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RETRACTION

STAT-1 facilitates the ATM activated checkpoint pathway following DNA damage

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Retraction of: *J. Cell Sci.* **118,** 1629-1639.

We were recently made aware of errors in our paper, which include misrepresentation of western blot data in Figs 4, 5 and 6 as detailed below. The misuse and re-use of western blot bands breaches the editorial policy of *Journal of Cell Science*, and so we must retract this article. The corresponding author, A.S., regrets the inappropriate figure manipulations of which the co-authors were completely unaware. We deeply regret that the majority of sound research presented in the rest of the paper has been invalidated in this manner, and the concern this will cause to the research community. The co-authors are repeating the affected experiments to determine whether the overall conclusions of the paper remain valid.

1. Fig. 4A, Panel B MDC1 Input lane and Panel A p53BP1 Input lane are the same (flipped horizontally).

2. Fig. 5A (pNBS1), Fig. 5B ATM and Fig. 5G (p53) blots are the same.

3. Fig. 5A (Chk2), Fig. 5C (pChk2) and Fig. 5G (Chk2) blots are the same.

4. Fig. 5B actin and Fig. 5E actin blots are the same.

5. Fig. 6A ATM and pChk2 are the same blot.

6. Fig. 6C and Fig. 6E actin blots are the same.

STAT-1 facilitates the ATM activated checkpoint pathway following DNA damage

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Summary

STAT-1 plays a role in mediating stress responses to various stimuli and has also been implied to be a tumour suppressor. Here, we report that STAT-1-deficient cells have defects both in intra-S-phase and G2-M checkpoints in response to DNA damage. Interestingly, STAT-1-deficient cells showed reduced Chk2 phosphorylation on threonine 68 (Chk2-T68) following DNA damage, suggesting that STAT-1 might function in the ATM-Chk2 pathway. Moreover, the defects in Chk2-T68 phosphorylation in STAT-1-deficient cells also correlated with reduced degradation of Cdc25A compared with ST[illegible]expressing cells after DNA damage. We also show that STAT-1 is required for ATM-dependent phosphorylation of NBS1 and p53 but not for BRCA1 or H[illegible]X phosphorylation following DNA damage. Expression levels of BRCT mediator/adaptor proteins MDC1 and 53BP1, which are required for ATM-mediated pathways, are reduced in cells lacking STAT-1. Enforced expression of MDC1 into STAT-1-deficient cells restored ATM-mediated phosphorylation of downstream substrates. These results imply that STAT-1 plays a crucial role in the DNA-damage-response by regulating the expression of 53BP1 and MDC1, factors known to be important for mediating ATM-dependent checkpoint pathways.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/118/8/1629/DC1

Key words: ATM, Cell cycle, Chk2, 53BP1, MDC1, STAT-1

Introduction

To ensure that cells pass on accurate [illegible]ies of their genomes on to the next generation, a series of sur[illegible]llance pathways – the so called cell-cy[illegible]e-checkpoint protein kinases – are activated following DNA dama[illegible]e to allow appropriate time for DNA repair to take place. The ATM kinase in conjunction with adaptor proteins (mediators/mediato[illegible]) plays a pivotal role in initiating the check[illegible]t-cascade pathway following DNA damage by phosphory[illegible]g a[illegible] activating a subset of ATM substrates including C[illegible]-T68, p53-S15, NBS1-S343 and BRCA[illegible]-S1387 (M[illegible]ochionna et al., 2000; Chehab et al., 2000; Lim[illegible]al., 2000[illegible]001; Shiloh, 2003). In mammalian cells, p[illegible]ing 53BP1 and MDC1 (mediator of DNA damage checkpoint) that contain a BRCA1 C-terminal (BRCT), have been termed a[illegible]tors/mediators, because they play a central role in regulating ATM activation and ATM-mediated pathways (Shultz et al., 2000; DiTullio et al., 2002; Fernandez-Capetillo et al., 2002; Wang et al., 2002; Abraham, 2002; Goldberg et al., 2003; Lou et al., 2003; Stewart et al., 2003; Xu and Stern, 2003; Peng and Chen, 2003).

Following DNA damage, Chk2 kinase is also effective in phosphorylating its own subset of substrates on alternative sites to those phosphorylated by ATM, including p53-S20, BRCA1-S988, Cdc25A-S123, Cdc25C-S216, PML and E2F-1 (Chehab et al., 2000; Hirao et al., 2000; Lim et al., 2000; Bartek and Lukas, 2003; Yang et al., 2002; Stevens et al., 2003). Activation of Chk2-Cdc25A has been implicated in both G1-S phase and G2-M transition checkpoint-control (Abraham, 2001; Falck et al., 2001; Falck et al., 2002; Bartek and Lukas, 2003; Shiloh, 2003), whereas Chk2-p53, Chk2-PML and E2F-1 activated pathways have been reported to play a role in the apoptotic pathway (Hirao et al., 2000; Yang et al., 2002; Takai et al., 2003; Stevens et al., 2003).

In addition to BRCA1, ATM also phosphorylates NBS1 on serine 343 (Lim et al., 2000). NBS1 is a component of the multifunctional MRE11-RAD50-NBS1 (MRN) complex that is involved in the repair of DNA double-strand breaks (DSBs) and is also required for Chk2 phosphorylation (D'Amours and Jackson, 2001; Buscemi et al., 2001). Following DNA damage, the histone variant H2AX is one of the earliest proteins to be phosphorylated and forms positive nuclear foci at sites of DSBs (Fernandez et al., 2002; Coleste et al., 2003). This is followed by recruitment of 53BP1 and MDC1 that colocalise with phosphorylated γH2AX at DNA DSBs (Fernandez et al., 2002; Wang et al., 2002; Stewart et al., 2003). Finally, the recruitment of the MRN complex facilitates the binding of DNA repair

factors (D'Armours and Jackson, 2001). Recent studies have reported that the response to DNA damage leads to distinct, branched pathways that are activated via the phosphorylation of specific ATM downstream targets and shows a regulatory hierarchy that converges to control-processes such as DNA repair, cell cycle or apoptosis (Wang et al., 2002; Foray et al., 2003).

The signal transducer and activator of transcription 1 (STAT-1) protein is essential for signalling of interferons (IFNs) (Broomberg and Darnell, 2000; Ihle, 2001), which, in addition to their role in innate immunity, serve as potent inhibitors of cell growth and promoters of apoptosis. Although STAT-1-deficient mice develop no spontaneous tumours, they are highly susceptible to chemical, carcinogen-induced tumourigenesis (Durbin et al., 1996; Kaplan et al., 1998). Crossing the STAT-1 knockout into a p53-deficient background yields animals that develop tumours more rapidly, and with a broader spectrum of tumour types than is seen with p53 single-mutants (Kaplan et al., 1998), suggesting that STAT-1, like p53, may have tumour suppressor properties.

p53 plays an important role in mediating the apoptotic programme (Vousden and Lu, 2002). Recently, STAT-1, like p53, has been directly implicated in modulating apoptosis. For example, cells lacking STAT-1 are less susceptible to tumour necrosis factor α-induced cell death than cells containing STAT-1 (Kumar et al., 1997). STAT-1-deficient cells are also resistant to hypoxia-induced cell death (Janjua et al., 2002) and STAT-1 promotes apoptosis in cardiac myocytes exposed to ischaemia/reperfusion injury (Stephanou et al., 2000; Stephanou et al., 2001; Stephanou et al., 2002).

Our recent work has shown that STAT-1 can interact with p53, modulate its activity by enhancing p53-specific gene expression and can induce apoptosis (Townsend et al., 2004). Moreover, levels of p53 are reduced in cells lacking STAT-1, and STAT-1 is a negative regulator of the p53 inhibitor Mdm2 (Townsend et al., 2004). However, the mechanism of how STAT-1 can inhibit cell growth is unclear. In this study, we investigated the role of STAT-1 in the DNA-damage response pathway in both murine and human cells lacking STAT-1, and found that its absence is associated with defects in the cell-cycle checkpoint and also with a reduction in a subset of ATM-dependent, phosphorylated downstream substrates after DNA damage.

Materials and Methods

Cell culture

Wild-type STAT-1$^{+/+}$ and STAT-1$^{-/-}$ mouse embryonic fibroblasts (Durbin et al., 1996) were kindly provided by David E. Levy (National Institutes of Health, Bethesda, MD) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The human fibrosarcoma cell lines 2fTGH and U3A, and U3A-derived cells stably expressing STAT-1 were kindly provided by Ian Kerr (Cancer Research UK, London) (McKendry et al., 1991) and cultured in DMEM supplemented with 10% FBS. HCT-116 cells, stably expressing HA-Chk2-wt or HA-Chk2-kd, were maintained as described previously (Lou et al., 2003).

The STAT-1 RNA interference (RNAi) vector was constructed using the protocol described previously (Paddison et al., 2002). Briefly, the forward and reverse primers were, 5′-ccagaacgaa tgagggtcctc-3′ and 5′-gagggaccctcattcgttctgg-3′, respectively. The MDC1 RNAi and green fluorescent protein (GFP)-MDC1 vectors were constructed as described previously (Peng et al., 2003). The MDC1 promoter (2.0 kb) was PCR-amplified from human DNA using the following forward and reverse primers 5′-gtaccttgggtgcgctgggc-3′ and 5′-gatctgggaaggatacacatt-3′, respectively, and cloned into the pGL3-basic luciferase reporter construct (Promega, UK).

Radioresistant DNA synthesis, BrdU, G2-M checkpoint and cell death assay

Rates of DNA synthesis after γ-irradiation (IR) of 2 and 10 Gy were measured with the two-isotope radioresistant DNA synthesis (RDS) assay. Twenty hours after plating, cells were pulsed with 5 nCi/ml [^{14}C]-thymidine (Amersham Biosciences, UK) and incubated a further 24 hours. The medium was then removed and the cell were washed and exposed to IR of 2 or 10 Gy. After a 30-minute incubation, the medium was replaced with fresh medium containing 20 μCi/ml [^{3}H]-thymidine (Amersham Biosciences, UK) and cells were incubated a further 30 minutes. Cells were then harvested and the ^{3}H:^{14}C ratios were measured to assess the rates of DNA synthesis.

To assess in more detail changes in S phase, we performed the standard BrdU assay. Briefly, for BrdU dynamic cell-cycle analysis, cells were first pulsed for 30 minutes at 37°C with 10 mM BRDU (Sigma) followed by extensive washing with PBS containing 1% BSA, 10 mM Azide (PBA). Cells were then irradiated or not, followed by second incubation for 0-48 hours. After this, cells were trypsinised, fixed in ice-cold 70% ethanol and washed with PBA. 2M HCl was added for 20 minutes, washed off and excess acid neutralised with 0.1 M $Na_2B_4O_7$. Cells were washed one further time in PBA before adding anti-BrdU monoclonal antibody (mAb) (BD Pharmingen) diluted in PBS with 0.5% BSA and 0.5% Tween-20 (PBT) for 45 minutes at room temperature. Cells were washed again with PBA and incubated with 10 μg/ml propidium iodide (PI) for 30 minutes to stain DNA before assessment by flow cytometry. Analysis of cells was performed with a FACScan flow cytometer (Becton Dickinson) using a 488 nm argon laser for excitation, and a 560 nm dichroic mirror and 600 nm band-pass filter (bandwidth 35 nm) for detection. Red fluorescence data was expressed on a linear scale, and green FL1 on a log scale.

To measure changes in mitosis specifically, we used the standard two-parameter flow cytometry assay to measure DNA and phosphorylated histone H3. Briefly, cells were plated, incubated, fixed and harvested in the same way as detailed above, incubated with a specific rabbit polyclonal antibody (Upstate) in PBT for 45-120 minutes, washed and incubated with a goat anti-rabbit FITC-conjugated Ab (Sigma). Cells were then washed, stained with PI and assessed by flow cytometry as before.

For survival assays, cells (2.0×10^{6}) were exposed to 10 Gy γ-IR and incubated for 72 hours. Cells were then washed with 1× PBS before staining with Crystal Violet (0.2% Crystal Violet 2% EtOH). Viable cells were calculated as a percentage to control cells that were not exposed to γ-IR.

Antibodies, western blotting and immunostaining

Cells were exposed to γ-IR (2 or 10 Gy) or left untreated and cell extracts were prepared in lysis buffer (150 mM NaCl, 50 mM Tris base, 0.5% SDS, 1% NP-40). Samples were then boiled in SDS sample buffer for 5 minutes and separated on a 10% SDS PAGE gel. Samples were transferred to nitro-cellulose filters and subjected to western blotting. Antibodies against Chk2, and the phosphorylated forms Chk2^{-T68} and p53^{-S20} were purchased from Cell Signaling. Antibody against phosphorylated NBS1^{-S343} was purchased from Oncogene. Antibodies against phosphorylated γ-H2AX, phosphorylated BRCA1^{-1497}, ATM and phosphorylated ATM-1981 were purchased from Upstate. Anti-STAT-1 and anti-Cdc25A antibodies were purchased from Santa Cruz.

For immunostaining, cells were grown on gelatin-coated coverslips and left either untreated or were treated with 50 ng/ml IFN-γ or 10 μM cisplatin for 4 hours. After fixation in –20°C methanol, coverslips

were incubated 60 minutes in PBS with 3% BSA at room temperature, followed by incubation in PBS with 1% BSA containing mouse anti-Chk2^{-T68} Ab(1:200) and rabbit anti-STAT-1 Ab (1:200) (Santa Cruz) for 60 minutes. After three washes in PBS, Alexa488-goat anti-mouse Ab (1:2000) (Molecular probes) and Alexa568-goat anti-rabbit Ab (1:1000) (Molecular probes) were added together in PBS with 1% BSA, with Hoechst 33258 (Sigma) for 30 minutes. After three washes in PBS, coverslips were mounted with DAKO fluorescent mounting medium. Images were collected using a Leica TCS SP2 confocal microscope, and absence of antibody cross-reaction and bleed-through of fluorophore was verified on control slides.

Results

Cells lacking STAT-1 exhibit defective S-phase and G2-M checkpoints in response to DNA damage

We previously reported that STAT-1-deficient cells showed reduced p53-mediated responses (Townsend et al., 2004). Since cells that lack p53 exhibit various defects in the cell cycle following DNA damage, we examined whether cells lacking STAT-1 also displayed similar defects. Initially, we assessed whether cells that lack STAT-1 showed any defects in S-phase checkpoint response to DNA damage. Radioresistant DNA synthesis (RDS) normally occurs in ATM- and Chk2-deficient cells exposed to DNA-damaging agents owing to a defective S-phase checkpoint. 2fTGH parental cells exposed to 5 Gy γ-IR resulted in approximately 80% inhibition of DNA synthesis (Fig. 1A). By contrast, U3A cells lacking STAT-1 showed only a 40-50% inhibition of DNA synthesis, consistent with an RDS phenotype. U3A-ST1 cells that had STAT-1 stably reintroduced, had similar γ-IR-ind[illegible]ition of DNA synthesis to that seen for 2fT[illegible] parental [illegible]ntrol cells. Moreover, the effects on DNA [illegible]hibition followi[illegible] γ-IR were dose-dependent (Fig. 1B). STA[illegible] expression in 2[illegible]GH, U3A or U3A-ST1 cells and also [illegible]eir res[illegible]siveness t[illegible]-interferon is shown in the upper pa[illegible] of Fig. 1A. [illegible] lev[illegible] of STAT-1 in 2fTGH and U3A-ST1 [illegible]ells wa[illegible] very s[illegible] and also the induction of STAT-1 [illegible]nosory[illegible]ion was very comparable in 2fTGH and U3A-S[illegible]ells. [illegible]so notice[illegible]at, no expression or induction of S[illegible]AT-1 [illegible]bserved i[illegible] the U3A cell line, demonstratin[illegible]at the 2f[illegible]L and [illegible]A-ST1 cells respond to STAT-1 acti[illegible]on to a similar[illegible]e. These data suggest that STAT-1 h[illegible] a r[illegible] in the RDS checkpoint-response following DNA da[illegible]age.

We next examined [illegible]ether STAT-1 has a role in the G2-M ch[illegible]t responses t[illegible] DNA damage. Once again we mpared the STAT-1-expressing 2fTGH and U3A-ST1 cells ith the STAT-[illegible]deficient U3A cells and measured the level of itosis followi[illegible] exposure to γ-IR. As shown in Fig. 1C (and

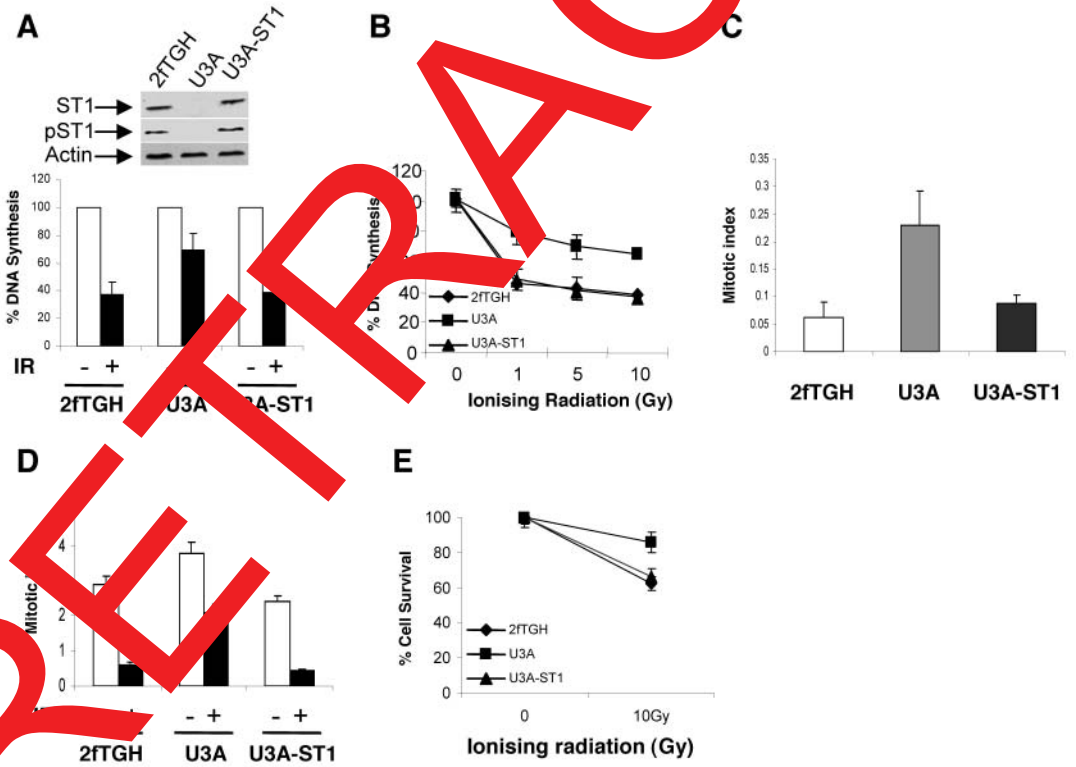

Fig. 1. STAT-1 [illegible]ctions in the S-phase and G2/M checkpoint. (A) U3A cells lacking STAT-1 display an RDS phenotype. The method involves pre-pulsing 2fTG[illegible]U3A or U3A-ST1 cells with ^{14}C thymidine, irradiation, and then assessing ^{3}H uptake after 2-5 Gy γ-irradiation (IR); DNA synthesis was assessed 2 hours later. The upper panel shows expression levels of STAT-1 (ST1) and also induction of phospho-STAT-1^{Y701} (pST1) in response to γ-interferon for 30 minutes in 2fTGH, U3A or U3A-ST1 cells. (B) Dose effect of ionising γ-irradiation (1-10 Gy) and DNA synthesis in 2fTGH, U3A or U3A-ST1 cells. (C) Analysis of the G2/M checkpoint in 2fTGH, U3A or U3A-ST1 cells exposed to 10 Gy γ-irradiation (IR). The mitotic index of cells was assessed by histone H3 phosphorylation 4 hours after irradiation. (D) Analysis of the G2/M checkpoint in 2fTGH, U3A or U3A-ST1 cells exposed to 10 Gy γ-irradiation (IR), where the mitotic index of cells was assessed by DAPI staining of chromosomal metaphase spreads of treated versus untreated cells. (E) Cell survival was assessed following exposure to 10 Gy γ-irradiation in 2fTGH, U3A or U3A-ST1 cells for 72 hours. After Crystal Violet staining, the percentage of cell survival was determined. Data are representative of three separate experiments.

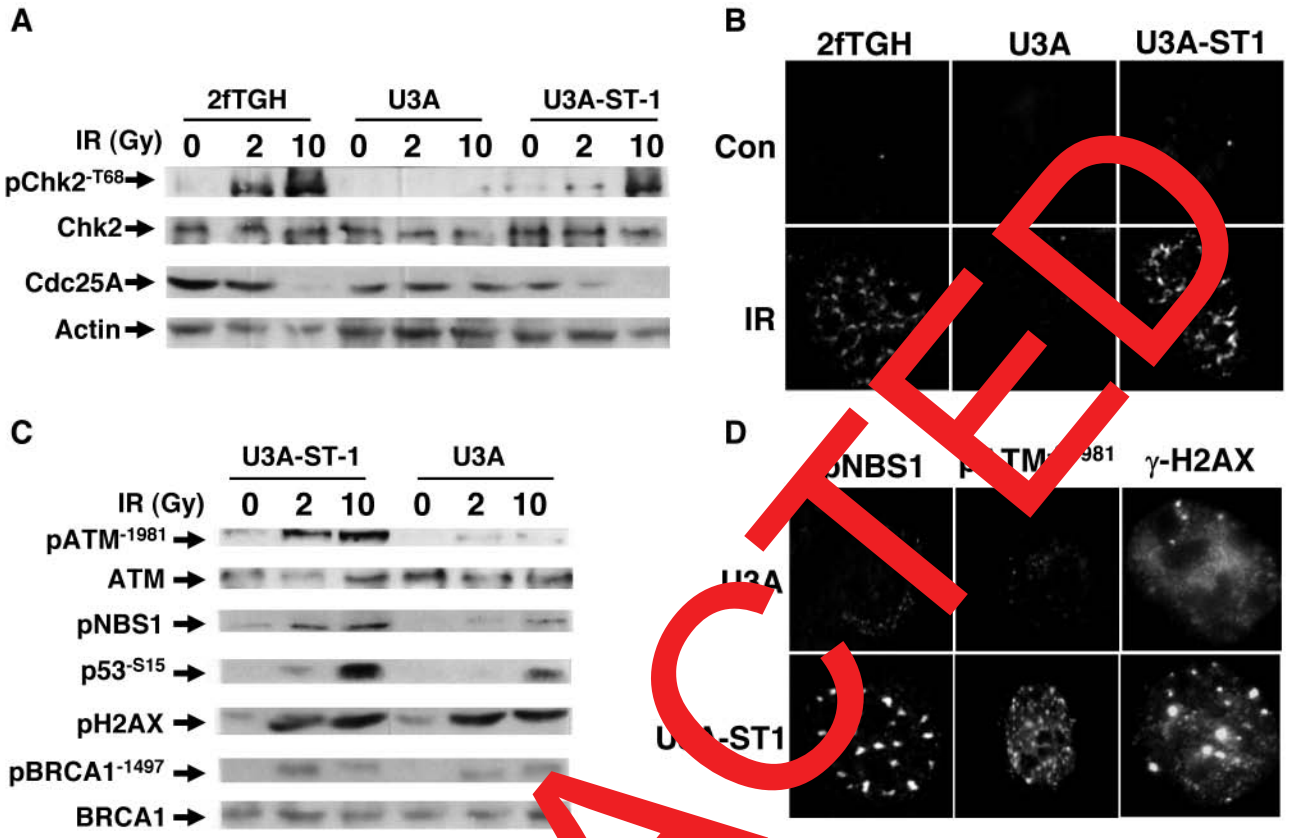

Fig. 2. STAT-1 is required for activation of the ATM-Chk2-Cdc25 and ATM-NBS1 pathways. (A) 2fTGH, U3A and U3A-ST1 cells were exposed to 0, 2 or 10 Gy γ-IR. Cells were harvested after 2 hours and extracts were incubated with relevant antibodies against the proteins indicated on the western blot. (B) 2fTGH, U3A and U3A-ST1 cells were exposed to γ-IR (2 Gy), fixed after 2 hours and immunofluorescence analysis with antibody against phosphorylated Chk2-T68 was carried out. (C) U3A-ST1 and U3A cells were exposed to 2 or 10 Gy γ-IR; cells were harvested 2 hours later and extracts immunoblotted with antibodies against the proteins indicated. (D) U3A-ST1 and U3A cells were exposed to γ-IR (2 Gy), fixed after 2 hours and immunofluorescence analysis was carried out with antibodies against the proteins indicated. The dimensions of the field of view are 40 mM×40 mM.

Fig. S1 in supplementary material for FACScan data), the levels of mitotic cells were much higher in U3A cells lacking STAT-1 when compared with 2fTGH and U3A-ST1 cells, which were similar following DNA damage.

To confirm whether a delayed G2-arrest was apparent in U3A cells, dynamic cell-cycle analysis was performed in a pulse-chase BrdU assay. Following a short pulse with BrdU, cells were irradiated and then incubated again for a further 24 hours. Using BrdU-specific antibodies, S-phase cells, which incorporate BrdU during the short pulse period, were identified and tracked over time after γ-IR. It was clear that 24 hours after γ-IR, approximately twice as many BrdU-labelled U3A cells were in the G2-M compartment, compared with the 2fTGH or U3A-ST1 cells (~62% and ~34%, respectively). By contrast, approximately twice as many BrdU-labelled STAT-1 expressing cells had reached G1 compared with the U3A cells (46% compared with 21%), indicating that they had bypassed G2-arrest (supplementary material, Fig. S1; Fig. 1D). These data demonstrate that STAT-1 expression facilitates a bypass of G2-arrest after γ-IR. Overall, these results demonstrate that cells lacking STAT-1 have both an enhanced RDS phenotype indicative of a defective intra-S-phase checkpoint, and an enhanced G2-M checkpoint.

Because many studies reported γ-IR hypersensitivity in cells with defects in the their ATM pathway, we also tested whether cells lacking STAT-1 are radiosensitive following exposure to γ-IR. As shown in Fig. 1E, cells lacking STAT-1 were more resistant to cell death than cells expressing STAT-1, suggesting in our case that STAT-1-deficient cells are not radiosensitive.

Defective Chk2-T68 phosphorylation in STAT-1-deficient cells after DNA damage

The ATM-Chk2-Cdc25A pathway plays an active role in both S-phase and G2-M-phase checkpoint responses to DNA damage (Abraham, 2001; Shiloh, 2003). To evaluate the molecular nature of the cell-cycle defects observed in STAT-1-deficient cells, we examined whether STAT-1 has a role in modulating the ATM-Chk2 pathway. Therefore, we examined Chk2-T68 phosphorylation after DNA damage in cells that express or lack STAT-1. Western blot analysis demonstrated enhanced Chk2-T68 phosphorylation in both the 2fTGH and U3A-ST1 cells after γ-IR (Fig. 2A). By contrast, Chk2-T68 phosphorylation was completely absent in U3A cells lacking STAT-1. Levels of unphosphorylated Chk2 were similar whether cells expressed or lacked STAT-1, suggesting that, in STAT-1-deficient cells, the defect in Chk2-T68 phosphorylation is not owing to STAT-1 regulating endogenous Chk2 levels.

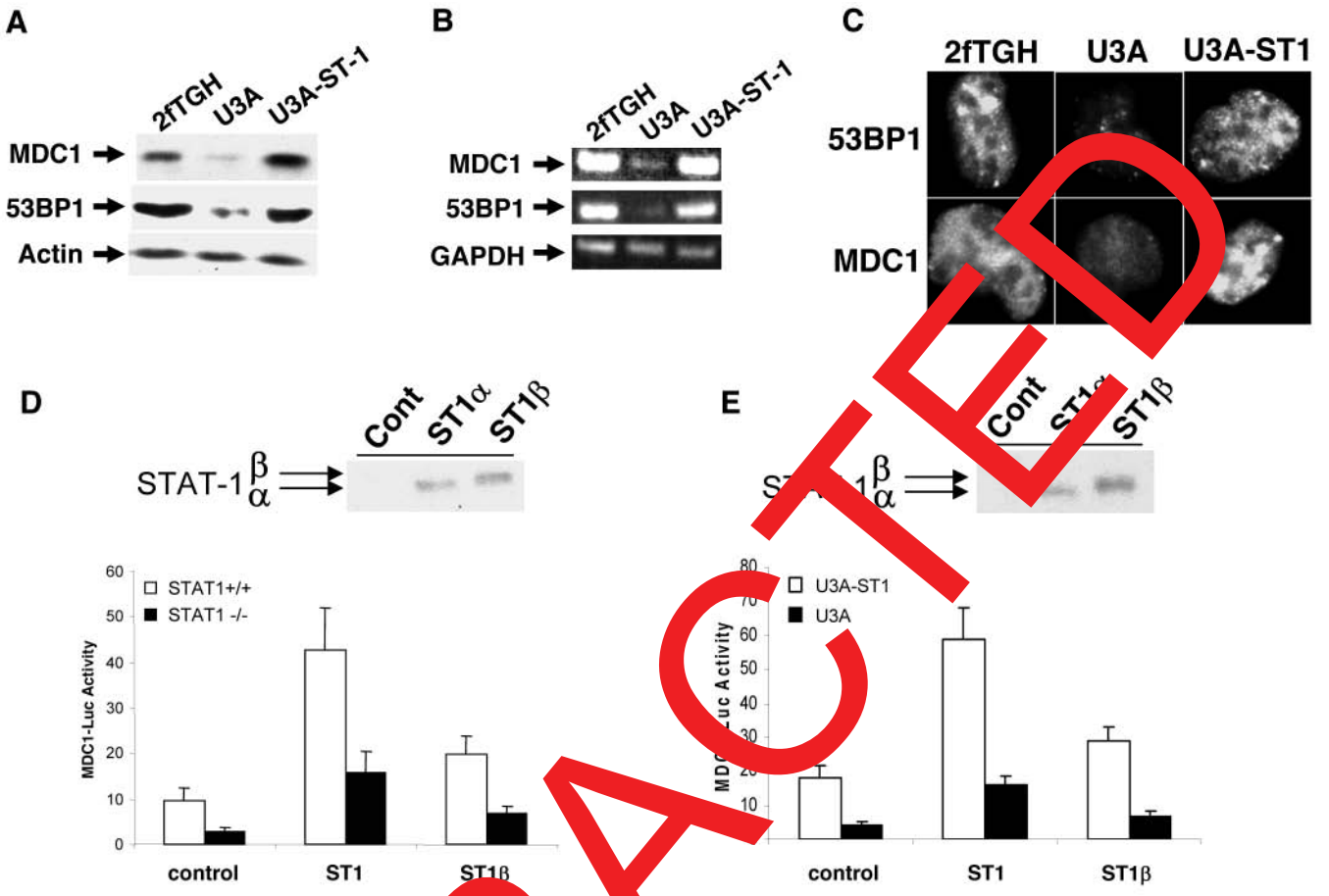

Fig. 3. Modulation of 53BP1 and MDC1 levels by STAT-1. (A) Cell lysates from 2fTGH, U3A and U3A-ST1 cells were immunoblotted for MDC1 and 53BP1. 53BP1 and MDC1 expression is reduced in STAT-1 deficient U3A cells. (B) RT-PCR showing reduced 53BP1 and MDC1 mRNA levels in U3A cells. MDC1 and 53BP1 mRNA levels assessed from 2fTGH, U3A or U3A-ST1 cells. (C) 2fTGH, U3A or U3A-ST1 cells were exposed to γ-IR (2 Gy), fixed after 2 hours and stained with anti-53BP1 or anti-MDC1 antibody. The dimensions of the field of view are 40 mM×40 mM. (D,E) The MDC1 promoter is modulated by STAT-1. The MDC1-reporter construct was transfected into STAT-$1^{+/+}$ and STAT-$1^{-/-}$ MEF cells, and U3A and U3A-ST1 cells together with either full length STAT-1 (ST1), STAT-1β (ST1B) or a control vector. Upper panels in D and E show immunoblots of transfected cells for STAT-1α (ST1α) or STAT-1β (ST1β).

Similarly, reduced nuclear staining of phosphorylated $Chk2^{-T68}$ was also observed in single-cell immunofluorescence analysis after γ-IR of U3A cells as compared with 2fTGH cells (Fig. 2B).

Since activated phosphorylated $Chk2^{-T68}$ is involved in phosphorylation and degradation of Cdc25A (Falck et al., 2001, Falck et al., 2003, Bartek and Lukas, 2003), we also examined the levels of Cdc25A in 2fTGH and U3A cells exposed to γ-IR. As shown in Fig. 2A, the defect in U3A cells was associated with sustained Cdc25A levels – compared with 2fGT and U3A cells – after γ-IR (Fig. 2A). These studies suggest that STAT-1 may be important in regulating the ATM-Chk2-Cdc25A pathway in response to DNA damage.

Defective $NBS1^{-S343}$ and ATM^{-S1981} phosphorylation in cells lacking STAT-1

An important early event in response to γ-IR-induced DNA DSBs is activation of ATM, involving autophosphorylation on serine 1981 and the conversion of inactive ATM dimers into active monomers (Bakkenist and Kastan, 2003). The Mre11-Rad50-NBS1 (MRN) complex is also recruited to DNA DSBs very early, and recent studies have shown that the MRN complex is required for activation of ATM (Carson et al., 2003; Uziel et al., 2003; Lee and Paull, 2004). Recently, NBS1 and BRCA1 have been shown to function via two independent, branched pathways that require H2AX to initiate both NBS1 and BRCA1 phosphorylation events (Fernadez-Capetillo et al., 2002). By contrast, 53BP1 is required for phosphorylation of BRCA1 but not NBS1 (Wang et al., 2002).

We therefore examined whether ATM activation and other components of the ATM pathway is modulated by STAT-1. After γ-IR, ATM phosphorylation on serine 1981 is dramatically reduced in U3A cells (without STAT-1) compared with U3A-ST1 cells (Fig. 2C). The levels of total ATM remained unchanged in U3A-ST1 and U3A cells, suggesting that the defect in ATM phosphorylation is not attributable to differences in ATM expression (Fig. 2C). Interestingly, the reduced activation of ATM after γ-IR-exposure was also associated with decreased phosphorylation of $p53^{-S15}$ and $NBS1^{-S343}$ but not H2AX or $BRCA1^{-1497}$ in STAT-1-deficient U3A cells versus U3A-ST1 cells (Fig. 2C). Similarly, immunostaining demonstrated reduced nuclear levels of the

phosphorylated forms of NBS1^{-S343} and ATM^{-S1981} but not γ-H2AX (Fig. 2D). These results show that STAT-1 can modulate distinct ATM regulatory pathways. The DNA-damage checkpoint pathway has been suggested to be branched and shows regulatory hierarchical pathways. The complexity of this hierarchical checkpoint pathway could be because of other ATM-like members (ATR or DNA-PK) that compensate when one pathway is blocked and/or the extent of DNA damage.

STAT-1 modulates the expression of 53BP1 and MDC1

The so-called DNA-damage adaptors/mediators 53BP1 and MDC1 have been reported to play a role in the initial activation of ATM as well as in phosphorylation of downstream ATM mediated pathways following DNA DSBs (Lou et al., 2003; Xu and Stern, 2003; Peng and Chen, 2003; Mochan et al., 2003). To investigate therefore, the mechanism of how STAT-1 is able to modulate the ATM–Chk2 and/or ATM–NBS1 pathways we examined whether the lack of ATM activation and ATM-mediated pathways is associated with changes in 53BP1 and MDC1 after DNA damage in STAT-1-deficient cells. Western blot analysis shows that the levels of both 53BP1 and MDC1 are reduced in cells lacking STAT-1 but were restored in U3A-ST1 cells (Fig. 3A). Similar results, showing reduced expression of *53BP1* and *MDC1*, were also obtained at the mRNA level in STAT-1-deficient U3A cells (Fig. 3B). Likewise, immunfluorescent staining of 53BP1 and MDC1 was also reduced in U3A cells compared with 2fTGH cells. Furthermore, 53BP1 and MDC1 expression levels were restored in U3A-ST1 cells (Fig. 3C). Because Chk2^{-T68}, p53 and NBS1 phosphorylation is abolished in cells lacking 53BP1 or MDC1 (Lou et al., 2003; Peng and Chen, 2003), our data suggest that STAT-1 regulates the expression of these crucial upstream mediators/adaptors that are required after DNA damage for ATM activation and for separate ATM branched downstream pathways.

Examination of the *MDC1* promoter region using the Transfact programme (version 3.0) showed the presence of several potential DNA binding sites of STAT-1. To determine whether STAT-1 directly regulates the transcription of the *MDC1* gene, we cloned a 2-kb fragment of the MDC1 promoter upstream of the transcriptional start site into the pGL3-basic luciferase reporter construct. As shown in Fig. 3D, the basal activity of the MDC1-reporter construct was much higher in STAT-1$^{+/+}$ than in STAT-1$^{-/-}$ MEF cells. Co-transfection of a full-length STAT-1 construct enhanced MDC1-reporter activity in both STAT-1$^{+/+}$ and STAT-1$^{-/-}$ cells. However, a STAT-1 construct without the C-terminal transactivation domain (STAT-1β), only partially enhanced MDC1-reporter activity. Similar data were also obtained in STAT-1-expressing U3A-ST1 and the STAT-1-deficient U3A cells (Fig. 3E). These data agree with previous reports by us and others that the C-terminal domain of STAT-1 is required for its effects on transcription (Levy, and Darnell, 2002; Stephanou et al., 2002; Stephanou et al., 2003); but most importantly, these findings indicate that the *MDC1* gene is a direct target of STAT-1.

STAT-1 forms a complex with Chk2 and MDC1 following DNA damage

Because STAT-1 binds to p53 and modulates its activity

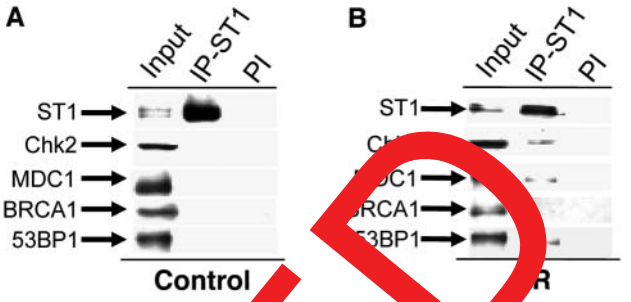

Fig. 4. Association of STAT-1 with Chk2, MDC1, 53BP1 following DNA damage. Immunoprecipitations were carried out with an anti-STAT-1 antibody (IP-ST1) in untreated 2fTGH cells (A) or 2fTGH cells exposed to γ-IR and immunoblotted with antibodies against the target proteins indicated.

(Townsend et al., 2004) and because 53BP1 has been shown to interact with p53 and Chk2, we investigated whether STAT-1 also associates with Chk2, 53BP1, MDC1 or BRCA1. Under non-stressed conditions STAT-1 did not interact with any of these factors in 2fTGH-cell lysates. However, following γ-IR, STAT-1 interacted with Chk2, MDC1 and 53BP1 but not with BRCA1 (Fig. 4B). An association between STAT-1 and Chk2 was also detected after γ-IR in HCT15 cells that stably expressed HA-Chk2 (Fig. S2a, supplementary material). Furthermore, a Chk2 kinase-dead (Chk2-KD) construct, containing a mutated kinase domain, was still able to interact with STAT-1 (supplementary material Fig. S2b), suggesting that the kinase activity of Chk2 is not essential for the interaction with STAT-1 following DNA damage. In addition, we found that STAT-1 signalling in HCT15 cells that stably express wild-type Chk2, is similar in HCT15 cells that stably express the Chk2 kinase-dead construct, which also suggests that the kinase activity of Chk2 does not affect the functional activity of STAT-1 (data not shown). We also performed similar STAT-1 immunoprecipitation experiments in U3A STAT-1-deficient cells and found no co-precipitation with any of the above factors (data not shown), which confirms that the binding of STAT-1 is specific to the above interacting factors in STAT-1-expressing cells. These data thus demonstrate that, STAT-1 can interact with Chk2, MDC1 and 53BP1 and this association is probably important for the regulation and function of Chk2.

Enforced synthesis of MDC1 in STAT-1-deficient cells restores the ATM phosphorylation of downstream substrates

To determine whether MDC1 is required for mediating ATM phosphorylation of downstream substrates in cells lacking STAT-1, we examined the effects of overexpressing MDC1 in U3A cells and measured the phosphorylation levels of p53, Chk2 and NBS1 mediated by ATM after inducing DNA damage. Enforced synthesis of MDC1 increased levels of phosphorylated ATM, and the phosphorylation of Chk2^{-T68}, p53^{-S15} and NBS1^{-S343}. However, enforced synthesis of a mutated MDC1 that lacks the forkhead-homology-associated (FHA) domain, stimulated phosphorylated ATM, and Chk2^{-T68} p53^{-S15} and NBS1^{-S343} phosphorylation only partially (Fig. 5A), indicating that the FHA domain is important for mediating distinct phosphorylation of downstream substrates

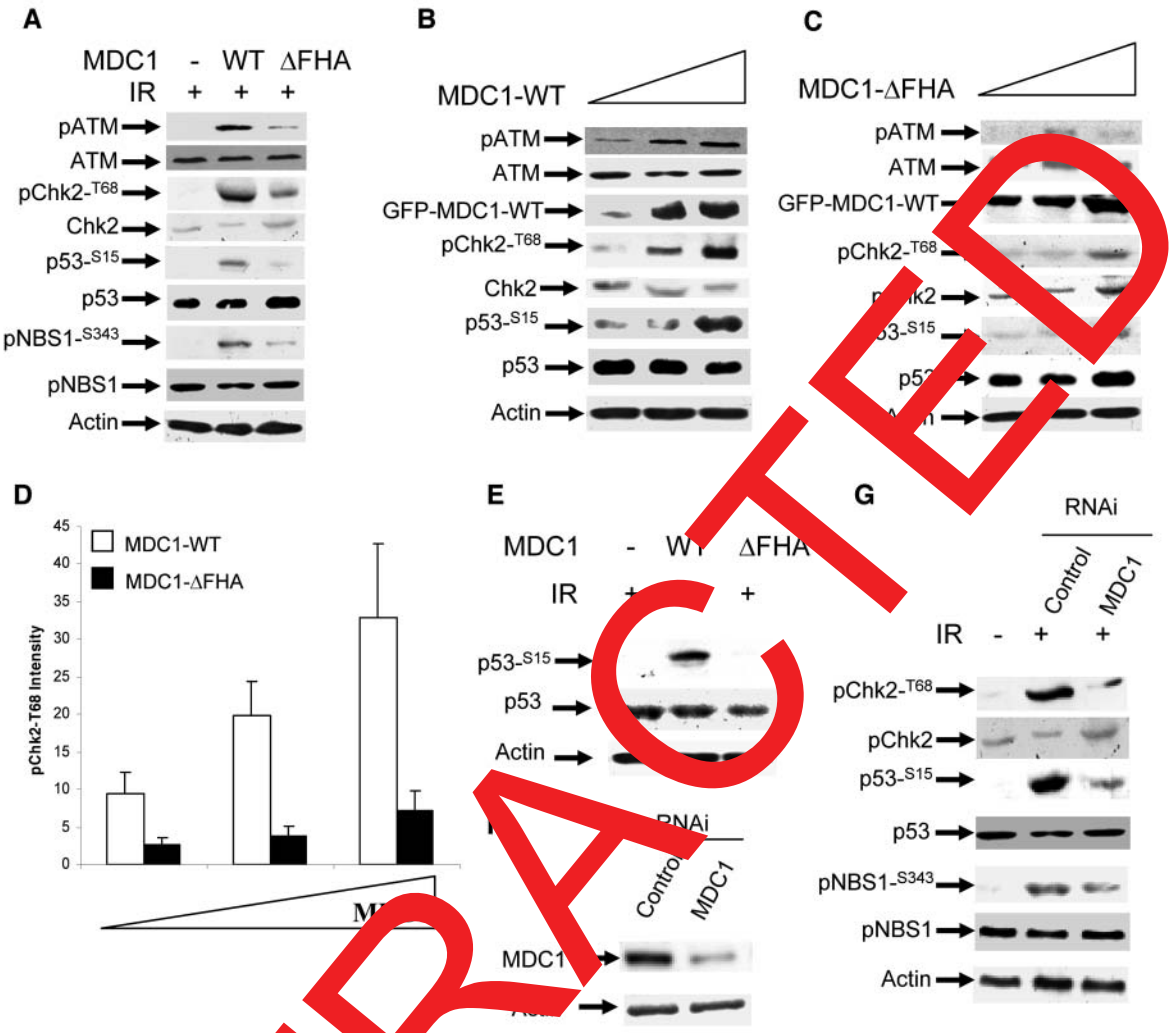

Fig. 5. In cells lacking STAT-1, overexpression of *MDC1* restores ATM-dependent phosphorylation, which requires the FHA domain. (A) U3A cells were transfected with GFP control, wild-type GFP-MDC1 (WT) or a mutant GFP-MDC1-ΔFHA (ΔFHA), lacking the FHA domain. Cells were immunoblotted with antibodies against the proteins indicated. (B,C) Effect of increasing amounts of (B) wild type (MDC1-WT) or (C) mutant (MDC1-ΔFHA) on ATM dependent phosphorylation as assessed by immunoblotting with antibodies against the proteins indicated. (D) Quantification of the Chk2^{-T68} phosphorylation shown in B and C by densitometry. Data represent three independent experiments. (E) MEF STAT-1$^{-/-}$ cells were transfected with GFP control, wild-type GFP-MDC1 (WT) or a mutant GFP-MDC1-ΔFHA (ΔFHA). Cells were immunoblotted with an antibody against phosphorylated p53^{-S15}, p53 and actin (control). (F) MDC1 RNAi reduces expression of endogenous MDC1 in 2fTGH cells. (G) Transfection of MDC1 RNAi in 2fTGH cells reduced ATM-dependent phosphorylation following γ-IR (5 Gy) as assessed by immunoblotting with antibodies against the proteins indicated.

by ATM. Overexpression of wild type *MDC1* had a dose-dependent effect in enhancing ATM^{-S1981}, Chk2^{-T68} and p53^{-S15} phosphorylation (Fig. 5B). By contrast, MDC1 lacking the FHA domain had a weaker dose-dependent effect on enhancing the phosphorylation of ATM[1], Chk2 and p53 (Fig. 5C,D). To demonstrate whether the defects in ATM phosphorylation of downstream substrates in the U3A cells can also be observed in other STAT-1-deficient cells, we also performed similar *MDC1* overexpression experiments in STAT1$^{-/-}$ MEF cells after γ-IR. Once again, enforced expression of wild-type *MDC1* but not of the mutant *MDC1* gene lacking the FHA domain enhanced p53^{-S15} phosphorylation (Fig. 5E).

To further investigate the effects of MDC1 in enhancing the activity of ATM, we inhibited the expression of MDC1 with RNAi. As shown in Fig. 5F, cells overexpressing the MDC1-RNAi construct showed a significant reduction in the expression of MDC1. In 2fTHG cells that expressed STAT-1, transfection of the MDC1-RNAi construct reduced the phosphorylation of Chk2^{-T68}, p53^{-S15} and NBS1^{-S343} following DNA damage (Fig. 5G). These results further support the notion that STAT-1 and its association with MDC1 plays a role in mediating DNA damage checkpoint responses.

STAT-1 expression is enhanced in cells defective in p53 and associated with enhanced MDC1 and 53BP1 levels

Interestingly, constitutive activation of phosphorylated Chk2^{-T68} and 53BP1 has been reported in p53-deficient or mutant cell lines, whereas inhibition of 53BP1 by RNAi

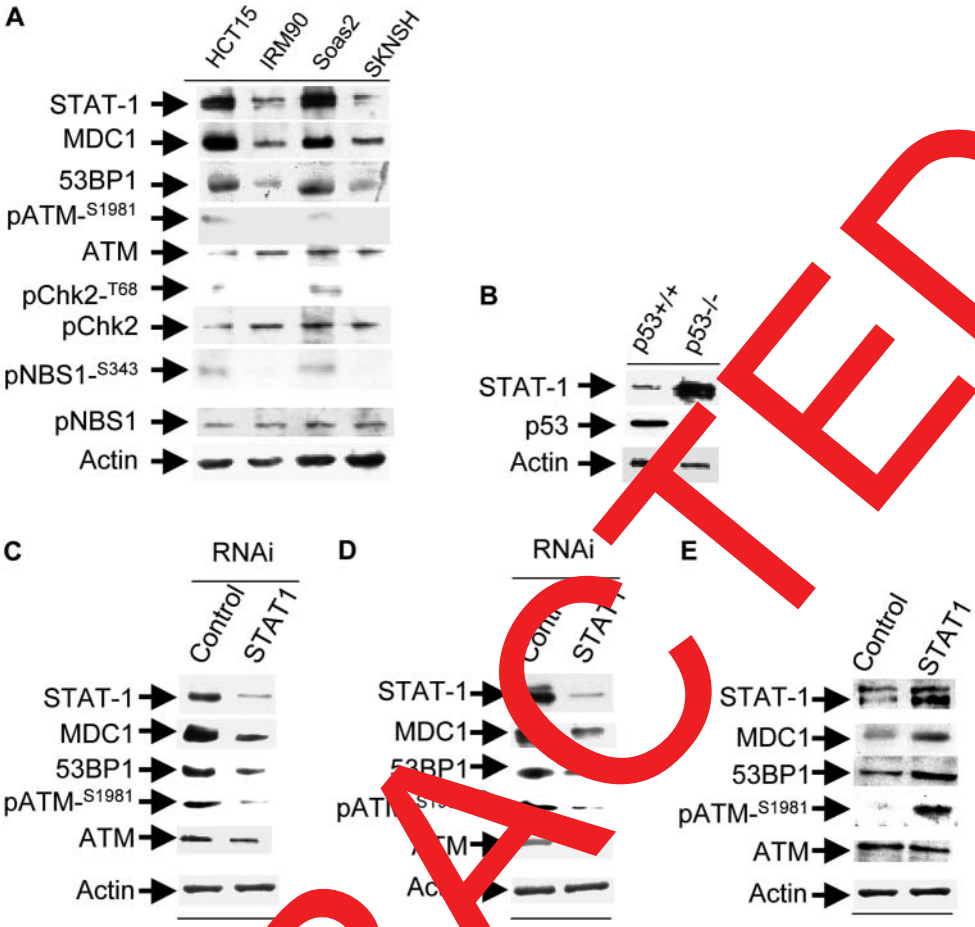

Fig. 6. Western blot analysis demonstrates that elevated expression of *STAT-1* is associated with enhanced expression of MDC1 and 53BP1, and constitutive phosphorylation of ATM, Chk2 and NSB1 in cells that lack p53 or carry a mutation for p53. (A) Lysates of Soas2 (p53-deficient), HCT15 (p53 mutant), IMR90 (p53 wild type) and SKNSH (p53 wild type) cells were immunoblotted with antibodies against the proteins indicated. (B) STAT-1 levels are enhanced in MEF $p53^{-/-}$ cells compared with MEF $p53^{+/+}$ cells as assessed in a western blot. (C,D,E) Western blots show that, transfection of HCT15 and Soas2 cells (C and D, respectively) with STAT-1 RNAi reduces levels of STAT-1 protein, levels of MDC1 and 53BP1, and also reduces the level of phosphorylated ATM (pATM-S1981). Overexpression of *STAT-1*, by contrast, increases levels of phosphorylated ATM and the phosphorylated forms of downstream phosphorylation-substrates of ATM. IMR90 cells were transfected with a *STAT-1* expression vector; cells were harvested 48 hours later and lysates immunoblotted with antibodies against the proteins indicated.

reduced phosphorylation of $Chk2^{-T68}$ in these cells (DiTullio et al., 2002). Moreover, staining of constitutively phosphorylated $Chk2^{-T68}$ was also observed in both lung and other cancer tissues that were p53 mutant, thus implying that 53BP1 is important for initiating ATM-dependent checkpoint pathways (DiTullio et al., 2002). Since our studies show that STAT-1 is also required for mediating a subset of ATM-dependent checkpoint pathways, we examined whether the STAT-1 status was altered in p53-deficient or mutant cell lines. As shown in Fig. 6A, expression of STAT-1 was significantly enhanced in Soas2 and HCT15 cells lacking functional p53, which correlated with increased expression of MDC1 and 53BP1 levels compared to IMR90 and SKNSH cells which both have functional p53 (Fig. 6A). Furthermore, in both HCT15 and Soas2 cells the increased MDC1 and 53BP1 levels were also associated with constitutively phosphorylated ATM^{-S1981}, $Chk2^{-T68}$ and NBS1. Additionally, expression of STAT-1 was also significantly enhanced in $p53^{-/-}$ MEF cells compared with $p53^{+/+}$ MEF cells of the same genotype (Fig. 6B) These studies show that STAT-1 levels are enhanced in cells that lack or have an inactive p53, which correlates with our finding that STAT-1 expression is necessary for the activation of ATM-dependent pathways by regulating the expression of MDC1 and 53BP1.

To determine whether the expression-status of *STAT-1* is indeed associated with the constitutively phosphorylated ATM^{-S1981}, we inhibited the expression of STAT-1 in Soas2 and HCT15 cells that had been transfected with a *STAT-1* RNAi construct (Fig. 6C,D). In these cells, phosphorylated ATM^{-S1981} was significantly reduced compared with cells that had been transfected with a control RNAi construct (Fig. 6D), which also correlated with the reduced expression of MDC1 and 53BP1. Overall, these studies demonstrate that, in cells defective in p53, overexpression of *STAT-1* is associated with enhanced ATM activity. Furthermore, we also found that, in IMR90 cells, suppression of *STAT-1* with STAT-1 RNAi also resulted in an RDS-like phenotype following DNA damage (data not shown). We therefore exclude the possiblility that the results of the RDS assays in U3A cells are artifacts arising from the use of an immortalised cell line.

To show whether the STAT-1 status is involved in modulating ATM activation in cells with are wild-type p53, we overexpressed *STAT-1* in the IMR90 cell line. As shown in Fig. 6E, overexpression of *STAT-1* resulted in enhanced expression of MDC1 and 53BP1, which also associated with constitutive phosphorylation of ATM. Thus, STAT-1 is able to modulate ATM activity presumably via the increased protein expression of the ATM mediators MDC1 and 53BP1.

Discussion

In response to DNA DSBs, distinct ATM-mediated regulatory pathways are activated and appear to play an important role in transducing DNA-damage signals to downstream effectors to control processes such as DNA repair, checkpoint arrest or apoptosis (Abraham, 2001; Shiloh, 2003). Distinct checkpoint pathways involved in DNA-damage-dependent S-phase responses are known to cooperate following DNA damage by inhibiting DNA replication. These include the ATM-Chk2-Cdc25A pathway (Falck et al., 2001; Falck et al., 2002; Bartek and Lukas, 2003) and the ATM-NBS1 pathway which jointly contribute to the inhibition of DNA synthesis after γ-IR. The mechanistic role of Cdc25A in the inhibition of DNA synthesis is well known; phosphorylated NBS1 seems to mediate the phosphorylation of the downstream structural maintenance of chromosome-1 (SMC1) protein following DNA damage (Yazdi et al., 2002).

Our data demonstrate that STAT-1 is able to modulate the phosphorylation of ATM and its downstream substrates $Chk2^{-T68}$ and $NBS1^{-S343}$, suggesting that the RDS phenotype, observed in cells lacking STAT-1, may be attributed to a defect in both the ATM-Chk2-Cdc25A and the ATM-NBS1-SMC1 pathways. Previous studies have shown that cells that lack functional NBS1 still have an intact ATM-Chk2-Cdc25A pathway in response to DNA damage (Yazdi et al., 2002), implicating the existence of a separate and distinct ATM-NBS1-SMC1 pathway involved in S-phase checkpoint-control. Furthermore, the ATM-NBS1-SMC1 pathway seems to be BRCA1-independent, because in cells that lack functional BRCA1, phosphorylation of SMC1 is not affected (Yazdi et al., 2002). Our studies show that activation of either the ATM-Chk2-Cdc25A or the ATM-NBS1-SMC1 pathway partly depends on STAT-1. How STAT-1 can mediate these effects is not clear, but it might involve activating the expression of both MDC1 and 53BP1 factors known to be required for mediating downstream activation of ATM-dependent pathways.

A role of MDC1 in mediating $Chk2^{-T68}$ phosphorylation was previously reported in cells following studies using RNAi to silence the *MDC1* gene (Lou et al., 2003; Peng and Chen, 2003). By contrast, we have shown that RNAi silencing of *MDC1* had no effect on activation of the $Chk2^{-T68}$-Cdc25A pathway (Goldberg et al., 2003). This discrepancy may be owing to cell-type-specific effects or the RNAi protocol from different studies, which showed a variable effect on MDC1 suppression. However, *MDC1* silencing has also been shown to reduce the phosphorylation of Chk2 on serines 33 and 35 (Mochan et al., 2003). Interestingly, MDC1 and 53BP1 function in parallel pathways, and suppression of both these factors has a greater effect on abolishing $Chk2^{-T68}$ phosphorylation than those seen by inhibition of either 53BP1 or MDC1 (Peng and Chen, 2003). Moreover, MDC1 physically associates with ATM and the MRN complex, and studies have suggested that MDC1 is an ATM/ATR-dependent organizer that recruits DNA-checkpoint-signalling- and repair-proteins to the sites of DNA damage (Goldberg et al., 2003; Xu and Stern, 2003). This is consistent with our data here, which shows that cells lacking STAT-1 showed reduced expression of both *53BP1* and *MDC1*, and this is associated with reduced ATM-dependent activated pathways following DNA damage. Our studies also confirm that STAT-1 is a direct activator of the MDC1 promoter and that STAT-1 probably is an important regulator of the *MDC1* gene.

The MRN complex together with ATM activation, is the earliest event that occurs at DNA DSBs (D'Amours and Jackson, 2001). The order in which ATM and MRN act in the early phase of DSB response is unclear. However, recent studies have shown that functional MRN is required for ATM activation and for ATM-mediated pathways because, after DNA damage cells lacking active MRE11 or NBS1 show a weaker response of activated–autophosphorylated–ATM and its downstream (ATM-dependent) pathways activated by phosphorylation (Carson et al., 2003; Uzeil et al., 2003; Lee and Paull, 2004). Furthermore, MDC1 is required for recruitment of NBS1 to sites of DNA DSBs, and the MRN complex is required for ATM activation (Xu and Stern, 2003). Both MDC1 and the MRN complex, together with ATM all form a large complex at sites of DNA DSBs (Xu and Stern, 2003) that mediates autophosphorylation of ATM at serine 1981 and dissociation of inactive ATM dimers into active monomers (Bakkenist and Kastan, 2003).

More recent studies have placed 53BP1 and MDC1 upstream of ATM by showing that both factors are independently recruited to sites of DNA DSBs and that these events are independent of ATM (Mochan et al., 2003). In cells with wild-type NBS1, suppression of 53BP1 expression had no effect on phosphorylation of ATM^{-S1981} but was associated with increased recruitment of MDC1 and NBS1 to sites of DNA DSBs, demonstrating that a reduction of 53BP1 is associated with a compensatory increase in MDC1-NBS1 activity (Mochan et al., 2003). By contrast, suppression of MDC1 resulted in a decrease of ATM^{-S1981} phosphorylation in cells expressing NBS1 following DNA damage (Mochan et al., 2003). Thus, 53BP1 and MDC1-NBS1 function in parallel pathways, which are able to cross-talk in order to activate the ATM-response to DNA damage. Additionally, these data demonstrate that the components of the MRN complex have a function upstream of ATM. Activation of ATM can then phosphorylate the MRN-complex-component NBS1, which mediates events downstream of ATM (ATM-NBS1-SMC1 pathway). Thus, depending on the phosphorylation status of NBS1, it can function upstream or downstream of ATM.

Our data also demonstrate that STAT-1 interacts with MDC1, 53BP1 and Chk2 following DNA damage. This is in contrast to previous data, showing that MDC1 or 53BP1 associate with Chk2 under normal conditions and that this association is abolished in response to γ-IR (Lou et al., 2003; Wang et al., 2002). Thus, STAT-1 might be recruited to sites of DNA DSBs together with MDC1, thereby facilitating the recruitment and phosphorylation of Chk2 through activated ATM.

Recently, we have shown that STAT-1 can interact with p53 and modulate p53-mediated transcriptional effects as well as

modulate apoptosis (Townsend et al., 2004). Our previous work and the data presented here demonstrate that, STAT-1, like p53, is involved in processes that mediate cell-cycle arrest or apoptosis. An important finding from this study is the mechanism of how STAT-1 may inhibit cell growth after genotoxic stress: inhibition might be mediated by STAT-1 regulating the ATM-Chk2-Cdc25A and ATM-NBS1-SMC1 pathways, which jointly contribute to the rapid inhibition of DNA synthesis after DNA damage. Interestingly, defects in the ATM pathways have been shown to be associated with radiosensitivity (Falck et al., 2001). We show here, that STAT-1-deficient cells are more resistant to cell death following γ-IR than STAT-1 expressing cells (Fig. 1D), indicating that cells that lack STAT-1 are not radiosensitive.

Both MDC1 and 53BP1 have been suggested to play a role as an adaptor protein in a similar fashion to the yeast proteins Rad9 and Rad53 (Rad53 is a homologue of human Chk2), which play a central role in transducing and amplifying DNA-damage-signals by activating the kinase Rad53 (Toh and Lowndes, 2003). Our data show that STAT-1 might also play a role as an adaptor protein for Chk2 by modulating its kinase activity after DNA damage. This is consistent with our data: cells lacking STAT-1 show reduced phosphorylation of $p53^{-S20}$, a downstream substrate of Chk2 kinase that is activated following γ-IR (data not shown). However, further studies are required to confirm whether STAT-1-Chk2 interaction can enhance the functional activity of Chk2 or whether Chk2/STAT-1 association alters STAT-1 functional activity.

A further key finding from this study is that the reduced ATM-dependent checkpoint-pathway in cells lacking STAT-1, is associated with reduced expression of *MDC1* and *53BP1*. This implies that STAT-1 modulates the [illegible]expression levels of *53BP1* and *MDC1*. Furthermore, we show for the first time that STAT-1 can transactivate the MDC1 promoter. Thus, STAT-1 is probably an important regulator of the *MDC1* gene, which plays a crucial role in transducing the DNA damage checkpoint response.

The ATM-Chk2-p53 pathway contributes to apoptosis following genotoxic stress. The stabilization of phosphorylation of p53 is known to be crucial in the apoptotic programme following genotoxic stress (Vousden and Lu, 2002). Our present data shows reduced levels of phosphorylated $p53^{-S15}$ in STAT-1-deficient cells after γ-IR, which correlates with resistance to apoptosis following DNA damage. Other established substrates for Chk2, include PML and E2F-1 (Yuan et al., 2002; Stevens et al., 2003). Both factors have been reported to play a role in promoting apoptosis in cells exposed to DNA-damaging agents. Furthermore, the functional activity of Chk2 to promote apoptosis, as well as its effects on RDS, requires the presence and association of MDC1 via its forkhead-homolgy-associated domain (Lou et al., 2003; Goldberg et al., 2003). Recent studies have suggested that the FHA domain is a phosphoamino acid binding-domain and interacts with other factors involved in DNA damage response pathways (Huang and Elledge, 2000). This is also consistent with our data in that, STAT-1 plays a role in regulating the expression of *MDC1* as well as interacting with MDC1, which may therefore be crucial to sensitise cells towards undergoing apoptosis.

Taken together, these findings identify STAT-1 as a major player in modulating the cell-cycle-checkpoint responses following DNA damage. Our data also show an enhanced *STAT-1* expression in cells that are defective in p53 and also have constitutively phosphorylated $Chk2^{-T68}$. This combination of events might be common in human cancer, especially, because *STAT-1* and *STAT-3* are were shown to be overexpressed in different cancers (Bromberg, and Darnell, 2000; Turkson and Jove, 2000). The [illegible]levels of STAT-1 or STAT-3 might also be important to determine cell-fate in response to a number of stress and stimuli. This implies that STAT-1 is a novel and important molecular target for the development of cancer therapy warranting further investigation in how STAT-1 deregulation and cancer are linked in checkpoint processes.

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