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ATR mediates cisplatin resistance in a *p53* genotype-specific manner

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Abstract

The protein kinase encoded by the *ataxia-telangiectasia and Rad3-related* (*ATR*) gene is activated by DNA damaging agents that are frequently employed as anticancer therapeutics. Inhibition of ATR expression in cultured cancer cells has been demonstrated to increase sensitivity to chemotherapeutic drugs, including the DNA crosslinking agent cisplatin. Cisplatin is a widely employed and effective drug, but its use is associated with significant toxicity. Here, we demonstrate that genetic inhibition of ATR expression selectively enhanced cisplatin sensitivity in human colorectal cancer cells with inactivated p53. A knockin strategy was employed to restore wild type p53 in cells harboring wild type or mutant *ATR* alleles. Knockin of functional p53 in ATR-deficient cells restored checkpoint function, suppressed apoptotic pathways, and dramatically increased clonogenic survival after cisplatin treatment. These results suggest that a strategy that combines specific inhibitors of ATR and conventional therapies might promote synthetic lethality in p53-deficient tumors while minimizing toxicity to normal tissues.

Keywords

ATR; p53; DNA damage; survival; chemotherapy; cisplatin; synthetic lethality; gene targeting

Introduction

The most widely used anticancer agents prevent cell growth by damaging chromosomal DNA or inhibiting DNA replication (Kastan and Bartek, 2004). Diverse types of DNA lesions and DNA structures trigger the activation of the phosphatydlinositol kinase-like kinases ATM and ATR. ATM is primarily activated by the double strand DNA breaks caused by ionizing radiation and radiomimetic drugs. ATR functions downstream of ATM in response to DNA damage (Jazayeri *et al.*, 2006), but is also activated independently of

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ATM by a wide range of agents that inhibit DNA replication and cause the accumulation of replication intermediates (Osborn *et al.*, 2002; Hurley and Bunz, 2007; Cimprich and Cortez, 2008). Replication inhibitors that can robustly activate ATR include antimetabolites that alter nucleotide metabolism and alkylating agents that cause DNA lesions that physically impede DNA replication forks. ATM and ATR directly phosphorylate over 700 downstream substrates that collectively control cell growth and survival (Matsuoka *et al.*, 2007).

Prominent among the regulatory proteins activated after DNA damage is the tumor suppressor p53, a transcription factor that is stabilized upon phosphorylation and thereby activated (Tibbetts *et al.*, 1999; Shiloh, 2006). Genetic alterations that cause loss of p53 function - which occur in a large proportion of all human cancers - cause defective regulation of cell growth and death in response to DNA damage, and therefore present a potential obstacle to effective therapy (El-Deiry, 2003; Meek, 2009). Because the toxicity of most therapeutic agents to normal tissues limits the doses that can be safely administered to patients, strategies for selectively sensitizing p53-deficient cancer cells to existing anticancer drugs and radiation would have significant clinical impact.

Recent studies have demonstrated parallel interactions between upstream DNA damage signaling pathways and p53 that may be exploited to selectively impair coordinated cell cycle arrest (Chung and Bunz, 2010) and improve therapeutic responses (Jiang *et al.*, 2009) in *p53*-mutant cells. For example, targeted inhibition of ATM and its substrate Chk2 has been shown to increase the sensitivity of $p53^{-/-}$ human cancer cells to the radiomimetic drug doxorubicin, while increasing the resistance of $p53^{+/+}$ cells (Jiang *et al.*, 2009). It is currently unknown whether specific targeting of ATR might similarly increase the sensitivity of p53-deficient cells.

ATR is a particularly attractive target for combination therapies as it is robustly activated by many different types of drugs. Inhibiting ATR activity, either by RNAi-mediated knockdown of *ATR* expression (Collis *et al.*, 2003) or by overexpression of a dominant-negative mutant ATR protein (Cliby *et al.*, 1998), has been shown to confer sensitivity to diverse anticancer agents, including ionizing radiation, methyl methanesulfonate and cisplatin.

To rigorously study the role of ATR in therapeutic responses, we generated a genetic model system wherein a human colorectal cancer cell line was engineered to harbor the hypomorphic mutation at the *ATR* locus that causes ATR-Seckel syndrome (Hurley *et al.*, 2007). At the cellular level, the ATR-Seckel mutation causes aberrant splicing of the *ATR* transcript and markedly decreased ATR expression (Alderton *et al.*, 2004; O'Driscoll *et al.*, 2003). Cancer cells homozygous for ATR-Seckel alleles ($ATR^{S/S}$) exhibit greatly reduced clonogenic survival in response to many commonly used anticancer agents, particularly to DNA crosslinking agents such as cisplatin (Wilsker and Bunz, 2007). Decreased ATR expression also causes differential and highly reproducible sensitization to antimetabolites, ionizing radiation and radiomimetic drugs in this in vitro system (Hurley *et al.*, 2007; Wilsker and Bunz, 2007). An important question that arises from these studies concerns the

potential efficacy of anti-ATR therapy. Would inhibiting ATR preferentially sensitize cancer cells with loss of p53 function?

Recent studies in mice suggest that the effects of ATR inhibition on cell survival are antagonized by p53. In a mouse model of ATR-Seckel syndrome, the deficiency of ATR causes impaired DNA replication during embryogenesis and accelerated aging in adult mice (Murga *et al.*, 2009). Homozygous disruption of *p53* in the ATR-Seckel background aggravates this aging phenotype. In a mosaic mouse model, the conditional disruption of *ATR* in a *p53*-mutant background causes the accumulation of DNA damage and tissue degradation (Ruzankina *et al.*, 2009). Together, these studies demonstrate that p53 functions to protect against the detrimental effects of ATR deficiency. In this study, we examined whether p53-mutant human cancer cells might be preferentially chemosensitized by genetic ATR inhibition. We show that RNAi-mediated knockdown of ATR preferentially sensitized $p53^{-/-}$ cells to the effects of cisplatin, and that knockin of wild type *p53* into the ATR-Seckel background suppressed apoptotic pathways, restored checkpoints and increased cisplatin resistance to the level exhibited by cells with wild type *ATR*. These data support specific ATR inhibition as a therapeutic strategy to target p53-deficient tumors.

Results

Increased sensitivity of p53-deficient cells to cisplatin after ATR knockdown

Our previous studies have shown that ATR-mutant colorectal cancer cells are dramatically sensitized to the alkylating agent cisplatin, as well as to other drugs that similarly cause DNA crosslinks (Wilsker and Bunz, 2007). To explore whether the inhibition of ATR might selectively sensitize p53-deficient cancer cells to this important class of therapeutic agent, we used gene-specific siRNA to reduce ATR expression in $p53^{+/+}$ and $p53^{-/-}$ derivatives of the colorectal cell line HCT116 (Fig. 1a). ATR is an essential protein required for efficient DNA replication. As expected, transient inhibition of ATR expression caused a reduction in clonogenic survival in both HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells (Fig. 1b). A low dose of cisplatin also suppressed clonogenic growth, irrespective of p53 genotype. The combination of ATR knockdown and treatment with cisplatin reduced the survival of $p53^{-/-}$ cells to a significantly greater extent than $p53^{+/+}$ cells, which were relatively resistant.

Restoration of p53 function by knockin

To examine in detail the combined effects of ATR and p53 in a stable genetic system, we used a knockin strategy (Fig. 2a) to restore normal p53 function in cells with previously engineered ATR-Seckel alleles. The parental DLD-1 cell line from which the *ATR*-mutant ($ATR^{S/S}$) cells were derived harbors a naturally-occurring inactivating point mutation in *p53* exon 7 (S241F) and expresses only mutant *p53* transcripts (Fig. 2b). The remaining *p53* allele in this diploid colorectal cell line has a wild type coding sequence, but is not expressed (Sur *et al.*, 2009). We used a recombinant adeno-associated virus (rAAV)-based gene targeting vector containing a synthetic exon promoter trap (SEPT) (Topaloglu *et al.*, 2005) to replace the mutant exon 7 with wild type sequence, and thereby reverse the inactivating mutation. The SEPT selection cassette, flanked by LoxP sites, was excised by transient

expression of the cre recombinase, thereby restoring the functional architecture of the *p53* locus (Fig. 2a).

Following infection of target cells with the *p53*-specific rAAV (Sur *et al.*, 2009), and stable selection of transgenic colonies, we identified three clones that had independently integrated the targeting construct into the *p53* locus, replacing the expressed mutant *p53* allele (Fig. 2b). One of these clones was chosen for detailed analysis; all effects described were reproduced in an independent clone. Clonal derivatives with the same genotype were phenotypically indistinguishable, as is typically the case for knockin/knockout cell derived by homologous recombination (Rago *et al.* 2007; Chung and Bunz, 2010).

Excision of the SEPT cassette resulted in the expression of wild type *p53* transcripts (Fig. 2b), and increased expression of the p53 target proteins p53R2 and p21 (Fig. 2c). The majority of inactivating *p53* mutations cause increased stability of the encoded protein. Accordingly, restoration of functional p53 resulted in a decrease in steady state p53 protein expression (Fig. 2c).

To further assess p53 function in knockin ($p53^{+/Sil}$) cells, we examined well-characterized responses to ionizing radiation (IR). After upregulation by IR, p53 activates the G_1/S checkpoint that controls entry into S-phase and stabilizes arrest at the G_2/M checkpoint, thereby inhibiting the onset of mitosis (Waldman *et al.*, 1995; Bunz *et al.*, 1998). Cells were irradiated and then immediately treated with nocodazole to prevent cell division and trap G_2/M checkpoint-defective cells in mitosis. Control treatment with nocodazole alone (no irradiation) revealed a modest increase in cells with a stable 2N DNA content in the *p53*-knockin population (Fig. 2d). Presumably, this failure of a proportion of cells to enter S-phase after 24 h was related to the increased levels of basal p21 induced by p53 pathway restoration (Fig. 2c). Asynchronous $ATR^{S/S}$ cells with mutant p53 ($p53^{-/Sil}$) have been shown to be markedly checkpoint-defective (Hurley *et al.*, 2007). Accordingly, after treatment with both nocodazole and IR, $ATR^{S/S}p53^{-/Sil}$ cells failed to accumulate at a 2N peak corresponding to G_1/S (Fig. 2d) and entered mitosis in large numbers (Fig. 2e). In contrast, the $ATR^{S/S}p53^{+/Sil}$ cells exhibited restored function of both G_1/S and G_2/M checkpoints.

ATR-deficiency selectively sensitized p53-mutant cells to cisplatin

We next used our new isogenic cell panel to assess the combined effects of *ATR* and *p53* status on cell survival after cisplatin treatment. Consistent with our previously published results (Wilsker and Bunz, 2007), $ATR^{S/S}p53^{-/Sil}$ cells exhibited markedly decreased survival after treatment with cisplatin across a broad dose range (Fig. 3a). The highest dose tested (1 μM) reduced survival of *ATR*-deficient cells nearly one hundred-fold, compared with isogenic DLD-1 cells that are ATR-proficient. Strikingly, the response of p53-knockin $ATR^{S/S}p53^{+/Sil}$ cells to cisplatin at all doses was similar to that of ATR-proficient parental DLD-1 cells, demonstrating that p53 could counteract the drug sensitization caused by ATR deficiency.

To evaluate the effect of p53 restoration on different drug responses, we separately compared the effects of p53 and ATR on survival after cisplatin and the DNA synthesis inhibitor hydroxyurea (HU). In a p53-deficient background, ATR is required for clonogenic

survival in response to both of these drugs (Wilsker and Bunz, 2007). Restoration of p53 function in parental DLD-1 with wild type *ATR* alleles caused a significant sensitization of these cells to both cisplatin and HU (Fig. 3b), consistent with the well known role of p53 as an inhibitor of cell proliferation after DNA damage and DNA replication inhibition. Cells with the $ATR^{S/S}$ genotype were rendered more resistant to cisplatin and HU when wild type p53 function was restored. Interestingly, the desensitizing effect of restored p53 was significantly more pronounced in cisplatin-treated cells. While $ATR^{S/S}p53^{+/Sil}$ cells treated with HU exhibited similar survival to HU-treated $ATR^{+/+}p53^{+/Sil}$ cells, the survival of $ATR^{S/S}p53^{+/Sil}$ cells after cisplatin most resembled cells with the $ATR^{+/+}p53^{+/Sil}$ genotype (Fig. 3b). We conclude that p53 most potently modified ATR-mediated survival pathways in cisplatin-treated cells, and propose that anti-ATR therapy might uniquely sensitize p53-deficient tumor cells to cisplatin. These results complement previous studies demonstrating that ATM inhibition can differentially promote survival or sensitivity to DNA damage, depending on *p53* status (Jiang *et al.*, 2009).

It is interesting to note that the p53-mediated response to cisplatin was more striking in cells with restored p53 function (*p53*-knockin DLD-1; Fig. 3b) than in cells that retained wild type *p53* alleles (HCT116); *p53*-knockout in HCT116 caused a minimal difference in survival (Fig. 1b). It has been reported that some p53-dependent responses may not be well maintained in *p53*-wild type cells such as HCT116 (Zhang *et al.*, 2006). Like all human cancer cell lines that have been adapted to *in vitro* culture and extensively propagated following explantation, HCT116 and DLD-1 cells have been selected for robust growth. The growth-suppressive pathways downstream of p53 are inactive in *p53*-mutant DLD-1 cells (Sur *et al.*, 2009), and presumably not subjected to negative selective pressure. One might therefore predict that growth suppressive pathways would be retained but inactive in p53-mutant DLD-1 and then robustly reactivated by wild type *p53* knockin. In support of this view, the upregulation of growth inhibitory p53 target genes that encode PUMA and Ferrodoxin reductase were more strongly upregulated by DNA damage in the DLD-1 $p53^{+/Sil}$ derivative than in other colorectal cancer cells lines that naturally harbor wild type *p53* alleles (Sur *et al.*, 2009). We have observed the p53-dependent phenotypes in knockin DLD-1 cells to be highly stable and infer that, in p53-wild type cells, strong selection against p53 phenotypes might occur during the initial establishment of new cell lines, rather than during routine passaging. We therefore predict that human cell lines with restored p53 function will be valuable reagents for the study of the p53-dependent responses to anticancer agents.

Suppression of cisplatin-induced apoptosis in *ATR*-mutant cells by restoration of functional p53

We tested whether the decrease in clonogenic survival exhibited by $ATR^{S/S}p53^{-/Sil}$ cells in response to cisplatin (Fig. 3a,b) might be related to the induction of apoptosis. Indeed, morphologic evidence of apoptosis was apparent in a large fraction of $ATR^{S/S}p53^{-/Sil}$ cells after cisplatin treatment (Fig. 4a). Cisplatin-induced apoptosis occurred independently of functional p53, and was in fact suppressed by p53 restoration. To confirm the biochemical activation of apoptotic pathways, we assayed the cleavage of caspase-3 and the phosphorylation of p53 on S46 (p-p53S46), a marker of upstream apoptotic signaling (Oda

et al., 2000). These biochemical markers of apoptosis were coordinately expressed in cisplatin-treated $ATR^{S/S}p53^{-/Sil}$ cells and suppressed in all isogenic cells with functional ATR or p53 (Fig. 4b). The small amount of p-p53S46 detected in cells of the other genotypes corresponded well with the relatively smaller proportion of apoptotic cells (1-3%) observed in these populations (Fig. 4a). Interestingly, phosphorylation of p53 S46 after cisplatin treatment was impaired in cells in which p53 had been functionally restored (Fig. 4b), suggesting the presence of a negative feedback loop. Overall, isogenic cells with proficient ATR and/or p53 pathways were resistant to the apoptotic pathways stimulated by cisplatin (Fig. 4a,b). These data suggest that increased apoptotic signaling in response to cisplatin may have contributed to the reduced clonogenic survival of $ATR^{S/S}p53^{-/Sil}$ cells after cisplatin treatment.

Downstream interactions between ATR and p53 pathways after cisplatin treatment

The chemoprotective effects of ATR have been largely attributed to the activation of its downstream effector kinase, Chk1 (Cimprich and Cortez, 2008). Recently, the stress activated kinase pathway composed of p38MAPK and MAPKAP2 (MK2) has emerged as another important responder to DNA damaging agents, including cisplatin (Reinhardt *et al.*, 2007; Reinhardt *et al.* 2010). Like Chk1, p38MAPK is controlled by ATR (Jirmanova *et al.*, 2005; Reinhardt and Yaffe, 2009). Yaffe and colleagues (Reinhardt *et al.*, 2007) have reported that loss of p53 functionally “rewires” the cellular response to cisplatin, thereby increasing the requirement for MK2 in the control of downstream survival pathways. Because the effect of p53 restoration in our cell lines differed in *ATR*-wild type and –mutant cells (Fig. 3b), we examined whether *p53* status affected relative activation of Chk1 and MK2 by cisplatin. Two isogenic cell pairs were tested: HCT116 cells and their *p53*-knockout derivative (Bunz *et al.*, 1998), and DLD-1 cells and their derivative harboring the wild type *p53* knockin (Sur *et al.*, 2009). Of these two cell lines, DLD-1 exhibited a greater p53-dependent effect on cisplatin sensitivity (Figs. 1a, 3a,b). Irrespective of cisplatin treatment, DLD-1 cells with restored p53 exhibited a decrease in total Chk1 protein and an increase in MK2 protein and T334 phosphoprotein (Fig. 5a). The decreased Chk1 protein in the p53-restored DLD-1 cells correlated with decreased levels of Chk1 phosphoproteins after cisplatin treatment (Fig. 5a). The HCT116 lines, in which the effect of *p53* status alone (in the absence of ATR manipulation) on cisplatin sensitivity was minimal (Fig. 1b) exhibited only a modest decrease in Chk1 phosphorylation in $p53^{+/+}$ compared with the $p53^{-/-}$ derivative, but no apparent difference in total Chk1 or MK2 proteins.

We next examined the combined effects of *ATR* and *p53* status on the p38MAPK-MK2 signaling pathway. Levels of MK2 were highest in cells with both wild type *ATR* and wild type *p53* (Fig. 5b). While the levels of p38MAPK protein did not vary with genotype, increased p38MAPK activation by cisplatin could be observed in the cells with restored p53. The re-introduction of p53 into DLD-1 cells thus caused an apparent shift in ATR-dependent signaling from Chk1 to MK2 (Fig. 5a). This rewiring of ATR responses after restoration of p53 correlated with decreased survival after DNA damage (Fig. 3b).

Discussion

ATR promotes cell survival after DNA damage and impeded DNA replication, and is therefore a logical target for sensitization to commonly employed anticancer therapeutics (Cimprich and Cortez, 2008; Wagner and Kaufmann, 2010). A critical question is whether the p53-deficient cells that compose a high proportion of human cancers would be selectively killed by such a combinatorial strategy. The answer to this question is not obvious. Depending on the context in which it is activated, p53 can trigger cell cycle arrest - and an overall increase in cell survival - or cell death by apoptosis (Vousden and Lu, 2002). We observed that knockdown of ATR preferentially sensitized *p53*-knockout colorectal cells to cisplatin (Fig. 1a,b) and that cells with inactivating mutations in both *ATR* and *p53* exhibited increased apoptosis (Fig. 4a,b) and reduced survival (Fig. 3a, b) after cisplatin treatment. Restoration of functional p53 dramatically suppressed these phenotypes. These results suggest that specific targeting of ATR would be an effective means of increasing the sensitivity of *p53*-mutant tumor cells to cisplatin while preserving the resistance of normal tissues that retain p53 function, thereby minimizing toxicity. The ultimate test of this hypothesis in patients awaits the development of highly specific ATR inhibitors (Wagner and Kaufmann, 2010).

Recent efforts to enhance the effects of DNA damaging agents and DNA replication inhibitors on p53-deficient cancer cells have largely focused downstream of ATR, on Chk1 (Tse *et al.*, 2007). While genetic inhibition of Chk1 and chemical Chk1 kinase inhibitors have been reported to be effective in sensitizing cancer cells to drugs such as gemcitabine, HU and 5-fluorouracil (Cho *et al.*, 2005; Karnitz *et al.*, 2005; Robinson *et al.*, 2006; Blasina *et al.*, 2008; Wilsker *et al.*, 2008), targeting Chk1 has proven a relatively ineffective approach to reduce cell survival in combination with cisplatin (Wagner and Karnitz, 2009; Zenvirt *et al.* 2010). In response to cisplatin, ATR activates multiple downstream effectors (Reinhardt *et al.*, 2007), including p53 (Pabla *et al.*, 2008), that may cumulatively affect survival. The rewiring of these downstream pathways that occurs upon loss of p53 function (Reinhardt *et al.*, 2007) may create an enhanced dependence on ATR. Studies arising from diverse experimental systems therefore suggest that targeting upstream ATR activity may represent a more effective means of sensitizing p53-deficient tumor cells to the distinct effects of cisplatin.

In cellular models and in actual human tumors, functional p53 can confer resistance to some types of DNA damage and sensitivity to others (Bunz *et al.*, 1999; Pirollo *et al.*, 2000). These distinct outcomes are probably related to the alternative cell cycle arrest or cell death pathways that can be activated by p53 (Vousden and Lu, 2002). Which of these downstream pathways predominates in a given cell appears to depend upon many factors, including the type of cell and the DNA damaging agent employed. One mechanism by which the choice between arrest and apoptosis is determined involves the kinase HIPK2, which phosphorylates p53 on S46 (Rui *et al.*, 2004). Stimuli that promote cell cycle arrest over apoptosis trigger the suppression of HIPK2, thereby suppressing p53 S46 phosphorylation (Li *et al.*, 2009). The p53 S46 site can also be phosphorylated directly by ATM (Kodama *et al.* 2010) and by DYRK2, an ATM-responsive kinase (Taira *et al.* 2007). Understanding

how ATR might influence these upstream pathways should provide significant insight into the manner in which p53 mediates different cell fates in response to DNA damage.

Materials and Methods

Cell lines and drugs

Cisplatin (*cis*-Diammineplatinum(II) dichloride) and hydroxyurea were purchased from Sigma-Aldrich (St. Louis, MO). The human colorectal cell lines HCT116 and DLD-1and their isogenic derivatives with targeted alterations in *p53* (Bunz *et al.*, 1998; Sur *et al.*, 2009) and *ATR* (Hurley *et al.*, 2007) were cultured in McCoy's 5A medium (Invitrogen, Carlsbad CA) supplemented with 6% fetal calf serum (Hyclone) and penicillin/streptomycin (Invitrogen).

Knockdown of ATR expression

For the transient knockdown of ATR, 3×10^4 cells in 24-well plates were transfected with 8 μmol of ATR-targeted siRNA (AACCUCCGUGAUGUUGCUUGA, synthesized by Integrated DNA Technologies, Inc, Coralville, IA) or non-targeting control siRNA (Dharmacon, Lafayette, CO), using 2μl Lipofectamine 2000 (Invitrogen) for 72 h.

Genotyping

The restoration of wild type *p53* in the $ATR^{S/S}$ DLD-1 cells was confirmed by sequencing amplified genomic DNA- and cDNA-derived PCR products from selected clones using the primers 100-P53 exon7 FOR: CTTGGGCCTGTGTTATCTCC and 101-p53 exon 7 rev: ATGGAAGAAATCGGTAAGAGG.

Drugs and assessment of clonogenic survival

Cells in 24-well plates were treated with drug under the conditions described, washed, harvested and replated at low density in 100 mm dishes. Following 14 d of growth, surviving cells were stained with crystal violet (Sigma-Aldrich). Colonies containing more than 50 cells were scored and the total number of colonies per dish was normalized to untreated controls. Each data point represents the average of three separate dishes.

Cell cycle analysis and quantification of apoptosis

To assess IR-dependent cell cycle checkpoints, cells were treated with a single 12 Gy dose delivered by a ^{135}Cs irradiator and then immediately incubated in medium containing 0.2 μg/ml nocodazole. After 24 h, cells were harvested, fixed, stained with Hoechst 33258, and analyzed by flow cytometry and fluorescence microscopy, as described (Jallepalli *et al.*, 2003). After cisplatin treatment, the proportion of Hoechst 33258-stained cells with blebbing of the nuclear membrane, and fragmented and condensed nuclei was assessed by fluorescence microscopy. At least 300 nuclei from different fields were scored for each data point.

Antibodies and Immunoblotting

Whole-cell lysates were denatured and fractionated on NuPAGE gels (Invitrogen). Proteins were transferred to PVDF membranes (Millipore, Billerica, MA) which were probed with antibodies directed against ATR, α-tubulin, p53, p53R2, Chk1, p21 (Santa Cruz Biotechnology, Santa Cruz, CA), phoshpo-p53Ser46, phospho-Chk1S345, phospho-Chk1S317, MK2, phospho-MK2T334, p38MAPK, phospho-p38MAPKT180/Y182 and cleaved caspase-3 (Cell SignalingTechnologies, Danvers, MA) under conditions recommended by the manufacturers. Blots were developed using enhanced chemiluminescence (GE Healthcare, Piscataway, NJ).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ATM	Ataxia telangiectasia mutated
ATR	ATM and Rad3-related
IR	ionizing radiation
HU	hydroxyurea

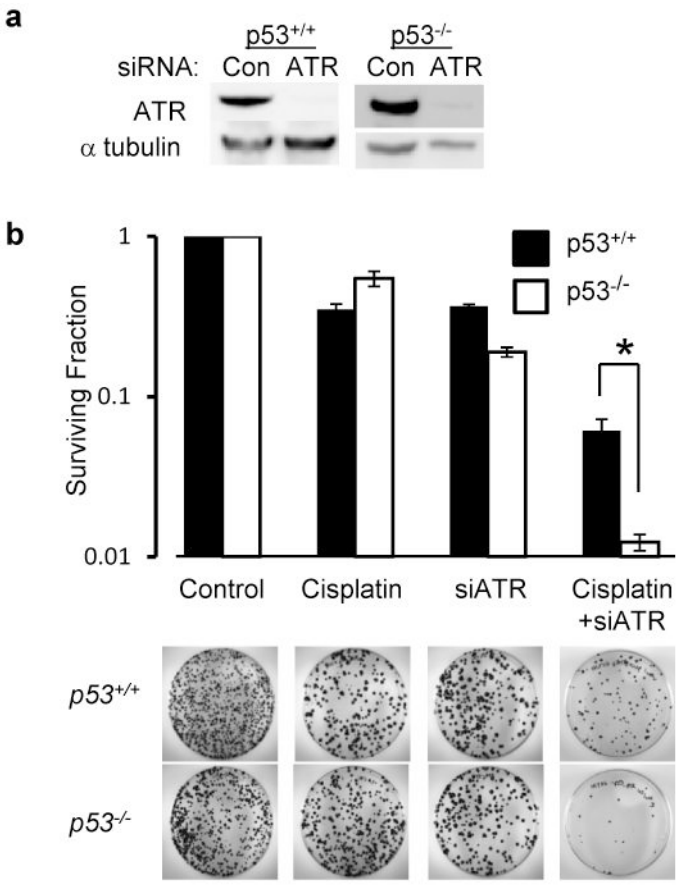

Figure 1.

Clonogenic survival after ATR knockdown and cisplatin treatment. (**a**) Levels of ATR protein in HCT116 *p53*$^{+/+}$ and *p53*$^{-/-}$ cells were assessed by immunoblot 48 h after siRNA transfection. (**b**) Following knockdown, untreated controls and transfected cells were mock treated or treated with 1 μM cisplatin for an additional 48 h, as indicated. After treatment, cells were washed three times, replated at low density and incubated for 14 d. Error bars represent SEM; representative plates are shown.

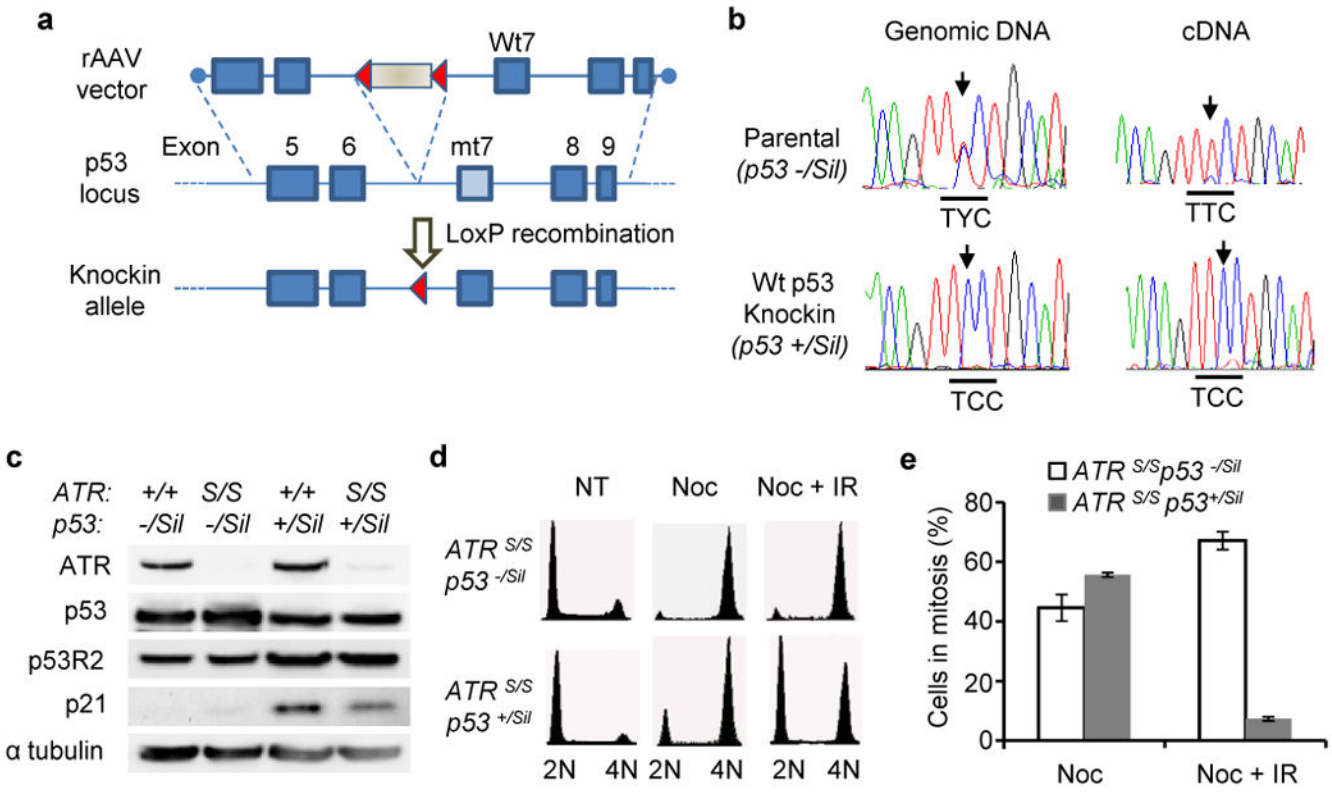

Figure 2.

Restoration of functional p53 by exon 7 knockin. (**a**) An rAAV-based knockin strategy (Sur *et al.*, 2009) was employed to replace mutant exon 7 (mt7) in DLD-1 $ATR^{S/S}$ cells with wild type exon 7 (Wt7) sequences. Cre-mediated recombination between LoxP sites flanking the SEPT cassette (gray box) allowed excision of the SEPT cassette. (**b**) The homologous introduction of wild type codon 241 (TCC, encoding an S residue) in targeted clones was confirmed by sequence analysis of the *p53* genomic locus and cDNA. (**c**) Expression of ATR, p53, p53R2 and p21 in DLD-1 derived cells with the indicated genotypes was assessed by immunoblot. α tubulin was probed as a loading control. To assess the function of p53-dependent checkpoints, $ATR^{S/S}$ cells differing in p53 genotype were treated with 12 Gy IR (Noc + IR) or mock irradiated (Noc) and then immediately incubated in media containing nocodazole (0.2μg/μl). Cells were fixed and stained with Hoescht 33258 dye 24 h after nocodazole addition. The G_1/S checkpoint was assessed by measuring the population of cells with 2N DNA content (2N) by flow cytometry (**d**). The Y-axis represents cell number. (**e**) The G_2/M checkpoint activated by IR was assessed by counting the number of cells trapped in mitosis, by fluorescence microscopy.

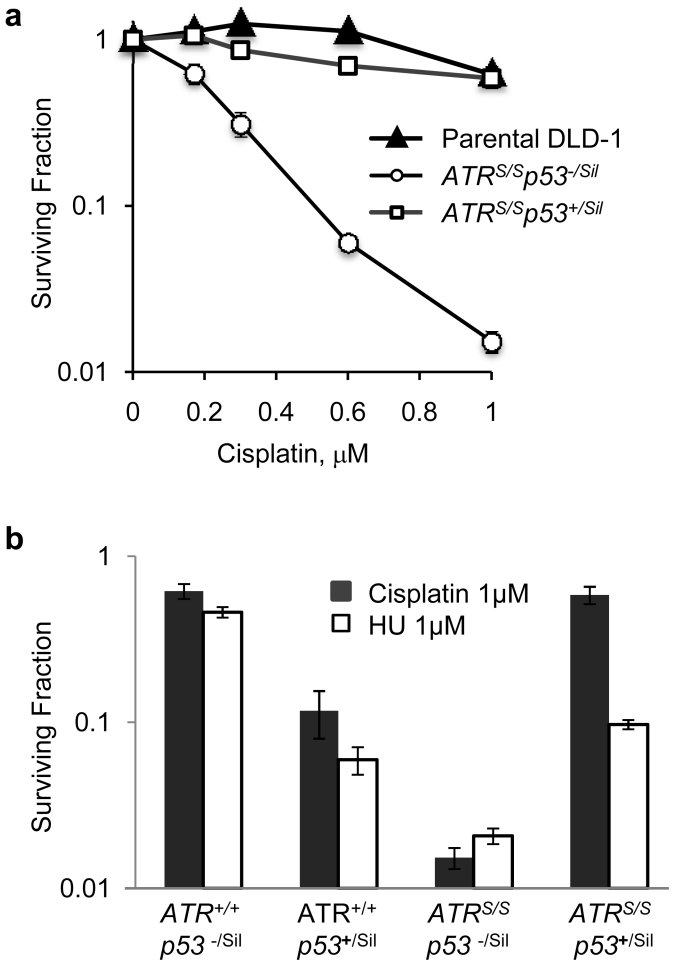

Figure 3.

The combined effects of *ATR* and *p53* genotype on clonogenic survival after drug treatment. (**a**) ATR-deficient ($ATR^{S/S}$) cells expressing mutant p53 ($p53^{-/Sil}$) or wild type p53 ($p53^{+/Sil}$) and parental DLD-1 controls ($ATR^{+/+}p53^{-/Sil}$) were treated with cisplatin at doses ranging from 0-1 μM for 48 h, and then replated in drug-free medium. (**b**) Cells with the indicated genotypes were treated with a single dose (1 μM) of either cisplatin or hydroxyurea (HU) for 24 and 48 h, respectively. Clonogenic survival was assayed as described in Materials and Methods. Error bars represent SEM

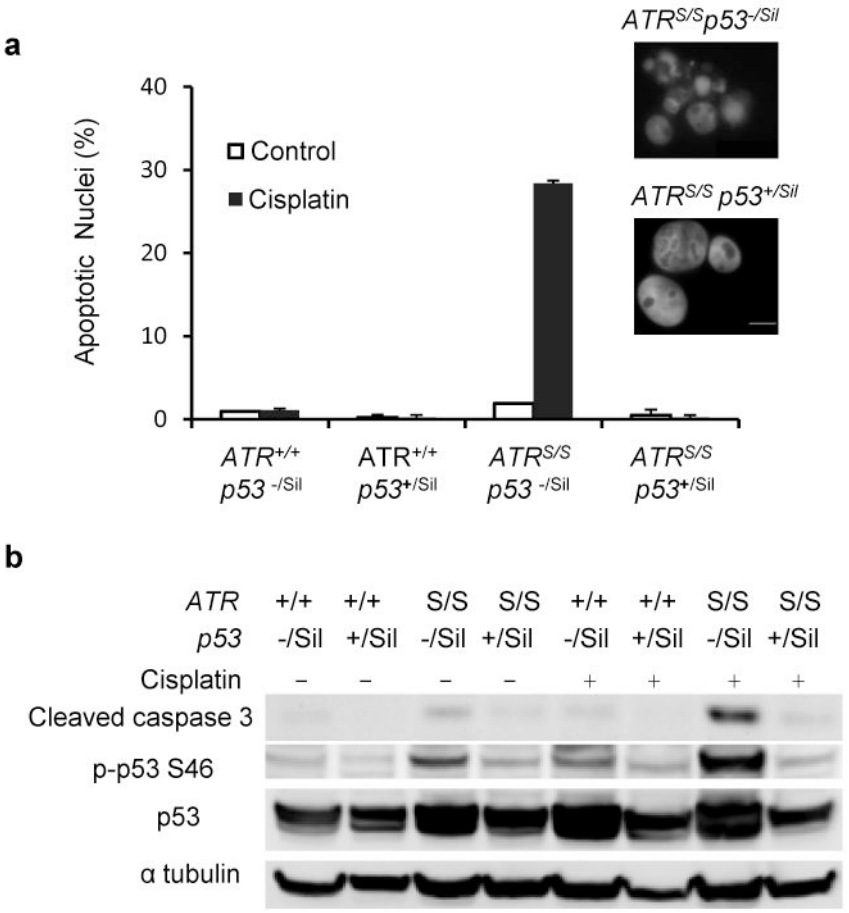

Figure 4.

Apoptotic responses to cisplatin. (**a**) Cells with the indicated genotypes were untreated (Control) or treated with cisplatin for 48 h. The fraction of apoptotic nuclei was assessed by fluorescence microscopy. Representative nuclei are shown at 40× magnification (inset, scale bar 10 μm). (**b**) Cells treated as in (a) were lysed and probed with the indicated antibodies.

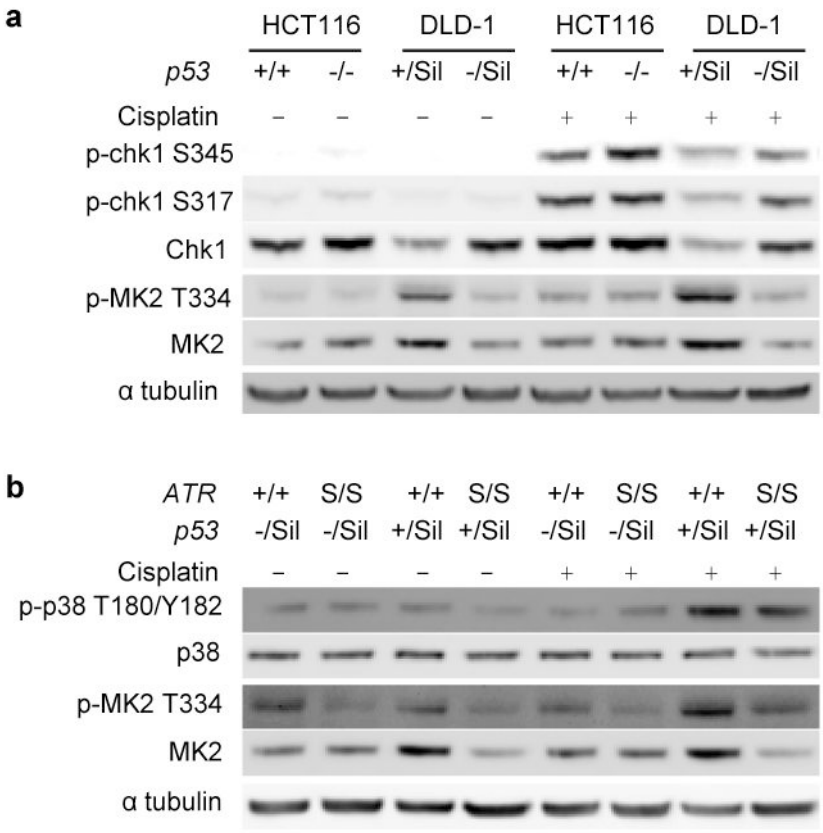

Figure 5.

Signaling pathways activated by cisplatin. (**a**) Isogenic HCT116 and DLD-1 cells differing in *p53* status were untreated (-) or treated (+) with cisplatin for 48 h. The indicated signaling proteins and phosphoproteins were assessed by immunoblot. (**b**) Isogenic DLD-1 cells differing in *ATR* and *p53* status were treated and analyzed as in (a). α tubulin was probed as a loading control.