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ATDC/TRIM29 phosphorylation by ATM/MAPKAP kinase 2 mediates radioresistance in pancreatic cancer cells

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is characterized by therapeutic resistance for which the basis is poorly understood. Here we report that the DNA and p53 binding protein ATDC/TRIM29, which is highly expressed in PDAC, plays a critical role in DNA damage signaling and radioresistance in pancreatic cancer cells. ATDC mediated resistance to ionizing radiation in vitro and in vivo in mouse xenograft assays. ATDC was phosphorylated directly by MAPKAP kinase 2 (MK2) at Ser550 in an ATM-dependent manner. Phosphorylation at Ser-550 by MK2 was required for the radioprotective function of ATDC. Our results identify a DNA repair pathway leading from MK2 and ATM to ATDC, suggesting its candidacy as a therapeutic target to radiosensitize PDAC and improve the efficacy of DNA-damaging treatment.

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Keywords

pancreatic cancer; ATM; MK2; DNA repair; DNA damage response

Introduction

Irradiation of cells triggers a complex cellular response resulting in the processing of DNA damage, the activation of cell cycle checkpoints, and in some circumstances, induction of apoptosis. The DNA damage sensor ATM is rapidly activated following exposure of cells to ionizing radiation (IR) by a mechanism suggested to involve alterations in chromatin topology/structure (1). Activated ATM phosphorylates more than 700 substrates specifically on SQ/TQ amino acid motifs (2). The histone variant H2AX is one of the earliest substrates to be phosphorylated by ATM and the phosphorylated SQ motif on H2AX nucleates the assembly of DNA damage response complexes consisting of NBS1, MDC1, 53BP1 and BRCA1 at sites of DNA damage (3–5). Other ATM substrates include p53 and Chk1/2, which regulate cell cycle checkpoints following IR (6). Despite recent discoveries, the full spectrum of proteins participating in the DNA damage response (DDR) and the mechanisms by which these multi-protein complexes execute DNA damage processing and signaling are not fully known.

In contrast to the DNA damage-specific activation of ATM, the p38 MAPK and MAPKAP kinase-2 (MK2) pathway, a global stress-response signaling cascade, responds to many types of stress stimuli, including cytokines, IR, UV radiation, and oxidative stress and is part of the ATM/ATR dependent cell-cycle checkpoint machinery (7–9). In response to IR, p38 is rapidly activated in an ATM-dependent manner. Inhibition of p38 MAPK impairs p21 accumulation and attenuates cell cycle arrest after IR (10). Furthermore, MK2 kinase, a direct downstream target of p38, is responsible for phosphorylating CDC25A/B/C and maintaining the G1, S, and G2/M checkpoints in DDR (11). A recent study indicated that, in the absence of p53, cells depend on a third cell-cycle checkpoint pathway involving p38/MK2 kinase for cell-cycle arrest and survival after DNA damage. MK2 depletion in p53-deficient cells, but not in p53 wild-type cells, caused abrogation of the CDC25A-mediated S phase checkpoint and elimination of G2 checkpoint by loss of CDC25B binding to 14-3-3 proteins in DDR (9). Since many cancers harbor defective p53, MK2 signaling might play a critical role in DDR to protect p53-defective cancer cells against radiation or chemotherapy.

Pancreatic cancer is a highly lethal disease which is often diagnosed in an advanced state, for which there are little/no effective therapies. It is the fourth most common cause of cancer death in the United States, with an annual death rate of 43,140 people (12). Recent advances in surgical and medical therapy have had only a modest impact on the mortality rate. One of the major hallmarks of pancreatic cancer is its extensive local tumor invasion and early systemic dissemination. Pancreatic cancer is also notoriously resistant to many types of cytotoxic chemotherapy and ionizing radiation, therapies commonly used in the management of locally advanced disease (13). Using Affymetrix gene profiling, we previously identified that pancreatic cancers overexpress the Ataxia-Telangiectasia Group D

Associated gene (ATDC) at a level at least 20 fold higher than normal pancreas and chronic pancreatitis (14).

ATDC, also known as TRIM29, is a member of the tripartite motif (TRIM) protein family consisting of 70 members. TRIM proteins have a series of conserved domains, which include a B-box type 1 (B1) and B-box type 2 (B2), followed by a coiled-coiled (CC) region (15). The TRIM family of proteins has been implicated in a variety of physiological processes, such as development, oncogenesis, apoptosis and antiviral defense (16–18). The ATDC gene was initially described as a candidate gene responsible for the genetic disorder ataxia telangiectasia (AT) since its expression increased radiation resistance of AT5BI (AT-D) cells (19, 20). However, ATDC was later dismissed as an AT gene after the gene responsible for ataxia telangiectasia (ATM) was identified (21). We previously reported that ATDC, highly expressed in pancreatic cancer cells, promotes pancreatic tumor growth via stimulation of the β -catenin pathway (18). Since pancreatic cancer cells are commonly resistant to radiation therapy and ATDC may play a role in the cellular response to IR, we hypothesized that the high expression of ATDC in pancreatic cancer cells may contribute to the radioresistant phenotype of pancreatic cancer and sought to define the molecular mechanisms by which this occurs.

Materials and Methods

Cell Lines, Reagents and Constructs

Panc1 Mia PaCa2, and BxPC3 pancreatic ductal adenocarcinoma and the human embryonic kidney (HEK293) cell lines were purchased from American Type Culture Collection and used within six months of attainment. No additional authentication was performed on the cell lines following receipt. The human keratinocyte HaCat cell line was a gift from James Elder (University of Michigan). The ATM inhibitor KU55933 was provided by Graeme Smith (Cambridge University) and the Chk1/2 inhibitor AZD7762 by Meredith Morgan (University of Michigan). The MK2 inhibitor was purchased from EMD (Darmstadt, Germany) and the p38 inhibitor SB203580 from Cell Signaling Technology (Beverly, MA), the complementary DNA of human ATDC (provided by John Murnane, University of California, San Francisco). ATDC mutants were generated using the QuikChange® II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). HEK293 cells with stable expression of ATDC, and Panc1 and BxPC3 cells with ATDC knockdown using shRNA have been described (18).

Proliferation Assay

Cell proliferation was measured using a CellTiter 96 AQ nonradioactive cell proliferation assay (Promega Corporation, Madison, WI) as described previously (18).

Apoptosis Assays

To detect apoptotic cells, an ApoAlert V-FITC kit was used (BD Biosciences, Palo Alto, CA). After treatments, cells were rinsed and stained with annexin V-FITC/propidium iodide (PI) according to manufacturer's instructions. Annexin V and PI staining (fluorescence intensity) were assessed by fluorescence-activated cell sorting (FACS) following the

manufacturer's protocol (Becton Dickinson, San Jose, CA). Detection of Annexin V-positive cells was assessed using BD CELLQuest software (Becton Dickinson, San Jose, CA). Experiments were performed in triplicate and repeated three times.

***In Vitro* Kinase Assays**

Cell extracts containing wild type (WT) or kinase dead (KD) Flag-ATM were prepared from transfected 293T cells. Supernatants were immunoprecipitated with anti-Flag M2 antibody (Sigma St Louis, MO) and protein A/G-agarose. Flag immunoprecipitates were subjected to *in vitro* kinase assays as described (22). Further details are provided in the Supplemental Material and Methods.

Development of a Phospho-ATDC (p-ATDC) Antibody

To generate an anti-phospho-ATDC (p-ATDC) antibody, we designed ATDC peptides with the sequence (amino acid: 543–557) as CGYPSLMRSpQSPKAQP (phospho-peptide) or CGYPSLMRSQSPKAQP (non-phospho-peptide). Peptides were synthesized by the Peptide Synthesis Core (University of Michigan). The cysteine residue at the N-termini of both peptides was used for peptide conjugation. The phospho-peptide was coupled to keyhole limpet hemocyanin (Inject Maleimide Activated Immunogen Conjugation Kit with mCKLH and BSA, Pierce). The conjugated phospho-peptide was used to immunize rabbits by Cocalico Biologicals, Inc. (Reamstown, PA).

GST-ATDC Fusion Protein Production

For GST fusion peptide expression vectors, complementary oligonucleotides encoding desired peptides were cloned into the BamHI/EcoRI site of pGEX-4T1 (GE Healthcare Biosciences Pittsburgh, PA). Further details are provided in the Supplemental Material and Methods.

p-ATDC Antibody Purification

For p-ATDC antibody purification, the rabbit serum first went through a column conjugated with a non-phospho-ATDC peptide five times, and then the column with phospho-ATDC peptide. The column was washed extensively with PBS before elution with 0.1 M Glycine (pH 2.5). 1 M Tris-HCl, pH 8.0 was immediately added to eluted fractions to neutralize the pH. The pATDC antibody was then concentrated and tested. Further details are provided in the Supplemental Material and Methods.

Luciferase Reporter Gene Assays

Assays were performed to measure β -catenin activity using the TOPflash or FOPflash reporter constructs as we have described previously (18).

Clonogenic Survival Assays

Cells with or without altered ATDC expression were cultured for 24 hours in 60 mm petri dishes and irradiated with varying doses of IR. Cells were then counted to produce the final cell number required for each colony forming experiment in dishes. To result in 50–150 colonies per well in control dishes after the incubation period, 500 cells were seeded for

HEK293 cells, and 250 cells were seeded for Panc1 cells. Cells for each condition were set up in triplicate dishes and incubated at 37°C for 8–14 days depending on the cell growth rate. The cells were then fixed with methanol and stained with 2% methyl blue in 50% ethanol. The number of colonies with more than 50 cells was counted and the percentage of cell survival was calculated as the ratio of colonies in dishes exposed to IR compared to non-irradiated samples. Three independent experiments were carried for each cell line. The cell survival curves were drawn by plotting the means of three experiments. Data are displayed as mean $\pm$ standard deviation.

Immunoblot Analysis

Immunoblot was performed as previously described (18). Detailed information is included in the Supplemental Methods.

Targeted Therapy of Orthotopic Pancreatic Tumors Using ATDC shRNA-Nanovectors and Ionizing Radiation

In vivo orthotopic implantation of BxPC3 (BxPC3-Luc) cells infected with a lentiviral construct pLentiloxEV-Luc encoding luciferase was performed as we have previously described (18). Briefly, BxPC3-Luc cells (5×10^6) were injected into the tail of pancreas in NOD-SCID mice. 14 days later, when the tumors grew up to 70–100 mm³, the mice were randomized. ATDC shRNA-nanoimmunoliposome (nano-) containing 30 μ g ATDC shRNA plasmid in 300 μ l were freshly prepared using our previously described technique (23), and injected via tail vein 3 times a week for total of 5 weeks. The ionizing irradiation was administrated with 1 Gy daily for 2 weeks while the mouse was secure with lead restraint which only permitted the tumor area exposed in a Phillips 250 orthovoltage unit (24–26). Six treatment groups were established, with 8 animals in each group. The treatment groups included: 1) Non-treated (Basal), 2) nano-control shRNA, 3) nano-ATDC shRNA, 4) ionizing radiation (IR) treatment, 5) nano-control shRNA+IR and 6) nano-ATDC shRNA + IR. Tumor growth was measured twice a week using Xenogen in vivo bioluminescent imaging systems at the University of Michigan Small Animal Imaging Resource facility (<http://www.med.umich.edu/msair/>). Tumor volume was calculated as $V = a \times b^2/2$, where a and b presented the long and vertical short diameter of the tumor. All animal experiments were done according to protocols approved by the University of Michigan Committee for Use and Care of Animals.

In vivo Study of ATDCS550 mutant on radio-resistance

Mia PaCa2 cells (5×10^6) stably expressing empty vector, flag tagged ATDC or ATDC(S550A) were suspended in a 1:1 mixture of DMEM/Matrigel (BD Biosciences) and injected subcutaneously into the flanks of NOD-SCID mice using previously described techniques (18). Further details are provided in the Supplemental Material and Methods.

Statistical Analysis

Statistical analysis was performed using the StatMate software package (GraphPad Software, Inc., San Diego, CA) and two-way analysis of variance (ANOVA). Results were expressed as the mean $\pm$ standard deviation. In case of statistically significant differences, a

Student's t-test was used to determine which groups statistically differed from each other. Statistical significance was accepted if $p < 0.05$.

Results

ATDC Confers a Survival Advantage in Response to IR

Ionizing radiation is a treatment option frequently used to manage locally advanced or borderline resectable pancreatic cancers (13). Based on previous findings that ATDC overexpression rescued the radiosensitive phenotype of AT group D cells (19, 20), we hypothesized that high levels of ATDC in pancreatic cancer may contribute to the resistance of pancreatic cancer cells to IR. To test this hypothesis, we first transfected FLAG-tagged ATDC into human embryonic kidney cells (HEK293) that have no detectable expression of ATDC. Stable expression of ATDC in HEK293 cells (HEK^{ATDC}) (Figure 1A, left) resulted in higher clonogenic cell survival following irradiation when compared to empty-vector transfected HEK293 cells (HEK^{Vector}) (Figure 1A, middle). Similarly, the ability of IR (10 Gy) to induce apoptosis was reduced in ATDC-expressing cells compared to control cells (Figure 1A, right). Conversely, using ATDC-targeting shRNA to reduce ATDC expression in the Panc1 pancreatic cancer cell line (Figure 1B, left) rendered them more sensitive to IR-induced clonogenic cell death (Figure 1B, middle) and apoptosis (Figure 1B, right). Similar results were obtained with BxPC3 pancreatic cancers and immortalized skin keratinocyte HaCAT cells, both expressing high ATDC levels (Figure 1C and Supplemental Fig. S1). Thus, ATDC enhances resistance to cell killing by IR in multiple cell lines. Gemcitabine chemotherapy is a standard treatment for metastatic pancreatic cancers which acts by induction of apoptosis via DNA and replicative stress. To determine if ATDC also induced resistance to gemcitabine, we treated BxPC3 cells transfected with either control or ATDC-targeting shRNA with 10 μ M of gemcitabine for 48 hours, measured apoptosis and found that ATDC knockdown sensitized cells to gemcitabine as well (Supplemental Fig. S2).

Silencing of ATDC Sensitizes Pancreatic Cancer Xenografts to IR

We next wanted to test if the radioprotective effect of ATDC was present in tumors *in vivo*. To demonstrate the feasibility of knocking down ATDC in established orthotopic tumor xenografts in NOD/SCID mice, we performed injections with nanovectors containing ATDC-targeting shRNA. These nanovectors were engineered to carry transferrin in its liposome layer to specifically target transferrin receptor-expressing pancreatic cancer cells. Nanovectors containing control or ATDC-targeting shRNA were injected i.v. into mice with established BxPC3 orthotopic tumors (n=5 per group). Xenografts were harvested 1 week later and the cells were used for protein isolation and western blotting (Figure 2A) or tumor sections were fixed and stained with anti-ATDC antibodies (Figure 2B and C). Injection of nanovectors containing ATDC-targeting shRNA decreased ATDC expression in the majority of tumor cells.

To investigate whether ATDC knockdown in BxPC3 orthotopic xenografts increased sensitivity to IR, we used the same nanovector approach in combination with IR (ten 1 Gy fractions over 2 weeks). ATDC-targeting shRNA significantly reduced tumor growth and in combination with IR completely blocked tumor growth (Figure 2D and E). These results

show that targeting ATDC in pancreatic cancer orthotopic xenografts *in vivo* inhibits tumor growth and sensitizes tumors to IR.

IR Induces Phosphorylation of ATDC in ATM-Dependent Manner

Following IR exposure, ATM is rapidly activated and phosphorylates over 700 substrates (2). The phosphorylation targets of ATM share a common motif of a serine residue followed by glutamine (SQ motif) (2). ATDC harbors one SQ motif at amino acids 550-551 at the C-terminus of the protein (Figure 3A). The SQ motif in ATDC is highly conserved across multiple species, highlighting a potential critical function in the molecule (Supplemental Fig. S3). To examine whether ATDC becomes phosphorylated following exposure to IR, we irradiated HEK^{ATDC} and following different periods of incubation, lysed the cells and immunoprecipitated ATDC with an anti-FLAG antibody. Immunoprecipitates were then subjected to immunoblotting with a phospho-Ser/Thr antibody. Exposure of cells to IR significantly induced ATDC phosphorylation as quickly as 15 minutes after IR (Figure 3B). We next generated rabbit polyclonal phospho-specific anti-ATDC antibodies against Ser550-phosphorylated peptides (p-ATDC). The specificity of the p-ATDC antibody was verified by treating the immunoprecipitated ATDC with λ -phosphatase (λ PPase) or calf intestinal phosphatase (CIPase) prior to immunoblotting (Supplemental Fig. S4). The p-ATDC antibody detected IR-induced phosphorylation of ATDC from irradiated HEK^{ATDC} and BxPC3 cells (Figures 3C, D). To determine whether the phosphorylation of ATDC was dependent on ATM, we knocked down ATM using targeting shRNA in BxPC3 cells (Supplemental Fig. S5). ATM knockdown significantly blocked IR-induced phosphorylation of ATDC in BxPC3 cells (Figure 3D). Furthermore, the specific ATM inhibitor KU55933 (20 μ M) blocked ATDC phosphorylation in response to IR in HEK^{ATDC} cells (Supplemental Fig. S6A). In contrast, knockdown of ATDC had no effect on the ability of ATM to autophosphorylate in response to IR, supporting the notion that IR-induced ATDC phosphorylation is directly mediated by ATM or another effector downstream of ATM (Supplemental Fig. S6B). To test if the SQ motif is critical for ATDC's radioprotective function, we transfected HEK293 cells with ATDC or a ATDC550A mutant and examined responses to IR. The ability of ATDC to protect cells from radiation-induced cell death was dependent on the serine 550 site (Figure 3E). To explore whether ATM directly phosphorylates ATDC, we conducted *in vitro* kinase assays using wild type (WT) or kinase dead (KD) FLAG-tagged ATM kinase, GST-ATDC fusion peptides containing the C-terminal region of ATDC (amino acids 534-559), and a positive control GST-p53-S15 peptide. *In vitro* kinase assays showed that the ATM kinase was not able to phosphorylate ATDC at S550 *in vitro* (Figure 3F), yet phosphorylated the control GST-p53-S15 peptide, suggesting that ATM indirectly mediates IR-induced phosphorylation of ATDC.

p38 Functions Downstream of ATM to Facilitate IR-Induced ATDC Phosphorylation

Since ATM did not directly phosphorylate ATDC in *in vitro* kinase assays, we explored whether ATM might activate other kinases, such as Chk1/2 and p38 (27), which in turn may directly phosphorylate ATDC. Experiments using pharmacological inhibitors of p38 or Chk1/2 at effective doses (Supplemental Fig. S7A, C and D) showed that the p38 inhibitor SB202196 (50 μ M), but not the Chk1/2 inhibitor AZD7762 (100 nM), inhibited IR-induced ATDC phosphorylation in ATDC-transfected HEK 293 (Figure 4A) and BxPC3 (Figure 4B)

cells. This suggests that p38, but not Chk1/2, is a mediator of ATM signaling facilitating IR-induced ATDC phosphorylation.

MK2 Kinase Phosphorylates ATDC on Ser-550 and Ser-552

It has been reported that MAPKAP kinase-2 (MK2) kinase is a downstream target for p38 kinase in the DDR following treatment with chemotherapeutic agents (9, 10). MK2 regulates the G2/M and G1 checkpoints by phosphorylating CDC25B and CDC25C directly at Ser-323 and Ser-216 respectively after DNA damage (9, 11). To test if MK2 is responsible for ATDC phosphorylation after IR treatment, we pretreated BxPC3 cells with a MK2 inhibitor (100 nM) (Supplemental Fig. S7B) and then exposed cells to 10 Gy IR. Following a 1 hour incubation, the cells were lysed and ATDC phosphorylation was examined. The MK2 inhibitor effectively abolished ATDC phosphorylation following IR (Figure 4C). To explore whether MK2 is capable of directly phosphorylating ATDC, we performed *in vitro* MK2 kinase assays using GST-ATDC fusion proteins containing the C-terminal region of ATDC (amino acids 534-559) and GST-CDC25C(200-256) as a positive control. MK2 kinase was able to directly phosphorylate ATDC *in vitro* (Figure 4D). We reasoned that the serine 550 site might be a phosphorylation site for MK2 as mutation to this site had abolished the radioprotective effect of ATDC. To determine the exact site of phosphorylation of ATDC by MK2, we utilized the Ala substitution mutant of serine 550 (S550A) and created several other Ala substitution mutants of serines nearby: residues 546 (S546A), 552 (S552A) and a double mutation of 550 and 552 (S550/552A). We observed that both the GST-ATDC(S550A) and GST-ATDC(S552A) mutants attenuated MK2-induced phosphorylation, while the GST-ATDC(S546A) mutant appeared to be as good of a substrate for MK2 as the wild type sequence (Figure 4D). When we used the GST-ATDC(S550/552A) double mutant, we observed a complete lack of MK2-mediated phosphorylation of the ATDC peptide (Figure 4D). Taken together, our results indicate that MK2 directly phosphorylates ATDC at both the Ser-550 and Ser-552 sites.

Phosphorylation at Ser-550 is Required for the Radioprotective Function of ATDC

To explore the contributions of phosphorylation at Ser550, Ser552 or both sites to the radioprotective function of ATDC, we utilized FLAG-tagged CMV expression vectors of the Ala substitution mutants at Ser-550(S550A), Ser-552(S552A) and Ser550/552(S550/552A). HEK293 cells transfected with these constructs (Supplemental Fig. S8A) were irradiated (5 and 10 Gy) and apoptosis and clonogenic survival was assessed. The S552A mutant retained a protective function against both IR-induced apoptosis and clonogenic cell killing, while the S550A and the S550/552A double mutants lost their protective effects (Figures 5A and B). Furthermore, knockdown of MK2 using a MK2 shRNA lentiviral vector (Supplemental Fig. S8B) significantly blocked the protective function of ATDC on both IR-induced apoptosis and clonogenic cell killing in ATDC-expressing but not parental HEK293 cells (Figure 5C and D).

To demonstrate the *in vivo* significance of these findings, we compared the impact of ATDC and the ATDC S550A mutant on the growth of Mia PaCa2-derived xenografts in response to IR. Mia PaCa2 pancreatic cancer cells (low endogenous ATDC expression) were stably transfected with empty vector, ATDC or the ATDC(S550A) mutant (Figure 5E) and were

inoculated subcutaneously in the flanks of NOD-SCID mice (n=6 per group). When tumor size reached approximately 70~100 mm³, mice were randomized to either no treatment or treated with a clinically relevant dose of IR (1.5 Gy, 5 days per week for 3 weeks). All mice tolerated IR treatments well without significant weight loss or change in physical activity (data not shown). ATDC significantly enhanced tumor growth and mediated resistance to IR. (Figure 5F, Supplemental Table 1). ATDC(S550A) promoted tumor growth similar to wild type ATDC, but did not confer resistance to IR. (Figure 5F, Supplemental Table 1). These results demonstrate that ATDC mediated radioresistance requires MK2 induced phosphorylation on S550, but this event is not required for ATDC induced pancreatic tumor growth.

IR-Induced Phosphorylation at Ser-550 Inhibits ATDC and Disheveled2 Interaction

We have previously demonstrated that ATDC promotes pancreatic cancer cell growth via β -catenin activation by binding with Disheveled2 (Dvl2) (18). To evaluate the impact of Ser-550 phosphorylation on ATDC-mediated effects on β -catenin signaling, we first compared the effects of ATDC and the S550A mutant on cellular proliferation and TCF-Luc activity as readout of active β -catenin signaling. Mutation of S550 to alanine had no impact on ATDC's ability to promote growth or activate β -catenin signaling (Figure 6A and B). Next, we examined the ability of both ATDC and the S550A mutant to bind Myc tagged Dvl2 in the absence and presence of IR. Both ATDC and the S550A mutant could bind equally to Dvl2 in the absence of IR. However, IR inhibited ATDC binding to Dvl2, an effect not observed with the S550A mutant (Figure 6C). To examine if IR-induced inhibition of ATDC-Dvl2 binding might have downstream effects on β -catenin signaling, we examined responses to IR in control, ATDC- and S550A mutant-transfected HEK 293 cells and measured TCF-Luc activity. IR alone did not affect TCF-Luc activity (Figure 6D). Transfection of ATDC increased TCF- Luc activity, which was diminished when cells were treated with IR (Figure 6D). While the S550A mutant was able to induce TCF-Luc activity to a level similar to that of wild type ATDC in the absence of IR, the IR- induced decrease in TCF-Luc activity was significantly diminished with the S550A mutant. These data suggest that IR-induced Ser-550 phosphorylation may be important for ATDC's radioresistance by driving dissociation of ATDC from Dvl2.

Discussion

Pancreatic adenocarcinoma is highly resistant to radiation and chemotherapy, making it one of the most lethal malignancies. Despite decades of research, the biologic mechanisms that promote pancreatic cancer therapeutic resistance are incompletely understood. We have previously shown that ATDC is highly expressed in pancreatic cancers where it enhances proliferation and invasion (18). ATDC was originally identified by its ability to restore radioresistance to AT fibroblasts (28) which led us to hypothesize ATDC may mediate radioresistance in pancreatic cancers. In this study, we describe ATDC as a novel determinant of resistance to IR which participates as a downstream signaling molecule in the ATM signaling cascade. ATDC's ability to mediate radioresistance requires phosphorylation of S550 by MK2 kinase, leading to the disassociation of ATDC from Dvl2. These data suggest that ATDC performs two important but separate functions-it drives proliferation

through β -catenin signaling and promotes radioresistance through the ATM/MAPKAP kinase 2 signaling pathway.

Genotoxic insults provoke a complex cellular response which involves protein phosphorylation, cell-cycle arrest, DNA repair or cellular death. Activation of ATM kinase is a crucial initial step which occurs immediately after DNA damage induced by IR. In normal cells, ATM activates both Chk2 and p53 which reside primarily within the nucleus. Chk2 and p53 then facilitate the activation of G1/S or G2/M cell cycle arrest to allow DNA repair and restore genomic integrity prior to DNA replication and mitosis (9). Recently, it was also determined that ATM also activates p38/MAPK, a sensor of cellular stress, which subsequently phosphorylates the MK2 kinase, relocating it from the nucleus to the cytoplasm (9). MK2 can phosphorylate and activate CDC25 family members as well as RNA-binding and regulatory proteins in the cytoplasm which collaborate to induce a sustained G1 or G2/M cell-cycle arrest allowing repair of DNA damage (11). The G2/M checkpoint is thought to be the final 'net' that catches cells that have escaped the earlier checkpoints, protecting cells from undergoing mitosis until the damage has been repaired.

In this study, we show that following IR, the MK2 kinase is activated in an ATM-dependent manner. MK2 phosphorylates ATDC on Ser550, an evolutionary conserved site in the protein, which is crucial for the role of ATDC in the DDR after IR. Knockdown of ATDC or expression of the S550A mutant sensitizes pancreatic cancer cells to radiation treatment in vitro and in vivo. Our data suggest that ATDC is an important member of the DDR downstream of the MK2 kinase. Further, we provide data showing that phosphorylation of ATDC by MK2 at the Ser550 site promotes the release of ATDC from DVL2, allowing ATDC to increase cellular radioresistance. Although ATDC lacks intrinsic kinase or enzymatic activity, it has been previously demonstrated to bind and sequester p53 in the cytoplasm, abrogating IR-induced p21 accumulation and blocking cell cycle arrest (29). The role of ATDC in mediating MK2's effects on cell cycle checkpoints remain unclear and additional experiments are planned to define the exact role of ATDC in effecting the signaling effects of MK2. Nonetheless, it is now clear that ATDC's physical interactions are not limited to p53 and also include MK2. In addition to its ability to mediate resistance to ionizing radiation, ATDC mediates resistance to UV irradiation (30). It is therefore reasonable to postulate that ATDC participates, either directly or indirectly in DNA repair processes, although we found no evidence that ATDC localizes to sites of DNA damage following laser irradiation (unpublished observations). The specific mechanism(s) by which ATDC facilitates DNA repair are a focus of future studies.

These actions identify ATDC as a global survival factor which aids in cellular survival and tolerance of DNA damage and cellular stress. Indeed, ATDC is normally expressed at high levels in tissues such as skin, which are subject to constant genotoxic insult by UV and IR and may thus provide a mechanism to allow these tissues to avoid premature or excessive cell death. However, when aberrantly expressed in tumor cells, this same mechanism induces cellular resistance to the DNA damaging agents commonly used to treat malignancy and may explain why malignancies such as pancreatic cancer with high levels of ATDC also demonstrate resistance to IR or chemotherapy.

In this study, ATDC expression levels correlate with cellular resistance to IR both *in vitro* and *in vivo*. These results imply that ATDC may be a predictive marker of tumor responsiveness to radiotherapy in human patients. We postulate that assessment of ATDC levels in tumor tissues may identify patients unlikely to benefit from radiotherapy. More importantly, our work identifies the ATDC/p38/MK2 pathway as a potential therapeutic target in pancreatic tumors and other tumor types, as ATDC expression has been reported to be upregulated in lung, bladder, colorectal, ovarian and endometrial cancers as well as multiple myeloma (<http://www.oncomine.org/>). Since ATDC-mediated radioresistance requires Ser-550 phosphorylation by MK2, treatment of patients with inhibitors targeting this pathway may provide sensitization to IR and the efficacy of MK2 inhibitors is already an area of active investigation in other tumor types (9). Although ATDC lacks an enzymatic function which could be targeted by small-molecule inhibitors, here we have described an *in vivo* strategy to deliver ATDC-targeting shRNA to pancreatic tumors in mice. If optimized for delivery to human patients, this method may allow radiosensitization of tumors with high levels of ATDC, allowing more effective treatment. Further investigation into the mechanism of ATDC up-regulation may also lead to additional “druggable” targets which can reduce ATDC expression and sensitize tumors to radiation.

In summary, we have demonstrated that ATDC is a novel MK2 kinase substrate phosphorylated by MK2 in an ATM-dependent manner in response to DNA damage. Phosphorylation occurs on the evolutionary conserved site Ser-550, which is crucial for the role of ATDC in the DDR after IR (Figure 7). Knockdown of ATDC or expression of the S550A mutant sensitizes pancreatic cancer cells to radiation treatment *in vitro* and *in vivo* in orthotopic pancreatic tumors. Our data suggest that ATDC is an important member of the DDR downstream of the MK2 kinase and represents a promising new therapeutic target for the sensitization of pancreatic adenocarcinoma and potentially other ATDC-overexpressing cancers to radiation therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ATDC	ataxia telangiectasia group D complementing gene
ATM	ataxia telangiectasia mutated
MK2	MAP kinase-activated protein kinase 2 (MAPKAP kinase 2)
Chk2	CHK2 checkpoint homolog
IR	Ionizing radiation
DSBs	double strand breaks

DDR DNA damage response

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Figure 1. ATDC protects cells against ionizing radiation

(A, D, G) Western blotting demonstrating ATDC expression levels in ATDC-stably transfected HEK293 (HEK^{ATDC}) cells (A), or Panc1 (D) and BxPC3 (G) cells expressing ATDC shRNA. (B, E, H) Clonogenic cell survival and (C, F, I) apoptosis assays performed in HEK293^{Vector} and HEK^{ATDC} cells (B, C), or Panc1 (E, F) and BxPC3 (H, I) cells expressing control or ATDC shRNA following varying doses of IR (n=4, mean $\pm$ SE, *p<0.05).

Figure 2. Silencing of ATDC inhibits orthotopic tumor growth and sensitizes tumors to IR
 (A) Immunoblot indicates silencing of ATDC in BxPC3 orthotopic xenografts by treatment of nanovectors carrying ATDC shRNA. (B, C) ATDC expression levels in BxPC3 xenografts treated with nanovectors (nano) carrying control (cont) or ATDC shRNA (scale bar, 50 μm). (D) Mice with BxPC3 orthotopic tumors were randomized and treated with nano-cont shRNA or nano-ATDC shRNA, 30 μg/mouse, i.v., 3/week for 5 weeks. Tumor size was measured twice weekly. (E) Mice with BxPC3 orthotopic tumors were randomized and treated with nano-cont shRNA or nano-ATDC shRNA, 30 μg/mouse, i.v., 3/week for 5 weeks, radiation (IR) (1 Gy, q.d., 5 × 2 weeks), or the combination of both treatments. Tumor size was measured twice weekly (*p<0.05 vs. nano-cont shRNA + IR, n=8 animals/group).

Figure 3. IR induced ATDC phosphorylation is dependent on ATM

(A) Schematic of the domains of ATDC. The SQ motif is located at the Ser-550 site. (B) At various times following IR (10 Gy), cell lysates of HEK 293 with or without ATDC expression were immunoprecipitated with Flag antibody and immunoblotted with a phospho-Ser/Thr antibody. (C) At various times following IR (10 Gy), cell lysates of HEK^{ATDC} or BxPC3 cells were immunoprecipitated with an ATDC antibody and immunoblotted with a p-ATDC antibody. (D) BxPC3 cells expressing control or ATM shRNA were exposed to IR (10 Gy) for indicated times and ATDC phosphorylation was measured by immunoprecipitation using an ATDC antibody and immunoblotting using a p-ATDC antibody. (E) Apoptosis assays performed in HEK293 cells expressing empty vector, ATDC or the ATDC(S550) mutant 24 hours after IR (10Gy) (*p<0.05 vs vector). (F) In *in vitro* kinase assays, wild type (WT) or kinase dead (KD) ATM was incubated with GST-ATDC peptides or positive control GST-p53-S15 for 30 min. All experiments were repeated three times.

Figure 4. IR mediates MK2 kinase-dependent phosphorylation of ATDC

(A, B) One hour after pretreatment with AZD7762 (100 nM), KU55933 (20 μ M) or SB203580 (50 μ M), HEK293 (A) or BxPC3 (B) cells were exposed to IR (10 Gy) for 1 hour. Cell lysates were immunoprecipitated with ATDC antibody and immunoblotted with p-ATDC antibody. (C) One hour after pretreatment with MK2 inhibitor (100 nM), BxPC3 cells were exposed to IR (10 Gy) for 1 hour. Cell lysates were immunoprecipitated with ATDC antibody and immunoblotted with pATDC antibody. (D) *In vitro* kinase assays with recombinant MK2 kinase were performed with wild type GST-ATDC(WT), mutant GST-ATDC: (S550A), (S552A), (S546A) and (S550/552A), and positive control GST-CDC25C. All experiments were repeated 3 times with similar results.

Figure 5. Ser-550 phosphorylation is required for ATDC's radio-protective function
 (A) Apoptosis assays were performed in HEK293 cells expressing ATDC or ATDC(S550A), (S552A) and (S550/552A) mutants 24 hours after IR (10 Gy) (* $p < 0.05$ vs vector). (B) Clonogenic survival assays using HEK293 cells expressing ATDC or ATDC(S550A), (S552A) and (S550/552A) mutants 14 days after IR (5 Gy) (* $p < 0.05$ vs vector). (C) Apoptosis assays performed 24 hr after IR in HEK293 cells with and without ATDC transfected with control or MK2 shRNA (* $p < 0.05$ HEK293 transfected with control shRNA with ATDC vs without ATDC, # $p < 0.05$ HEK293 cells with ATDC- control vs MK2 shRNA). (D) Clonogenic survival assays 14 days after IR (5 Gy) using control or ATDC expressing HEK293 cells with or without MK2 shRNA ($n = 3$, mean $\pm$ SE, * $p < 0.05$ HEK293 transfected with control shRNA with ATDC vs without ATDC, # $p < 0.05$ HEK293 cells with ATDC- control vs MK2 shRNA). (E) Immunoblotting indicated the expression of empty vector, flag-tagged wild type ATDC and mutant ATDC (S550A) in stably transfected Mia PaCa2 cells. (F) After establishing Mia PaCa2 subcutaneous xenografts, mice were randomized and left untreated or treated with IR (1.5 Gy, 5 d/week for 3 weeks). Tumors were measured twice weekly (* $p < 0.05$ vs. Vector, # $p < 0.05$ vs. S550A) ($n = 6$ animals/group). All experiments were repeated three times and data is expressed as the mean $\pm$ SE.

Figure 6. IR-Induced Phosphorylation at Ser-550 Inhibits ATDC and Disheveled2 Interaction (A) Cell proliferation of HEK293 cells expressing control vector, ATDC or the S550A mutant measured by the MTS assay (* $p < 0.05$ vs vector). (B) TCF reporter activity in HEK293 cells expressing control vector, ATDC and the S550A mutant (* $p < 0.05$ vs control vector). (C) HEK293 cells expressing ATDC or S550A mutant were transfected with Myc-tagged Dvl2. 48 hours after transfection, cells were irradiated (10 Gy) for 1 hr. Cells were lysed and immunoprecipitated with Myc antibody and then immunoblotted with an ATDC antibody. (D) HEK293 cells expressing empty vector, ATDC or the S550A mutant with the TCF reporter genes, TOPFlash or FOPFlash. 24 hours after reporter transfection, cells were irradiated (IR, 10 Gy), Five hours after irradiation, luciferase activity in cell lysates were assessed. Cont: Control. TCF-Luc activity was measured as the ratio of TOPFlash/FOPFlash. All experiments were repeated three times (* $p < 0.05$ vs. non-irradiated samples).

Figure 7. ATDC Activates β -Catenin Signaling and Participates in the IR-induced DNA Damage Response via Distinct Mechanisms

Left: In the absence of IR, ATDC binds to stabilized Dvl2, bringing it to the β -catenin destruction complex (GSK3 β -APC-Axin). Binding of ATDC and Dvl2 to the destruction complex inhibits destruction complex function, resulting in the release of β -catenin from the destruction complex, increased β -catenin levels and subsequent activation of downstream target genes and cell growth. Right: In the presence of IR, ATM is activated, resulting in MK2 kinase phosphorylation in a p38 kinase-dependent manner. Activated MK2 binds to and induces ATDC phosphorylation at Ser-550 (p-ATDC). p-ATDC disassociates from Dvl2 and increases the radioresistance of pancreatic cancer cells.