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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

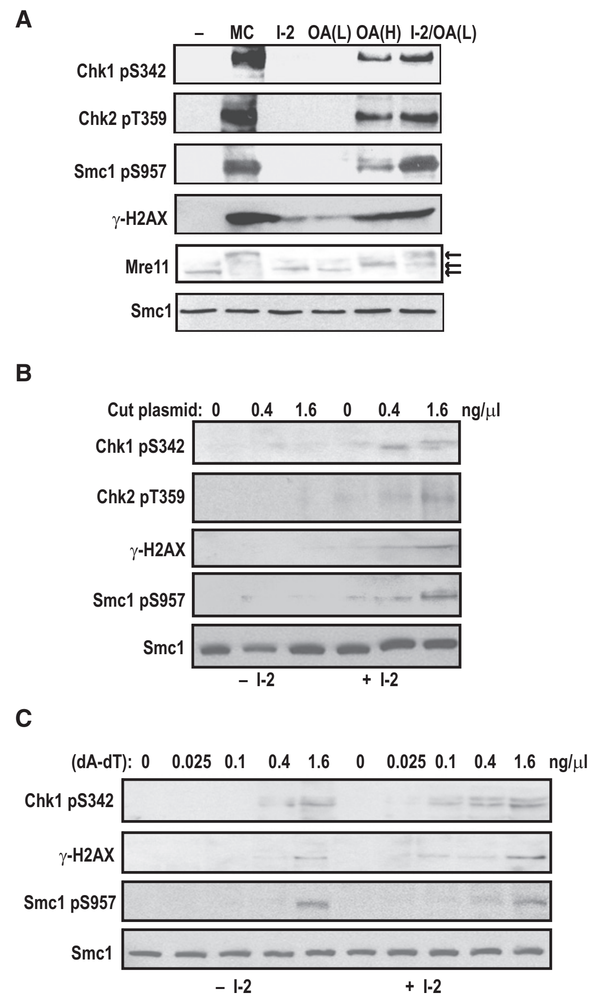

Figure 1. Inhibition of PP1 Enhances DNA Damage Checkpoint Signaling

(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.

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

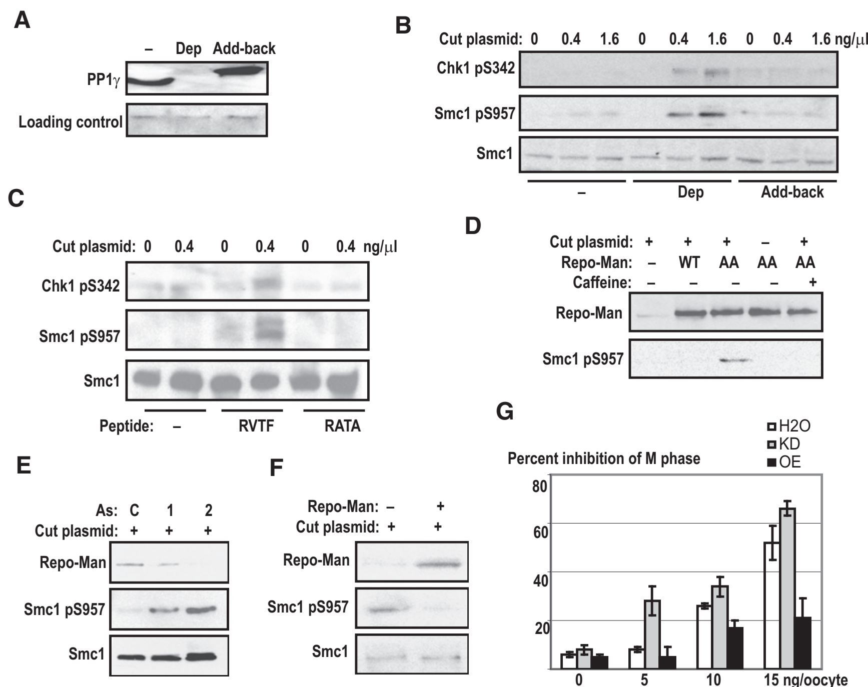

Figure 2. Repo-Man/PP1γ Regulates DNA Damage Checkpoint Activation

(A) Extracts were mock treated with beads alone (–), immunodepleted of PP1γ with anti-PP1γ beads (Dep), or PP1γ-depleted and then reconstituted with purified recombinant His$_6$-PP1γ (Add-back). The level of PP1γ was monitored by western blotting, and a nonspecific band served as a loading control.

(B) Extracts in panel (A) were supplemented with cut plasmid DNA at the indicated concentrations and monitored by western blotting as indicated. Depletion of PP1γ increased DNA damage response (DDR) signaling.

(C) Egg extracts were supplemented with water (–), RATA, or RVTF peptides (Figure S2B) and then treated with or without 0.4 ng/μl of cut plasmid DNA and analyzed by western blotting. Disruption of PP1γ binding by the RVTF peptide increased the DDR.

(D) Immature *Xenopus* oocytes were preinjected with mRNA encoding full-length wild-type (WT) or mutant (AA) Repo-Man and incubated overnight. Oocyte extracts were prepared and supplemented with 2 ng/μl of cut plasmid DNA with or without 10 mM caffeine, an ATM inhibitor, and analyzed by western blotting.

(E and F) Oocytes were preinjected with two different Repo-Man antisense oligonucleotides (1 and 2), a control (c) oligonucleotide (E), or Repo-Man mRNA (F) and incubated overnight. Oocyte extracts were prepared and supplemented with cut plasmid DNA for 30 min, followed by western blotting of the indicated proteins.

(G) Immature *Xenopus* oocytes preinjected with an antisense oligonucleotide (Figure 2E, "2") to knock down (KD) Repo-Man or with mRNA to overexpress (OE) Repo-Man were incubated overnight and then injected again with the indicated concentrations of cut plasmid DNA. Oocytes were then stimulated with progesterone to undergo M-phase progression, and after 200 min the percentage of oocytes that remained blocked in interphase was scored. The mean ± standard error of the mean (SEM) (n = 3) of replicate experiments is shown. See also Figures S1 and S2.

DNA (Figure 2C). In addition, expression of a mutant Repo-Man, in which the RVTF motif was changed to RATA, also reduced PP1 association (Figure 3A) and enabled Smc1 phosphorylation with a lower amount of damaged DNA (Figure 2D). The enhanced response was DNA damage induced and caffeine sensitive, confirming that it results from ATM- or ATR-dependent signal transduction (Figure 2D). Moreover, reducing the level of Repo-Man expression with two different antisense oligonucleotides increased sensitivity to DNA damage in a fashion correlated with the efficiency of the knockdown (Figure 2E). Conversely, ectopic expression of Repo-Man suppressed DNA damage-induced signaling in egg extracts, as shown by phosphorylation of Smc1 (Figure 2F). To assess DNA damage checkpoint activation by induction of cell-cycle arrest in vivo, we studied progesterone-induced *Xenopus* oocyte maturation, a model of the G2/M transition. Injection of damaged DNA activated the checkpoint and blocked the transition to M phase (Figure S2I). When the Repo-Man level was reduced by antisense oligonucleotides, the minimal amount of DNA damage required to affect cell-cycle progression was lowered, whereas oocytes with overexpressed Repo-Man underwent maturation despite the presence of DNA damage at doses sufficient to activate the checkpoint in control oocytes (Figure 2G). Therefore, these results establish Repo-Man as a negative regulator of the DDR. By targeting PP1γ to chromatin to inhibit checkpoint signaling, Repo-Man dictates the cellular response to low-dose DNA damage and sets a threshold for checkpoint activation.

Repo-Man Targets PP1 to Modulate ATM Activation

Among components of the DNA damage signaling pathway, especially those that bind to chromatin, we observed that ATM, but not Chk1 or H2AX, was copurified from egg extracts in a pull-down assay with His-tagged Repo-Man, wild-type or mutated in the PP1 binding site (Figures 3A and 3B), suggesting that Repo-Man association with ATM is independent of its association with PP1. The extracts were not supplemented with sperm DNA, and thus Repo-Man-PP1 association is not mediated by chromatin. The interaction was confirmed with endogenous Repo-Man and ATM coimmunoprecipitation in *Xenopus* egg extracts (Figure 3C). The ATM-interacting domain of Repo-Man was mapped to its C terminus, whereas the middle part of Repo-Man, which contains the RVTF motif, was sufficient to bind PP1γ (Figure 3D). By binding to PP1γ in one region of the molecule and to ATM in another, Repo-Man acted as a bridge for PP1-ATM interaction, which was reduced in extracts with Repo-Man knockdown (Figure S3A). In light of DDR regulation by protein phosphatases, we speculate that Repo-Man brings PP1γ adjacent to ATM, leading to regulation of ATM. ATM autophosphorylated at Ser 1981 was efficiently dephosphorylated by PP1γ in vitro (Figure S3B), and, more importantly, extracts with a reduced level of Repo-Man exhibited augmented ATM autophosphorylation with or without DNA damage (Figure 3E, top). Consistently, ATM kinase activity in extracts was enhanced by Repo-Man knockdown (Figure 3E, bottom). Taken together, our evidence pinpoints ATM as a key substrate of the Repo-Man-PP1γ enzymatic complex.

Repo-Man Is Associated and Colocalized with ATM and Regulates ATM Activation in Human Cells

The interaction between ATM and Repo-Man identified in *Xenopus* egg extracts (Figures 3A–3D) is conserved in human cells. As shown in Figure 4A, Repo-Man

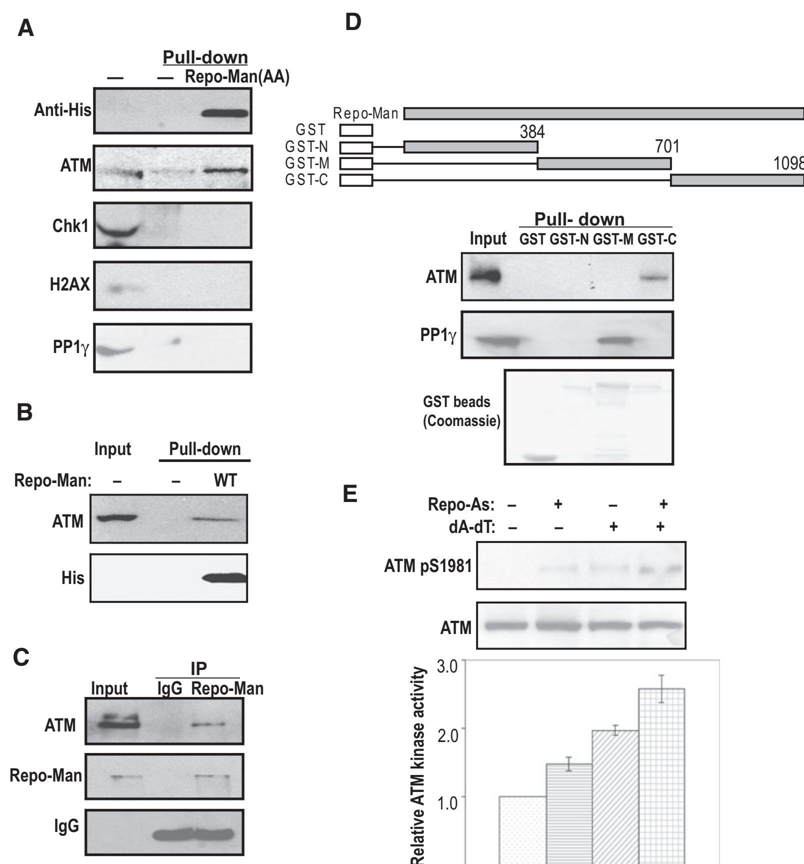

Figure 3. Repo-Man Targets PP1γ to Regulate ATM

(A and B) His-tagged AA mutant (A) or wild-type (B) Repo-Man was expressed by adding mRNAs into *Xenopus* egg extracts, as described in the Supplemental Experimental Procedures, and then reisolated on Talon beads (right). Ten percent control extract without addition of mRNA was loaded in the left column as an input control; the middle shows a parallel pull-down in the control extract. The beads were eluted in sample buffer and blotted with the indicated antibodies. ATM was pulled down with Repo-Man.

(C) Immunoprecipitation (IP) was performed in egg extracts with Repo-Man antibody, and samples were analyzed by western blotting with the indicated antibodies. The left column is a 10% extract input control. ATM was copurified with Repo-Man.

(D) Three segments of Repo-Man (N, M, and C, as indicated) were subcloned, bacterially expressed with an N-terminal GST tag, and purified on glutathione Sepharose beads. The beads were then used for a pull-down assay in egg extracts, eluted with sample buffer, and analyzed by Coomassie staining and western blotting. ATM and PP1γ bind to C and M regions of Repo-Man, respectively.

(E) Top: *Xenopus* oocytes were injected with Repo-Man antisense oligonucleotides, incubated overnight, and subjected to a second injection of water or 5 ng damaged DNA (dA-dT). The oocytes were incubated for 30 min and homogenized, and extracts were analyzed by western blotting for ATM and its autophosphorylation at Ser 1981. Bottom: the same oocyte extracts were also analyzed by an in vitro kinase assay for ATM kinase activity, as described in the Supplemental Experimental Procedures. Relative activities are shown as mean ± SEM (n = 3) of replicate experiments. Repo-Man knockdown resulted in a higher level of ATM activation in vivo. See also Figure S3.

coimmunoprecipitated with ATM in nontumorigenic human MCF10A epithelial cells. To study the subcellular localization of ATM and Repo-Man, we first extracted MCF10A cells to remove soluble but not chromatin-bound proteins and then immunostained for ATM or Repo-Man. Chromatin-bound ATM extensively colocalized with Repo-Man (Figure 4B). Generation of chromatin fibers provided higher resolution for studying chromatin localization of proteins, and the same pattern of chromatin distribution was observed for ATM and Repo-Man on chromatin fibers (Figure 4C). The specificity of the Repo-Man immunostaining of cells and chromatin fibers was confirmed in Figure S4. Our studies in *Xenopus* egg extracts and oocytes show that Repo-Man targets PP1 to regulate ATM phosphorylation and activation (Figure 2). This function is also conserved in human cells. Ectopic expression of Repo-Man tagged by GFP reduced etoposide-induced ATM activation, as judged by the different level of ATM autophosphorylation at Ser 1981 in GFP-positive and control cells (Figure 4D, top). In agreement with the PP1 dependence of this regulation, similar expression of a PP1 binding-deficient Repo-Man mutant (AA), in which the RVTF binding motif was mutated to RATA, did not reduce DNA damage-induced ATM activation, but instead further stimulated the response (Figure 4D, bottom).

Repo-Man Is Dissociated from Active ATM at DNA Damage Sites

In *Xenopus* egg extracts, chromatin association of both Repo-Man and PP1γ was reduced by high-dose Neocarzinostatin (NCS), a DNA-damaging agent known to cause DSBs on genomic DNA [28] (Figure 5A). On the other hand, chromatin binding of ATM increased after DNA damage, possibly reflecting recruitment to DNA damage sites (Figure 5A). A similar dissociation of Repo-Man from chromatin after DNA damage was observed in MCF10A cells (Figure 5B). An interesting hypothesis is that release of Repo-Man near DNA damage sites facilitates activation of the DDR, and consequently, active ATM at DNA damage sites may not be associated with or inhibited by Repo-Man. To test this idea, we subjected MCF10A cells to DNA damage, extracted them to remove soluble proteins, and immunostained the residual chromatin for active ATM and Repo-Man. In contrast to extensive colocalization between ATM and Repo-Man in unperturbed cells (Figures 4B and 4C), Repo-Man did not colocalize with active, phosphorylated ATM (Figure 5C). Measurement of fluorescence intensity further suggests that the level of Repo-Man was generally reduced at foci where active ATM was concentrated (Figure 5C, bottom). These foci have been shown to reflect DNA damage sites where many DDR factors are recruited [29], and thus the removal of Repo-Man from these areas supports our hypothesis. Another line of supporting evidence was provided by immunostaining of chromatin fibers. As previously shown [30], double-strand breaks are not directly visible but can be marked on chromatin fibers by detection of DDR factors. Interestingly, Repo-Man is excluded from chromatin domains enriched with active ATM (Figure 5D). Dissociation of Repo-Man from active ATM was also directly monitored by immunoprecipitation with phospho-specific ATM pS1981 antibody. As shown in Figure 5E, Repo-Man did not coimmunoprecipitate with phospho-ATM in DNA damage-treated cells.

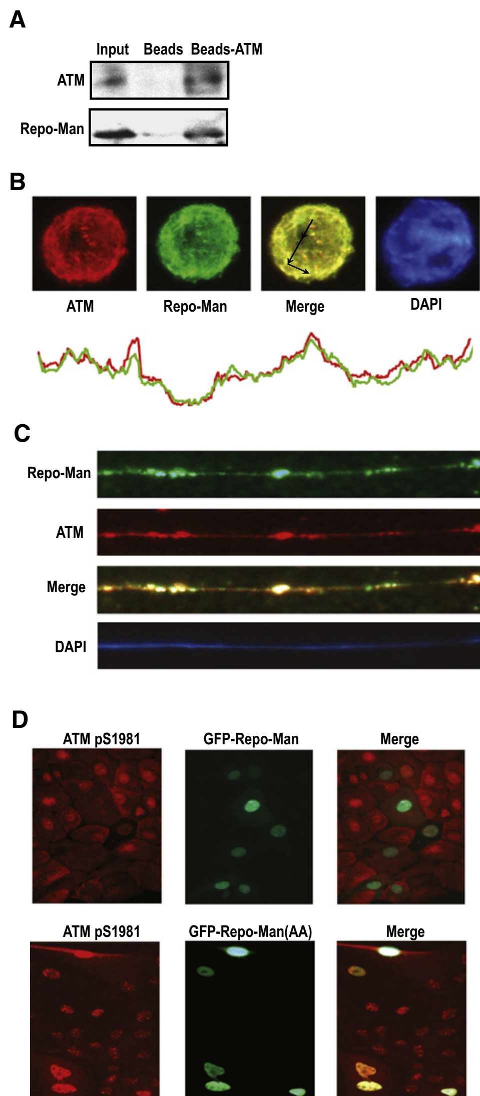

Figure 4. Repo-Man Associates with ATM and Regulates Its Activation in Human Cells

(A) Immunoprecipitation (IP) was performed in MCF10A cell extracts with control agarose beads (beads) or beads conjugated to ATM antibody (beads-ATM). The immunoprecipitates and 10% cell extract input were analyzed by western blotting for ATM and Repo-Man.

(B) Top: MCF10A cells were extracted, immunostained for ATM and Repo-Man, and costained with DAPI. Bottom: red and green curves represent fluorescence intensity in corresponding colors following the black trail marked on the merged panel.

(C) Chromatin fibers were generated from MCF10A cells, immunostained for ATM and Repo-Man, and costained with DAPI.

(D) MCF10A cells were infected with recombinant adenovirus expressing GFP-wild-type Repo-Man or GFP-RATA mutant Repo-Man. These cells were then treated with 500 nM etoposide for 2 hr and analyzed by direct fluorescence (green) for GFP and indirect immunofluorescence (red) for ATM pS1981. See also Figure S4.

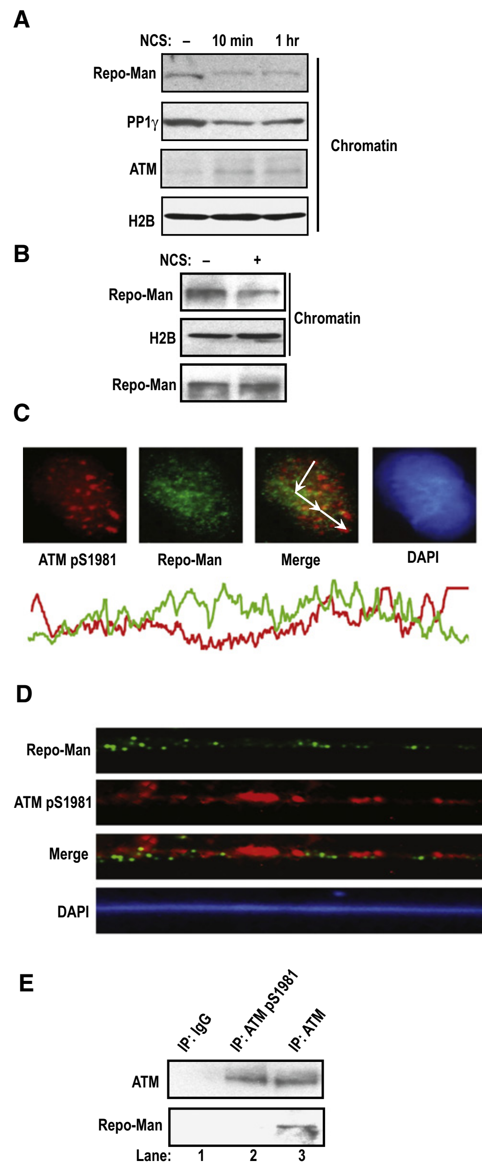

Figure 5. Dissociation of Repo-Man from Chromatin and Active ATM at DNA Damage Sites

(A) *Xenopus* egg extracts were treated with water (–) or 20 μg/ml Neocarzinostatin (NCS) for 10 min or 1 hr. Chromatin fractions were isolated from the extracts by centrifugation and analyzed by western blotting. DNA damage induced partial dissociation of Repo-Man and PP1γ from chromatin, whereas ATM was recruited to chromatin.

(B) MCF10A cells were treated with water or 2 μg/ml NCS for 2 hr. Chromatin fractions (top panels) and total extracts (bottom) were analyzed by western blotting as indicated.

(C) Top: MCF10A cells were treated with 1 μM etoposide for 2 hr, extracted, immunostained for ATM pS1981 and Repo-Man, and costained with DAPI. Bottom: red and green curves represent fluorescence intensity in corresponding colors following the white trail marked on the merged panel.

(D) Chromatin fibers were generated from MCF10A cells, immunostained for ATM pS1981 and Repo-Man, and costained with DAPI.

(E) Immunoprecipitation (IP) was performed in etoposide-treated MCF10A cell extracts with ATM pS1981 antibody (middle). A similar IP with nonimmune IgG served as a negative control (left), and an IP with total ATM antibody in undamaged cell extracts served as a positive control (right). The immunoprecipitates were analyzed by western blotting for ATM and Repo-Man.

Expression of Repo-Man Is Frequently Elevated in Cancer Cells

Repo-Man was first identified as a gene whose expression correlates with known cell-cycle genes [31]. More recently, it has been classified by two independent high-throughput screens into a unique group of genes that are upregulated during progression of neuroblastomas and melanomas

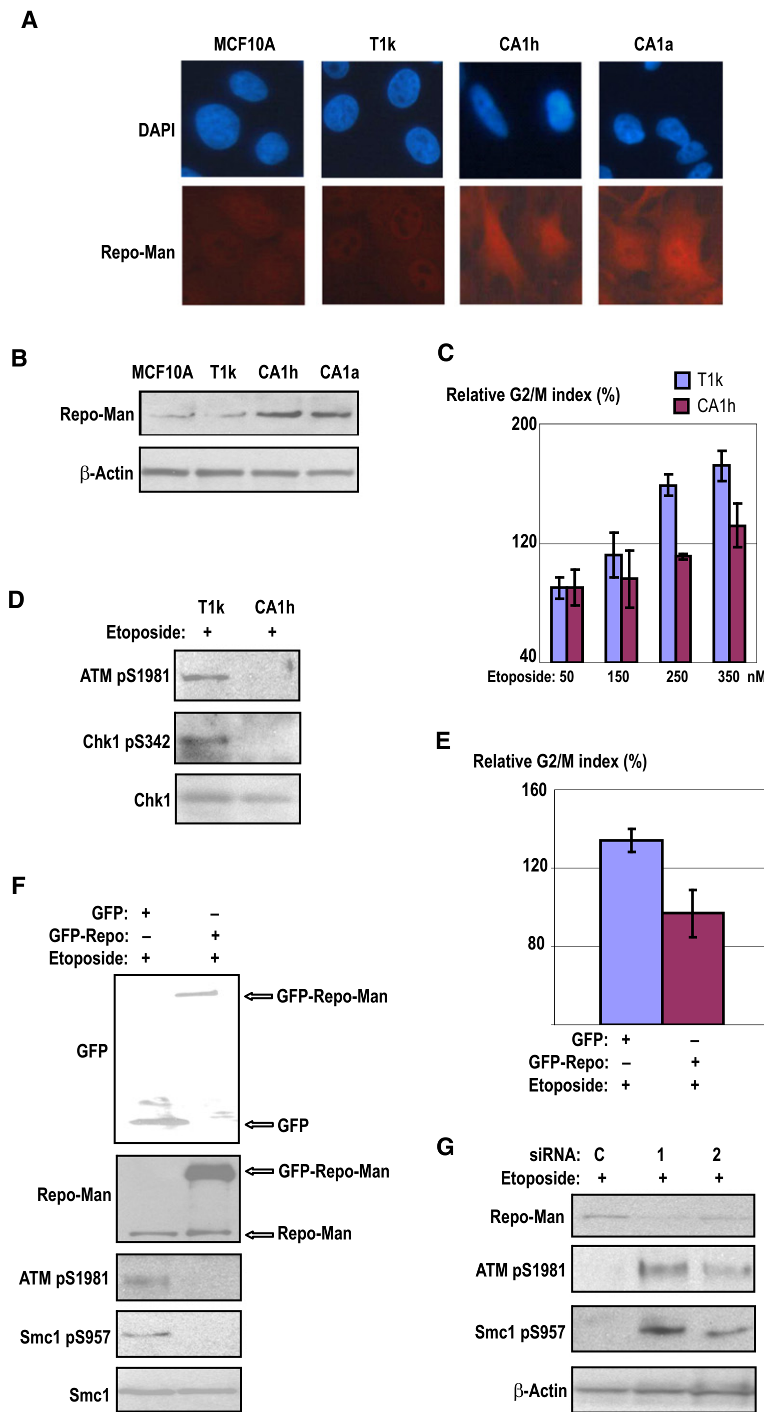

Figure 6. Overexpression of Repo-Man during Tumor Progression Blunts the DNA Damage Response

(A) The MCF10A parental cell line and its progressively more oncogenic variants (T1k, CA1h, and CA1a) were immunostained with Repo-Man antibody and costained with DAPI.
(B) The cell lines in (A) were assayed by western blotting for Repo-Man and β-actin.
(C) T1k and CA1h cells were treated with etoposide at the indicated concentrations for 16 hr and analyzed by fluorescence-activated cell sorting (FACS) to determine the percentage of cells in the G2/M phase. The actual G2/M percentages of etoposide-treated T1k and CA1h cells were normalized to those of control T1k and CA1h cells without etoposide treatment for a relative G2/M index. Increases in the G2/M index reflect etoposide-induced activation of the G2/M checkpoint. The mean ± SEM (n = 3) of replicate experiments is shown.
(D) Tlk and CA1h cells treated with 250 nM etoposide were analyzed by western blotting for DDR signaling, as indicated by ATM and Chk1 phosphorylation.
(E) T1k cells were infected with recombinant adenovirus expressing either GFP or GFP-Repo-Man. These cells were then treated with 250 nM etoposide and analyzed by FACS for cell-cycle arrest as in (C).
(F) Cells in (E) were analyzed by western blotting with the indicated antibodies.
(G) CA1h cells were transfected with control small interfering RNA (siRNA) (C) or two different Repo-Man siRNAs (1 and 2), treated with 250 nM etoposide, and analyzed by western blotting for the indicated proteins. See also Figure S5.

[32, 33]. These results prompted us to test whether Repo-Man overexpression is generally evident during cancer progression. Using immunohistochemistry, we observed enhanced expression of Repo-Man in many, but not all, cancer tissues as compared to adjacent normal tissues (Figures S5A and S5B). As summarized in Figure S5C, significantly increased Repo-Man expression was found in roughly half of all cancer tissues tested. To extend this analysis, we obtained a series of staged and graded human breast tumors and matched normal tissues. As shown in Figure S5D, analysis in these tissue lysates shows that Repo-Man was frequently overexpressed in late stage and higher-grade tumors. Consistently, Repo-Man level was also elevated in a panel of tumorigenic breast cancer cell lines compared to nontumorigenic lines (Figure S5E). Finally, the National Cancer Institute-supported mRNA expression database (http://www.oncomine.org;) shows that Repo-Man transcription is frequently upregulated in various cancers (Figure S5F). We thus speculate that increased Repo-Man levels during cancer progression result at least partly from activated gene transcription. Taken together, a comprehensive set of evidence shows that Repo-Man is overexpressed in a large proportion of late-stage cancers.

Elevated Repo-Man Expression Blunts the DNA Damage Response during Cancer Progression

To investigate whether overexpression of Repo-Man renders early-stage tumor cells less sensitive to DNA damage, we analyzed the well-established MCF10A human breast cancer model system consisting of a series of transformed mammary epithelial cell (MEC) lines that share a common genetic ancestry. Each line represents a distinct stage of breast cancer development, ranging from normal (i.e., MCF10A) to premalignant (i.e., MCF10A-T1k) to malignant (i.e., MCF10A-CA1h) to invasive and metastatic (i.e., MCF10A-CA1a) [34]. These lines can be studied in cell culture and also as human tumor xenografts in nude mice. Extensive gene array, SAGE, and single-nucleotide polymorphism analyses have indicated that this system accurately recapitulates the genetic changes that are found during human breast cancer progression [35, 36]. Interestingly, expression of Repo-Man is greatly elevated during the transition from T1k to CA1h, as confirmed by both immunofluorescence and western blotting (Figures 6A and 6B). In concert with this change in Repo-Man

level, the DDR to etoposide in malignant CA1h cells is less sensitive than that in premalignant T1k cells, as judged by both phosphorylation of DDR components and G2/M arrest (Figures 6C and 6D). Importantly, ectopic expression of Repo-Man significantly reduced the DDR in T1k cells to the CA1h level (Figures 6E and 6F), indicating that upregulation of Repo-Man is sufficient for the reduced DDR in CA1h cells. Conversely, knockdown of Repo-Man expression resensitized the DDR in late-stage cancer cells, including CA1h (Figure 6G) and CA1a in the MCF10A breast cancer model, and also in BT-20, a well-established breast cancer cell line initially derived from an advanced invasive ductal carcinoma (Figure S6A). These results confirm the critical role of Repo-Man in modulating DNA damage signaling.

Repo-Man Expression Is Required for CA1h Cell Growth in Soft Agar

To assess whether upregulation of Repo-Man is necessary to promote tumorigenesis, we studied growth of tumor cells in soft agar assays. Malignant CA1h cells grow into large colonies in soft agar, whereas precancerous T1k cells only form very small ones. Interestingly, CA1h cells stably infected with lentiviral small hairpin RNA (shRNA) that knocks down Repo-Man expression were unable to form large colonies in soft agar (Figure 7A; Figure S6B), despite little effect on proliferation in regular culture. On the other hand, ectopic expression of Repo-Man was not sufficient to promote soft agar growth of T1k, although some increase in colony size was evident (Figures 7B and 7C). Therefore, upregulation of Repo-Man is necessary but not sufficient for anchorage-independent/soft agar growth in the MCF10A breast cancer model.

Discussion

Serine/threonine phosphatases are less studied compared to their functional counterparts, serine/threonine kinases. Both PP1 and PP2A rely on additional targeting subunits to perform specific functions. Recent publications have begun to identify these targeting subunits and the underlying mechanisms and to present an attractive picture in which the complexity, specificity, and importance of protein phosphatases are generally comparable to that of protein kinases [10, 11]. In the DDR pathway, several groups of protein phosphatases have been shown to dephosphorylate DDR components and promote recovery from checkpoint arrest. Some key questions, however, remain to be answered. For example, do protein phosphatases regulate the initial activation of the DDR, and how is regulation specifically achieved? In this study, we show that inhibition of PP1 and PP2A efficiently turns on DDR signaling in *Xenopus* egg extracts. These findings underscore the essential role of both PP1 and PP2A in suppression of the DDR. The involvement of PP2A reported here supports its previously revealed role as an important DDR regulator that promotes checkpoint recovery through dephosphorylation of multiple DDR factors, including H2AX, Chk2, Chk1, ATM, and p53, etc. [9, 14–16]. Data presented here clearly indicate that PP1 also plays a critical role in DDR regulation. Inhibition of PP2A or PP1 alone is not sufficient to activate DDR signaling, but simultaneous inhibition of both phosphatases fully turns on the DDR. Moreover, inhibition of PP1 results in a sensitized response to DNA damage. This study reveals the first specific PP1-dependent regulation of the DDR mediated by a chromatin-bound targeting subunit, Repo-Man. Importantly, Repo-Man interacts with PP1 and ATM through

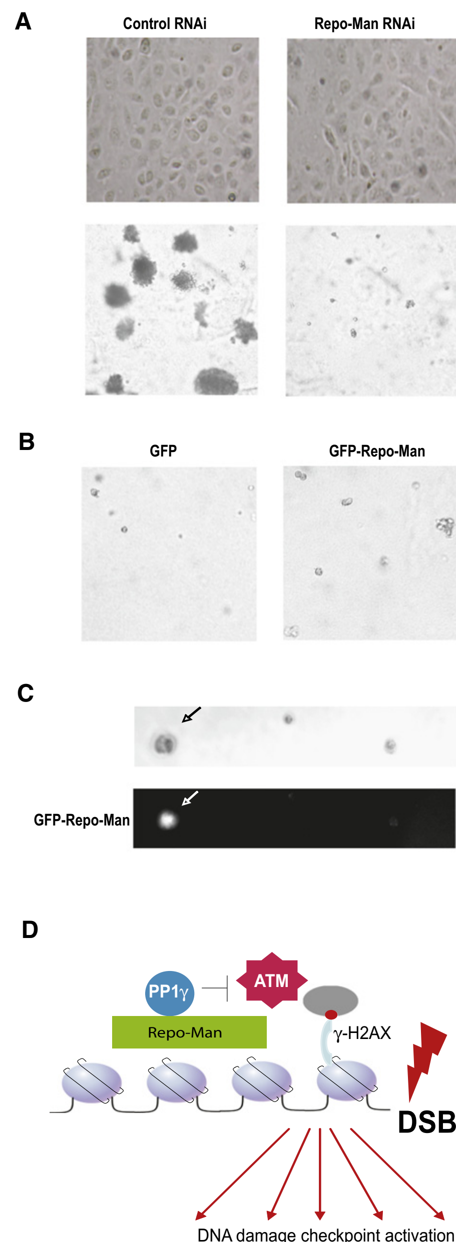

Figure 7. Repo-Man Expression Is Required for Growth of T1k Cells in Soft Agar

(A) CA1h cells treated with control or Repo-Man small hairpin RNA were grown on standard tissue culture dishes (top) or in soft agar for 14 days. Representative pictures of the colonies formed in soft agar are shown (bottom).

(B and C) T1k cells were infected with adenovirus encoding GFP or GFP-Repo-Man and then grown in soft agar. Representative pictures of the colonies formed after 14 days are shown. As indicated by arrows, the larger colony (C, top) was expressing GFP-Repo-Man, as detected by direct GFP fluorescence (C, bottom).

(D) A model in which Repo-Man recruits PP1γ to chromatin to antagonize activation of ATM-dependent signal transduction. The Repo-Man-dependent DDR regulation illustrated in this model is strengthened by Repo-Man overexpression during cancer progression, resulting in reduced DDR sensitivity. See also Figure S6.

distinct motifs, thus targeting PP1γ to antagonize ATM activation and negatively regulate DNA damage-induced signal transduction. In this scenario, Repo-Man controls both the

localization and substrate specificity of the PP1 holoenzyme complex and sets the threshold for checkpoint activation in both *Xenopus* oocytes and egg extracts (Figure 7D). The functional relationship between ATM and Repo-Man was further strengthened by studies in human cells. Repo-Man associates with ATM and extensively colocalizes with ATM on chromatin. Overexpression of Repo-Man in cells reduces DNA damage-induced ATM activation, whereas a PP1 binding-deficient Repo-Man dominant-negative mutant enhances the response. Another important question regarding specific phosphatase complexes is how they are regulated by DNA damage. We report here that Repo-Man is released from DNA damage sites and dissociated from active ATM, presumably to facilitate DDR activation by DNA damage above the threshold level. This is a striking result because it argues that activation of the DDR involves removal of inhibitory regulators. Though the underlying mechanism is unclear, the chromatin dissociation of Repo-Man-PP1 may be phosphorylation dependent, because MC treatment removed PP1 and Repo-Man from chromatin (data not shown).

The DDR pathway is essential to protect genomic stability and suppress tumorigenesis. Inherited mutations in DDR factors, including ATM, Chk1, Chk2, Mre11, Nbs1, etc., predispose patients to various cancers. However, most cancer patients are born with an intact DDR pathway, and little is known about how their tumor cells escape from the DDR. Recent reports show that the DDR is activated in early, precancerous cells as a barrier to suppress cell proliferation and cancer progression [2]. DDR activation is greatly reduced in late-stage cancer cells, presumably resulting in uncontrolled cell proliferation [2, 37, 38]. The mechanism of this modulation in DNA damage responsiveness is unknown. Our study presented here discovered a mechanism that may contribute to this process. We found that Repo-Man expression is significantly enhanced in many, although not all, cancers. Repo-Man overexpression is sufficient to modulate DDR sensitivity during progression of cancer because elevated Repo-Man blunted the DDR in early-stage precancerous cells and, conversely, knockdown of Repo-Man restored DDR sensitivity in advanced-stage cancer cells. We propose that during early stages of cancer progression, the DDR is activated in response to elevated genomic instability to prevent further cell proliferation. Such a process poses a selection pressure for cells with a reduced DDR, such as those in which Repo-Man is upregulated. These cells resume proliferation bearing a high level of spontaneous DNA damage, resulting in acquisition of additional mutations and further cancer progression. The involvement of Repo-Man in cancer progression is supported by the soft agar assay showing that Repo-Man expression is essential for colony formation. However, several aspects of this model still remain to be further clarified. For example, it is unclear whether Repo-Man upregulation is a consequence of oncogenic stimuli. Moreover, although not all cancers exhibit Repo-Man overexpression, a similar deregulation of the DDR could be achieved in some cancers through parallel mechanisms that involve other protein phosphatase complexes [12, 14, 16, 39]. Of particular interest to us, inhibition of PP1, PP2A, or PP2Cδ/Wip1 has been shown to affect cell survival post radiation or after chemotherapy (reviewed in [40]). We report here that merely reducing Repo-Man expression resensitized advanced cancer cells to the DDR and restrained their growth, suggesting that Repo-Man may be a potentially valuable target in cancer therapy.

Experimental Procedures

cDNA Cloning of *Xenopus* Repo-Man

Total RNA was extracted from *Xenopus laevis* oocytes with a QuickPrep total RNA extraction kit (Amersham) following the protocol recommended by the manufacturer. The 5′ and 3′ sequences of *Xenopus* Repo-Man were determined by rapid amplification of cDNA ends (RACE) with a SMART RACE cDNA amplification kit (Clontech). The full-length cDNA of *Xenopus* Repo-Man was then generated by end-to-end polymerase chain reaction (with primers 5′-ATCTCCAGAAAGGTTTTGCAGGAG-3′ and 5′-AATGTAA CGGTCCATGGGTTCTGC-3′). The sequence has been deposited in GenBank under accession number FJ532285.

Cell Extraction and Chromatin Fiber

Cell extraction was carried out via a protocol described previously, with minor modifications [41]. Cells on coverglasses were treated with extraction buffer (0.5% NP40 in phosphate-buffered saline [PBS]) for 5 min on ice and washed once with extraction buffer and twice with PBS. Immunostaining was then performed as described below. Chromatin fibers were generated following a previously established protocol [42]. Briefly, 2×10^5 cells were resuspended in 0.5% sodium citrate for 10 min and spun onto a coverglass. The coverglass was then dipped in salt/detergent buffer (25 mM Tris 7.5, 500 mM NaCl, 1% Triton X-100) for 10 min and removed for immunostaining.

Chromatin Fractionation

As described previously [6], to reisolate sperm chromatin from egg extracts, extracts were diluted 10-fold with chromatin dilution buffer (50 mM HEPES, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, 0.5 mM spermine 3 HCl, 0.15 mM spermine 4 HCl, 0.1% Triton X-100). The diluted extract was then layered onto 3 ml of chromatin dilution buffer containing 30% sucrose and centrifuged at $6000 \times g$ for 15 min. The pellet was resuspended as the chromatin-enriched fraction.

Repo-Man Knockdown

Small interfering RNA (siRNA) duplexes targeting human Repo-Man (target sequences UCAGAAGCAUGCCGAAUUA and GUUCAAAGGCUACCGGA GA) and a nontargeting control siRNA were purchased from Dharmacon and transfected into CA1h, CA1a, or BT-20 cells, following the protocol recommended by the manufacturer. Lentiviral shRNAs targeting Repo-Man were utilized to infect CA1h cells, following the protocol recommended by the manufacturer (Sigma, NM_152562). Morpholino antisense oligonucleotides targeting *Xenopus* Repo-Man were designed and produced by Gene Tools. Both morpholino oligonucleotides (TCTGGAAGTCATCACCT CCTCTCCA and AGATTCAAATTCAAATCTGCCGCCG) were suspended in nuclease-free water to a concentration of 0.1 mM, and 40 nl was injected into each oocyte.

Soft Agar Assay

The growth of T1k and CA1h cells in soft agar was performed as described [43]. In brief, cells (10,000 cells per plate) were grown in 0.3% agar on a cushion of 0.6% agar in 35 mm plates for 14 days, after which the colonies were observed under a light microscope.

Xenopus Oocytes and Cytostatic Factor Extracts

Oocyte maturation was induced in vitro by addition of progesterone (10 μg/ml), and entry into M phase was monitored by germinal vesicle breakdown with a dissecting microscope, as described previously [6]. Cytostatic factor extracts, prepared as previously described [6], were stably released into interphase by supplementation with 0.4 mM $CaCl_2$ and 100 μg/ml cycloheximide, followed by incubation for 30 min at room temperature prior to use. Oocyte extracts were generated by homogenization of the oocytes, followed by centrifugation at $15,000 \times g$ for 4 min at 4°C. NCS was purchased from Sigma and used to generate DNA DSBs in extracts at concentrations indicated in the figure legends. Extracts used in this study were supplemented with 1,000 demembranated sperm nuclei/μl, unless otherwise indicated. I-2 and MC were purchased from New England Biolabs and Calbiochem, respectively.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at doi:10.1016/j.cub.2010.01.020.

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