RETRACTION

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

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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 STAT-1-expressing cells after DNA damage. We also show that STAT-1 is required for ATM-dependent phosphorylation of p53 but not for BRCA1 or H2AX phosphorylation following DNA damage. Expression levels of BRCT-mediated/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.

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

Introduction

To ensure that cells pass on accurate copies of their genomes on to the next generation, a series of surveillance pathways – the so called cell-cycle-checkpoint protein kinases – are activated following DNA damage to allow appropriate time for DNA repair to take place. The ATM kinase in conjunction with adaptor proteins MDC1 and 53BP1 plays a pivotal role in initiating the checkpoint-cascade pathway following DNA damage by phosphorylating and activating a subset of ATM substrates including Chk2-T68, p53-S15, NBS1-S343 and BRCA1-S1387 (Melochionna et al., 2000; Chehab et al., 2000; Lim et al., 2000; Takai et al., 2003; Shiloh, 2003). In mammalian cells, proteins 53BP1 and MDC1 (mediator of DNA damage checkpoints that contain a BRCA1 C-terminal (BRCT), have been termed adaptors/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).

In addition to BRCA1, ATM also phosphorylates NBS1 on serine 343 (Lim et al., 2000). NBS1 is a component of the multifunctional 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; Buscemii 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 BRCA1-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).

Key words: ATM, Cell cycle, Chk2, 53BP1, MDC1, STAT-1
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 (Yousden 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-dependent 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 substrates for 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 2TGH-p53−/− and U3A-derived cells stably expressing STAT-1 were kindly provided by Jan Kerr (Cancer Research UK, London) (McKendry et al., 1991) and cultured in DMEM supplemented with 10% FBS. HT1080 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′-ccagaacgaaaggtgttggtgcgctgggc-3′ and 5′-gagggaccctcattcgttctgg-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 or 10 Gy were measured with the two-isotope radioresistant DNA synthesis (RDS) assay. Twenty hours after plating, cells were pulsed with 5 nCi/ml [14C]-thymidine (Amersham Biosciences, UK) and incubated a further 24 hours. The medium was then replaced, all the cell were exposed to 2 or 10 Gy IR. After a 30-minute incubation, the medium was replaced with fresh medium containing 20 μCi/ml [3H]-thymidine (Amersham Biosciences, UK) and cells were incubated a further 30 minutes. Cells were then harvested and the 3H:14C ratios were measured to assess rates of DNA synthesis.

To assess in more detail changes in S phase, we performed the standard BrdU assay. Briefly, for a 24 hour dynamic cell-cycle analysis, cells were 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 a second incubation for 0–48 hours. After this, cells were fixed, in ice-cold 70% ethanol and washed with PBA. 2M HCl was added for 30 minutes, washed off and excess acid neutralised by a second incubation for 0–48 hours. After this, cells were rinsed, fixed, in ice-cold 70% ethanol and washed with PBA. 2M HCl was added for 30 minutes, washed off and excess acid neutralised with 0.1 M Na2B4O7. 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 (PBS T). Cells were incubated at room temperature. Cells were washed again with PBS and incubated with 10 μg/ml propidium iodide (PI) for 30 minutes to stain DNA before assessment by flow cytometry. Analysis of samples 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 (2.0×10^4) 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-S168 and p53-S20 were purchased from Cell Signaling. Antibody against phosphorylated NBA1-S343 was purchased from Oncogene. Antibodies against phosphorylated γ-H2AX, phosphorylated BRCA1-S1811, 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 processed as described previously.
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, 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. Radioreistant 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-induced inhibition of DNA synthesis to that seen for 2fTGH parental control cells. Moreover, the effects on DNA inhibition following γ-IR were dose-dependent (Fig. 1B). STAT-1 expression in 2fTGH, U3A or U3A-ST1 cells and also their responsiveness to γ-interferon is shown in the upper panel of Fig. 1A. The level of STAT-1 in 2fTGH and U3A-ST1 cells was very similar, and also the induction of STAT-1 phosphorylation was very comparable in 2fTGH and U3A-ST1 cells. Also notice that, no expression or induction of STAT-1 was observed in the U3A cell line, demonstrating that the 2fTGH and U3A-ST1 cells respond to STAT-1 activation to a similar degree. These data suggest that STAT-1 has a role in the RDS checkpoint-response following DNA damage.

We next examined whether STAT-1 has a role in the G2-M checkpoint responses to DNA damage. Once again we compared the STAT-1-expressing 2fTGH and U3A-ST1 cells with the STAT-1 deficient U3A cells and measured the level of mitosis following exposure to γ-IR. As shown in Fig. 1C (and Fig. 1.
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 incorporated the BrdU during the short pulse period, were identified and traced over time after γ-IR. It was clear that 24 hours after γ-IR, approximately twice as many BrdU-labelled U3A cells were present 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 U3A-ST1 and U3A cells 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 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\textsuperscript{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\textsuperscript{T68} phosphorylation after DNA damage in cells that express or lack STAT-1. Western blot analysis demonstrated enhanced Chk2\textsuperscript{T68} phosphorylation in both the 2fTGH and U3A-ST1 cells after γ-IR (Fig. 2A). By contrast, Chk2\textsuperscript{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\textsuperscript{T68} phosphorylation is not owing to STAT-1 regulating endogenous Chk2 levels.
STAT-1 modulates ATM-dependent pathways

Similarly, reduced nuclear staining of phosphorylated Chk2-T68 was also observed in situ by immunofluorescence analysis after γ-IR of U3A cells and 2fTGH cells (Fig. 2B). Since activated phosphorylated Chk2 -T68 is involved in phosphorylation and degradation of Cdc25A (Falck et al., 2001, Falck et al., 2002; Bartek and Lukas, 2003), we also examined the levels of Cdc25A in 2fTGH and U3A cells 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 μM × 40 μM. (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).
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, immunofluorescent 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 γ-IR 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 the crucial upstream mediators/adaptors that are required for DNA damage for ATM activation and for separate ATM-branched downstream pathways.

Examination of the MDC1 promoter region using the Transfact programme (version 4.0) showed the presence of several potential DNA binding sites for 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–/– U3A cells. Transfection of a full-length STAT-1 construct in U3A cells resulted in a 10-fold increase in luciferase activity compared to the empty vector controls. The transfection efficiency of STAT-1 was confirmed by western blot analysis using an anti-STAT-1 antibody (IP-ST1) in untreated 2fTGH cells (A) or 2fTGH cells exposed to γ-IR (B) and immunoblotted with antibodies against the target proteins indicated.

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.
STAT-1 modulates ATM-dependent pathways

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by ATM. (A) In cells lacking STAT-1, overexpression of MDC1 WT had a dose-dependent effect on enhancing ATM-S1981, Chk2-T68 and p53-S15 phosphorylation (Fig. 5B). By contrast, MDC1 lacking the FHA domain had a much weaker dose-dependent effect on activating 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 2TGH cells. (G) Transfection of MDC1 RNAi in 2TGH cells reduced ATM-dependent phosphorylation following γ-IR (5 Gy) as assessed by immunoblotting with antibodies against the proteins indicated.

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
reduced phosphorylated Chk2-T68 in these cells (DiTullio et al., 2002). Moreover, staining for 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 mediating 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 possibility 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 γIR. 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 Cdc25A phosphorylation of ATM and its downstream substrates Chk2T68 and NBS1S343, 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 alternate and distinct ATM-NBS1-SMC1 pathway involved in phase checkpoint control. Furthermore, the ATM-NBS1-SMC1 pathway is active in both 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 STAT-1 in modulating Chk2T68 phosphorylation was previously reported in cell lines. In doing studies using RNAi to silence the MDC1 gene (Lou et al., 2003; Peng and Chen, 2003) and 53BP1, which was shown to have a specific effect on activation of the Chk2T68-Cdc25A pathway (Goldberg et al., 2003). This discrepancy may be due 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 Chk2T68 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 show a 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 activating role of the MDC1 promoter and that STAT-1 probably is an important regulator of the MDC1 promoter.

The MRN complex, together with ATM, 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 SB repair is unclear. However, recent studies have shown that functional MRN is required for ATM activation in both ATM-mediated pathways because, after DNA damage, cells lacking active MRE11 or NBS1 show a weaker response to 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 function in a large complex at sites of DNA DSBs (Xu and Stern, 2003). This complex 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 γIR (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 radiosensitivity (Falck et al., 2001). We show here, that STAT-1 is involved in processes that mediate cell-cycle arrest or apoptosis, as well as interacting with MDC1, which may therefore be crucial in modulating the cell-cycle-checkpoint responses to sensitise cells towards undergoing apoptosis.

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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