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Brief report

Dissection of Rad9 BRCT domain function in the mitotic checkpoint response to telomere uncapping

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ABSTRACT

In *Saccharomyces cerevisiae*, destabilizing telomeres, via inactivation of telomeric repeat binding factor Cdc13, induces a cell cycle checkpoint that arrests cells at the metaphase to anaphase transition—much like the response to an unrepaired DNA double strand break (DSB). Throughout the cell cycle, the multi-domain adaptor protein Rad9 is required for the activation of checkpoint effector kinase Rad53 in response to DSBs and is similarly necessary for checkpoint signaling in response to telomere uncapping. Rad53 activation in G1 and S phase depends on Rad9 association with modified chromatin adjacent to DSBs, which is mediated by Tudor domains binding histone H3 di-methylated at K79 and BRCT domains to histone H2A phosphorylated at S129. Nonetheless, Rad9 Tudor or BRCT mutants can initiate a checkpoint response to DNA damage in nocodazole-treated cells. Mutations affecting di-methylation of H3 K79, or its recognition by Rad9 enhance 5′ strand resection upon telomere uncapping, and potentially implicate Rad9 chromatin binding in the checkpoint response to telomere uncapping. Indeed, we report that Rad9 binds to sub-telomeric chromatin, upon telomere uncapping, up to 10 kb from the telomere. Rad9 binding occurred within 30 min after inactivating Cdc13, preceding Rad53 phosphorylation. In turn, Rad9 Tudor and BRCT domain mutations blocked chromatin binding and led to attenuated checkpoint signaling as evidenced by decreased Rad53 phosphorylation and impaired cell cycle arrest. Our work identifies a role for Rad9 chromatin association, during mitosis, in the DNA damage checkpoint response to telomere uncapping, suggesting that chromatin binding may be an initiating event for checkpoints throughout the cell cycle.

1. Introduction

In metazoans, unrepaired DNA double strand breaks (DSBs) are a deleterious form of DNA damage that can lead to inactivation of tumor suppressors, activation of oncogenes and the promotion of carcinogenesis. In response to DSBs, cells initiate a checkpoint response that senses DNA lesions and signals to downstream effectors to induce cell cycle arrest and DNA repair. Signal transduction in response to DNA damage has been reviewed extensively and many of the checkpoint signaling proteins are conserved from yeast to humans [1–3]. In *Saccharomyces cerevisiae*, DSB induction recruits the Mre11–Rad50–MRXrs2 complex (MRE11–RAD50–NBS1 in humans) and Tel1 (ATM) to breaks [1,4] where Tel1 may phosphorylate histone H2A (H2AX) at S129 [5,6]. Next, the multi-domain adaptor protein Rad9 (53BP1, MDC1, BRCA1) localizes to chromatin adjacent to DSBs [7–10] and DNA ends undergo 5′–3′ resection in the late S and G2/M phases. The Rfa1–Rfa2–Rfa3 heterotrimer (RPA) binds to ssDNA and recruits the Rad24 (RFC) clamp loader to assemble the Rad17–Mec3–Ddc1 clamp (Rad9–Rad1–Hus1) at the junction of ssDNA and dsDNA [11]. The RFA, Rad24 and Rad17 complexes recruit Ddc2 (ATRIP) and the Mec1 (ATR) kinase to breaks [4,12–15]. Mec1 phosphorylates Rad9, which recruits Rad53 (CHK2) to DSBs and leads to Mec1-dependent phosphorylation and auto-phosphorylation of Rad53 [16–18]. Finally, Rad53 kinase activity propagates a DNA damage signal that leads to cell cycle arrest and DNA repair.

The nucleosome, the basic unit of chromatin, is an octameric DNA–protein complex consisting of histones H3, H4, H2A, H2B and 146 bps of DNA [19,20]. Histones can be modified by ubiquitination, phosphorylation, methylation and acetylation [21]. These chromatin modifications alter the interaction among histones, DNA and other proteins, and thereby play significant roles in the DNA damage response [1,22–27]. For example, the mammalian

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phosphoinositide-3-kinase-related protein kinases (PIKKs) ATM, ATR and DNA-PK, phosphorylate H2A variant H2AX in response to DNA damage [28–30]. In budding yeast, histone H2A is phosphorylated by Mec1 and Tel1 kinases to form a chromatin domain that extends up to 50 kb from DNA lesions and recruits chromatin modifiers including NuA4, Ino80 and SWR [23,31–33]. H2A phosphorylation may lead to changes in chromatin structure [34,35] but also mediates recruitment and retention of checkpoint proteins, including Rad9 homologs 53BP1, MDC1 and BRCA1, at DNA lesions [36]. Recruitment of MDC1, like *Schizosaccharomyces pombe* homolog Crb2, is mediated by direct binding to phosphorylated H2A via tandem BRCA1 carboxyl-terminal (BRCT) domains [37,38]. Moreover, *hta1, 2-S129A* point mutants are sensitive to DSB-inducing agents, deficient in non-homologous end joining and have G1 and S phase checkpoint defects [9,10,35].

The biology of telomeres, specialized chromatin domains that protect chromosome ends, has been reviewed extensively [39–43]. When the telomere is "uncapped" by the loss of one or more protein components that bind to $G_{1-3}T::C_{1-3}A$ repeats, the resulting structure resembles one end of a natural DSB and activates a checkpoint response during mitosis. Cdc13 [44], a telomere-specific protein in yeast that binds to single stranded $G_{1-3}T::C_{1-3}A$ overhangs, plays a key role in the recruitment of telomerase [45] and telomere capping [46]. The temperature sensitive *cdc13-1* P371S allele binds single stranded telomeric repeats at permissive temperatures (<25 °C) but dissociates at restrictive temperatures (>26 °C). When held in G1 with α-factor and shifted to non-permissive temperature, *cdc13-1* mutants do not activate Rad53 [47]. However, with the accumulation of Cdc28 activity in late S or G2/M phases, uncapped telomeres undergo 5′–3′ resection to generate long regions of ssDNA [46], activating Rad53 and a sustained mitotic checkpoint arrest at the metaphase to anaphase transition. Thus, the characteristic changes in gene expression and cell cycle progression in *cdc13-1* mutants at non-permissive temperature resemble the response to ionizing radiation [46,48–50]. As such, the *cdc13-1* mutation has served as a powerful tool to elucidate molecular mechanisms mediating the G2/M checkpoint response to DNA damage [39,44,51–53].

Seminal work characterizing the DNA damage checkpoint in budding yeast identified Rad9 as a key mediator in signaling [54,55]. When challenged with genotoxic agents or ionizing radiation, mutants deleted for the *RAD9* gene display DNA damage sensitivity, fail to activate Rad53, and display checkpoint defects at each phase of the cell cycle. Similarly, *rad9Δ cdc13-1* cells fail to arrest, instead dividing to form microcolonies of inviable cells after temperature shift [44,51,56–60]. Rad9, 53BP1 and Crb2 share a domain structure characterized by tandem BRCT and Tudor domains at the carboxyl terminus. Although the Rad9 BRCT domains have been implicated in oligomerization [61,62], the Rad9 BRCT domains also interact with phosphorylated H2A peptides *in vitro* and disrupting this interaction leads to G1 and intra-S phase checkpoint defects [9,10,61]. Consistent with dual recognition of chromatin by Rad9, chromatin immunoprecipitation studies demonstrate that H3 K79 di-methylation and H2A S129 phosphorylation are both necessary for Rad9 chromatin association [9,10,61]. The Rad9, 53BP1 and Crb2 Tudor domains have been shown to bind di-methylated histone H3 at K79 and/or H4 K20 *in vitro* [7,63]. Yeast strains deficient in the conserved H3 K79 methyl-transferase Dot1 or expressing the Tudor domain mutant *rad9-Y798Q* display G1 and intra-S phase checkpoint defects after ionizing radiation [8]. In addition, *rad9Δ, rad9-Y798Q* Tudor domain mutants and *dot1Δ* strains harboring the *cdc13-1* mutation also have an increased rate of ssDNA accumulation at uncapped telomeres [51,56,57,59].

While Rad9 is necessary for checkpoint signaling in response to telomere uncapping, a role for Rad9 chromatin association in signaling damage to telomeres remains unclear. In this study, we targeted the putative Rad9 phospho-H2A binding site to analyze the role of H2A phosphorylation in checkpoint signaling at uncapped telomeres. Comparative protein modeling was used to identify residues in the Rad9 BRCT domains that define a phospho-H2A binding pocket. As expected, mutation of these residues resulted in checkpoint defects in G1 and S phase after ionizing radiation. However, contrary to nocodazole-based assays, *rad9* BRCT mutants displayed an attenuated or impaired checkpoint response to telomere uncapping. In addition, chromatin immunoprecipitation analysis shows that Rad9 binds to sub-telomeric chromatin and this interaction is dependent on both chromatin-binding domains. Taken together, these results demonstrate that Rad9 chromatin association at sub-telomeric chromatin is important for DNA damage checkpoint activation in the response to telomere uncapping during mitosis.

2. Materials and methods

2.1. Western blot analysis

For Western blot analysis, OD_{600} of yeast cultures were measured and volumes corresponding to 2–2.5 OD_{600} units were collected. Cell pellets were treated with 0.2N NaOH for at least 5 min and resuspended in 100 μl of 1× SDS sample buffer (125 mM Tris–HCl pH 6.8, 20% glycerol, 4% SDS, 1.43 M β-mercaptoethanol). Samples were incubated at 95 °C for 5 min and centrifuged at 16,100 × *g* for 1 min to clarify the lysate. 15 μl of cell lysate was fractionated with NUPAGE 3–8% TA gels (Invitrogen) and transferred to nitrocellulose. Rad53-FLAG blots were probed using M2 mouse anti-FLAG (Sigma, 1:1000), and Rad9–13Myc blots using 9E10 mouse anti-MYC (1:200, Santa Cruz), and detected with goat anti-mouse IgG HRP conjugate secondary antibody. Phospho-H2A Western blotting was performed with rabbit anti-yeast histone H2A phospho-Ser129 (Millipore 1:100 in 1× PBS) and tubulin was detected using 1:500 YL1/2 rat anti-tubulin (Millipore) and HRP conjugated goat anti-rat IgG secondary antibody. Figures are representative of two or more replicate experiments.

2.2. cdc13-1 checkpoint assays

Rad53 activation and flow cytometry analysis: Strains carrying the *cdc13-1* allele were incubated at 25 °C in α-factor (WHWLQLKPGQPNleY) [64] for 1 h, transferred to 37 °C for 1 h and then released into 37 °C media for an additional 3 h. Samples were collected hourly and analyzed for Rad53 activation and DNA content.

Microcolony analysis: Sonicated and serially diluted *cdc13-1* strains were plated and incubated at 25, 30 or 37 °C for 6.5–7 h. Next, plates were transferred to 4 °C until scored, as previously described [55]. Briefly, microcolonies with ≤four cell bodies were scored as arrested and microcolonies with >four cell bodies were scored as cycling. The data shown are the average of three replicates, mean ± SD.

2.3. Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were performed as described previously [8] with minor modifications. Briefly, overnight cultures grown in minimal media were pelleted, washed with sterile water and grown in YPD to OD_{600} 0.3–0.4 at 20 °C. α-Factor was added to the final concentration of 15 μg/ml and the culture was grown for 1 h at room temperature and then shifted to 37 °C for 30 min. Cells were pelleted and washed once with sterile water and twice with pre-warmed media. Cells were resuspended in 200 ml YPD and grown at 37 °C. Samples were collected at 30 min, 1 h and 2 h time points and cross-linked with 1% formaldehyde for 20 min at 20 °C. Cross-linked chromatin was sonicated (Diagenode