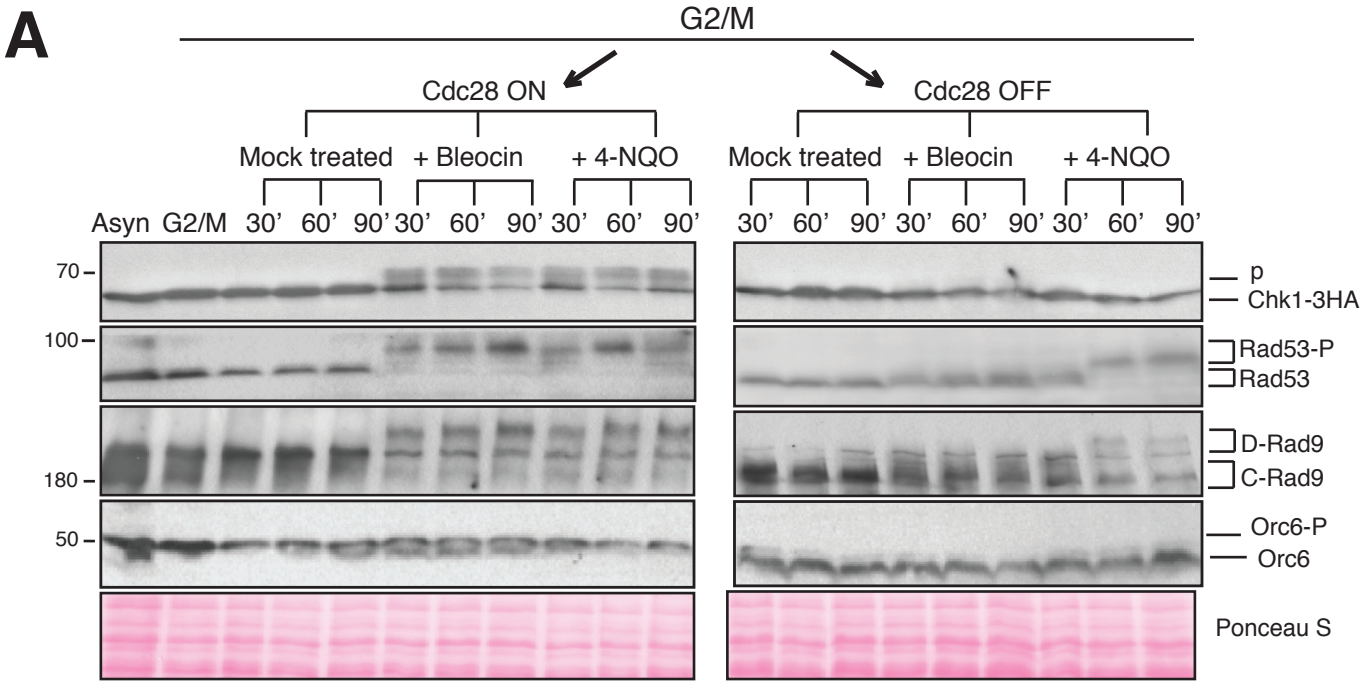
Kumar - Figure 2

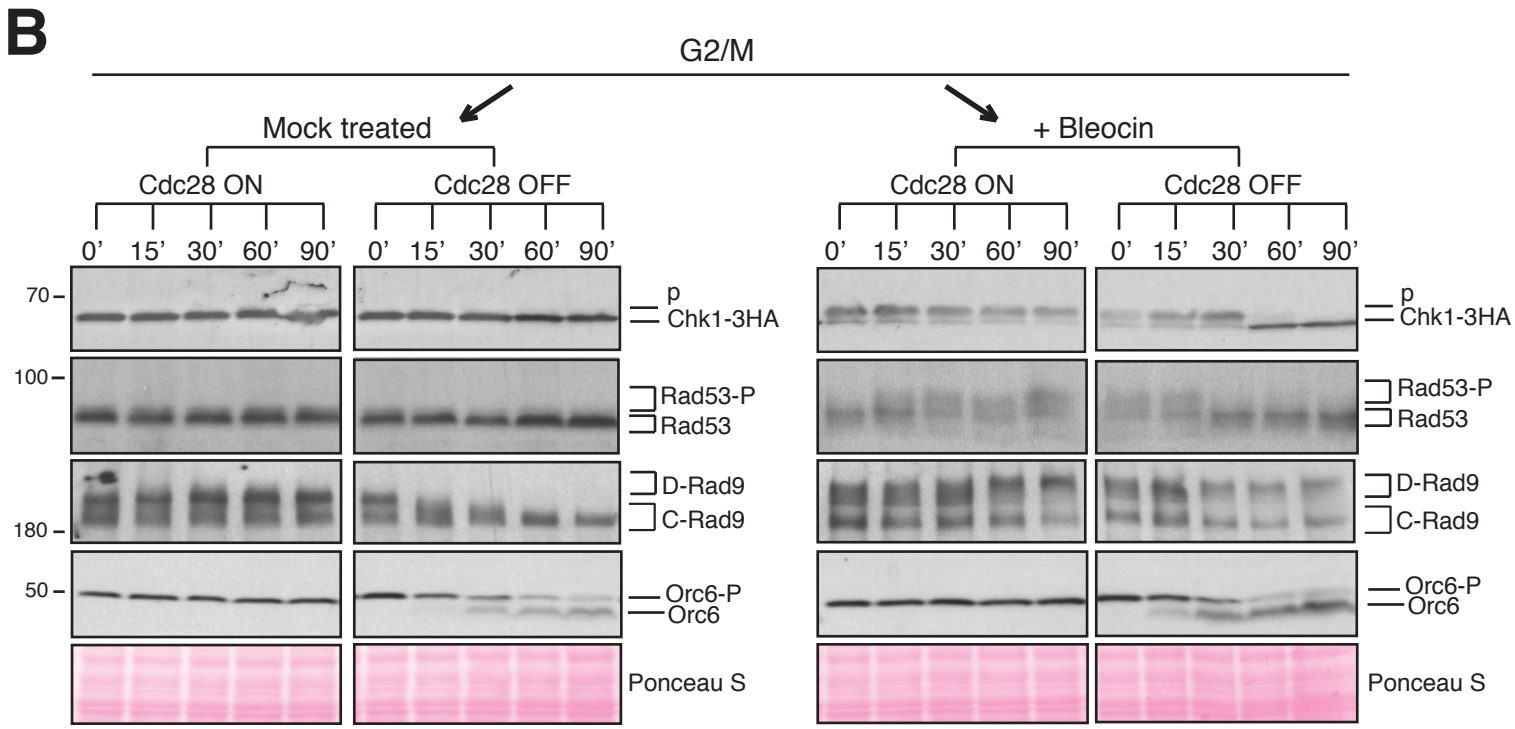
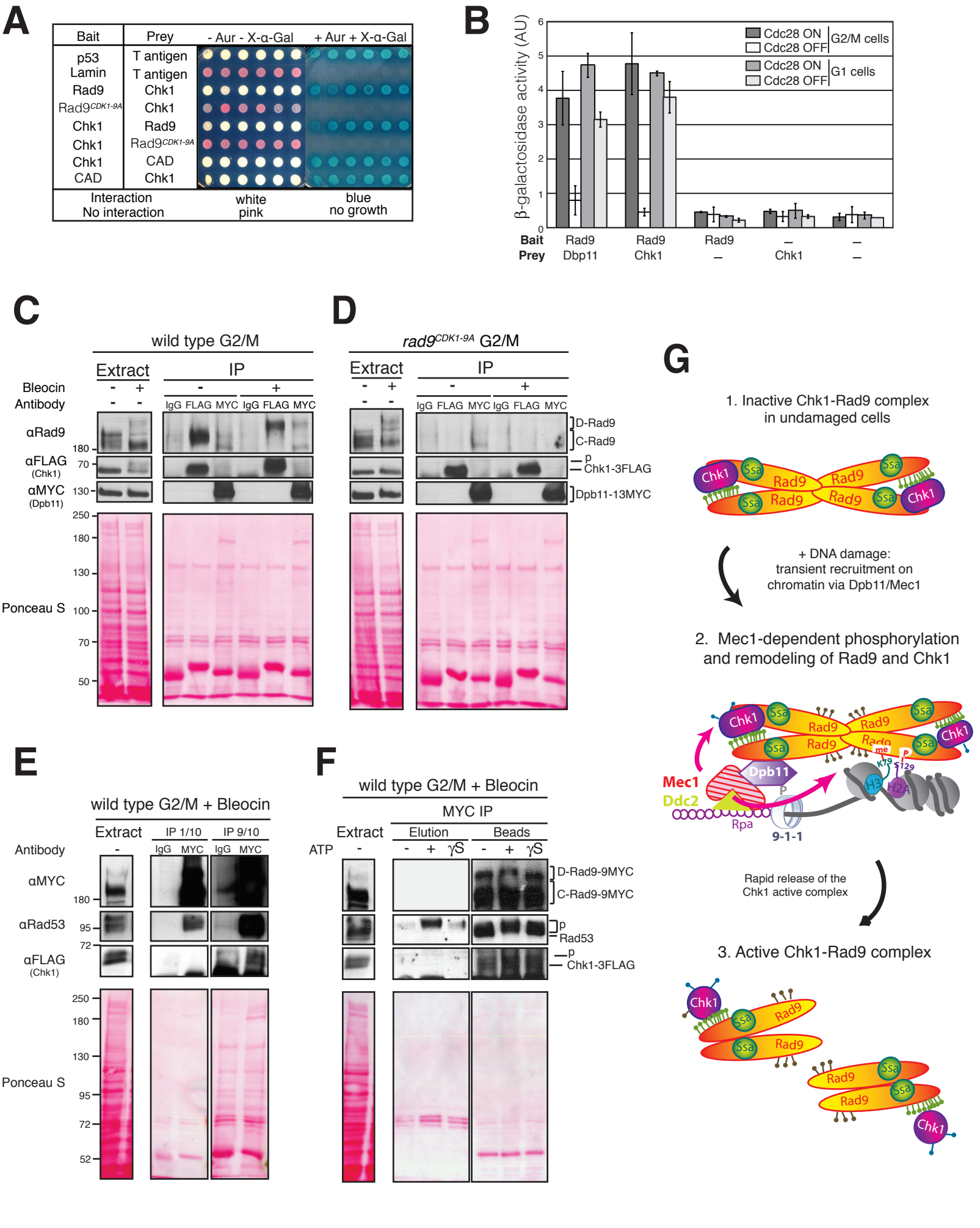


Kumar - Figure 3



Kumar - Figure 4





Kumar - Figure 5