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Article

Repo-Man Controls a Protein Phosphatase 1-Dependent Threshold for DNA Damage Checkpoint Activation

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Summary

Background: In response to DNA damage, cells activate checkpoints to halt cell-cycle progression and prevent genomic instability. Checkpoint activation induced by DNA double-strand breaks (DSB) is dependent on the ATM kinase, a master regulator of the DNA damage response (DDR) that is activated through autophosphorylation and monomerization.
Results: Here we show that either protein phosphatase 1 or 2A is sufficient to suppress activation of the DDR and that simultaneous inhibition of both phosphatases fully activates the response. PP1-dependent DDR regulation is mediated by its chromatin-targeting subunit, Repo-Man. Studies in *Xenopus* egg extracts demonstrate that Repo-Man interacts with ATM and PP1 through distinct domains, leading to PP1-dependent regulation of ATM phosphorylation and activation. Consequently, the level of Repo-Man determines the activation threshold of the DNA damage checkpoint. Repo-Man interacts and extensively colocalizes with ATM in human cells. Expression of wild-type, but not PP1 binding-deficient, Repo-Man attenuates DNA damage-induced ATM activation. Moreover, Repo-Man dissociates from active ATM at DNA damage sites, suggesting that activation of the DDR involves removal of inhibitory regulators. Analysis of primary tumor tissues and cell lines demonstrates that Repo-Man is frequently upregulated in many types of cancers. Elevated Repo-Man expression blunts DDR activation in precancerous cells, whereas knockdown of Repo-Man in malignant cancer cells resensitizes the DDR and restrains growth in soft agar.
Conclusions: We report essential DDR regulation mediated by Repo-Man-PP1 and further delineate underlying mechanisms. Moreover, our evidence suggests that elevated Repo-Man contributes to cancer progression.

Introduction

To protect genomic integrity after DNA damage, cells have evolved surveillance mechanisms, generally termed the "DNA damage response (DDR)," that encompass both DNA repair and signal transduction pathways, activating cell-cycle checkpoints and arresting cell-cycle progression [1, 2]. The DDR to DNA double-strand break (DSB) is initiated by activation of the ataxia telangiectasia mutated (ATM) Ser-Thr kinase, which triggers multiple mechanisms of signal amplification. Activation of ATM involves intermolecular autophosphorylation so that a small pool of activated ATM at the site of DSBs rapidly induces ATM autophosphorylation throughout the cell [3]. Moreover, ATM anchoring to chromatin by γ-H2AX and adaptors, such as Mdc1 and the Mre11-Rad50-Nbs1 complex, results in expansion of H2AX phosphorylation to large chromatin regions flanking DSBs [4]. A potential consequence of these amplification mechanisms is that minimal DNA damage may eventually cause full activation of the DDR. However, recent studies indicate that a threshold level of DNA damage has to be reached for the checkpoint to affect cell-cycle progression. In *Xenopus*, 2–4 ng/μl of damaged DNA is required to elicit DNA damage checkpoint signaling in egg extracts and to slow cleavage cycles in embryos [5, 6]. Similarly, a defined G2/M checkpoint threshold of 10–20 DSBs per cell has been reported in human cells, and DNA damage below that threshold level neither efficiently activates the checkpoint nor sustains it prior to completion of DNA repair [7, 8]. It is currently unclear how DNA damage thresholds are achieved and regulated; a credible speculation is that cells have evolved inhibitory mechanisms to prevent checkpoint activation by a subthreshold level of DNA damage.

Recently, several Ser-Thr protein phosphatases have been found responsible for deactivation of the DNA damage checkpoint during recovery from cell-cycle arrest [9–11]. A well-studied example is PP2Cδ/Wip1, which has been shown to dephosphorylate multiple phospho-S/TQ sites targeted by ATM or ATR, including those in ATM itself and its substrates [12, 13]. Also connected to the checkpoint recovery pathway are PP1 and PP2A, which together account for 95% of total cellular Ser-Thr phosphatase activity. Unlike Wip1 or other PP2C family members, specific functions of PP1 and PP2A are conferred by additional targeting subunits that control their subcellular localization and substrate specificity [10, 11]. Studies in yeast and mammalian cells show that specific PP2A and PP4 (PP2A-like) complexes are responsible for γ-H2AX dephosphorylation during checkpoint recovery [14–16]. The involvement of PP1 in checkpoint recovery is less well studied, but in *S. pombe*, dephosphorylation of Chk1 by Dis2 (a PP1 homolog) allows mitotic entry upon completion of DNA repair in G2 [17]. The specific targeting subunit in yeast or higher eukaryotes that mediates PP1 regulation of DDR factors has yet to be identified.

Given that activation of the DDR relies on protein phosphorylation by ATM and other kinases, it is possible that protein phosphatases, particularly PP1 and PP2A, create a sensitivity threshold for DNA damage checkpoint activation in addition to promoting checkpoint recovery. In this study, we show that the chromatin-bound Repo-Man-PP1γ complex modulates ATM activation, thereby setting the threshold for checkpoint activation. Importantly, Repo-Man (*re*cruits *P*P1 *o*nto *m*itotic chromatin at *an*aphase, also known as Cdca2) is frequently upregulated in various cancers, and overexpression is both necessary and sufficient for reduced DDR sensitivity during cancer progression. Reduction of Repo-Man expression attenuates growth of breast cancer cells in soft agar, arguing that upregulation of Repo-Man is essential for anchorage-independent growth of at least some tumor cells.

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Results

PP1 and PP2A Both Regulate DNA Damage Response Activation

PP1 and PP2A are the two major forms of Ser-Thr phosphatase in *Xenopus* egg extracts [18, 19]. To investigate whether PP1 and PP2A are required to suppress DDR activation, we utilized microcystin-LR (MC) to inhibit PP1 and PP2A phosphatases in undamaged *Xenopus* egg extracts, which have been widely used to study the DDR [20, 21]. Quite strikingly, MC induced robust phosphorylation of Smc1, H2AX, Chk1, Chk2, and Mre11, as judged by phospho-antibody blotting or retarded electrophoretic mobility (Figure 1A). Okadaic acid (OA) at 2 μM inhibits most PP2A and PP1 activity [18, 22] and was sufficient to activate responses similar to those induced by MC (Figure 1A). In contrast, OA at 0.4 μM that inhibits only PP2A activity [18, 22], or Inhibitor-2 (I-2) at 0.4 μM that specifically inhibits PP1 [23], did not elicit significant activation of Smc1, Chk1, Chk2, or Mre11 phosphorylation, despite minimal H2AX phosphorylation (Figure 1A). Interestingly, extracts treated with both OA at 0.4 μM and I-2 exhibit strong phosphorylation of Smc1, Chk1, Chk2, H2AX, and Mre11 (Figure 1A). Taken together, these results indicate that both PP1 and PP2A are involved in DDR regulation. Either PP1 or PP2A alone is sufficient to suppress spontaneous DDR activation, and inhibition of both phosphatases synergistically induces DDR signaling without actual DNA damage. The critical involvement of PP1 in DDR regulation is also supported by evidence that PP1 inhibition sensitizes DDR activation. When *Xenopus* egg extracts were supplemented with I-2 to inhibit PP1, we observed an elevated response to low-dose DNA damage, added as either cut plasmid DNA (Figure 1B) or double-stranded oligonucleotides (Figure 1C).

Repo-Man Recruits PP1 to Chromatin to Suppress DNA Damage Response Activation

MC-induced Chk2 and Chk1 phosphorylation was more pronounced in extracts supplemented with sperm DNA (see Figure S1A available online), which itself is undamaged and does not activate the checkpoint on its own [6]. The DNA dependence of MC-induced Chk1 and Chk2 phosphorylation suggests that inhibition of protein phosphatases produces chromatin-based signal transduction like that induced by actual DNA damage [24]. An attractive hypothesis is that a chromatin-bound protein phosphatase suppresses DDR activation and that its inhibition promotes DDR signaling on chromatin. The γ isoform of PP1 has been reported to be present on chromatin [25], and we confirmed that a portion of PP1γ binds to chromatin in *Xenopus* egg extracts, whereas neither PP1α nor PP2A were detectable on chromatin (Figure S1B). To investigate the relevance of PP1γ to activation of the DNA damage checkpoint, we immunodepleted PP1γ from egg extracts and observed that the extracts became more sensitive to DNA damage, whereas add-back of recombinant PP1γ abolished the hypersensitivity (Figures 2A and 2B). These results suggest that PP1γ is involved in suppression of DNA damage signaling.

Unlike other protein phosphatases, specific functions of PP1 are achieved through interactions with targeting subunits that contain a consensus PP1-interacting motif, RVxF [10]. A PP1γ-specific interacting protein, Repo-Man, has been reported to recruit PP1γ onto mitotic chromosomes [26, 27]. The interaction between Repo-Man and PP1γ persists through interphase, implying that Repo-Man could also be responsible for chromatin localization of PP1γ in interphase [26]. We cloned the Repo-Man homolog in *Xenopus* (GenBank accession number FJ532285) and raised an antibody against its C terminus (Figures S2A–S2C). Affinity-purified PP1γ antibody coimmunoprecipitated Repo-Man from interphase egg extracts, confirming interaction between PP1γ and Repo-Man in *Xenopus* (Figure S2D). Furthermore, specific chromatin binding of PP1γ and Repo-Man was evident in *Xenopus* egg extracts (Figure S2G), whereas knockdown of endogenous Repo-Man with antisense oligonucleotides (Figure S2C) or disruption of Repo-Man-PP1γ interaction with a consensus RVTF peptide (Figures S2E and S2F) reduced binding of PP1γ to chromatin (Figure S2H). We thus confirm in *Xenopus* that Repo-Man interacts with PP1 through its RVTF motif and recruits PP1 onto chromatin. Importantly, addition of the RVTF peptide, but not the control RATA peptide, led to Chk1 and Smc1 phosphorylation with a lower dose of damaged

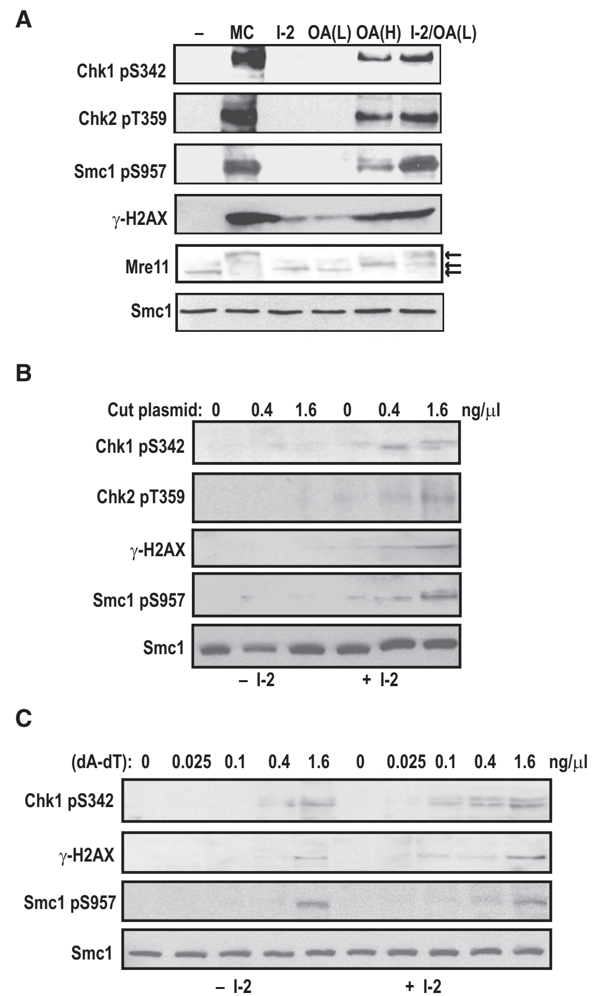

Figure 1. Inhibition of PP1 Enhances DNA Damage Checkpoint Signaling

(A) Interphase *Xenopus* egg extracts containing 1000 sperm nuclei/μl were treated with microcystin (MC) at 3 μM, okadaic acid (OA) at 2 or 0.4 μM (marked as H or L), or Inhibitor-2 (I-2) at 0.4 μM as indicated and analyzed by western blotting with the indicated antibodies.

(B and C) Interphase egg extracts as in (A) were mock (buffer) or I-2 treated and then supplemented with EcoR1-linearized pGEX 4T-1 plasmid (cut plasmid) DNA (B) or a double-stranded oligonucleotide (dA-dT) (C) at the indicated concentrations. Samples were then analyzed by western blotting as indicated. See also Figure S1.

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