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Supplemental Data

ATM Regulates a RASSF1A-Dependent DNA Damage Response

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Supplemental Experimental Procedures

Tissue Culture

All cell culture reagents were purchased from Invitrogen. MCF7, HeLa, U2OS and H1299 cells (purchased from Cancer Research UK, London or LGC Promochem (ATCC)) were cultured in complete DMEM (DMEM, 10% fetal calf serum, 1 mM sodium pyruvate, 2 mM glutamine, 0.1 mM MEM non essential amino acids, 100 U/ml Penicillin and 100 μg/ml streptomycin. PEBS, YZ3 and YZ5 were cultured in complete DMEM supplemented with 200 μg/ml Hygromycin B. Cells were cultured in humidified incubator with 5% CO_2 at 37^{o}C. All chemical reagents were purchased from Sigma unless stated otherwise. Caffeine and the ATM inhibitor, KU55399 (2-Morpholin-4-yl-6-thianthren-1-yl-pyran-4-one) were purchased from Merck Biosciences/Calbiochem. All irradiation were carried out in a Caesium-137 irradiator (GmbH).

Transfection of Plasmid DNA and siRNA Duplexes

All cell lines were seeded at a density of 2 x 10^6 cells per 10 cm dish 16 hr before transfection and cultured in complete medium without antibiotics. Cells were transfected with 2 μg plasmid DNA using Lipofectamine 2000 (Invitrogen) according to the manufacturers protocol. For siRNA, cells were transfected with 50 nM siRNA duplexes using either Lipofectamine 2000 (Invitrogen) or Hiperfect (Qiagen). The sequences of the siRNA's used in this study are:
LATS1 (Sense) 5′-GGUUCUGAGAGUAAAAUUAtt (Ambion);
LATS1 (Sense) 5′-UAGCAUGGAUUUCAGUAAUUU-3′ (Dharmacon);
MST2 Sense: 5′-GGAUAGUUUUUCAAAUAGGtt-3′(Ambion);
Sense: 5′-GCCCAUAUGUUGUAAAGUAu-3′ (Dharmacon);
RASSF1A (Sense) GACUCCAGUGGUAAUCUACtt-3′ (Eurofins MWG OPERON);
RASSF1A Sense: 5′-CUACAUAACUUCCUACGUAUU-3′ (Dharmacon);
YAP1 (Sense) 5′-GACAUCUUCUGGUCAGAGAtt-3′ Dharmacon;
P73 (Sense) 5′-AGCGCACCAUCUUCUUCAtt-3′ (Eurofins MWG OPERON).
The nontargeting control used was siGENOME Non-Targeting siRNA #2 (Dharmacon).

Mutagenesis

All site directed mutagenesis experiments were carried out using the QuikChange II Site-Directed Mutagenesis Kit according to the manufacturer's protocol.

Antibodies

Anti-Flag (M2 clone), anti-Flag M2 agarose, anti-ATM (A6218) and anti-γ-tubulin antibodies were purchased from Sigma. Anti-RASSF1A clone 3F3 (sc-58470), anti-MST2 (sc6211), anti-YAP (sc-15407), anti-calnexin (sc-70481), anti-LATS1 (sc-9388) were purchased from Santa Cruz Biotechnology. Anti-RASSF1A clone eB114-10H1 was purchased from eBioscience. Phospho-(Ser/Thr.) ATM/ATR substrate antibody (2851s) and phospho-p53 (Ser15) antibody (9284) were purchased from Cell Signalling Technology. Anti-MST2 (1943-1) was purchased from Epitomics. Anti-Lats1 (A300-478A) was purchased from Bethyl. Anti-HA (05-904) was purchased from Millipore. Anti-p73 (A300-126A) was purchased from Universal Biologicals, anti-p73 (MS-764-PO) was purchased from Lab vision. Anti-ATM (MAB2290) was purchased from R&D systems and anti-DNA-PKcs (MS-423-PABX) from Stratech. The phosphospecific antibody RASSF1A S131 was custom made by Eurogentec. Rabbits were immunized with the phosphor-Ser (underlined) peptide (WETPDL<u>S</u>QAEIE) corresponding to residues 125-136 of RASSF1A. Secondary antibodies coupled to horseradish peroxidise were purchased from Pierce. Lambda protein phosphatase (P0753S) was purchased from New England Biolabs (UK).

Immunoprecipitations and Western Blotting

Cells were washed twice with ice cold PBS before lysis. All cells were lysed by scrapping in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EGTA, 5 mM $MgCl_2$, 1% v/v NP40, 1% w/v sodium deoxycholate, 0.1 % w/v SDS 10 mM sodium β-glycerophosphate, 50 mM NaF, 1 mM Na_3VO_4, 5 mM sodium pyrophosphate and "Complete" proteinase inhibitor cocktail EDTA free (Roche one tablet per 50 ml). Immunocomplexes were precipitated with Protein G sepharose (Ge Healthcare). Immunoprecipitates were washed three times with 50 mM Tris-HCl, 150 mM NaCl, 1% v/v NP40, 1% w/v sodium deoxycholate, 0.1% w/v SDS.

When MST2 was immunoprecipitated for the in-gel kinase assay and LATS1 co-immunoprecipitation, cells were cultured in complete media containing 0.1% FCS for 24 hr before treatment. Cells were washed twice with ice cold PBS and lysed in 0.5% (v/v) NP40 lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EGTA, 5 mM $MgCl_2$, 0.5% v/v NP40, 10 mM sodium β-glycerophosphate, 50 mM NaF, 1 mM Na_3VO_4, 5 mM sodium pyrophosphate and "Complete" proteinase inhibitor cocktail EDTA free (1 tablet per 50 ml). Proteins were resolved on 4%-12% Bis-Tris Nu-PAGE gels (Invitrogen) and transferred onto PVDF (polyvinylidene fluoride) membrane (Millipore) before immunoblotting with the appropriate antibodies. In gel kinase assays were performed as previously described.

Cell Assays

HeLa cells were transfected with siRNA and replated for apoptosis assays after 48 hr. Cells were treated with 15-25 μM cisplatin, etoposide (10 μM) or doxorubicin (10 μM) harvested at the

times indicated in the figure legends and analyzed by flow cytometry. Apoptosis was assessed by evaluating caspase activation (according to the manufacturers protocol) using the CaspACE FITC-VAD-fmk in situ marker (Promega). All error bars represent standard error.

For Clonogenic Assays; HeLa, MCF7 or H1299 cells were seeded at a density of 1 x 10^5 per well in a six well dish 16 hr before transfection. Cells were transfected with plasmid DNA (0.1 μg/well) or siRNA. 24 hr following transfection with plasmid DNA or 48 hr following siRNA transfection cells were seeded at a density of 400 cells/6 cm dish and irradiated at the indicated dose or treated with cisplatin 3 hr after plating. Plates were stained with crystal violet (0.5% w/v crystal violet, 50% v/v MeOH and 10% v/v EtOH) 14-21 days later and colonies counted. All conditions were done in triplicate and error bars represent standard error.

Cell Viability Assay

H1299 cells were transfected with pcDNA3, RASSF1A or RASSF1A-S131A together with pBabe Puro. 24 hr after transfection, cells were trypsinized and replated in 96 well dishes at a density of 3 x 10^3 cells/well. The cells were then exposed to 1.5 μg/ml puromycin for 48 hr to select for transfected cells before being treated with 15 μM cisplatin. 48 hr later, cell viability was determined using the resazurin assay. The cells were incubated with media containing to 10 μg/ml resazurin at 37^{o}C for 1 hr. Resazurin reduction was then measured fluorometrically using a plate reader (Wallace Perkin Elmer) at excitation 530 nm and emission wavelength 590 nm. Viability experiments were performed in triplicate and repeated at least twice.

Kinase Assays

The MST2 in gel kinase assay was performed as previously described. Briefly myelin basic protein (MBP) is copolymerised into the separating gel and when MST is denatured and refolded in the gel MBP presents as a substrate for MST2 kinase activity which can be detected by incubation with γ32-P-ATP. MST2 auto-kinase activity is not detected in the absence of MBP. ATM kinase assay was performed by incubating Flag-ATM immunoprecipitates with HA-RASSF1A immunoprecipitated from MCF7 cells using anti-HA antibodies and protein eluted by incubation of beads in 0.1M Glycine pH2.5. Kinase assays were performed in a buffer consisting of 25 mM Tris-HCl (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol (DTT), 0.1 mM Na3VO4, 10 mM MgCl2, 50 μM ATP, and ^{32}P-ATP. Reactions were incubated for 90 min at 30 °C prior to SDS-PAGE (SDS polyacrylamide gel electrophoresis) electrophoresis and detection on a BioRad PhosphorImager.

Image Analysis

X-ray film from western blot experiments were scanned on a flat bed scanner (Kodak). To quantify the bands obtained via western blot analysis, we applied ImageJ software based analysis (http://rsb.info.nih.gov/ij/). All bands were normalised against the loading controls.
Statistics; all p values were calculated by Student's t test, error bars indicate standard error.

Figure S1.
(A) LATS1 was immunoprecipitated from MCF7 cells transfected with either RASSF1A (R1A) or RASSF1A-S131A and treated with 10 Gy of ionizing radiation as indicated. LATS1 immunoprecipitates were incubated in kinase assay buffer containing γ^{32}P-ATP and subsequently resolved by SDS-PAGE. LATS1 autophosphorylation was detected by a phosphorImager (PMI-imager Bio Rad).
(B) HeLa cells transfected with siRNA against RASSF1A, MST2, LATS1, or a nontargeting control (NT) as described previously. Results are representative of two independent siRNAs for each target.

Figure S2.
(A) Endogenous RASSF1A was immunoprecipitated (Anti-RASSF1A [ebiosciences]) from HeLa cells treated with ionizing radiation (4 Gy) and harvested at the indicated time points. Cells were pretreated with DMSO or 10 mM caffeine for 1 hr prior to irradiation. Immunoprecipitates were probed with the generic ATM substrate antibody (Cell signalling 2851s) and anti RASSF1A (3F3, Santa Cruz) antibodies.
(B) MCF7 cells transfected with pcDNA3 or FLAG-RASSF1A were irradiated (10 Gy) and harvested 30 min following irradiation. FLAG tagged proteins were immunoprecipitated and immunoprecipitates were treated with 200 units lambda protein phosphatase (New England Biolabs, UK) or buffer according to the manufacturer's instructions.

Figure S3.

(A) MCF7 cells were transfected with 2 μg pcDNA3, FLAG-RASSF1A or FLAG-RASSF1A-S131A. Cells were preteated with 10 μM LY94002, 10 μM 2-Morpholin-4-yl-6-thianthren-1-yl-pyran-4-one (KU55933; calbiochem) 10 μM NU7026 or an equivalent volume of DMSO prior to ionising radiation (10Gy). Cells were harvested 30 min post irradiation and FLAG containing proteins immunoprecipitated from lysates. Immunoprecipitates were resolved on SDS-PAGE gels and western blotted with the indicated antibodies. Phospho-S131 bands were quantitated via Image J analysis and normalised to the loading control. Data is representative of four independent experiments. Error bars represent standard error.

(B) U2OS cells transfected with Flag-RASSF1A were harvested 1 hr following irradiation (4 Gy). Immunoprecipitations of ATM, DNA-PKcs or control (IgG) were addressed for co-precipitation of FLAG-RASSF1A by western blot. Lysates and immunoprecipitates were blotted with the indicated antibodies.

(C) 50 μg of total protein from Hela, MCF7, PEBS (ATM null) and YZ3 (ATM reconstituted) was separated on a NuPage Tris Acetate gel (Invitrogen) and lysates probed by western blot for ATM expression levels.

(D) MCF7 cells were transfected with 5 μg pcDNA3, HA-RASSF1A (2.5 μg HA RASSF1A and 2.5 μg pcDNA 3), FLAG-RASSF1A (2.5 μg FLAG RASSF1A and 2.5 μg pcDNA 3) or HA-RASSF1A and FLAG RASSF1A (2.5 μg HA RASSF1A and 2.5 μg FLAG RASSF1A) as indicated. FLAG tagged proteins were immunoprecipitated from MCF7 cells irradiated (10 Gy) as indicated 30 min post irradiation and probed for the HA tag by western blot. Immunoprecipitates and lysates were probed as indicated.

(E) MCF7 cells were transfected with either pcDNA3, FLAG-RASSF1A or mutated FLAG-RASSF1A-S131A. Cells were incubated with either DMSO or 10 μM ATM inhibitor (2-Morpholin-4-yl-6-thianthren-1-yl-pyran-4-one, calbiochem (ATMi) for 1 hr prior to irradiation 4 (Gy) and endogenous MST2 was immunoprecipitated 2 hr post irradiation. Lysates and immunoprecipitates were blotted with the indicated antibodies.

Figure S4.
(A) H1299 cells were transfected with pcDNA3, FLAG-RASSF1A or the S131A mutant FLAG-RASSF1A. Cells were cultured for 9 hr with 25 μM cisplatin. Lysates were probed with the indicated antibodies.
(B) H1299 cells were transfected with pcDNA3, FLAG-RASSF1A or the S131A mutant FLAG-RASSF1A. Cells were cultured for 24 hr with 25 μM cisplatin and the amount of P21 (santa cruz) expression in lysates samples determined by western blot. Blots are representative of three experiments.
(C) MCF7 cells were transfected with siRNA against RASSF1A. Cells were irradiated (10 Gy) and endogenous MST2 immunoprecipitated 30 min following irradiation. The activity of MST2 in immunoprecipitates was determined via the in gel kinase assay. MST2 immunoprecipitates were probed by western blot for MST2.