Identification and characterization of FUS/TLS as a new target of ATM

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ATM (ataxia-telangiectasia mutated), ATR (ATM- and Rad3-related) and DNA-PK (DNA-dependent protein kinase), important regulators of genome stability, belong to the PIKKs (phosphoinositide 3-kinase-like kinase) family of protein kinases. In the present study, DNA-affinity chromatography was used to identify DNA-binding proteins phosphorylated by these kinases. This resulted in the identification of FUS (fused in sarcoma)/TLS (translocated in liposarcoma) as an in vitro target of the PIKKs. FUS is a member of the Ewing’s sarcoma family of proteins that appears to play a role in regulating genome stability, since mice lacking FUS show chromosomal instability and defects in meiosis. The residues in FUS that are phosphorylated in vitro and in vivo were identified, and phospho-specific antibodies were generated to demonstrate that FUS becomes phosphorylated at Ser42 in vivo, primarily in response to agents that cause DSBs (double-strand breaks). DSB-induced FUS phosphorylation in vivo at Ser42 requires ATM and not DNA-PK. Although Ser42 is retained in the oncogenic FUS–CHOP [C/EBP (CCAAT/enhancer-binding-protein)-homologous protein 10] fusion generated by a t(12;16)(q13;p11) chromosomal translocation, Ser42 in FUS–CHOP is not phosphorylated after DNA damage. These results identify FUS as a new target of the ATM-signalling pathway and strengthen the notion that FUS regulates genome stability.

Key words: ataxia-telangiectasia mutated (ATM), DNA damage, Ewing’s sarcoma (EWS), fused in sarcoma (FUS), kinase, phosphorylation.

INTRODUCTION

ATM (ataxia-telangiectasia mutated), ATR (ATM- and Rad3-related) and DNA-PK (DNA-dependent protein kinase) are key regulators of cellular responses to DNA damage [1]. They belong to a conserved family of protein kinases termed the PIKKs (phosphoinositide 3-kinase-like kinases), because the catalytic domains of these proteins show similarity to phosphoinositide 3-kinase [2]. ATM and DNA-PK respond to DSBs (double-strand breaks), whereas ATR responds to almost all types of DNA damage, as well as to perturbations during DNA replication [3]. These kinases are recruited to sites of DNA damage by targeting of subunits with which they physically associate [4]. Binding at sites of DNA damage is thought to activate these kinases, enabling phosphorylation of multiple target proteins on serine or threonine residues that are followed by glutamine residues [5]. These S/TQ motifs often lie in clusters referred to as SCDs (S/TQ cluster domains) [6].

DSBs are particularly dangerous DNA lesions that can lead to genome instability if not rapidly detected and repaired. DSBs arise as a result of chemical attack of the DNA backbone by reactive species, such as oxygen free radicals, as a result of replisome stalling and as a result of nicks in the chromosomes that are converted into DSBs during DNA replication [7,8]. Under laboratory conditions, DSBs can be induced by exposure of cells to IR (ionizing radiation) or chemotherapeutic agents, such as the topoisomerase II inhibitor etoposide. DSBs arise during normal cellular processes, such as meiotic recombination and V(DJ) recombination [9]. The two main mechanisms to repair DSBs in all organisms are NHEJ (non-homologous end-joining) and HR (homologous recombination). NHEJ involves the processing and re-ligation of DNA ends and is potentially error-prone, since processing of the ends can involve the loss of sequence information [10]. In contrast, HR, which appears to be restricted to S-phase and G2-phase [11], is an error-free process. HR uses an intact copy of the broken DNA template to repair the DSB, and starts with exonucleolytic resection of both DNA ends at a DSB to generate a single-stranded 3’ overhang. Coating of the ssDNA (single-stranded DNA) overhang with Rad51 leads to the formation of a nucleoprotein filament that invades the homologous duplex, usually the sister chromatid [12], to form a D-loop. The invading strand then acts as a primer for new DNA synthesis. There are different models for the completion of HR, and some involve the formation of Holliday junctions, four-way DNA structures that are resolved, or dissolved, by Holliday-junction-processing proteins [13].

PIKKs regulate DNA repair. Cells lacking DNA-PK show defects in NHEJ [14]. However, these defects are not as pronounced as those seen in cells lacking DNA ligase IV [15], and, in this sense, DNA-PK appears to play a facilitatory role. ATM regulates NHEJ [16], but there are reports that efficient HR also requires ATM [17]. How ATM regulates DNA repair at the molecular level is not entirely clear, in that key proteins/residues that must be phosphorylated by ATM or DNA-PK for efficient DSB repair remain to be identified. Recent proteomic studies have identified a large number of new substrates of PIKKs, but the significance of most of these phosphorylation events is not yet clear [18,19].

Mice lacking the protein FUS [fused in sarcoma; also known as TLS (translocated in liposarcoma)], like cells lacking ATM or

Abbreviations used: ATM, ataxia-telangiectasia mutated; ATR, ATM- and Rad3-related; C/EBP, CCAAT/enhancer-binding protein; CHOP, C/EBP-homologous protein 10; Ddx5, DEAD-box protein 5; DNA-PK, DNA-dependent protein kinase; DSB, double-strand break; dsDNA, double-stranded DNA; EWS, Ewing’s sarcoma; FUS, fused in sarcoma; GST, glutathione transferase; HA, haemagglutinin; HEK-293, human embryonic kidney cell; HR, homologous recombination; HU, hydroxyurea; IR, ionizing radiation; LDS, lithium dodecyl sulfate; MALDI, matrix-assisted laser-desorption ionization; MMS, methylmethyl sulfonate; NHEJ, non-homologous end-joining; PIKK, phosphoinositide 3-kinase-like kinase; RNP, ribonucleoprotein; RRM, RNA-recognition motif; ssDNA, single-stranded DNA; SMC1, structural maintenance of chromosomes 1; STAT, signal transducer and activator of transcription 3; TAF, TATA-box-binding protein-associated factor; TFA, trifluoroacetic acid; TOF, time-of-flight; TLS, translocated in liposarcoma.

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DNA-PK, are hypersensitive to IR [14,20,21]. FUS/TLS was first identified because its N-terminus is fused to the DNA-binding domain of the transcription factor CHOP [C/EBP (CCAAT/enhancer-binding protein)-homologous protein 10] in human myxoid and round-cell liposarcomas as a consequence of a t(12;16)(q13;p11) translocation [22,23]. This results in the expression of a FUS–CHOP fusion protein that is sufficient for cell transformation, and this requires the FUS portion of the oncogenic fusion protein [24,25]. It was subsequently shown that FUS and its two close relatives, EWS (Ewing’s sarcoma) and TAF (TATA-box-binding protein-associated factor) 15/TAF15, are fused to a variety of transcription factors, resulting in the expression of transforming oncoproteins in various human sarcomas and leukaemias [26]. The normal cellular functions of wild-type FUS (or EWS or TAF15) are unknown. These proteins have features typical of RNA-binding proteins, including a highly conserved RRM (RNA-recognition motif) flanked by Arg-Gly-Gly repeats [27,28]. FUS has been shown to bind RNA in vitro and in vivo [22,29], but the relevance of these observations to the function of FUS is unknown. FUS also has a zinc-finger motif and may bind to DNA [30]. A variety of cellular interactor proteins have been reported for FUS, including hRNP [heterogeneous nuclear RNP (ribonucleoproteins)], splicing factors and transcription factors [29,31,32].

FUS-deficient mice show increased sensitivity to IR, meiotic defects resulting in male sterility and high levels of chromosomal abnormalities [20,33]. Intriguingly, FUS has been shown to be able to stimulate the formation of DNA D-loops between complementary DNAs in vitro [34]. This type of DNA transaction corresponds to one of the first steps in HR, strand invasion. Taken together, these results suggest that FUS might play a role in the cellular response to DNA damage, and the ability of FUS to stimulate D-loop formation has led to speculation about a role for FUS in HR [35].

We identified FUS in a screen for DNA-binding proteins that are phosphorylated in vitro by members of the PIKK family. Exposure of cells to agents that induce DSBs, such as IR and the topoisomerase II inhibitor etoposide, caused FUS to become phosphorylated [20,36]. The sites of FUS phosphorylation were identified and phospho-specific antibodies were used to demonstrate that FUS is a target of ATM in vivo when DSBs arise. Intriguingly, although Ser42 is retained in the oncogenic FUS–CHOP fusion caused by a t(12;16)(q13;p11) chromosomal translocation, Ser42 in FUS–CHOP is not phosphorylated after DNA damage. These results clearly show that FUS is a novel target of the ATM-signalling pathway.

MATERIALS AND METHODS

Cell lines and treatments

HEK-293 cells (human embryonic kidney cells) were grown in DMEM (Dulbecco’s modified Eagle’s medium) (GIBCO) supplemented with 10% (v/v) FBS (foetal bovine serum) (HyClone). Growth medium also contained 1% penicillin/streptomycin and 1% L-glutamate (both from Invitrogen). All cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. The ATM inhibitor KU55933 and the DNA-PK inhibitor NU7441, prepared at stock concentrations of 10 mM in DMSO, were a gift from Dr Graeme Smith (KuDOS Pharmaceuticals, Cambridge, U.K.) [36,37]. To induce DNA damage, exponentially growing cells that were treated (or untreated) with 10 mM KU55933, 10 mM NU7441 or with vehicle (0.1% DMSO) for 1 h were exposed to the indicated doses of IR, delivered from a Cs₁³⁷ source at a dose rate of 2.9 Gy per min, or were incubated with etoposide (10 μM), MMS (methylmethane sulfonate) (0.01%) or HU (hydroxyurea) (10 mM). Samples were taken immediately prior to and at different time points after treatment. For Western blot analysis and immunoprecipitation, cells were lysed in lysis buffer [50 mM Tris/HCl (pH 7.4), 270 mM sucrose, 1% Triton X-100, 1 μM microcystin-LR and protease inhibitors]. Lysates for immunoprecipitations were snap-frozen and stored at −80°C until required. For Western blotting, lysates were denatured in LDS (lithium dodecyl sulfate) sample buffer (Invitrogen) containing 10% (v/v) 2-mercaptoethanol. Proteins were separated by electrophoresis using Bis-Tris gels (4–12% gels) (Invitrogen), transferred on to nitrocellulose and subjected to Western blotting with the indicated antibody.

Antibodies and plasmids

The primary antibodies used in this study were: anti-FUS, anti-SMC1 (pherophospho-Ser666 (where SMC1 is structural maintenance of chromosomes 1) and anti-SMC1 antibodies (Axoxra), anti-HA antibody (where HA is haemagglutinin) (12CA5, Roche), anti-FLAG antibody (Sigma), anti-STAT3 (signal transducer and activator of transcription 3) antibody (Santa Cruz Biotechnology), anti-RPA antibody (where RPA is replication protein A) (Abcam), anti-Ku70 antibody (Abcam) and anti-MUS81 antibody (Abcam). All primary antibodies were used at a dilution of 1:1000. A phospho-specific antibody against Ser42 of FUS was raised by immunizing sheep with the peptide QQSYSGYPQSQDTSG coupled to KLH (keyhole-limpet haemocyanin), where pS represents phospho-Ser42. The antibody was purified from sheep serum by affinity chromatography on CH-Sepharose to which the phosphopeptide immunogen had been covalently coupled. Immunoblots with this antibody were performed in the presence of 40 μg/ml non-phosphopeptide to neutralize any antibodies that recognized unphosphorylated FUS. HRP (horseradish peroxidase)-conjugated secondary antibodies were obtained from Pierce and were used at a dilution of 1:4000 for 1 h. Full-length FUS was amplified with an N-terminal HA tag, sub-cloned into pCR2.1 and cloned into the NotI and BamHI sites of pCMV5. FUS was also cloned into pGEX-6P1 from pCR2.1, again using the NotI and BamHI sites. FUS–CHOP was amplified with an N-terminal FLAG tag from a Bluescript vector [a gift from Dr Terence Rabbitts (MRC Laboratory of Molecular Biology, Cambridge, U.K.)] and cloned into the BamHI and XhoI sites of pcDNA3. Mutations were introduced into pCMV-HA-FUS using the QuiKChange multi-site-directed mutagenesis kit (Strategene). Plasmids were transfected into HEK-293 cells using a calcium phosphate precipitation method [39].

DNA-affinity chromatography

Linear dsDNA (double-stranded DNA), Holliday junction and ‘Y-fork’ structures were constructed by annealing the following oligonucleotides: 888, 889, 890 and 891 (Holliday junction), 888 and 891 (Y-fork) and P1 (TGCAAACACTTTTGCGTCCTCCCGATAGTGATCGTATTTTTAGCTGACCCAGGTTTTCCCCGATTTTTGACG and P2 (GTCCTGAAACATCGGGAACCTGGCTCACGAAAAATTACGACTCTATCTCGGGACCGAAAAAGTGTGTTGAGC) to generate dsDNA. P1 was also used to generate ssDNA. Oligonucleotides 888, 889, 890 and 891 have been described previously [38]. Oligonucleotides 888 and P1 were synthesized to contain a 5′-biotin moiety, and all the bases in each oligonucleotide were phosphorothioate substituted. Oligonucleotides were purchased from Sigma-Genosys and were HPLC-purified. To generate each structure, 100 μg of the

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relevant oligonucleotide was incubated in 100 μl of annealing buffer [50 mM Tris/HCl (pH 7.6), 10 mM MgCl₂ and 0.1 mM EDTA] and boiled at 95°C for 5 min before cooling at room temperature (23°C) for a minimum of 2 h. Streptavidin-coated Dynabeads (Invitrogen) (300 μl) were washed three times in annealing buffer, added to the annealed oligonucleotides, and the mixture was incubated at room temperature for 30 min. The beads were then washed five times in annealing buffer and resuspended in 300 μl of annealing buffer and stored at 4°C.

In small-scale chromatography experiments (Figure 1A) 10 μl of DNA–beads was incubated with 300 μg of HeLa whole-cell extract, and in large-scale experiments (Figure 1B), 30 μl of DNA–beads was incubated with approx. 50 mg of HeLa whole-cell extract. Incubations were performed for 1 h at 4°C, followed by four washes in lysis buffer. The beads were then subjected to phosphorylation reactions or were denatured in SDS sample buffer, heated at 95°C for 5 min and then subjected to SDS/PAGE, followed by either Coomassie Brilliant Blue staining or Western blot analysis.

In vitro DNA-PK phosphorylation reactions

Recombinant GST (glutathione transferase)–FUS, anti-FUS immunoprecipitates or FUS bound to DNA structures was incubated in kinase buffer (1x) in the presence of the DNA-PK catalytic subunit (60 ng/μg of substrate), Ku70/80 (20 ng/μg of substrate), calf thymus DNA (20 ng/μg of substrate) and [γ-32P]ATP (0.1 mM, GE Healthcare; 1000–2000 c.p.m./pmol in analytical kinase assays, approx. 10000 c.p.m./pmol for phosphorylation-site analysis). Assays were incubated at 30°C for the indicated times and stopped by the addition of LDS sample buffer. Samples were heated for 5 min at 95°C and subjected to SDS/PAGE.

Stable transfection of HEK-293 cells with FLAG-tagged FUS–CHOP fusion protein

Early-passage HEK-293 cells were transfected with pcDNA3-FLAG FUS–CHOP using the calcium phosphate method [39]. After 16 h, the transfected cells were seeded at 50% confluence and were allowed to adhere to dishes overnight before the addition of G418 (3 mg/ml). After 8 days, G418-resistant colonies were isolated and expanded. FLAG–FUS–CHOP expression was tested by Western blotting.

Identification of FUS residues phosphorylated by DNA-PK in vitro

FUS was immunoprecipitated from 25 mg of cell extract protein by incubating the cell extract with 5 μg of anti-FUS antibody non-covalently bound to Protein G-coated Dynabeads for 90 min at 4°C. The beads were then washed four times in ice-cold lysis buffer containing 0.5 M NaCl and then lysis buffer containing 0.15 M NaCl before in vitro phosphorylation of FUS to stoichiometry with DNA-PK for 60 min (see above). After phosphorylation, samples were boiled in 15 μl of 2× LDS sample buffer (Invitrogen) and subjected to electrophoresis on polyacrylamide gels (4–12% gels), followed by fixation and staining with Colloidal Blue (Invitrogen). FUS, which was evident as a 66 kDa band after staining, was excised, and the amount of 32P incorporated was determined by Cerenkov counting. For phospho-mapping, the protein was reduced with 10 mM dithiothreitol, alkylated with iodoacetamide (50 mM in 0.1 M ammonium bicarbonate) and digested with chymotrypsin (5 μg/ml proteinase in 25 mM triethylammonium bicarbonate). The resulting peptides were applied to a Vydc 218TP215 C18 column equilibrated with 0.1% TFA (trifluoroacetic acid) and the column was used as a positive control in the kinase assay. (C) Extracts of HeLa cells were subjected to immunoprecipitation (IP) with anti-FUS or non-specific (anti-HA) antibodies. Immunoprecipitates were incubated with [γ-32P]ATP in kinase buffer in the presence (+) or absence (−) of DNA-PK as described in the Materials and methods section. Proteins bound to beads were subjected to autoradiography. Recombinant p53 was used as a positive control in the kinase assay. (E) Extracts of HeLa cells were subjected to immunoprecipitation (IP) with anti-FUS or non-specific (anti-HA) antibodies. Immunoprecipitates were incubated with [γ-32P]ATP in kinase buffer in the presence (+) or absence (−) of DNA-PK as described in the Materials and methods section. Where indicated, the DNA-PK inhibitor NU7441 or the ATM inhibitor KU55933 was included in the phosphorylation reaction. Immunoprecipitates were subjected to SDS/PAGE, followed by Western blotting, staining with Colloassie Brilliant Blue or autoradiography. Recombinant PHAS-1 (PHASI) was used as a positive control for DNA-PK activity. RPA, replication protein A.
was developed with a linear gradient of acetonitrile/0.1% TFA at a flow rate of 0.2 ml/min with 0.1 ml fractions collected. $^{32}$P radioactivity was recorded with an on-line monitor. Phosphorylation-site mapping was performed essentially as described previously [40]. Identification of $^{32}$P-labelled peptides was performed by MALDI (matrix-assisted laser-desorption ionization)-TOF (time-of-flight) and MALDI-TOF-TOF MS on an Applied Biosystems 4700 Proteomics Analyzer using a matrix of 10 mg/ml α-cyano-4-hydroxycinnamic acid in buffer [50% (v/v) acetonitrile, 0.1% TFA and 10 mM ammonium phosphate]. Sites of phosphorylation within the peptides were determined by a combination of MALDI-TOF-TOF MS and solid-phase Edman sequencing. Solid-phase sequencing was performed on an Applied Biosystems Procise 494C sequencer after the peptide was covalently coupled with a Sequenylamine, membrane, and the $^{32}$P radioactivity released after each cycle was measured by Cerenkov counting.

Q-Trap MS identification of in vivo sites of FUS phosphorylation

HEK-293 cells (10-cm diameter dishes, 15 dishes per treatment) were exposed to IR (20 Gy) (or untreated) and left to recover for 1 h. Cells were lysed in ice-cold lysis buffer [50 mM Tris/HCl (pH 7.4), 0.27 M sucrose, 1% Triton X-100, 1 μM microcin Lin-0 and a cocktail of protease inhibitors] and centrifuged at 20,000 g for 5 min at 4 °C. FUS was immunoprecipitated from 25 mg of cell extract protein by incubating with 5 μg of anti-FUS antibody bound to Protein-G-coated Dynabeads for 90 min at 4 °C. Beads were washed four times in ice-cold lysis buffer containing 0.5 M NaCl and once in buffer containing 0.15 M NaCl before boiling in 15 μl of 2× LDS sample buffer (Invitrogen). Proteins were subjected to SDS/PAGE on Bis-Tris gels (4–12% gel) and electroblotted to a nitrocellulose membrane. The gel pieces were then extracted using 2.5% (v/v) acetic acid and 50% (v/v) acetonitrile for 15 min before combining the supernatant with the original dried sample and drying once under vacuum. The gel pieces were then extracted using 2.5% (v/v) acetic acid and 50% (v/v) acetonitrile for 15 min before combining the supernatant with the original dried sample and drying once under vacuum. The gel pieces were then extracted using 2.5% (v/v) acetic acid and 50% (v/v) acetonitrile for 15 min before combining the supernatant with the original dried sample and drying once under vacuum.

**RESULTS**

DNA-affinity chromatography to enrich DNA-binding proteins

Since ATM, ATR and DNA-PK bind to sites of DNA damage, where they become activated, we reasoned that important targets of these kinases might also be bound at these sites. With this in mind, we carried out affinity chromatography using a variety of synthetic DNA structures to enrich for potential targets of these kinases. Linear dsDNA, Holliday junction and Y-fork structures were constructed by annealing oligonucleotides. One oligonucleotide in each DNA structure contained a 5′-biotin group. In addition, all oligonucleotides were synthesized with phosphorothioate-substituted bases to prevent their degradation by nuclease in cell extracts. Each structure, and ssDNA, was immobilized on streptavidin-coated magnetic beads and incubated with whole-cell extracts of HeLa cells, followed by Western blotting with different antibodies.

We first tested the ability of the immobilized DNA structures to retain the known structure-specific DNA-binding factors Mus81 [41] and hSli1, the human orthologue of yeast Sli1 [38] and J. Rouse, unpublished work). These are structure-dependent nucleases that cleave Holliday junctions and branched DNA structures, at least in vitro. As shown in Figure 1(A), endogenous Mus81 or transiently transfected FLAG–hSli1 bound to the Y-fork and Holliday junction, but not to dsDNA, ssDNA or empty beads. However, Ku70, which specifically binds to the ends of DNA [42], also bound to the Y-fork and Holliday junction as well as to linear DNA, presumably due to the presence of free DNA ends. The large subunit of replication protein A (which should bind only to ssDNA) [43] was found to be associated not only with ssDNA, but also with the Y-fork and Holliday junction. To reduce non-specific binding of factors like Ku to the ends of the Holliday junction and Y-fork, and enrich structure-specific binding factors, competitor calf thymus dsDNA was included in the binding reaction. As shown in the last two panels of Figure 1(A), competitor dsDNA prevented binding of Ku70, but not hSli1, to the Holliday junction and Y-fork. These conditions were used to enrich for DNA-binding factors that may be targets for the PIKKs.

Identification as FUS as an in vitro substrate of PIKKs

ATM, ATR and DNA-PK all phosphorylate target proteins on serine and threonine residues that are followed by a glutamine residue (referred to as S/TQ motifs) and all three kinases appear to have identical specificity in vitro [44,45]. Since DNA-PK is an abundant protein that can be purified to homogeneity, whereas ATM and ATR are present at low-copy numbers in cells (results not shown), DNA-PK was used as a tool to identify DNA-binding proteins that are phosphorylated by PIKKs.

DNA-affinity chromatography of HeLa whole-cell extracts was carried out using dsDNA and Holliday junctions as described above, and beads were incubated with $\gamma$-32P-labelled ATP–Mg$^{2+}$ in the presence or absence of DNA-PK holoenzyme and dsDNA (necessary for DNA-PK to be active). Samples were then denatured and subjected to SDS/PAGE, stained with Colloidal Blue, followed by autoradiography (Figure 1B). In the absence of DNA-PK, weak phosphorylation of a protein bound to both dsDNA- and Holliday-junction-coated beads that migrated slightly more slowly than the 66 kDa protein marker was observed (Figure 1B). This was probably due to one or more contaminating kinases that associated with these beads. However, when DNA-PK was added to the beads, much stronger phosphorylation of a protein of approx. 66 kDa was observed (Figure 1B). This radiolabelled species was excised from the gel, digested with trypsin, and the resulting peptides were analysed on a 4000 Q-Trap mass spectrometer. This revealed the presence of two proteins: Ddx5 (DEAD-box protein 5), a member of the DEAD-box family of RNA helicases and FUS/TLS (results not shown). To determine which of these proteins is phosphorylated by DNA-PK, Ddx5 and FUS were both immunoprecipitated from cell
extracts and the immunoprecipitates were incubated with γ-<sup>32</sup>P-labelled ATP–Mg<sup>2+</sup> in the presence or absence of DNA-PK holoenzyme and dsDNA. A band of approx. 66 kDa in anti-FUS immunoprecipitates was strongly phosphorylated by DNA-PK, and this was inhibited by inclusion of the DNA-PK inhibitor NU7441 [36], but not by inclusion of the ATM inhibitor KU55933 [37] (Figure 1C). This band was confirmed to be FUS by Western blotting (Figure 1C). It is unlikely that the band phosphorylated in the absence of DNA-PK is FUS, since it had a slightly different molecular mass, and the basal phosphorylation of this protein was not prevented by inclusion of NU7441 or KU55933 (Figure 1C), so this protein was not investigated further. DNA-PK did not catalyse detectable phosphorylation of any proteins in the anti-Ddx5 immunoprecipitates (results not shown).

To exclude the possibility that a protein co-immunoprecipitating with FUS, and not FUS itself, is phosphorylated by DNA-PK, FUS was expressed as a GST fusion protein in bacteria. GST–FUS, but not GST, was efficiently phosphorylated by DNA-PK in a time-dependent manner, with a stoichiometry of approx. 5 moles of phosphate incorporated per mole of FUS (results not shown), indicating that FUS is phosphorylated efficiently by DNA-PK <i>in vitro</i> on at least five residues.

We next wished to identify the FUS residues phosphorylated by DNA-PK. Inspection of the amino-acid sequence of FUS revealed 12 S/TQ motifs, all located within the N-terminal 132 amino acids (see Figure 3C). FUS was phosphorylated to stoichiometry with DNA-PK <i>in vitro</i> and digested with chymotrypsin, since no cleavage site for trypsin was present in the N-terminal 132 amino acids of FUS. Chymotryptic peptides were separated by reverse-phase HPLC chromatography (Figure 2A) and this revealed five radio-labeled peptides. The phosphorylated residue in each one was determined by solid-phase Edman sequencing (results not shown). This analysis identified the residues in FUS phosphorylated by DNA-PK as Ser<sup>26</sup>, Ser<sup>42</sup>, Ser<sup>61</sup>, Ser<sup>84</sup> and Ser<sup>131</sup> (Figure 2B). All of these residues lie in S/TQ motifs that conform to the consensus sequence for the PIKKs, and are all found in the serine/threonine-rich N-terminal region of FUS (Figure 3C).

**Identification of FUS residues phosphorylated <i>in vivo</i> after DNA damage**

To determine if FUS becomes phosphorylated <i>in vivo</i> under conditions where PIKKs are activated, cells were exposed (or not exposed) to IR that causes DSBs. Cells were lysed, FUS was immunoprecipitated and precipitates were subjected to SDS/PAGE (Figure 3A, upper panel). FUS was excised and digested with chymotrypsin, since there are no tryptic cleavage sites within the N-terminal serine/threonine-rich region of FUS that contains the S/TQ motifs (Figure 3C, lower panel). Chymotryptic peptides were analysed on a 4000 Q-Trap mass spectrometer with precursor ion scanning to identify phosphopeptides. As shown in Figure 3(A) (lower left-hand panel), several phosphopeptides were detected in FUS isolated from untreated cells. However, several extra phosphopeptides were detected after exposure of cells to IR (Figure 3A, lower right-hand panel). The sequences of these peptides is shown in Figure 3(B). It was not possible to use tandem MS to pinpoint the exact residue(s) phosphorylated in these chymotryptic peptides, as this is only possible with tryptic peptides since the positive charge of the arginine or lysine residues in tryptic peptides is required (N. Morrice, unpublished work). However, all of the DNA-damage-inducible FUS phosphopeptides mapped to the serine/threonine-rich N-terminal region of FUS (Figure 3C) that is also phosphorylated <i>in vitro</i> by DNA-PK (Figure 2). In fact, all of the residues phosphorylated by DNA-PK, except Ser<sup>131</sup>, are located in the DNA-damage-induced FUS phosphopeptides (Figure 3C). This strongly suggests that FUS is phosphorylated on one or more of these residues by PIKKs <i>in vivo</i> after exposure of cells to DNA damage.
Figure 3 Identification of residues in FUS phosphorylated after DNA damage in vivo

(A) HEK-293 cells were treated with IR (10 Gy) or untreated [Control (Ctl)] and, after cell lysis, FUS was immunoprecipitated and subjected to SDS/PAGE (upper panel). FUS was excised, digested with chymotrypsin and the resulting peptides were analysed by LC-MS with precursor-ion scanning on a 4000 Q-Trap system (lower panels). Each peak corresponds to a phosphopeptide, and those phosphopeptides that increase in abundance after exposure of cells to damaging agents are labelled 1–6 (lower right-hand panel). amu, atomic mass unit. (B) Sequences of the chymotrypic phosphopeptides analysed in (A). (C) Schematic diagram of the domain architecture of FUS and primary amino-acid sequence. Upper panel: the N-terminal S/TQ-rich region, the RRM domain, the Arg-Gly-Gly (RRG) repeat regions and the zinc-finger domain (Zn) are indicated. Lower panel: the amino-acid sequence of FUS. The region corresponding to the overlapping chymotryptic phosphopeptides is underlined, and the residues phosphorylated by DNA-PK in vitro are highlighted in grey.

FUS is phosphorylated at Ser^{42} in vivo after exposure of cells to agents that cause DSBs

To confirm that DNA-damage-induced phosphorylation of FUS occurred on one of the residues identified by MS and by in vitro analyses, phospho-specific antibodies were raised against Ser^{30} and Ser^{42} and affinity-purified using the phosphopeptide immunogen. Phosphopeptides corresponding to Ser^{50} failed to generate antibodies capable of recognizing the phosphopeptide immunogen (results not shown). In contrast, dot-blot analysis...
**Figure 4**

**FUS is phosphorylated at Ser42 in vivo primarily in response to agents that cause DSBs**

- (A) The indicated amounts of the relevant phosphopeptides and non-phosphopeptides (dephospho-peptides) were diluted in water, spotted on to nitrocellulose at various concentrations and allowed to dry. The membrane was then subjected to Western blot analysis with an FUS anti-phospho-Ser42 antibody.
- (B) HEK-293 cells were left untreated or exposed to IR (10 Gy) or UV light (30 J/m²) and allowed to recover for the times indicated. Alternatively, cells were treated with MMS (0.01%) or HU (10 mM) for the times indicated. After cell lysis, extracts were subjected to SDS/PAGE, followed by Western blot analysis with the indicated antibodies.
- (C) HEK-293 cells were transiently transfected with wild-type HA-FUS (WT) or HA-FUS S42A and exposed to IR (10 Gy) (+) or untreated as a control (−). Cell extracts were subjected to immunoprecipitation (IP) with anti-HA or with non-specific antibodies (anti-STAT3 antibody) as a control. Immunoprecipitates were subjected to SDS/PAGE, followed by Western blot analysis with a FUS anti-phospho-Ser42 antibody (upper panel). Cell extracts were also Western blotted with an anti-HA antibody (lower panel). pSer, phospho-serine.

showed that affinity-purified phospho-specific antibodies raised against Ser42 recognized the correct phosphopeptide immunogen, but not the corresponding non-phosphopeptide (Figure 4A). These antibodies were then used to investigate FUS phosphorylation in vivo. HEK-293 cells were exposed to IR or UV light and allowed to recover for different time periods, or cells were exposed to MMS or HU for the times indicated. Cell extracts were subjected to SDS/PAGE followed by Western blotting. The anti-phospho-Ser42 antibody recognized a single band in cell extracts of exactly the same molecular mass as FUS only after exposure of cells to DNA damage (Figure 4B). Phosphorylation of Ser42 of FUS was apparent 15 min after exposure to IR and was still evident 2 h post-irradiation (Figure 4B). The kinetics of FUS phosphorylation were similar to those of IR-induced phosphorylation of SMC1, a known target of ATM, at Ser966 (Figure 4B). In contrast, exposure of cells to MMS, a DNA-alkylating agent, UV light or HU (which slows down DNA replication) caused much weaker phosphorylation of FUS Ser42, even at the longest time points tested (Figure 4B and results not shown). Phosphorylation of SMC1 Ser966 was easily detectable in each case. A number of proteomic studies were carried out by other groups to identify targets of PIKK kinases, and these involved immunoprecipitation of proteins from cell extracts using generic anti-phospho S/TQ antibodies [18,19]. However, FUS was not identified in these screens.

To be certain that the antibodies that we raised recognized phospho-Ser42 of FUS specifically, HEK-293 cells were transiently transfected with HA-tagged wild-type FUS or a mutant FUS in which Ser42 was mutated to an alanine residue. Cell extracts were subjected to immunoprecipitation with anti-HA antibodies or with a non-specific antibody (anti-STAT3 antibody) used as a control. As shown in Figure 4(C), wild-type FUS, but not the S42A mutant, was recognized by the anti-phospho-Ser42 antibody after immunoprecipitation with the anti-HA antibody, but not with a non-specific antibody (anti-STAT3 antibody), after exposure of cells to IR. In contrast, the FUS S42A mutant is not recognized, although it is present in similar amounts in anti-HA immunoprecipitates (Figure 4C). These results indicate that FUS is phosphorylated at Ser42 in vivo primarily in response to IR that causes DSBs. Since etoposide, an inhibitor of topoisomerase II that causes DSBs in cells, also induced strong phosphorylation of FUS (see Figure 6B), it is likely that the phosphorylation of FUS is primarily triggered by DSBs. It is worth noting that cell transfection caused the basal phosphorylation of FUS to increase dramatically (results not shown), so Western blots were exposed for short time periods, and therefore the band intensity in Figure 4(C) is weaker than in Figure 4(B).

Ser42 of FUS–CHOP is not phosphorylated after DNA damage

The t(12;16)(q13;p11) chromosomal translocation in human myxoid liposarcoma results in fusion of the N-terminal 266 amino acids of FUS to CHOP, a member of the C/EBP family of transcription factors (Figure 5A) [22,23]. In the FUS–CHOP fusion protein, the RRM domain and the zinc finger of FUS are replaced by CHOP, but the N-terminal S/TQ-rich region of FUS (containing the RRM domain and zinc finger) may be required for phosphorylation by ATM. To test if the RRM domain or the zinc-finger domain is required for FUS phosphorylation by ATM, Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to Asp327, Cys427, Cys428 and Phe288 were all mutated to
Figure 5  Ser42 of FUS–CHOP fusion is not phosphorylated on exposure of cells to IR

(A) Schematic representation of the oncogenic FUS–CHOP fusion protein that arises as a result of a t(12;16)(q13;p11) chromosomal translocation. A part of the 5′-untranslated region of CHOP that is translated in FUS–CHOP is shown in black (bottom diagram). (B) FLAG–FUS–CHOP was immunoprecipitated (IP) from extracts from HEK-293 cells stably expressing this protein before (−) or after (+) exposure of cells to IR. Cell lysates (left-hand panels) and anti-FLAG immunoprecipitates (right-hand panels) were subjected to SDS/PAGE, followed by Western blot analysis with the anti-phospho-Ser42 (FUS pSer42) antibody. Membranes were stripped and re-probed with the indicated antibody (anti-FUS or anti-FLAG antibodies) to check for equal protein loading. (C) Wild-type HA–FUS or HA–FUS containing mutations in both the RRM domain and the zinc-finger domain (Cys427, Cys428, Phe288, Phe305 and Asp327) (Fus: Zn finger RRM mutant), or HA–FUS S42A (FUS: Ser42Ala) were immunoprecipitated (IP) from HEK-293 cells before (−) or after (+) exposure of cells to IR. Immunoprecipitates were subjected to SDS/PAGE, followed by Western blot analysis with an anti-phospho-Ser42 (FUS pSer42) antibody. Membranes were stripped and re-probed with an anti-HA antibody to check for equal protein loading. bZIP, basic leucine zipper; Chr, chromosome.

alanine residues and PheFUS was mutated to an arginine residue. Cys427 and Cys428 are conserved in all C2–C2 zinc fingers, whereas Phe288 is conserved in the RNP-2 sub-motif of RRM domains [46]. Mutation of the conserved phenylalanine and aspartic acid residues (PheFUS and Asp327 of FUS), which lie between the RNP-2 and RNP-1 regions in RRM, has been shown to abolish binding to RNAs [47]. HEK-293 cells were transiently transfected with the resulting compound mutant or wild-type FUS, fused to a HA epitope tag. The FUS S42A mutant was used as a negative control. Both HA–FUS and the HA–FUS RRM/zinc mutant are phosphorylated at Ser42 after exposure to IR (Figure 5C). Taken together, these results suggest that although the C-terminal region of FUS is required for phosphorylation of Ser42, the RRM and zinc-finger domains are not involved.

ATM, not DNA-PK, mediates IR-induced phosphorylation of FUS Ser42

We next sought to determine the kinase(s) responsible for DSB-induced phosphorylation of FUS. Ser42 lies in a consensus sequence for ATM, ATR and DNA-PK. ATM and DNA-PK are activated in response to DSBs, but not in response to UV light or MMS, for example, which activate ATR. Since FUS is phosphorylated in response to DSBs, but only very weakly in response to MMS or UV light, we reasoned that the most probable FUS kinases are ATM and DNA-PK. Pre-incubation of cells with NU7441, a specific inhibitor of DNA-PK [36], had no effect on the phosphorylation of FUS induced by IR (Figure 6A). In contrast, the ATM-specific inhibitor, KU55933 [37], severely reduced phosphorylation of FUS at Ser42 triggered by exposure of cells to IR or etoposide (Figure 6B). These results indicate that DSB-induced phosphorylation of FUS is catalysed by ATM.

DISCUSSION

In the present study, we carried out DNA-affinity chromatography to enrich for potential targets of PIKKs. Using this approach, we identified FUS and found that it is phosphorylated efficiently by DNA-PK in vitro. The ability of FUS to bind to the different DNA species used in this study was not pursued, and it is not clear if FUS really binds to DNA or if the binding we observed was a result of non-specific electrostatic interactions. It is also not clear if FUS really binds to DNA or if the binding we observed was indicative of binding to sites of DNA damage. However, the DNA-affinity chromatography approach allowed us to discover that DNA-PK can phosphorylate FUS efficiently, revealing FUS as a potential in vivo target of PIKK-family members.

Although FUS has a total of 12 S/TQ motifs, all located in the N-terminal 132 amino acids of FUS, DNA-PK phosphorylated FUS in vitro with a stoichiometry of 5 mol of phosphate per mol of protein, suggesting phosphorylation of five residues. In vitro mapping revealed the five residues phosphorylated by
DNA-PK; Ser\textsuperscript{26}, Ser\textsuperscript{42}, Ser\textsuperscript{61}, Ser\textsuperscript{84} and Ser\textsuperscript{131} (Figure 3C), and all of these residues lie in S/TQ motifs. Q-Trap MS analysis of FUS immunoprecipitated from cells after treatment with IR led to the identification of several chymotryptic phosphopeptides from the N-terminus of FUS that contained Ser\textsuperscript{26}, Ser\textsuperscript{42}, Ser\textsuperscript{61} and Ser\textsuperscript{84}. However, it was not possible to pinpoint precisely which residues in these peptides were phosphorylated, because the positive charge that occurs in tryptic peptides (trypsin cleaves immediately after lysine and arginine residues), but not in chymotryptic peptides, is essential for phosphorylation-site identification by tandem MS. Nonetheless, the good agreement between the FUS residues phosphorylated in vitro by DNA-PK and the serine residues in the chymotryptic phosphopeptides prompted further investigation. In this light, an affinity-purified antibody raised against phospho-Ser\textsuperscript{42} demonstrated clearly that FUS is phosphorylated at this residue primarily in response to IR or etoposide, agents that cause DSBs. In contrast, FUS was only weakly phosphorylated in response to MMS, UV light or HU (Figure 4B). The spectrum of agents that induced FUS phosphorylation indicated that ATM or DNA-PK were the most likely FUS kinases.

Although DNA-PK phosphorylates FUS efficiently in vitro, NU7441, a specific inhibitor of DNA-PK, has no effect on FUS Ser\textsuperscript{42} phosphorylation in vivo. In contrast, IR- or etoposide-induced FUS phosphorylation was prevented by pre-incubation of cells with KU55933, a specific inhibitor of ATM. Therefore ATM phosphorylates FUS at Ser\textsuperscript{42} in vivo. While the present study was underway, a number of proteomic studies were carried out by other groups to identify targets of PIKKs, and these involved immunoprecipitation of proteins from cell extracts using generic anti-phospho S/TQ antibodies \cite{18, 19}. However, FUS was not identified in these studies. In one of these studies, trypsin was used to cleave proteins in extracts before immunoprecipitation by the anti-phospho S/TQ antibody. The region of FUS phosphorylated by ATM is not cleaved by trypsin and therefore would have been too large for MS analysis and consequent identification as a PIKK target.

Ser\textsuperscript{42} of FUS lies in the region of FUS that becomes fused to transcription factors in sarcomas and leukaemias (Figure 5A) \cite{22, 23}. We tested the FUS–CHOP fusion protein, stably expressed in HEK-293 cells, for phosphorylation after IR. Intriguingly, although wild-type FUS was phosphorylated in response to IR, no phosphorylation of FUS–CHOP was detected (Figure 5B). The basis for this observation is not yet clear, but we reasoned that the C-terminal portion of FUS that is missing from oncogenic FUS fusion proteins could be required for N-terminal phosphorylation. The C-terminus of FUS contains two conserved domains, a zinc-finger domain and an RRM domain (Figure 5A). However, these domains do not appear to be required for phosphorylation of FUS, as mutation of conserved residues in these domains does not affect phosphorylation on Ser\textsuperscript{42} after exposure to IR (Figure 5C). The most likely explanation for these results is that the C-terminal region of FUS, missing from FUS–CHOP, contains a docking site for ATM, and this will be interesting to investigate. It is also possible that the C-terminus of FUS missing from FUS–CHOP is required to co-localize FUS with ATM at DSBs. However, we could not detect the nuclear foci which are typical of proteins that are stably retained at sites of DNA damage (results not shown).

Like mice lacking ATM, mice lacking FUS are hypersensitive to IR and show defects in spermatogenesis resulting from perturbations in meiotic synapses \cite{20}, and loss of FUS or ATM causes high levels of chromosomal instability \cite{33}. The defects in meiosis are consistent with the interruption of an early step in HR. In our
hands, however, the efficiency of DSB repair in cells from FUS−/− mice is indistinguishable from FUS+/− mice (M. Gardiner and J. Rouse, unpublished work).

We would like to test the impact of ATM-catalysed phosphorylation on FUS function. However, the role of FUS in the cellular response to DNA damage is not yet clear, but is currently under investigation in this laboratory. The IR sensitivity of embryonic fibroblasts isolated from FUS−/− mice is slight compared with cells from mice defective in other DNA-damage-responsive proteins [20] (results not shown), and the severe sensitivity of the FUS−/− mice to killing by IR may be due to effects on specific cell types. Given that FUS has been shown to bind RNA, and has RNA-binding motifs, it is possible that FUS regulates DNA-damage-induced transcription or splicing, or other aspects of RNA metabolism linked with DNA-damage responses. This is currently under investigation in this laboratory.

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