DNA replication defects, spontaneous DNA damage, and ATM-dependent checkpoint activation in replication protein A-deficient cells

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Running title: Phenotypic characterization of RPA deficiency

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SUMMARY

Replication protein A (RPA) is a heterotrimeric, single-stranded DNA (ssDNA)-binding complex comprised of 70 kDa (RPA1), 32 kDa (RPA2), and 14 kDa (RPA3) subunits that is essential for DNA replication, recombination, and repair in eukaryotes. In addition, recent studies using vertebrate model systems have suggested an important role for RPA in the initiation of cell cycle checkpoints following exposure to DNA replication stress. Specifically, RPA has been implicated in the recruitment and activation of the ATM-Rad3-related protein kinase, ATR which, in conjunction with the related kinase, ATM (ataxia-telangiectasia-mutated), transmits checkpoint signals via the phosphorylation of downstream effectors. However, the requirement of RPA for ATR recruitment and activation is controversial and the cellular consequences of RPA functional deficiency have not been investigated. In this report, we have explored the effects of RPA insufficiency on DNA replication, cell survival, and ATM/ATR-dependent signal transduction in response to genotoxic stress. RNA interference-mediated suppression of RPA1 caused a slowing of S phase progression, G2/M cell cycle arrest, and apoptosis in HeLa cells. RPA-deficient cells demonstrated high levels of spontaneous DNA damage and constitutive activation of ATM, which was responsible for the terminal G2/M arrest phenotype. Surprisingly, we found that neither RPA1 nor RPA2 were essential for the HU- or UV-induced phosphorylation of the ATR substrates CHK1 and CREB. These findings reveal that RPA is required for genomic stability and suggest that activation of ATR can occur through RPA-independent pathways in response to genotoxic stress.
Replication protein A (RPA) is a trimeric complex composed of 70 kDa (RPA1), 32 kDa (RPA2), and 14 kDa (RPA3) subunits that is essential for DNA replication in all organisms (1). RPA represents the major cellular single-stranded DNA (ssDNA)-binding activity in eukaryotic cells and coats ssDNA filaments stoichiometrically in vitro (1). Through its binding and stabilization of ssDNA, RPA facilitates the unwinding and destabilization of double-stranded DNA (dsDNA), which represents a critical step during DNA replication, recombination, and repair. The major DNA-binding activity of RPA resides within the 70 kDa RPA1 subunit, which contains a centrally-positioned, high affinity, bipartite DNA-binding domain (DBD) and a low-affinity carboxyl-terminal DBD (1,2). The RPA2 protein also contains a DBD, as well as a phosphorylation site-rich amino terminus that may regulate RPA activity in response to cell cycle phase transitions and DNA damage. Kinases implicated in the phosphorylation of RPA2 include cyclin-dependent kinases, and members of the PI3 kinase–related kinase superfamily, including DNA-dependent protein kinase (DNA-PK), ataxia-telangiectasia-mutated (ATM), and ATM/Rad3-related (ATR) (3-7). DNA-PK, ATM, and ATR are serine/threonine-glutamine (S/T-Q)-directed kinases with overlapping substrate specificities that regulate DNA repair, apoptosis, and cell cycle checkpoint responses to genotoxic stimuli (8,9). At least two S/T-Q residues of RPA2 (Thr-21 and Ser-33) are phosphorylated by DNA-PK, and most likely ATM and ATR, in vitro and within intact cells in response to DNA damage (4,7,10,11). The biochemical functions of individual RPA2 S/T-Q sites are not well understood. However, DNA damage-induced phosphorylation of RPA2 correlates with decreased binding of RPA to p53 (12,13), suggesting that modulation of protein-protein interactions represents one functional endpoint.

In addition to its role in the maintenance and processing of ssDNA, RPA has been implicated as a regulator of DNA damage-induced cell cycle checkpoints. Hypomorphic mutations in RPA1 are associated with hypersensitivity to DNA-damaging agents and cell cycle checkpoint defects in budding and fission yeasts (14,15). Immunodepletion of RPA from Xenopus oocytes abrogates an aphidicolin-induced DNA replication checkpoint, which functions to suppress mitosis in the presence of incompletely replicated DNA (16). Using the Xenopus system it was
also shown that RPA is required for the suppression of DNA synthesis in response to DNA strand breaks (17). The requirement of RPA for checkpoint activation in *Xenopus* extracts mirrors that for ATR (17-19), implying a functional interaction between these two proteins during the initiation of checkpoint signals. Consistent with this notion, RPA is required for the association of ATR with chromatin in the *Xenopus* system, suggesting that RPA recruits ATR, either directly or indirectly, to sites of genetic damage (16,20). Mammalian RPA was found to promote the chromatin association of ATR *in vitro* via the ATR-interacting protein, ATRIP (21). Furthermore, in that study it was concluded that RPA is required for the ATR-mediated phosphorylation of its effector kinase, CHK1, after exposure to hydroxyurea (HU) or UV light. However, it is clear that ATR also contains an intrinsic, RPA-independent, DNA binding activity (21-23) and the quantitative requirement of RPA for the chromatin association of ATR-ATRIP complexes *in vitro*, and for ATR activation *in vivo*, remains an unsettled matter. In addition, although RPA has been extensively studied *in vitro*, the cellular consequences of RPA functional deficiency have yet to be explored in mammalian cells.

In this report we have explored the genetic requirement of RPA for DNA replication, survival, and cell cycle checkpoint function in human cells. We demonstrate that RPA deficiency causes spontaneous DNA damage, apoptosis, and the induction of an ATM-dependent G2/M checkpoint. Surprisingly, we found that neither the RPA1 nor RPA2 subunits were required for ATR-dependent substrate phosphorylation in DNA-damaged HEK 293T cells. Our findings demonstrate a requirement for RPA in the maintenance of genomic integrity, and suggest that ATR is activated independently of RPA in response to genotoxic stress.
**EXPERIMENTAL PROCEDURES**

*Cell culture and antisera*—HEK 293T and HeLa cells were maintained in Eagle’s minimum essential medium containing 10% FCS. Antibody suppliers included: GeneTex (α-ATM), Oncogene Research (α-RPA1, α-RPA2, and α-BrdU), Santa Cruz Biotechnology (α-CHK1 (G-4), Upstate Biotechnology (α-tubulin, and α-pH2AX-139), Cell Signaling (α-CREB), Affinity Bioreagents (α-ATR), and R&D Systems (α-pATM-1981 and α-pCHK1-317). The α-pCREB-121 antibody has been previously published (24).

*Transfections and protein analysis*—Transfection-ready siRNA duplexes were purchased from Dharmacon Research. siRNAs used in this study included: RPA1 (5’-AACUGGUUGACGAAAGUGGUG-3’), ATR (5’-AACCCGCGUUGCGUGGUUGA-3’), RPA2, and ATM. The RPA2 and ATM siRNAs represented mixtures of four distinct RNA duplexes (SmartPool, Dharmacon). Three micrograms of siRNA was used for each transfection using the calcium phosphate DNA precipitation procedure. Cells were harvested 48-72 h later and extracts prepared as described (24). Seventy-five micrograms of total protein was separated on 10% SDS-PAGE gels and transferred to Immobilon PVDF membranes (Millipore). Membranes were blocked in Tris-buffered saline containing 0.2% Tween-20 (TBS-T) and 5% dried milk and incubated overnight at 4° C with the indicated primary antibodies diluted in blocking solution. After washing, the blots were incubated with HRP-conjugated sheep anti-mouse or goat anti-rabbit secondary antibodies (Jackson) and developed using SuperSignal chemiluminescent substrate (Pierce).

*Immunofluorescence microscopy*—For the phospho-H2AX analysis, HeLa cells were transfected with scrambled control (SCR) or RPA1 siRNAs and fixed 72 h later in 4% paraformaldehyde at room temperature. The cells were then permeabilized in PBS, 0.2% Triton-X 100 (PBS-T) for 10 min, washed once with PBS and blocked for 30 min in PBS containing 3% BSA and 2% goat serum. The cells were then incubated overnight at 4° C with 2 µg/ml of α-phospho-H2AX antibody diluted in blocking solution. The cells were washed three times in PBS-T and incubated for 1 h at room temperature with 0.4 µg/ml FITC-conjugated goat anti-mouse IgG (Caltag). The cells were washed twice with PBS-T, once in PBS, and mounted using
Vectashield containing DAPI (Vector Laboratories). A Carl Zeiss Axiovert 200 fluorescence microscope was used to visualize all samples.

Cell cycle analysis and viability assays—DNA synthesis was measured using a bromodeoxyuridine (BrdU) incorporation assay. HeLa cells were transfected with SCR or RPA1 siRNAs and then pulse-labeled 48 h later with 10 µM BrdU for 30 min. The cells were then cultured in BrdU-free medium and harvested at 4 h intervals over a 24 h hour period. Following fixation with ice-cold 70% ethanol, the cells were processed for immunostaining with α-BrdU as described (25). The cells were subsequently stained with propidium iodide (PI) and analyzed by two-parameter flow cytometry. Profiles of the BrdU- and PI-stained populations were plotted using the WinMDI shareware package (Stanford University). Cell viability was directly measured via trypan blue uptake. Following trypsinization, cells were diluted 1:1 in trypan blue solution (Sigma) and examined under a microscope using a hemacytometer. For each treatment, 200 total cells were scored for trypan blue uptake and the percent viability was calculated by dividing the number of trypan blue-positive cells by the total cell number.

RESULTS AND DISCUSSION

Phenotypic characterization of RPA1-deficient cells—We used an RPA1-specific small-interfering RNA (siRNA) to suppress RPA1 expression in HEK 293T or HeLa cells. Immunoblotting confirmed that the expression of RPA1 was reduced by more than 90% in both cell lines within 48 h of transfection, whereas the expression of RPA2 was unaffected (Fig. 1A). Transfection of a scrambled (SCR) siRNA had no effect on RPA1 expression. To examine the impact of RPA1 dosage suppression on DNA synthesis and cell cycle progression, we transfected HeLa cells with SCR or RPA1 siRNAs and then pulse-labeled the cells 48 h later with BrdU for 30 min. The cells were then incubated in BrdU-free medium and harvested at 4 h intervals for staining with α-BrdU and PI. FACS analysis of the stained cell populations revealed that RPA1-deficient cells incorporated BrdU at a reduced level in comparison to the control cells (Fig. 1B, zero time point). In addition to an acute DNA synthesis defect, RPA1-deficient cells also displayed abnormal S phase progression. By 8 h after BrdU pulse labeling,
virtually all of the control cells accumulated 4N DNA content, indicating the completion of S phase. In contrast, 60% of RPA1-deficient cells displayed a DNA content intermediate between 2N and 4N at this time point. By 16 h post-labeling, the difference between the control and RPA1-deficient cell populations was even more pronounced. At this time point, 79% of the BrdU-positive control cells, but only 7% of RPA1-deficient cells, had completed mitosis and re-entered G1 phase. The majority of the RPA1-deficient cells retained a 4N DNA content 16 h after BrdU pulse-labeling, suggesting that the cells had arrested in G2/M phase.

The G2/M arrest profile of RPA1-deficient HeLa cells was further examined over a 96 h time frame. HeLa cells were transfected with SCR or RPA1 siRNAs and then stained with PI either 48 h or 96 h later to measure DNA content by flow cytometry. The cell cycle profiles of HeLa cells transfected with SCR or RPA1 siRNAs were comparable at 48 h post-transfection. However, by 96 h post-transfection, the RPA1-deficient cells displayed a robust G2/M arrest phenotype (Fig. 1C). In addition, a substantial fraction (60-70%) of the cells detached from the tissue culture dish and underwent apoptosis, as assessed by trypan blue staining and morphologic analysis (Fig. 1D). The remaining attached cells exhibited an abnormal, elongated morphology, possibly indicative of terminal G2/M arrest (Sup. Fig. 1). In sum, we find that RPA1 functional deficiency causes a slowing of S phase, terminal G2/M arrest and apoptosis in HeLa cells.

**RPA1 deficiency causes spontaneous DNA damage and activates ATM**—Based on the above findings, we hypothesized that insufficient levels of RPA1 resulted in the generation of unrepaired DNA damage during S phase, with subsequent activation of a G2/M cell cycle checkpoint. To test this hypothesis we first examined whether RPA1-deficient cells displayed increased levels of DNA damage. HeLa cells were transfected with SCR or RPA1 siRNAs and stained 72 h later with an antibody that recognizes phosphorylated histone γ-H2AX, a specific marker of DNA double-strand breaks (DSBs) (26). Control cells displayed only weak phospho-H2AX immunoreactivity, with less than 5% of cells displaying brightly-staining phospho-H2AX nuclear foci that are characteristic of cells containing DSBs (Fig. 2A and data not shown). In contrast, 70% of RPA1 siRNA-transfected HeLa cells displayed intense phospho-H2AX staining (Fig. 2A). As noted above, the RPA1-deficient cells were also morphologically distinct from the
controls, with most of the cells displaying an elongated nucleus. From these findings we conclude that RPA1 deficiency causes spontaneous DNA damage, which activates a terminal G$_2$/M checkpoint in HeLa cells.

It is well established that ATM is required for optimal G$_2$/M checkpoint induction in response to IR and other agents that induce DSBs (27,28). To ascertain whether the G$_2$/M arrest observed in RPA1-deficient HeLa cells was ATM-dependent, we transfected HeLa cells with RPA1 and ATM siRNAs, and then examined the cell cycle profiles by PI staining 72 h later. Consistent with the earlier experiments, transfection of RPA1 siRNA alone caused a pronounced G$_2$/M arrest, whereas transfection of an ATM siRNA did not substantially affect the HeLa cell cycle (Fig. 2B). Cells that were co-transfected with RPA1 and ATM siRNAs displayed an attenuated G$_2$/M arrest phenotype relative to cells that were transfected with RPA1 siRNA alone (Fig. 2B), even though the RPA1 expression levels were comparably reduced in both cell populations (data not shown). This finding indicates that ATM contributes to the G$_2$/M arrest observed in RPA1-deficient cells. However, the finding that the G$_2$/M arrest was not completely abolished suggests that ATM-independent mechanisms may also contribute to checkpoint activation in response to RPA functional insufficiency.

The above finding implied that ATM is activated as a consequence RPA1 deficiency. To test this possibility, we measured the activation status of ATM in HeLa cells 48 h or 72 h after transfection with SCR or RPA1 siRNAs. Activation of ATM was assessed using an antibody (α-pATM-1981) that recognizes an activation-specific autophosphorylation site at Ser-1981 (29). This analysis revealed that ATM was autophosphorylated on Ser-1981 following transfection with RPA siRNA (Fig. 2C). Although autophosphorylation was detectable by 48 h, it was much more pronounced at 72 h post-transfection, which is consistent with the time course of DSB induction shown in Fig. 2A. The level of ATM autophosphorylation was about three-fold less than that induced by 10 Gy of IR. From the combined findings, we conclude that prolonged RPA1 deficiency induces spontaneous DNA damage, activates ATM, and induces an ATM-dependent G$_2$/M cell cycle checkpoint in HeLa cells.
Effects of RPA siRNA on ATM and ATR activation following DNA damage—We next sought to determine the relative requirement of RPA for the activation of ATM and ATR following DNA damage. The observation that ATM became autophosphorylated during prolonged RPA1 deficiency suggested that ATM activation does not require RPA. We formally tested this possibility and found that RPA1 siRNA had no effect on the autophosphorylation of ATM on Ser-1981 following exposure to 5 Gy of IR, validating that RPA is not required for the activation of ATM in response to this stimulus (Fig. 3).

To determine the requirement of RPA for ATR activation, we examined the effects of siRNAs specific for RPA1 or RPA2 on the ATR-dependent phosphorylation of CHK1 on Ser-317 (30,31). For comparative purposes, an ATR siRNA was employed as a side-by-side control. Interestingly, during the course of these experiments, we found that an RPA2 siRNA caused the coordinate down-regulation of RPA1, but not vice versa (Fig. 4A). The basis for this phenomenon is not known, but it was observed using several RPA2 siRNAs and may reflect a role for RPA2 as an RPA1 stability determinant. Immunoblot analysis using a CHK1 Ser-317 phospho-specific antibody revealed that neither RPA2 nor RPA1 siRNAs substantially inhibited the phosphorylation of CHK1 on Ser-317 following exposure of HEK 293T cells to HU (3 mM), UV light (25 J/m²), or IR (10 Gy) (Fig. 4B). This lack of effect was probably not due to incomplete RPA1 or RPA2 suppression, as the levels of both proteins were drastically reduced 48 h after siRNA transfection (Fig. 4A). In the same experiment, an ATR siRNA suppressed CHK1 phosphorylation in response to all three stimuli (Fig. 4B). From this experiment we conclude that CHK1 phosphorylation on Ser-317 in response to HU, UV light, and IR is ATR dependent, but RPA independent.

The requirement of RPA for the DNA damage-induced phosphorylation of other ATR substrates was also explored. The cyclic AMP response element-binding protein (CREB) is a transcription factor that is phosphorylated by ATM in vivo on Ser-121 in response to IR (24). We have also shown that both ATM and ATR phosphorylate CREB on Ser-121 in vitro (Ref. 24 and data not shown). In support of a role for ATR as a mediator of CREB phosphorylation in vivo, we found that an ATR siRNA abolished the HU- and UV-induced phosphorylation of the Ser-121 residue (Fig. 4C). In contrast, transfection with RPA1 or RPA2 siRNAs had no effect on CREB
phosphorylation in response to either stimulus. Thus, like CHK1, the UV- and HU-induced phosphorylation of CREB is ATR dependent, but RPA independent. Finally, in this experiment we also observed that the IR-induced phosphorylation of CREB was unaffected by an ATR siRNA, which contrasts with the CHK1 result (compare Figs. 4B and 4C). However, this finding is consistent with our previous report demonstrating that IR-induced phosphorylation of CREB is ATM dependent (24).

In this study we have demonstrated that RPA is required for genomic integrity in mammalian cells; RPA insufficiency causes spontaneous DSBs, ATM-dependent G2/M arrest, and apoptotic cell death. The mechanism of DNA damage induction following RPA suppression is unknown, but most likely involves the execution of an aberrant DNA replication cycle after RPA levels have declined below a critical threshold level. Consistent with this possibility, DSB induction and ATM activation were observed 72-96 h after RPA1 siRNA transfection, which is 24-48 h after RPA1 expression reached its minimum. It is possible that DSB induction reflects a structural role for RPA in protecting ssDNA filaments from spontaneous strand breakage. A non-exclusive possibility is that, in the prolonged absence of RPA, ssDNA present at replication forks is converted into DSBs through failed cycles of recombination. RPA functionally interacts with the enzymatic machinery of recombination, including RAD51, RAD52, and BLM, a helicase that contributes to the resolution of recombination intermediates present at stalled replication forks (32-35). It is therefore conceivable that DSBs arise in RPA-deficient cells secondarily to gross DNA recombination abnormalities.

In our hands an RPA1 siRNA did not substantially inhibit the IR-induced autophosphorylation of ATM, nor did RPA1 or RPA2 siRNAs suppress ATR-dependent substrate phosphorylation in response to HU or UV light. The RPA independence of ATR activation was somewhat surprising given several recent reports implicating RPA as an upstream regulator of the ATR pathway in Xenopus and mammalian cells (16,20,21). Nevertheless, we failed to observe inhibitory effects of RPA1 or RPA2 siRNAs on the HU- or UV-induced phosphorylation of three ATR substrates: CHK1, CREB, and RAD17 (Fig. 4 and unpublished results). In these same experiments, an ATR siRNA strongly inhibited substrate phosphorylation, even though the ATR siRNA was less efficacious than either the RPA1 or RPA2 siRNAs (Fig. 4). Because of inherent
caveats associated with RNAi-based experiments, we cannot rule out the possibility that a low threshold level of RPA was sufficient to activate ATR in our experiments. However, at minimum, our findings indicate that a reduction in RPA dosage does not cause a corresponding linear decrease in ATR activity, as assessed by phosphorylation of CHK1 on Ser-317 and CREB on Ser-121. This conclusion is at odds with recent reports that also used RNAi to implicate RPA in the ATR-dependent phosphorylation of CHK1 in HeLa cells (21,36). The basis for the conflicting findings is not known but could reflect the use of different CHK1 phospho-specific antibodies and/or cell type-dependent differences between the HEK 293T cells used here and HeLa cells used in the earlier studies. For unknown reasons, we did not observe robust phosphorylation of CHK1 in UV- or HU-treated HeLa cells (unpublished observation). Clearly, additional studies are needed to clarify the role of RPA in the recruitment and activation of ATR in response to genotoxic stress.
ACKNOWLEDGMENTS
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REFERENCES


**FIGURE LEGENDS**

**Fig. 1.** **RPA1-deficiency leads to defective S phase progression and G₂/M cell cycle arrest.**

*Panel A.* HEK 293T cells (left panel) or HeLa cells (right panel) were transfected with either scrambled control siRNA (SCR) or an RPA1-specific siRNA. Cell extracts were prepared 48 hours later and subjected to SDS-PAGE and immunoblot analysis using antibodies specific for RPA1 and RPA2 (left panel) or RPA1 (right panel). A non-specific band recognized by the RPA1 antibody is marked in the right panel as a loading control reference. 

*Panel B.* Defective S phase progression in RPA1-deficient cells. HeLa cells were transfected with SCR siRNA or RPA1 siRNA. Forty-eight hours later, the cells were pulse-labeled with BrdU and harvested at the indicated time intervals. The cells were then stained with α-BrdU mAb and PI and analyzed by two-parameter FACS to measure DNA synthesis (BrdU-FITC, Y axis) and DNA content (PI, X axis). Immunoblotting was used to confirm the suppression of RPA1 for each of the experiments presented in panels *B-D* (data not shown). 

*Panel C.* RPA1-deficient cells undergo terminal G₂/M arrest. HeLa cells were transfected with SCR or RPA1 siRNAs and subjected to PI staining and FACS analysis either 48 h or 96 h later. 

*Panel D.* Prolonged RPA1 deficiency causes loss of cell viability. HEK 293T cells were transfected with RPA1 or SCR siRNAs and cell viability was assessed at the indicated times post-transfection by staining with trypan blue (n=3).

**Fig. 2.** **RPA1-deficiency causes DNA damage and activates an ATM-dependent G₂/M checkpoint.**

*Panel A.* Spontaneous DNA damage in RPA1-deficient cells. HeLa cells were transfected with scrambled (SCR) or RPA1 siRNAs. Seventy-two hours later, cells were stained with a phospho-specific antibody against serine 139 of histone γ-H2AX (p-H2AX) and DAPI and then examined by immunofluorescence microscopy. 

*Panel B.* ATM promotes RPA1 siRNA-induced G₂/M arrest. HeLa cells were transfected with either SCR siRNA, or siRNAs specific for ATM and RPA1, either individually or in combination. Seventy-two hours later the cells were stained with PI and analyzed by FACS. Fractions of the cells were also analyzed by immunoblotting with α-ATM and α-RPA1 antibodies to confirm RPA1 and ATM suppression. The expression of RPA1 was reduced comparably between cells transfected with RPA siRNA and ATM/RPA1 siRNAs (data not shown). 

*Panel C.* RPA1 deficiency causes constitutive activation of ATM. HeLa cells were transfected with scrambled (S) or RPA1 (R1) siRNAs. Cell extracts were prepared 48 h or 72 h
later and subjected to immunoblot analysis using antibodies specific for RPA1, serine 1981-phosphorylated ATM (\(\alpha\)-pATM-1981), and total ATM. Where indicated (+), the cells were exposed to 10 Gy of IR 1 h prior to harvest to induce the phosphorylation of ATM on Ser-1981.

Fig. 3. **RPA is not required for ATM activation in response to IR.** HeLa cells were transfected with either scrambled siRNA (S) or RPA1 (R1) siRNAs. Forty-eight hours later, the cells were exposed to 5 Gy of IR or mock irradiated. Cell extracts were then subjected to SDS-PAGE and immunoblotting with \(\alpha\)-pATM-1981, \(\alpha\)-ATM, \(\alpha\)-RPA1, and \(\alpha\)-tubulin antibodies.

Fig. 4. **Relative effects of RPA1, RPA2, and ATR siRNAs on ATR substrate phosphorylation.** HEK 293T cells were transfected with scrambled (S), ATR (A), RPA2 (R2), or RPA1 (R1) siRNAs. Forty-eight hours later, the cells were treated either with 25 J/m\(^2\) UV light, 10 Gy of IR, 3 mM HU, or vehicle alone. Cell extracts were prepared either 1 h (IR, UV) or 4 h (HU) later and analyzed by SDS-PAGE and immunoblotting with antibodies specific for: RPA1, RPA2, ATR, and tubulin (A); CHK1 and Ser-317-phosphorylated CHK1 (\(\alpha\)-pCHK1-317) (B); CREB and Ser-121-phosphorylated CREB (\(\alpha\)-pCREB-121) (C).
A

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293T

siRNA:

SCR  |  RPA1

α-RPA1  |  

α-RPA2  |  

HeLa

siRNA:

SCR  |  RPA1

α-RPA1  |  

Non-specific

B

t (h):

0  |  4  |  8  |  16

SCR siRNA

BrdU-FITC

RPA1 siRNA

PI (DNA content)

C

siRNA:

SCR  |  RPA1

Events

48 h  |  96 h

48 h  |  96 h

PI (DNA content)

D

Percent viability

0  |  10  |  20  |  30  |  40  |  50  |  60  |  70  |  80  |  90  |  100

48  |  72  |  96

Hours post transfection

SCR

RPA1
A

siRNA:

SCR

RPA1

B

SCHEME

ATM

RPA1

RPA1 + ATM

C

t (h):

48

72

IR:

- + -

- + -

siRNA:

S S R1

S S R1

α-RPA1

α-pATM-1981

α-ATM
Dodson, et al. Figure 3

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A

siRNA: α-RPA1
α-RPA2
α-ATR
α-tubulin

B

siRNA: α-pCHK1-317
α-CHK1

C

siRNA: α-pCREB-121
α-CREB
Supplemental Figure 1. Abnormal morphology of RPA-deficient cells. HeLa cells were transfected with SCR or RPA1 siRNAs and analyzed 72 h later by phase-contrast microscopy. Several abnormally elongated cells are denoted by arrows.