Full activation of PKB/Akt in response to insulin or ionizing radiation is mediated through ATM

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Activation of PKB/Akt at Ser473 is mediated through ATM

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Summary

The gene mutated in Ataxia Telangiectasia (ATM) has been implicated in several cell functions such as cell cycle control, response to DNA damage and insulin. PKB/Akt has also been implicated in the cellular response to insulin, γ−radiation and cell cycle control. Interestingly, lack of PKB/Akt function in vivo is able to mimic some phenotypic abnormalities associated with Ataxia Telangiectasia (AT). Here we show that ATM is a major determinant of full PKB/Akt activation in response to insulin or γ−radiation. This effect is mediated through the Phosphatidylinositol-3 kinase (PI3k) domain of ATM which affects specifically Akt Serine 473 phosphorylation. This conclusion was inferred from the results obtained in transient transfection assays using exogenous PKB/Akt and ATM in Cos cells. Moreover, the use of ATM inhibitors or siRNA confirmed our observation. Further supporting these results we also observed that biological responses tightly regulated by Akt, such as FKHR activity after insulin treatment or γ−radiation response, were altered in cell lines derived from AT patients and Knock Out (KO) mice for ATM in which phosphorylation in Serine 473 was almost abolished. This study proposes new clues in the search of the unknown PDK2 and proposes new explanations for the radiosensitivity or insulin intolerance described more than 30 years ago in AT patients.
**Introduction**

Ataxia telangiectasia (AT) is an autosomal recessive disorder characterized by growth retardation, cerebellar ataxia, cancer susceptibility, oculotaneus telangiectasia, severe immunodeficiency, resistance to insulin and hypersensitivity to gamma radiation among other symptoms (for review see (1). At the cellular level, the role of the gene Mutated in Ataxia Telangiectasia (ATM) includes a wide spectrum of biological processes including, cell cycle control, genome stability, apoptotic machinery, response to genotoxic stress and others (2-5). The protein encoded by the ATM gene belongs to the super family of Phosphatidylinositol-3 kinase (PI3K) due to the presence of a p110 like domain but no lipid kinase activity has been demonstrated so far (6). This domain is responsible for the phosphorylation of proteins such as p53, ChK1, 4EBP, BRCA1 following a conserved domain –S/T-Q- in which S or T is the phosphoacceptor residue (for a review see (7). In fact, regulation of ATM is mediated by autophosphorylation in residue Ser1981, at the FAT domain, following this conserved motif as has been recently demonstrated (8).

The PKB/Akt, referred now as Akt, protein is a key player of a pathway related to survival, by inhibition of apoptotic signals and promoting cell cycle progression with a clear implication in cancer and other pathologies (9,10). Also, Akt has been shown to be potently activated in response to a wide variety of growth factors such as insulin or IGF-1, and more recently in response to DNA damage (11,12). Phosphorylation in two residues seems to be critical to total Akt activation, a Thr in position 308 mediated through PDK1, and Ser473 mediated by a yet unknown protein called PDK2 (13). Although several candidates have been proposed, including the PDK1 itself (14-17), few data are known about this enzymatic activity that seems to be stauroporin insensitive and PI3K inhibitors sensitive (18,19). Interestingly these two apparent independent signal transduction pathways, ATM and Akt, seem to converge at a physiological level in response to stimuli such as insulin or γ–radiation. Furthermore,
Knock Out (KO) models for Akt and ATM showed similarities in terms of phenotypic abnormalities such as growth retardation, defects in the maturation of the immune system, infertility, resistance to insulin or radiosensitivity (20-22).

We undertook the present study to explore the existence of a possible link between these two proteins and if the connection could explain some of the biological properties such as radiosensitivity or insulin resistance observed in AT disease.
**Experimental Procedures**

**Cell lines**

GM00637, GM09607 and GM08391 were purchased from Coriell Institute for Biomedical Research (Candem, NJ). MEF derived cell lines from Knock Out animals for p53 lines (p53 -/-, ATM +/-) referred as KO p53 and Double Knock Out for p53 and ATM (p53 -/-, ATM -/-) referred as DKO were kindly supplied by Dr. Philip Leder (HHMI at Harvard Medical school, Boston, MA, 02115). 293T, IMR90 and Cos7 cells were maintained in DMEM 10% FBS (Biowhittaker, Verviers, Belgium) in 5% CO₂ at 37C.

**DNAs and transfections**

Flag-6-His ATM (Wild type) wt in PEBS7-YZ5 was kindly provided by Dr. Y. Shiloh (Tel Aviv University, School of Medicine, Israel). Flag-6-His ATM wt and kd form in PCDNA3 was kindly provided by Dr. M.B. Kastan (St. Jude Children’s Research Hospital, Memphis, TN). Green Fluorescence Protein (GFP) tagged p53 was obtained from pEF1-p53-WT (23) and then subcloned into the BamH1/Not1 site of pCEFL-GFP. 6-His tagged p110α wt was kindly supplied by Dr. S. Gutkind (NIDCR, NIH, Bethesda, MD). 293T cells were transfected using standard techniques of Calcium Phosphate. Cos, Double KO and GM09607 were transfected with Lipofectamine (Invitrogen, Carlsbad, CA) following manufacturer instructions. In the case of stably transfected cells, cells were selected with appropriate dose of Hygromycin (Roche, Indianapolis, IN). In transient transfection assays cells were collected 48 hours post-transfection. FKHR wt, mutant and reporter for FKHR activity were kindly supplied by Dr. L del Peso (Hospital de la Princesa, Madrid, Spain). Glutathione S-transferase (GST) p53 wt has been previously described (23). Hemagglutinin (HA) tagged Akt wt and triple mutant (residues 473,308 and 179) in pCMV5 were kindly supplied by Dr. D. Alessi (MRC, Protein Phosphorylation Unit, Dundee, UK).

**Antibodies, Western blotting and Immunoprecipitation procedures**

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Specific Phosphoantibodies against Ser473 or Thr308 of Akt and phospho ERK1/2 were purchased from Cell Signaling Technologies (CST, Beverly, MA), antibody against total Akt and ERK1, IR-α were purchased from Santa Cruz Biotech (Santa Cruz, CA). Akt Kinase assays were performed using a non-radioactive kit from CST and following manufacturer instructions. Antibody against HA and GFP was from Covance (Richmond, CA) and Roche respectively. Antibody against 6His and Flag were from Sigma. Cells for Akt phosphorylation assays were treated and collected in lysis buffer (25mM HEPES pH 7.5, 0.3 M NaCl, 1.5mM MgCl$_2$, 0.2 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.5% Deoxycholic Acid, 20mM B-glycerophosphate) in the presence of protease and phosphatase inhibitors (20µg/ml Aprotinin, 20µg/ml Leupeptin, 1mM PMSF and 0.1mM Na$_3$VO$_4$, 20mM NaF, 1mM NaPPI) and protein quantity was quantified (Biorad assay, München, Germany). Cells for coimmunoprecipitation assays were collected in HNTG lysis buffer (23). Then, lysates were processed for western blotting (usually 50 µg) or IP using specific antibodies and Protein G (Amershan Pharmacia). Immunocomplexes were extensively washed and resuspended in Sample buffer 5x. Antibody detection was achieved by enhanced chemiluminescence (ECL, Amersham Pharmacia, Uppsala, Sweden).

**PCR protocols and generation of GST fusion proteins**

Primers and protocol for cloning PI3K domain from Y5Z DNA has been previously described (24). PCR product was cloned onto pEF1/His-C (Invitrogen). DNA was confirmed by automatic sequencing. To generate GST-Akt protein with the last 80 aa of Akt we used as a template pCVM5-HA-Akt forward primer 5´-GGGGGATCCTTCTTTGCCCGTATCGTG-3´ which contains a BamH1 site, and the reverse primer 5´- CCCGCGGCCGCTCAGGCGTGCTGCTGGC -3” which contains a Not1 site. PCR was performed using pfu Turbo DNA polymerase from Stratagene (La Jolla, CA) for 35 cycles (denaturation, 95ºC , 1min, annealing, 60ºC, 2min and extending, 72ºC, 3min) and a final extending cycle of 10 min . PCR product was
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subcloned into PGX4T-1 vector (Amersham) in the BamH1 and Not1 site. Positive constructs were tested for expression after IPTG (Sigma, St. Louis, MO) treatment in BL21 (DE3) bacteria (Invitrogen). DNA was confirmed by automatic sequencing. GST p53 wt has been previously described (23). All GST-fusion proteins were purified from E. coli BL21 (DE3) harbouring the corresponding plasmid) following the method previously described. GST-Akt and GST-p53 were used in the presence of Glutation Sepharose beads (Amersham).

Treatments

Insulin was from Novo Nordisk (Bagsvaerd, Denmark). Cells were treated for 30 minutes with indicated dose. Cells were gamma irradiated using a Cobalt source (Theratron 780 AECL, Canada) at SSD of 57 Cm at a dose rate of 285 cGy/min. Cells were kept at 37°C, 5% CO₂ immediately after Gamma radiation for 7 days in viability assays or 30 minutes for kinase assays. Cells were pretreated for 1 hour with either 200nM of wortmannin (Sigma), 20 µM Ly294002 (Cell Signaling Technology), 2mM caffeine (Sigma) in the cases indicated.

Luciferase assay

Reporter assays for FKHR were performed as previously described (25). Cells were transiently transfected in a 24 well/plate, O/N starving with or without insulin 10nM for 18 hours. In the case of ATM wt over-expression we used 1,5 µg. Extracts were collected in Promega Lysys buffer and samples measured using a Luminometer (Dynex). Luciferase activity was normalized with Renilla control (Promega, Madison, WI)

In vitro Kinase assay for ATM

Kinase reaction for ATM activity was performed as previously described (26), but using as a substrate GST-p53 (80 aa) or GST-Akt (80 aa) bound to Glutation Sepharose beads or the immunocomplex from Cos cells transfected with HA-Akt wt.
**RNA interference assays.**

Silencer Validated siRNA for ATM was obtained from Ambion (Massachusetts, USA). Transfection of the siRNA was performed with Lipofectamine as described before. Cells were treated with insulin after overnight starving, 24-36 hours after transfection.
Results

Overexpresion of ATM promotes Ser473 phosphorylation of Akt through the PI3K domain.

To assess the possible link between Akt and ATM we first over-expressed exogenous tagged ATM and Akt in Cos cells. Over expression of Flag-6His-tagged ATM wt induced phosphorylation in Ser473 of Akt in a dose dependent fashion, while Flag-6His-tagged ATM Kinase Dead (Kd) was unable. However, in residue Thr308 of Akt we only appreciate a slight increase (faint band in fig. 1a). As a control for Akt and ATM activity we used insulin and Ser 15 phosphorylation of p53 protein respectively (27,28). In fact, PI3K inhibitors, such as wortmannin, Ly294002 or caffeine were able to block ATM mediated Akt Ser473 phosphorylation (fig.1b). To confirm this observation an Akt kinase assay was performed, and we observed that ATM wt induced Akt kinase activity following a dose dependent kinetic, while we did not detect any effect in the presence of ATM Kd (fig. 1c). It has been reported kinase activity in Thr308 mutant Akt after MAPKAP2 in vitro phosphorylation of Ser473 residue, indicating that Ser473 activation can render kinase activity by itself supporting our observations (11). Interestingly, γ−radiation, a classical ATM stimulus, was also able to induce Akt kinase activity (fig.1c). However, all the previous experiments were performed by overexpression of ATM or using chemical inhibitors, known to block PI3K also implicated in Akt activation. Therefore, we decided to inhibit specifically ATM using RNA interference technology. Cos cells were transfected with exogenous ATM in the presence of increasing amounts of validated siRNA for ATM (ranging from 0 to 400 nM) and ATM expression was monitorized (upper panel fig. 1d). Therefore, we performed the same experimental approach with endogenous ATM stimulated with Insulin in the presence of HA-tagged Akt (lower panel fig.1d). While a marked decrease was observed in Ser473 phosphorylation, no effect was detected in Thr308. The same experiment was performed in Hela cells with exact results (data not shown).
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To evaluate the implication of the PI3K domain of ATM in the Ser473 phosphorylation, we decided to PCR-clone this domain, known to mediate some of the biological properties of ATM such as radiosensitivity (24). The 6-His tagged PI3K domain from ATM was also able to induce Ser473 and Ser15 phosphorylation of Akt and p53 respectively, in a dose dependent fashion inhibited by wortmannin (fig. 1e). Expression of the PI3K domain of ATM was evaluated by western blotting anti 6-His tag (data not shown). However p110α, the catalytic subunit of the conventional PI3K, was able to induce phosphorylation in both Akt residues, suggesting a clear difference between these two PI3K families (fig. 1f).

ATM binds to Akt and mediates Ser473 Akt phosphorylation

In order to gain further insight into the molecular mechanism of Akt activation mediated by ATM we decided to investigate the possibility of a physical interaction between ATM and Akt. Therefore, 293T cells were transiently transfected with HA-Akt plus Flag-6His-tagged ATM wt or Kd. Extracts were immunoprecipitated against 6-His and blotted against HA. A clear binding between the two molecules was detected, demonstrating that both ATM wt and ATM kd were in vivo associated with HA-Akt (fig. 2a). In addition, we decided to analyse the phosphorylation status of the Akt bound to ATM, we observed that it was only phosphorylated in Ser473, while no signal was detected in Thr308 (fig 2a). As a positive control, GFP-p53 was cotransfected with ATM and, we detected binding and p53 phosphorylation at Ser 15 (fig. 2b). In this scenario we decided to analyse the possibility of a direct phosphorylation in the Ser473 of Akt, a putative phosphoacceptor in the ATM phosphorylation consensus site, although it is not followed by a Q (7). In this context, and as a first approach, we decided to use the whole molecule as a substrate. Thus, HA-tagged Akt wt and a triple mutant in positions 473, 308 and 179 were transfected in Cos cells, immunoprecipitated and used as a substrate for in vitro kinase assay using ATM immunoprecipitated from 293T cells. In this case a 32P incorporation was detected in the presence of ATM wt form, while we
did not detect any signal using the mutant forms of ATM or Akt (fig. 2c). However, this experiments did not allow us to conclude that Serine 473 is the target of ATM kinase activity. Therefore, we decided to generate GST fusion protein containing the last 80 aa of Akt and mutant form in Ser473 changed to Ala that were used as a substrate for \textit{in vitro} ATM kinase assays. Unfortunately, we did not detect any incorporation of phosphate (fig. 2d). To assess the reliability of this approach we used, in the same conditions, a GST containing the first 80 aa of p53 and a mutant form in Ser 15 changed to Ala, behaving as a specific substrate of ATM (fig. 2d).

Cell lines from AT patients or KO mice are defective in Akt Serine 473 phosphorylation in response to insulin.

To confirm our previous biochemical observations we moved to an experimental model such as the cell line GM08931 (fibroblast derived from an AT patient) and as a control we used IMR90. Cells were starved overnight treated with insulin and Akt phosphorylation in Ser473 was evaluated. Cells lacking ATM protein showed a clear decrease in phosphorylation of Ser473 respect to the control cell line IMR90 (fig. 3a). However, genetic backgrounds could be extremely different between IMR90 and GM08391, so we decided to use another cell line from an AT patient and his closed normal counterpart, GM09607 (\textsuperscript{−/−}) and GM00637 (\textsuperscript{−/+}). Again exact results were obtained in terms of Ser473 phosphorylation with no differences in Thr308 (fig. 3b). To assess whether the differences observed were specifically due to lack of ATM expression, we transfected ATM wt in AT deficient cells (GM09607). As expected, Ser473 phosphorylation was restored in the cell line over-expressing ectopic ATM wt (fig. 3b). To exclude that the insulin receptor or intermediate molecules were affected in these ATM deficient cells we measured levels of the insulin Receptor (IR) and functionality of the pathway. No differences were observed in level of expression for the IR (fig. 3c) or in terms of MAPK activation (fig. 3b). However, all of our previous observations could be due to a different specific response in our experimental models (two cell lines with
different backgrounds respect to their control). Therefore, we decided to use a biological model with identical genetic background such as MEF from ATM KO mice (30). Again, exact results were obtained in terms of Akt phosphorylation, functionality of the pathway or expression of the IR (fig. 4a, b). In fact, restoration of ATM expression was able to render phosphorylation in Ser473 (fig. 4a, b).

To evaluate the biological implication of ATM in the insulin dependent Akt activation, we decided to check a specific target of Akt upon insulin stimulation such as FKHR inhibition (25,31). We used a transcriptional system in which luciferase expression was under the control of six FKHR response elements. As expected, cells null for ATM had clearly diminished the inhibitory effect mediated by Akt activation, 45% less than the ATM expressing cells (fig. 4c). Furthermore, over-expression of ATM rendered almost a complete block of FKHR activity in both cell systems (fig. 4c). As a control we used mutant form of FKHR in Akt phosphorylation sites in which no inhibitory effects were detected by insulin or ATM expression (data not shown).

**Defective Akt activation correlates with the AT associated radio sensitivity.**

However, using only one stimulus, insulin, could not allow us to propose a general mechanism in which ATM is controlling Akt activity. Therefore we challenged another very well established stimulus for ATM also able to activate Akt, which is γ−radiation (fig. 1c). Then GM09607 and GM00637 γ-irradiated cells were analysed for Akt activity using an *in vitro* kinase assay. Lack of Akt activity was detected in AT cells correlating with a sensitive phenotype and reverted by over-expression of ectopic ATM wt (fig. 5a). Exact results were obtained in the model derived from KO cells (fig. 5 b).
Discussion

In this report we demonstrate that ATM is implicated in Akt Ser473 phosphorylation and its PI3K domain is necessary for this effect. Also, our results show how Akt and ATM may originate an *in vivo* complex without an apparent direct phosphorylation of Akt at serine 473 by ATM, suggesting the existence of other protein/s that directly phosphorylate Ser473 Akt. However, is important to note that although ATM did not phosphorylate a small GST fusion protein containing the last 80aa of Akt, the whole molecule renders the opposite results. This apparent contradiction can be explained by the lack of conformational requirements in the small GST or by the presence of a third enzymatic activity in the whole Akt innumoprecipitated. Our data with the GST fusion protein containing the last 80aa of p53 or Akt, the low level of phosphorylation in the innumoprecipitated Akt versus the GST of p53 and the lack of a consensus site for a ATM phosphorylation in Akt strongly suggest and indirect mechanism of phosphorylation. Nonetheless, these data provide evidences for a new role for ATM that is controlling the unknown PDK2 protein, supporting the existence of a PDK2 activity that is sensitive to PI3K inhibitors but not to staurosporine (16-18,29) and exclude any dependence between Ser473 and Thr308 Akt phosphorylation.

The use of KO cells for ATM and cell lines derived from patients, in addition to our prior observation in Cos cells using siRNA for ATM that exclude a compensatory mechanism, support a physiological role for ATM in insulin mediated Akt activation. AT patients are insulin resistant and KO mice for Akt develop resistance to insulin (21). Therefore, insulin resistance in AT patients can be partially explained by the lack of Ser473 phosphorylation, lack of full Akt activity and the subsequent decrease in the downstream targets, such as FKHR. Therefore, we propose that resistance to insulin in AT patients could be the result of the deregulation of several mechanisms controlled by ATM, such as lack of 4EBPI (32) and Akt activity.
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Radiosensitivity, the classical hallmark of AT, can be explained by lack of Akt activity as has been observed in Akt1 deficient mice (22), while hyperactivation has been related to resistance (12). Indeed, the increase in the G2/M checkpoint after \( \gamma \)-radiation in AT cells (33), is probably due to the low Akt activity, that is a key protein in the control and transition of G2/M checkpoint after DNA damage (34). We propose a model in which Akt activation by \( \gamma \)-radiation is controlled mainly by ATM, and probably by other molecules, in a p110 independent fashion as has been previously demonstrated in squamous cell carcinoma (35). Our data using the PI3K domain from ATM support the existence of two types of PI3K. The conventional ones, able to control phosphorylation of Akt in Thr308 and Ser473, but probably without an important role in DNA damage, and the second one, here represented by ATM, able control Ser473 phosphorylation of Akt (fig.6). Furthermore, other biological properties associated to ATM dysfunction, such as hypogonadism or growth retardation, fit perfectly with the link observed between ATM protein and the Akt activity, as has been observed in KO mice for AKT1 (22).

Regarding the mechanism of Akt activation mediated by ATM is noteworthy that recent evidences suggest that ATM is a downstream effector of Akt through the novel kinase ARK5 (36). The role proposed for ARK5 is based only in biochemical evidences after glucose depletion, with no physiological implications. However, our data re-expressing ATM wt in ATM null cells, the use of specific siRNA for ATM and the biochemical response observed after insulin or \( \gamma \)-radiation, two well known stimuli for ATM activation, demonstrate that ATM is a major upstream activator of Akt through the control of Ser473 phosphorylation. Interestingly, no evidences have been reported showing that lack of Akt affects ATM activity. Nevertheless, a possible feed back loop of regulation between ATM and Akt could be also considered (fig. 6). Furthermore, other member of the PI3K super family extremely close to ATM, also implicated in the stress response (37), DNA-PK, has been recently proposed as the unknown PDK2...
Activation of PKB/Akt at Ser473 is mediated through ATM (38), supporting the role of this type of PI3K members in the control of Ser 473 phosphorylation. In fact, a possibility could be the functional convergence of these two proteins, DNA-PK and ATM, in the control of Akt as has been demonstrated for c-Abl or H2AX phosphorylation in response to ionizing radiation (39, 40).

Thus, as a major conclusion, the results shown here constitute the first evidence of a novel function of ATM as a main upstream activator of Akt activity through the control exerted on the Ser473 phosphorylation, suggesting a mechanistic model for some of the biological symptoms of AT disease such as radiosensitivity or resistance to insulin. Whether this model is applicable or not to other contexts is currently being studied, as it is the identification of the putative proteins that might interact with, and regulate, the ATM-Akt signalling pathway.
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Footnotes

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1 The abbreviations used are: ATM, mutated in Ataxia Telangiectasia; AT, Ataxia Telangiectasia; KO, Knock Out; PI3K, Phosphatidylinositol-3 kinase; HA, Hemagglutinin, GST, Glutathione S-transferase; Wt, Wild type; kd, Kinase death; GFP, Green Fluorescence Protein.

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Figure Legends

Figure 1. ATM mediates Akt Ser 473 phosphorylation through the PI3K domain in Cos cells

a Transient transfection in Cos cells introducing increasing amounts, 1-3µg, of Flag-6-His ATM and 1µg of HA-Akt or GFP-p53. Antibodies against phosphorylated form of Akt in Thr308 or Ser473 were used to detect Akt activation. Total Akt was used as a loading control. Phosphorylation of Ser 15 of p53 was used as a control of ATM functionality. ATM expression was detected with anti-Flag. As a positive control HA-Akt was stimulated with insulin (100nM) for 20 minutes and GFP-p53 with γ-radiation (20Gy) for 30 minutes.

b Transient transfection in Cos cells introducing increasing amounts, 1-3µg, of Flag-6-His ATM and 1µg of HA-Akt in the presence of the following PI3K inhibitors: 200nM wortmannin, 20 µM Ly294002, 2mM caffeine.

c Cos cells were transfected with increasing amounts, 1 to 4µg, of Flag-6-His ATM wt or Kd plus 1µg of HA-Akt. Kinase assay was performed to detect Akt activity using as a substrate GST-GSK3. As a positive control HA-Akt transfected cells were treated with insulin (100nM) for 20 minutes (left). Transfected HA-Akt (1µg) is activated in γ-radiated (20Gy, 30 minutes) Cos cells (right).

d Increasing amounts, (0-400 nM), of the siRNA were co-transfected in Cos cells with 1mg ATM. Expression of ATM was evaluated by Western Blot and as a control we measured endogenous Akt (upper panel). The fold change of ATM expression was estimated by densitometric analysis of filters. The values (given as arbitrary units) were normalized taking into consideration the ATM protein levels obtained without siRNA as determined by immunoblot analysis. Results show a representative blot out of three. Same amounts of the siRNA were co-transfected in Cos cells with 0.5µg HA-Akt. Cells were treated with insulin (100nM) for
20 minutes when indicated. HA-Akt was immunoprecipitated and phosphorylation status of HA-Akt was analysed (Lower panel).

e The 6His-tagged PI3K (1 and 3µg) domain of ATM was cotransfected with HA-Akt or GFP-p53 (1µg) in Cos cells. Wortmamin (200nM) was used to inhibit PI3K domain of ATM. Phosphorylation of Akt in Ser473 or P53 in Ser15 was evaluate.

f 1µg of HA-Akt was cotransfected in Cos cells with 3µg of Flag-6-His ATM wt, PI3K domain of ATM, p110 α or treated with insulin for 20 minutes.

The fold change of activation was estimated by densitometric analysis of filters. The values (given as arbitrary units) were normalized taking into consideration the Akt protein levels of each sample as determined by immunoblot analysis. Results show a representative blot out of three (SD: Standard Deviation).

**Figure 2. ATM binds but does not directly phosphorylate Akt in Ser 473**

a HA-Akt (4µg) was cotransfected with Flag-6His-ATM wt or kd (4µg) in 293T cells. Coimmunoprecipitation was done with 6-His antibody and WB was developed with HA antibody (left panel) or with phospho-Akt against Thr308 or Ser473 (right panel). Positive controls for Akt phosphorylation were performed with insulin (100nM) for 20 minutes.

b Coimmunoprecipitation experiments between p53 Flag-6-His ATM wt or kd following the same approach as in fig. 2a.

c Immunoprecipitated HA-Akt wt (wt) and triple mutant (mut) (2µg) from Cos cells was used as a substrate for in vitro kinase assays. Flag-6-His ATM wt or kd (7µg) were obtained by transfection and immunoprecipitation from 293T. 32P incorporation was detected by film exposure. Aliquots from kinase reaction were used to detect HA-Akt and Flag-ATM.
Activation of PKB/Akt at Ser473 is mediated through ATM

d GST-Akt wt (wt), GST-Akt Ser473Ala (mut), GST-p53wt (wt) or GST-p53ser15Ala (mut) were used as a substrate for in vitro kinase assays. Flag-6-His ATM wt or kd were obtained as in fig 2c. $^{32}$P incorporation was detected as in fig. 2c. Aliquots from kinase reaction were used to detect GST-Akt, GST-p53 and Flag-ATM.

Results show a representative blot out of three with nearly identical results.

Figure 3 AT patients derived cell lines are defective in Akt Ser 473 phosphorylation in response to insulin.

a IMR90 and GM08391 cells were treated for 30 min with increasing amounts of insulin. Akt Ser473 phosphorylation was assessed. Total Akt was used as loading controls.

The fold change of activation was estimated by densitometric analysis of filters. The values (given as arbitrary units) were normalized taking into consideration the Akt protein levels of each sample as determined by immunoblot analysis. Results show a representative blot out of three (SD: Standard Deviation).

b GM00637, GM09607 and GM09607 expressing Flag-6-His ATM wt were treated with insulin and phosphorylation of endogenous Akt and ERK1/2 was evaluated. Total Akt and Erk1 were used as loading controls.

The fold change of activation was estimated by densitometric analysis of filters. The values (given as arbitrary units) were normalized taking into consideration the Akt protein levels of each sample as determined by immunoblot analysis. Results show a representative blot out of three (SD: Standard Deviation).

c Insulin receptor α subunit levels were measured. Akt was used as a loading control.
Activation of PKB/Akt at Ser473 is mediated through ATM

Figure 4 KO cells for ATM are defective in Akt Ser 473 phosphorylation in response to insulin.

a Cell lines KO (p53 -/-, ATM +/+), Double KO (p53 -/-, ATM -/-) and DKO ATM (DKO expressing Flag-6-His ATM wt) were treated with insulin 100nM for 30 minutes and phosphorylation of endogenous Akt and ERK was evaluated. Total Akt and Erk1 were used as loading controls. The fold change of activation was estimated by densitometric analysis of filters. The values (given as arbitrary units) were normalized taking into consideration the Akt protein levels of each sample as determined by immunoblot analysis. Results show a representative blot out of three (SD: Standard Deviation).

b Insulin receptor α subunit levels were detected as in fig 3c.

c Reporter assays for FKHR performed in KO and Double KO in the presence of insulin or overexpressing Flag-6-His ATM wt. Double KO cells have drastically reduced the capacity of FKHR transcriptional inhibition upon insulin stimulation. The data represent Photinus luciferase activity standardized for the Renilla activity present in each cellular lysate, expressed as fold inhibition compared to control cells. The data represent the average and standard deviation of 3 separate assays performed in triplicate cultures.

Figure 5 Lack of Akt phosphorylation at Ser 473 in cell lines derived from AT patients or KO mice correlates with radio sensitivity.

a Akt kinase assay was performed in GM00637, GM09607 and GM09607 expressing ATM wt cell lines 30 minutes after treatment with 20 Gy (upper panel). The values (given as arbitrary units) were normalized taking into consideration the Akt protein levels of each sample as determined by immunoblot analysis. Results show a representative blot out of three (SD: Standard Deviation). Viability assays to γ-radiation in GM09607 (□) and GM09607 expressing ATM wt (▲) measured 7 days after irradiation. The data represent the average and standard deviation of 3 separate assays performed in triplicate culture (lower panel).
Akt kinase assay performed in KO p53, DKO, or DKO ATM cells 30 minutes after treatment with 20 Gy (upper panel). The values (given as arbitrary units) were normalized taking into consideration the Akt protein levels of each sample as determined by immunoblot analysis. Results show a representative blot out of three (SD: Standard Deviation). Viability assays to γ-radiation in KO (O), DKO (□) or DKO ATM (▲) measured 7 days after irradiation. The data give the average and standard deviation of 3 separate assays performed in triplicate culture (lower panel).

**Figure 6**

*Proposed model for the modulation of the AKT signalling pathway by ATM.*
Activation of PKB/Akt at Ser473 is mediated through ATM

Figure 1

**Diagram**

- **Panel e**: PI3K ATM
  - Wortmannin 200nM
  - p-Akt (Ser 473)
  - HA-Akt
  - p-p53 (Ser15)
  - GFP-p53

- **Panel f**: p-Akt (Thr 308)
  - p-Akt (Ser 473)

| PI3K ATM | Wortmannin 200nM | p-Akt (Ser 473) | HA-Akt | p-p53 (Ser15) | GFP-p53 |
|---|---|---|---|---|---|
| - | - | - | - | - | - |
| + | + | + | + | + | + |

| PI3K ATM | Wortmannin 200nM | p-Akt (Ser 473) | HA-Akt | p-p53 (Ser15) | GFP-p53 |
|---|---|---|---|---|---|
| - | - | - | - | - | - |
| + | + | + | + | + | + |

| PI3K ATM | Wortmannin 200nM | p-Akt (Ser 473) | HA-Akt | p-p53 (Ser15) | GFP-p53 |
|---|---|---|---|---|---|
| - | - | - | - | - | - |
| + | + | + | + | + | + |

Fold activation (+/- SD)

- Panel e: 1 2.3 4.4 1
- Panel f: 1 4.1 1.3 1.5 7.2 1 5.1 5.3 4.2 8.2 1 5.1 5.3 4.2 8.2
Activation of PKB/Akt at Ser473 is mediated through ATM

Figure 2

a) HA-Akt + 6His-ATM wt
HA-Akt + 6His-ATM kd
WB HA

b) GFP-p53 6His-ATM wt
GFP-p53 6His-ATM kd
WB GFP

c) ATM wt ATM kd
wt mut wt mut
12p HA-Akt
HA-Akt
Flag-ATM

d) ATM wt ATM kd
wt mut wt mut
12p GST-Akt
GST-Akt
12p GST-p53
GST-p53
Flag-ATM
Activation of PKB/Akt at Ser473 is mediated through ATM

**Figure 3**

**a**

| Insulin (nM) | IMR90 | GM08391 |
|--------------|-------|---------|
| 0            | 0     | 0       |
| 400          | 0     | 0       |

- p-Akt (Ser 473)

| Fold activation (±/− SD) | IMR90 | GM08391 |
|--------------------------|-------|---------|
| 1,6                       | 5,7   | 6,2     |
| 0,2                       | 0,4   | 0,1     |

**b**

| GM00637 | GM09607 | GM09607 ATM wt |
|---------|---------|---------------|
| -       | +       | -             |
| +       | -       | +             |

- p-Akt (Thr 308)
- p-Akt (Ser 473)
- Akt
- p-MAPK
- ERK1

**c**

| GM00637 | GM09607 | GM09607 ATM | IMR90 | GM08391 |
|---------|---------|-------------|-------|---------|

- IR-α
- Akt
Activation of PKB/Akt at Ser473 is mediated through ATM

Figure 4

| KO p53 | DKO | DKO ATM |
|--------|-----|---------|
| -      | -   | -       |
| +      | +   | +       |

Insulin 100nM

- p-Akt (Thr 308)
- Akt
- p-Akt (Ser 473)
- p-MAPK
- ERK1

Fold activation (+/-) SD

Fold Inhibition

Luciferase Arbitrary units

- KO
- DKO

control
Insulin
ATM wt
Activation of PKB/Akt at Ser473 is mediated through ATM

Figure 5

a

| γ-Radiation | GM00637 | GM09607 | GM09607 ATMwt |
|-------------|---------|---------|--------------|
| -           | +       | +       | +            |

p-GSK3 (Ser21/9)

Akt

1 5 1 1 1 1,1 1 3,2 Fold activation (+/-) SD

0 0,4 0 0,2 0 0,3

Survival (% of control cells)

γ-Radiation (Gy)

Survival (% of control cells)

γ-Radiation (Gy)

b

| γ-Radiation | KO p53 | DKO | DKO ATM |
|-------------|--------|-----|--------|
| -           | +      | -   | +      |

p-GSK3 (Ser21/9)

Akt

1 3,6 1 0,8 1 3,1 Fold activation (+/-) SD

0 0,2 0 0,3 0 0,3

Survival (% of control cells)

γ-Radiation (Gy)
Activation of PKB/Akt at Ser473 is mediated through ATM

Figure 6
Full activation of PKB/Akt in response to insulin or ionizing radiation is mediated through ATM

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