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HMGA1 protein is a novel target of the ATM kinase

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ARTICLE INFO

Article history:
Received 12 May 2008
Received in revised form 14 July 2008
Accepted 17 July 2008
Available online 8 September 2008

Keywords:
HMGA1
ATM
DNA damage

ABSTRACT

The high mobility group HMGA1 protein belongs to a family of architectural factors that play a role in chromosomal organisation and gene transcription regulation. HMGA1 overexpression represents a common feature of human malignant tumours and is causally associated with neoplastic transformation and metastatic progression. Recently, HMGA1 expression has been correlated with the presence of chromosomal rearrangements and suggested to promote genomic instability. Here, we report a novel interaction between HMGA1 protein and the ataxia-telangiectasia mutated (ATM) kinase, the major key player in the cellular response to DNA damage caused by several agents such as ionising radiation (IR). We identified an SQ motif on HMGA1, which is effectively phosphorylated by ATM *in vitro* and *in vivo*. Interestingly, confocal microscopy revealed that HMGA1 colocalises with the activated form of ATM (ATM S1981p). Moreover, HMGA1 ectopic expression decreases cell survival following exposure to IR as assessed by clonogenic survival in MCF-7 cells, further supporting the hypothesis that HMGA1 might act as a downstream target of the ATM pathway in response to DNA damage.

1. Introduction

The high molecular group A (HMGA) proteins are a class of nuclear, non-histone proteins involved in a wide range of cellular processes such as chromatin remodelling, gene transcription, differentiation and neoplastic transformation.[1]

The HMGA family consists of the *HMGA1* gene, which codes by alternative splicing for two major isoforms, the HMGA1a and

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doi:10.1016/j.ejca.2008.07.033

HMGA1b proteins, and the *HMGA2* gene. HMGA proteins contain three DNA binding domains, referred to as 'AT-hooks', that allow the binding to the minor groove of AT rich DNA sequences and a highly acidic carboxy-terminal region. HMGA proteins behave as architectural factors of gene transcription,[1] regulating, positively or negatively, the expression of a large number of genes in a way dependent on the cellular context.[2]

HMGA proteins are highly expressed during embryogenesis, whilst they are expressed only at low levels in normal adult tissues.[3] HMGA1 overexpression represents a common feature of human malignant tumours including thyroid,[4] breast,[5] ovary[6] and prostate,[7] and is causally associated with the acquisition of a transformed phenotype. In fact, HMGA1 protein suppression prevents thyroid cell transformation by the Kirsten murine sarcoma virus,[8] and an adenovirus carrying the *HMGA1* gene in the antisense orientation induces death of human thyroid carcinoma cells.[9] Moreover, HMGA1 overexpression induces the neoplastic phenotype in Rat1a cells and human CB33 lymphoid cells[10] and in the human breast epithelial MCF-7 cells.[11] Accordingly, transgenic mice overexpressing the wild-type form of the *Hmga1* gene develop pituitary adenomas and natural killer cell lymphomas.[13] Interestingly, in several human prostate cancer cell lines HMGA1 expression has been positively correlated to the extent of chromosomal rearrangements,[14] and its ectopic expression was able to enhance the presence and heterogeneity of unbalanced chromosomal rearrangements in LNCaP prostate cell line,[14] suggesting a role for HMGA1 proteins in the acquisition of genomic instability, one of the hallmarks of cancer cells. In human breast tumours HMGA1 overexpression has been correlated to the downregulation of *BRCA1*,[5] a gene involved in DNA repair following different types of DNA damage.[15] Moreover, HMGA1 overexpression was found to decrease cell survival following exposure to DNA-damaging agents of human breast cancer-derived MCF-7 cells, by inhibition of nucleotide excision repair (NER), through downregulation of XPA,[16] or by inhibition of double-strand breaks (DSBs) repair, through a mechanism involving *BRCA1* downregulation.[5] Recently, also HMGA2 expression has been shown to promote enhanced sensitivity in response to doxorubicin and other related DNA-damaging agents, likely through modulation of the signalling pathway responsible for the maintenance of genomic integrity.[17]

Genome stability is threatened by DNA-damaging agents that can either be endogenous, deriving from normal cell metabolism, or exogenous such as ionising radiation (IR). IR induces DNA double-strand breaks (DSBs) that can potentially lead to mutations, translocations, abnormal recombination and chromosome breakage or loss. Detection of damaged DNA triggers checkpoint pathways that prevent cell cycle progression and activate the DNA repair system. If the type or amount of damage overwhelm the survival response machinery, apoptosis is triggered.[18]

ATM, the gene mutated in the human disease ataxia-telangiectasia (AT),[19] is crucial for initiating signalling pathways following exposure to IR or other agents that cause DSBs. Like other syndromes that are caused by defects in the DNA-damage response, AT patients show an increased risk for cancer, chromosome fragility and radiosensitivity.[20] Once activated by DNA damage,[21] ATM phosphorylates numerous substrates to induce cell cycle arrest, to reduce chromosomal breakage and to enhance cell survival. ATM belongs to the 'PI3K-like protein kinases' (PIKKs) family of proteins, which all contain a domain with motifs typical of the phosphatidylinositol 3-kinase (PI3K).[18] ATM, similarly to other PIKKs, features a serine/threonine kinase activity. In particular, ATM targets serine or threonine residues followed by glutamine,[22] named the SQ/TQ (or S/TQ) motif, which is characteristic of DNA-damage response proteins.

Since HMGA proteins have been recently shown to play a role in the cellular response to DNA-damaging agents, we hypothesised that HMGA might function as adaptor mediators of the ATM-induced signalling pathway following IR. Our studies demonstrate the interaction between HMGA1b and ATM proteins and identify the HMGA1 protein as a novel target of the ATM kinase. Even though the physiological role of this interaction needs further studies, we provide evidence that HMGA play a role in the cellular response to DNA damage caused by IR.

2. Materials and methods

2.1. Cell cultures

Human embryonic kidney 293T cells were cultured in DMEM supplemented by 10% (v/v) foetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin (GIBCO-BRL). Human lymphoblasts GM2184 and GM1526 were obtained from Coriell Cell Repositories and cultured in RPMI supplemented by 15% (v/v) FBS, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin (GIBCO-BRL). Wild-type and *Hmga1* –/– AB2.1 ES cells, described elsewhere,[23] were cultured on a layer of mitomycin C-inactivated fibroblasts. Before γ-irradiation fibroblasts were removed by three passages onto 0.1% (w/v) gelatin-treated plates, and the undifferentiated state was maintained by the addition of 10^3 units/ml leukaemia inhibiting factor (Chemicon). Wild-type, *Hmga1* +/– and *Hmga1* –/– mouse embryonic fibroblasts (MEFs) were established from 14.5 dpc embryos following the standard procedures. MEFs were cultured in DMEM supplemented by 10% (v/v) FBS, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 1% (v/v) non-essential aminoacids (GIBCO-BRL).

For γ-irradiation experiments, cells were irradiated by 6 MV X-ray of a linear accelerator with doses ranging from 0 to 10 Gy.

2.2. Plasmid constructs and recombinant proteins

HMGA1b full-length and its deletion mutants were cloned into pCEFL-HA, in frame with the HA epitope, as described elsewhere.[24] Wild-type *HMGA1b* full-length was also cloned into pcDNA3.1/Hygro (Invitrogen) for co-immunoprecipitation experiments. The pFLAG-ATM-wild-type (wt) and kinase-dead (kd) expressing vectors previously described[25] were kindly provided by Dr. M.B. Kastan. The recombinant HMGA1b full-length and truncated (aa 1–79) proteins were generated by cloning the corresponding cDNAs in frame to the polyhistidine tag in pET21c expression vector (Novagen). Serine 88 of HMGA1b was mutated to alanine by PCR using pET-HMGA1b wild-type as template, and the resulting cDNA was cloned into pET21c vector in frame to the polyhistidine tag.

BL21/DE3 cells were transformed with each vector (pET-HMGA1b, pET-HMGA1b (1–79), pET-HMGA1b S88A), grown in Luria Broth (LB) and induced with isopropyl-β-D-thiogalactoside, then harvested and sonicated. Recombinant proteins were purified using the His-Trap purification kit (Amersham Biosciences), then dialysed and analysed by SDS–PAGE.

2.3. Transfections, immunoprecipitation and Western blots

Transient transfections into 293T cells were performed with FuGene6 (Roche) according to the manufacturers' specifications. Mammalian cell extracts were prepared and co-immunoprecipitations were performed as previously described,[23] in the presence or absence of 100 ng/μl ethidium bromide. Anti-FLAG M2 (Sigma) or anti-HA Clone 12CA5 (Roche) monoclonal antibodies were used for co-immunoprecipitation experiments along with protein A/G-sepharose (Amersham Biosciences). Anti-ATM/ATR substrate (phospho-Ser/Thr antibody) (Cell Signalling Technology) was used to evaluate the phosphorylation of HMGA proteins by ATM *in vivo*. Proteins were resolved by 3–8% (v/v) NuPAGE (Invitrogen) or by 15% (v/v) SDS–PAGE and transferred to high or low molecular weight nitrocellulose, respectively. Western blots were performed using the above-mentioned anti-FLAG and anti-HA antibodies, or anti-ATM polyclonal antibody (Novus Biologicals) and anti-HMGA1 polyclonal antibody (raised against the amino-terminal region of the protein). All the antibodies were diluted (1:200) in 5% (w/v) non-fat dry milk (Biorad).

2.4. In vitro kinase assays

For *in vitro* kinase assays, the ATM kinase was obtained by immunoprecipitation with an anti-FLAG antibody from 293T transfected cells or with an anti-ATM antibody (Novus Biologicals) from human lymphoblasts. Beads were washed twice with buffer A, twice with buffer A containing 0.5 M LiCl and twice with kinase buffer as previously described.[19] ATM kinase reactions were carried out at 30 °C for 15 min in 50 μl of kinase buffer containing 10 μCi of [γ-^{32}P]ATP and 10 μg of each substrate (for recombinant proteins) or 50 mM of cold ATP and 200 μM of each substrate (for peptides). 5 mM caffeine (Sigma) was used as a specific kinase inhibitor. Following the kinase reaction, HMGA1 peptides (produced by Neosystem) were spotted onto P81 phosphocellulose squares (Upstate) and washed extensively with 1% orthophosphoric acid. ^{32}P incorporation was measured by a β-counter scintillator (Beckman Coulter). Recombinant proteins, instead, were resolved by 15% SDS–PAGE and phosphorylation was revealed by autoradiography.

2.5. Immunofluorescence microscopy

Wild-type and *Hmga1* –/– MEFs were grown on glass coverslips, then fixed for 20 min in 4% (v/v) paraformaldehyde in PBS following mock or γ-irradiation. Cells were then permeabilised with 0.2% (v/v) Triton X-100 in PBS for 5 min at room temperature and blocked in PBS containing 1% (w/v) BSA for 20 min. Cells were incubated with primary antibodies for 1 h at room temperature in a humidified chamber, then washed with PBS and incubated for an additional hour with the secondary antibody. Primary antibodies used for immunostaining were: goat anti-HMGA1b sc-1564 (Santa Cruz), rabbit anti-HMGA1 (raised against the amino-terminal region of the protein), rabbit anti-ATM S1981p (Rockland) and mouse anti-γH2AX (Cell Signaling). As secondary antibodies we used anti-goat rhodamine-conjugated, anti-rabbit fluorescein-conjugated and anti-mouse rhodamine-conjugated (Jackson ImmunoResearch). All the antibodies were diluted (1:100) in PBS.

2.6. FACS analysis

Cells were plated in 6-well plates at a density of 2.5×10^5/well, incubated for 24 h and then exposed to a range of ionising radiations (0–10 Gy). Before harvesting at different timepoints following IR (0, 12, 24, 48 h), cells were pulsed for an hour with 10 μM BrdU (BrdU Flow kit, BD Pharmingen), then fixed and stained with a FITC-anti-BrdU antibody according to the manufacturers' instructions. 7-Amino-actinomycin D (7-AAD) fluorescent dye was used to label DNA. Cells were sorted on a FACScan flow cytometer (Becton Dickinson), and the results were analysed with FlowJo software, 4.3 version (TreeStar).

2.7. Clonogenic assay

Cell lines were plated in 6-well plates at a density of 300–800 cells/well, incubated for 24 h and then exposed to a range of doses of ionising radiations (0–10 Gy), followed by a two-week incubation. Prior to counting colonies, cells were fixed and stained with crystal violet. Populations of more than 50 cells were scored as surviving colonies. The mean of colony counts normalised for plating efficiency is reported.

3. Results

3.1. ATM and HMGA1 proteins interact in vivo

To determine whether ATM and HMGA1 interact *in vivo*, we transiently transfected 293T cells with expression vectors containing the full-length cDNAs for *ATM* and *HMGA1b* genes fused to the FLAG or HA tag, respectively. Total cell lysates were immunoprecipitated using an anti-HA antibody and analysed by immunoblotting with an anti-ATM antibody. A band corresponding to FLAG-ATM was effectively co-immunoprecipitated only in cells expressing HA-HMGA1b demonstrating that the two proteins are able to interact *in vivo* (Fig. 1a). Moreover, HA-HMGA1b is able to co-immunoprecipitate also the endogenous ATM protein, which is highly expressed in 293T cells[21] (Fig. 1a, middle lane). Since both ATM and HMGA1 are chromatin-associated proteins, we performed a co-immunoprecipitation experiment in the presence of ethidium bromide to exclude that their co-immunoprecipitation may be dependent on contaminating DNA (Fig. 1a).

The reverse co-immmunoprecipitation was carried out transfecting 293T cells with a vector containing the wild-type *HMGA1b* cDNA along with the FLAG-ATM-wt vector or a kinase-dead FLAG-ATM-kd mutant, in which the catalytic activity is impaired.[24] The analysis by immunoblot revealed that FLAG-ATM-wt is also able to co-immunoprecipitate the

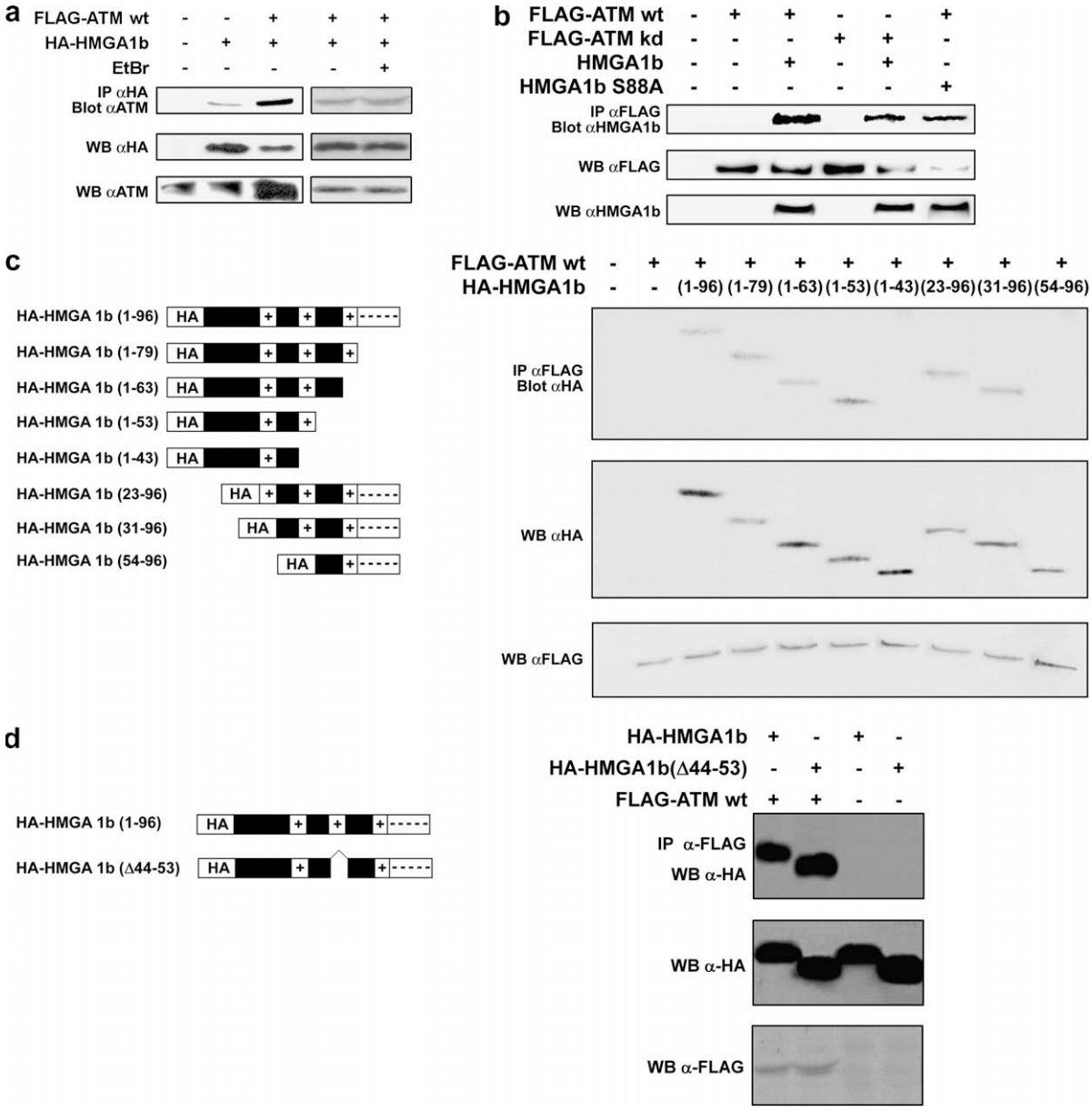

Fig. 1 – *HMGA1b and ATM protein interaction.* **(a) 293T cells were transiently transfected with a vector expressing HA-HMGA1b, alone or along with a FLAG-ATM expression vector. Total cell lysates were immunoprecipitated with anti-HA antibody and blotted with anti-ATM antibody (upper panel). To determine protein levels, Western blots were performed with anti-HA antibody (middle panel) and with anti-ATM antibody (lower panel). Immunoprecipitation in the presence of EtBr was also performed as a control of the specificity of the protein–protein interaction. (b) 293T cells were transiently transfected with vectors expressing FLAG-ATM, wild-type (wt) or kinase-dead (kd), alone or in combination with a vector expressing HMGA1b wild-type or HMGA1b S88A. Total cell lysates were immunoprecipitated with anti-FLAG antibody and blotted with anti-HMGA1b antibody (upper panel). To determine protein levels, Western blots were performed with anti-FLAG antibody (middle panel) and with anti-HMGA1b antibody (lower panel). (c) 293T cells were transiently transfected with a vector expressing FLAG-ATM wt alone or along with vectors expressing either the wild-type form of HMGA1b (1-96) or deletion mutants spanning several regions of the HMGA1b protein as shown in the diagram (AT-hooks (+) and the acidic tail (-----) are indicated). Total cell lysates were immunoprecipitated with anti-FLAG antibody and blotted with anti-HA antibody (upper panel). To determine protein levels, Western blots were performed with anti-HA antibody (middle panel) and with anti-FLAG antibody (lower panel). (d) 293T cells were transiently transfected with vectors expressing the wild-type form of HMGA1b or a deletion mutant (Δ44–53) lacking the second AT-hook, as shown in the diagram. Cells were also transfected with a vector expressing ATM wt and compared to cells not transfected. Total cell lysates were immunoprecipitated with anti-FLAG antibody and blotted with anti-HA antibody (upper panel). To determine protein levels, Western blots were performed with anti-HA antibody (middle panel) and with anti-FLAG antibody (lower panel).**

HMGA1b protein (Fig. 1b). Mutations of the ATM catalytic domain do not seem to impair the interaction since the FLAG-ATM-kd mutant retains its ability to co-immunoprecipitate HMGA1b (Fig. 1b). Likewise, mutation of the putative ATM target site on HMGA1b, serine 88 to alanine, (see below), does not affect the interaction (Fig. 1b).

3.2. *The presence of at least two AT-hook domains of HMGA1 is necessary for its interaction with ATM*

To identify the region of HMGA1 required for ATM binding, we used a series of amino- or carboxy-terminal deletion mutants of the HMGA1 proteins, fused to the HA tag (Fig. 1c). 293T cells were transiently transfected with each HMGA1 mutant along with a FLAG-ATM-wt expression vector. Total cell lysates were then immunoprecipitated with an anti-FLAG antibody and analysed by immunoblotting using an anti-HA antibody. Neither the progressive carboxy-terminal (1–79, 1–63, 1–53) nor the amino-terminal (23–96, 31–96) deletion mutants of HMGA1 showed reduced ability to co-immunoprecipitate ATM, compared with the full-length protein (1–96) (Fig. 1c). Conversely, no interaction was observed between ATM and the HMGA1 mutants 1–43 and 54–98, both containing just one AT-hook domain and both lacking the second AT-hook (Fig. 1c). To evaluate whether the second AT-hook domain of HMGA1 is required for the interaction, we generated a HMGA1 mutant lacking the second AT-hook domain (Δ44–53) and tested its ability to interact with FLAG-ATM. As shown in Fig. 1d, this HMGA1 mutant retains the ability to interact with ATM, indicating that the presence of at least two AT-hook domains, rather than just the second AT-hook, is required for HMGA1–ATM interaction.

3.3. *HMGA1 is phosphorylated by ATM in vitro and in vivo*

Since HMGA proteins are extensively post-translationally modified and phosphorylation has been frequently reported,[26–28] we decided to investigate whether HMGA1 is targeted by ATM kinase activity. By sequence analysis we found that HMGA1 contains in its COOH terminal region a consensus site for ATM phosphorylation, an SQ motif (serine 88/glutamine 89), which is highly conserved amongst different species and the different HMGA family members (Fig. 2a).

We then tested a 20 amino acid peptide, corresponding to the HMGA1 acidic tail (aa 77–96), which contains serine 88, as a substrate for ATM kinase activity. The endogenous wild-type ATM kinase was immunoprecipitated from the total protein extract of the human lymphoblastoid cell line GM2184. Before harvesting, cells were treated with a 10 Gy dose of IR to increase ATM kinase activation as previously described.[21] As shown in Fig. 2b, ATM was able to phosphorylate *in vitro* the C-terminal peptide of HMGA1. The phosphorylation was highly specific as it was strongly inhibited by 5 mM caffeine (1,3,7-trimethylxanthine), an ATM/ATR specific inhibitor,[29] and was impaired from the substitution of serine 88 with an alanine residue (Fig. 2b).

We then assayed the ability of ATM to phosphorylate the full-length HMGA1 recombinant protein, fused to a six-histidine tag. ATM kinase and its kinase-dead mutant were immunoprecipitated from transiently transfected 293T cells. HMGA1 was strongly phosphorylated by the FLAG-ATM-wt kinase and only to a lesser extent by the FLAG-ATM-kd mutant, in which the kinase activity is impaired. In both cases, the phosphorylation was strongly decreased by 5 mM caffeine and when serine 88 of HMGA1 was substituted by alanine (Fig. 2c, upper panel). The same amount of ATM and ATM kinase-dead proteins was used for the assays (Fig. 2c, lower panel). The above data indicate that HMGA1 phosphorylation by ATM is specific, as it is inhibited by both caffeine and a mutation impairing ATM kinase activity. Interestingly, some kinase activity was obtained when using an antibody cross-reacting with both ATM and ATR to immunoprecipitate extracts from lymphoblasts wild-type for ATM (GM2184) or ATM null (AT1526), respectively. In fact, a strong phosphorylation occurred to HMGA1 when the kinase was obtained from GM2184 cells (Fig. 2d, upper panel) but a fainter phosphorylation also occurred when the kinase was immunoprecipitated from AT1526 cells (Fig. 2d, middle panel). Western blot analysis using an antibody against ATR confirmed that the endogenous kinase was effectively immunoprecipitated from AT1526 cells (data not shown). Therefore, it is likely that also the ATR kinase, another member of the PIKKs family, has the ability to phosphorylate serine 88 of HMGA1. Phosphorylation was inhibited in the HMGA1 mutant in which serine 88 is mutated to alanine and in the HMGA1 mutant 1–79, that lacks the C-terminal of the protein containing serine 88. Phosphorylation was inhibited by 5 mM caffeine as well (Fig. 2d). The Coomassie staining indicates the amount of recombinant proteins used as substrates for the kinase assays (Fig. 2d, lower panel).

To verify that HMGA1 is effectively a substrate of ATM kinase activity in response to DNA damage *in vivo*, we treated HA-HMGA1-transfected 293 cells with a 10 Gy dose of IR to induce endogenous ATM activation. Cells were collected 5 min after treatment and extracted proteins immunoprecipitated with antibodies that recognise serine and threonine residues phosphorylated by ATM (Anti-P-Sub-ATM). Analysis of the immunoprecipitates through Western blot with an anti-HA antibody showed a band corresponding to the HA-HMGA1 protein, indicating that, following IR exposure, HMGA1 is phosphorylated *in vivo* by ATM (Fig. 2e). The specificity of HMGA1 phosphorylation by ATM was assessed by the absence of HMGA1 immunoprecipitation in the presence of 5 mM caffeine (Fig. 2e).

3.4. *Colocalisation of HMGA1b and ATMS1981p*

To support further the hypothesis that HMGA1b is a target of the ATM kinase we investigated whether the two proteins colocalise to the same regions of the nuclear compartment by double-immunofluorescence labelling. Mouse embryonic fibroblasts (MEFs), wild-type for both *ATM* and *Hmga1* genes, were either treated or not treated with a 2 Gy dose of IR. Following double staining with antibodies against HMGA1b (red channel) and against the activated, phosphorylated (serine 1981) form of ATM, ATMS1981p, (green channel), cells were analysed by confocal microscopy (Fig. 3a). As expected, ATM kinase was massively activated after irradiation and, intriguingly, it partially colocalises with the endogenous HMGA1b

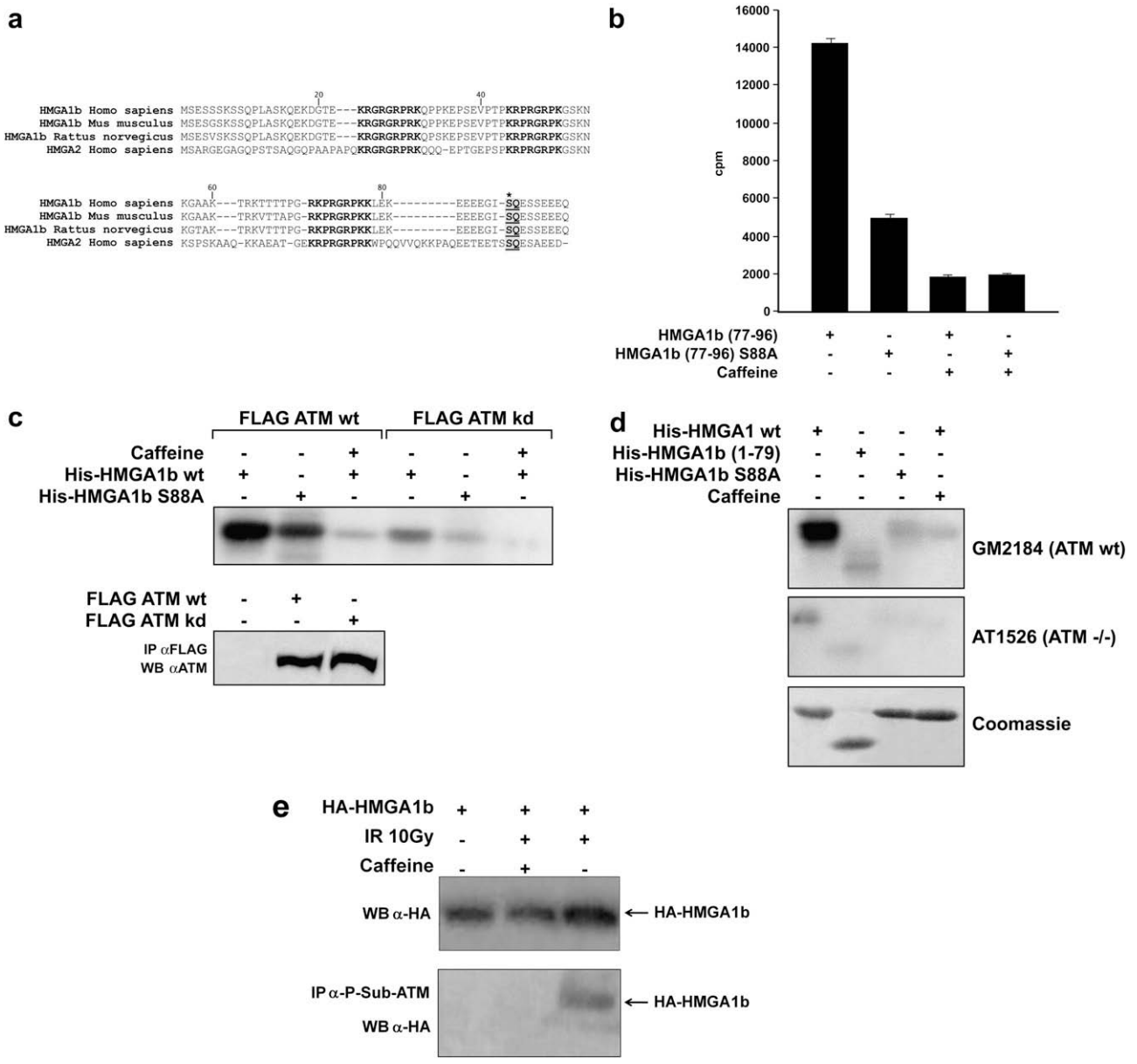

Fig. 2 – *HMGA1 phosphorylation by ATM kinase.* **(a) Amino acid sequence alignment of HMGA1b and HMGA2 from the indicated species (AT-hooks are shown in bold, ATM consensus site is underlined and serine 88 is marked with an asterisk). (b) *In vitro* kinase assay testing ATM kinase activity on the HMGA1 carboxy-terminal peptide (aa 77–96) wild-type or mutated (S88A). Phosphorylation was assessed with or without caffeine (5 mM). A representative experiment (out of three different ones, each performed in duplicate) is shown. (c) *In vitro* kinase assays using FLAG-ATM wild-type (wt) or its kinase-dead (kd) mutant on the histidine-tagged HMGA1b recombinant proteins both wild-type (with or without 5 mM caffeine) and mutated in the putative ATM consensus motif (S88A). Following the kinase reaction, proteins were resolved by SDS–PAGE and the autoradiography of the dried gel is shown (upper panel). FLAG-ATM wt and kd were immunoprecipitated from transiently transfected 293T cells with anti-FLAG antibody. One fifth of each immunoprecipitate was probed with anti-ATM antibody (lower panel). (d) *In vitro* kinase assays were performed using the endogenous ATM kinase immunoprecipitated from human GM2184 lymphoblasts or from AT1526 (ATM –/–) as negative control. Kinase activity was assayed on the histidine-tagged HMGA1b recombinant proteins either wild-type (with or without 5 mM caffeine), mutated in serine 88 or deleted of the carboxy-terminal tail containing serine 88. Following the kinase reaction, proteins were resolved by SDS–PAGE and the autoradiography of the dried gel is shown (upper and middle panels). Similar amounts of recombinant proteins were used for the assay as shown by Coomassie staining (lower panel). (e) 293T cells transfected with a vector expressing HMGA1b were untreated or treated with a 10 Gy dose of IR to induce activation of endogenous ATM. Total cell extracts were immunoprecipitated with an antibody able to recognise serine and threonine residues phosphorylated by activated ATM (Anti-P-Sub-ATM). Immunoprecipitates were analysed by SDS–PAGE followed by Western blot with anti-HA antibody. 293T cells were also treated with 5 mM caffeine as a control of the phosphorylation specificity.**

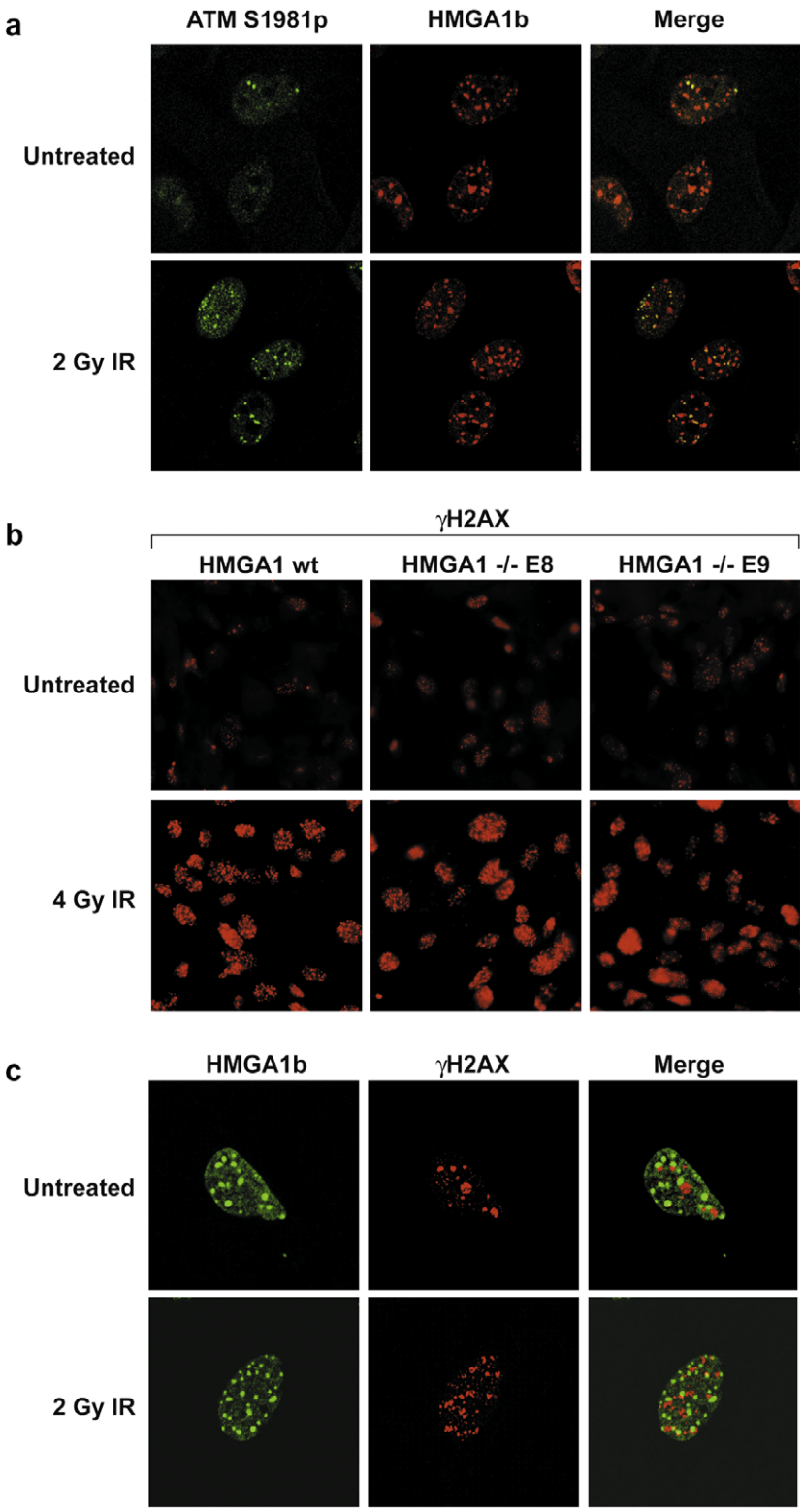

Fig. 3 – *Colocalisation of HMGA1, ATM and γH2AX foci.* (a) HMGA1 colocalises with the ATM active kinase. MEFs were untreated or treated with a 2 Gy dose of IR, and then analysed by confocal microscopy following double-immunofluorescence staining with antibodies against ATM S1981p (green channel) and HMGA1b (red channel). (b) γH2AX foci formation in *Hmga1* null MEFs. MEFs wt and –/– for *Hmga1* were untreated or treated with a 4 Gy dose of IR. Immunofluorescence staining is with an antibody against γH2AX (red channel). (c) HMGA1b does not colocalise with γH2AX foci. Wild-type MEFs were untreated or treated with a 2 Gy dose of IR, and then analysed by confocal microscopy following double-immunofluorescence staining with antibodies against HMGA1b (green channel) and γH2AX (red channel).

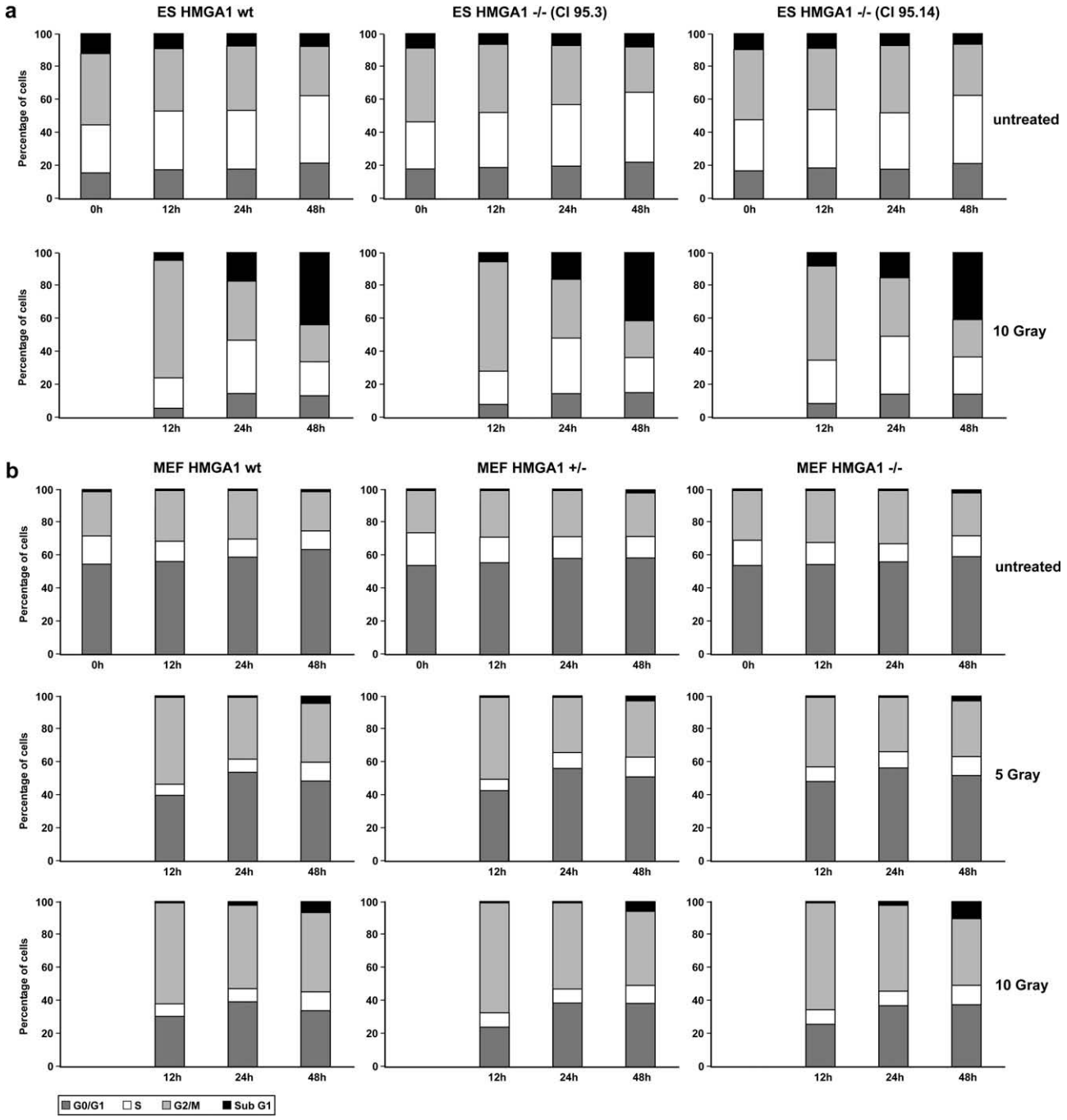

Fig. 4 – *Cell cycle checkpoints are not impaired in Hmga1 null cells following IR.* (a) ES *Hmga1* wt or *Hmga1* –/– (Clones 95.3 and 95.14) were either untreated or treated with a 10 Gy dose of IR. Cells were harvested at different timepoints following BrdU treatment and stained with a FITC-conjugated anti-BrdU antibody and 7-AAD. Cell cycle profiles were obtained by FACS analysis and the distribution of the cells in each different phase is reported from a representative experiment (out of three different ones, each performed in duplicate). (b) MEFs from *Hmga1* wild-type, +/– and –/– embryos were either untreated or treated with doses of 5 and 10 Gy IR. Cells were harvested at different timepoints following BrdU treatment and stained with a FITC-conjugated anti-BrdU antibody and 7-AAD. Cell cycle profiles were obtained by FACS analysis and the distribution of the cells in each different phase is reported from a representative experiment (out of two different ones, each performed in triplicate).

protein (Fig. 3a, merge), both when activated in untreated cells and when activated by γ-irradiation. This colocalisation provides additional evidence that HMGA1b may act *in vivo* as a substrate of the functional ATM kinase.

3.5. *HMGA1 does not localise with IR-induced γH2AX foci*

The phosphorylation of histone H2AX (γH2AX) is amongst the earliest responses to DNA damage, and it is considered the

earliest detectable marker for DSBs.[30] Since many proteins involved in DNA repair quickly relocalise to the γH2AX nuclear foci[31], we sought to investigate first whether γH2AX effectively forms foci in *Hmga1* null cells, then if HMGA1 relocalises to the γH2AX foci following DNA damage. Mouse embryonic fibroblasts (MEFs) wild-type or null for the *Hmga1* gene (*Hmga1* –/– E8 and E9) were either untreated or exposed to a 4 Gy dose of IR and after 3 h fixed and stained with an antibody against the phosphorylated form of histone H2AX. Immunofluorescence showed that, following IR treatment, γH2AX foci are effectively induced in *Hmga1* –/– as in wild-type cells (Fig. 3b, red channel).

To assess if HMGA1 is recruited to the same DSBs sites where γH2AX acts, we treated wild-type MEFs with a 2 Gy dose of IR. After three hours IR-induced DNA damage cells were fixed and double labelled with antibodies against HMGA1b (green channel) and γH2AX (red channel). Confocal microscopy revealed that in mouse embryonic fibroblasts HMGA1b does not localise with IR-induced γH2AX foci at least at the dose and timepoint used (Fig. 3c).

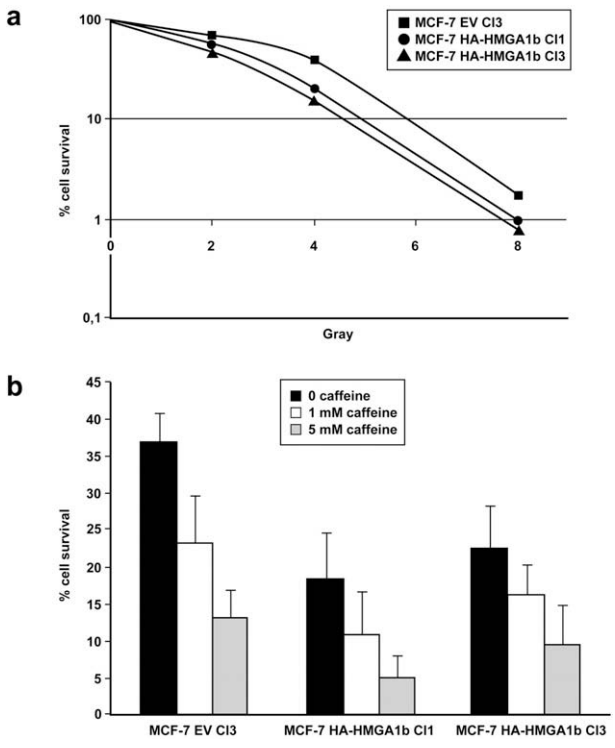

Fig. 5 – *Cell survival is decreased in HMGA1b expressing MCF-7 cells following IR*. (a) Clonogenic survival assay of the indicated MCF-7 clones exposed to 0, 2, 4, 8 Gy of IR. The percentage of survival is reported from a representative experiment (out of three independent ones, each performed in triplicate). (b) Clonogenic survival assay of MCF-7 clones exposed to a 4 Gy dose of IR and treated with the indicated amount of caffeine. The percentage of survival represents the mean of three different experiments (+s.d.), each performed in triplicate.

3.6. *Cell cycle checkpoints are not impaired in Hmga1 null cells following IR*

The ATM-mediated pathway is responsible for the activation of cell cycle checkpoints following DNA damage. The resulting process allows the proper assembly of the DNA repair machinery. To investigate whether HMGA1 might be involved in this pathway, we analysed the cell cycle profile of mouse embryonic stem (ES) cells or fibroblasts (MEFs) null for *Hmga1* in response to IR.

ES cells devoid of the feeder fibroblasts were exposed to a 10 Gy dose of IR and harvested at different timepoints (0, 12, 24 and 48 h) after 1 h of 5-bromo-2-deoxyuridine (BrdU) treatment (Fig. 4a). At 12 h, following IR treatment, both *Hmga1* –/– clones (95.3 and 95.14) and wild-type ES cells accumulate in G2/M. At 24 h cells restarted cycling or underwent apoptosis that was massive at 48 h. Anyway, no significant differences were observed between wild-type and *Hmga1* –/– cells at least at the IR dose tested.

We also analysed cell cycle profiles of MEFs obtained from *Hmga1* wild-type, +/– and –/– embryos at 14.5 dpc. MEFs at early passages were exposed to doses of 5 and 10 Gy IR, then treated with BrdU and analysed at 12, 24 and 48 h (Fig. 4b). At 12 h MEFs of all three genotypes arrested in G2/M in a dose-dependent manner. At 24 and 48 h, the block was slowly released and only a small percentage of cells underwent apoptosis (less than 10%). As for the ES cells we did not observe any statistically significant difference between wild-type or *Hmga1* null cells at the IR doses and timepoints analysed. However, even though it seems that lack of HMGA1 does not affect the ability of ES cells and MEFs to activate cell cycle checkpoints following IR, we cannot rule out the possibility that the other member of the HMGA family, HMGA2, might compensate for HMGA1 loss.

3.7. *Cell survival is decreased in HMGA1b expressing MCF-7 cells following IR*

Cells defective in genes involved in the response to DNA damage usually show an altered long-term survival following exposure to the damaging agent. Therefore, we sought to investigate whether HMGA1 was able to affect cell survival following IR treatment. To this aim we used a different cellular system such as the human breast cancer cell line MCF-7, in which neither *HMGA1* nor *HMGA2* genes are expressed. Moreover, HMGA1b expression has been previously shown to sensitise MCF-7 cells to damage-induced by UV and cis-platin treatment.[12] We compared two different clones of MCF-7 stably transfected with an HMGA1b expressing vector to the control cells, transfected with the empty vector (EV). Cells were exposed to doses of 2, 4 and 8 Gy of IR and after 2 weeks clonogenic survival was evaluated by colony counting. Both HMGA1b expressing clones (MCF-7 HA-HMGA1b Cl1 and Cl3) showed a decrease in the percentage of cell survival compared to the control MCF-7 EV Cl3 (Fig. 5a). Interestingly, this response was highly reproducible and described also in response to the radiomimetic antibiotic bleomycin.[12] To assess whether the enhanced radiosensitivity of HMGA1b expressing cells was correlated to the ATM/ATR pathway, cells were exposed to a 4 Gy IR dose, treated with two different

doses of caffeine (1 and 5 mM, respectively) and analysed after two weeks. Caffeine treatment effectively enhanced cell radiosensitivity in a dose-dependent manner, but no significant differences were observed between HMGA1b expressing MCF-7 clones and MCF-7 EV Cl3 control cells (Fig. 5b).

4. Discussion

Recently, several works correlated HMGA expression to enhanced cell sensitivity in response to different DNA-damaging agents.[5,16,17] Here, we report a novel interaction between the HMGA1 family member and the ATM protein kinase, the major key player in the activation of the cellular response aimed to safeguard genome integrity following DNA damage. We show that HMGA1b and ATM are able to co-immunoprecipitate in 293T cells and that at least two AT-hook domains of HMGA1 are necessary for this interaction. Since ATM phosphorylates its substrates on serine or threonine that precede a glutamine residue, we looked for the presence of these S/TQ motifs on the HMGA protein sequences. Interestingly, both the HMGA1 and the HMGA2 members of the HMGA family present such motifs in the carboxy-terminal region of the protein, and this SQ motif also appears to be highly conserved amongst different species making it tempting to speculate that it may be crucial to the protein function. Subsequently, we demonstrated that HMGA1 is indeed an ATM target *in vitro* using both the HMGA1 C-terminal peptide and the full-length protein. HMGA1 phosphorylation was strongly reduced when using caffeine or the ATM kinase-dead mutant. Moreover, we showed that HMGA1 phosphorylation was indeed site-specific since it was abolished when serine 88 was mutated to alanine. Intriguingly, we found trace HMGA1b phosphorylation when immunoprecipitating extracts from ATM –/– cells with an antibody cross-reacting with the ATR kinase. It is, therefore, likely that HMGA1 might be a target also of the ATR kinase that shares with ATM the same consensus sequence and several substrates,[18] and it has been recently shown to be activated by ATM following IR.[32]

Finally, using an antibody raised against a pool of ATM/ATR substrates to immunoprecipitate HMGA1b after IR exposure, we also demonstrated that ATM phosphorylates HMGA1b *in vivo*. Accordingly, this phosphorylation was inhibited by caffeine.

Following DNA damage, in fact, ATM is activated through autophosphorylation at serine 1981 resulting in the dissociation of inactive dimers.[21] The active ATM monomers in part are free to move throughout the nucleus and in part are recruited at the site of DNA damage.[18] The M/R/N complex appears to be responsible for the initial recruitment of ATM at DSBs sites,[33–35] then MDC1 has been recently shown to bind γH2AX and retain active ATM on the chromosome regions adjacent to the break sites,[36] leading to further expansion of H2AX phosphorylation. The phosphorylation of H2AX by ATM is amongst the earliest responses to DSBs and controls the accumulation of checkpoint/repair proteins to large chromatin regions surrounding DNA damage sites. γH2AX has been proposed to function as a docking protein for the retention of the DNA-damage response factors,[37] or it may modulate the chromatin structure to facilitate the accumulation of checkpoint proteins.[37] We observed efficient formation of γH2AX foci following IR in *Hmga1* –/– MEFs. Interestingly, as assessed by immunofluorescence and confocal microscopy there is no colocalisation between the HMGA1b and γH2AX proteins. Since HMGA and H2AX are both chromatinic proteins involved in chromatin remodelling it is tempting to speculate that HMGA1 phosphorylation might serve to displace HMGA and favour assembly of H2AX in the regions of DNA damage. This may be consistent with the recent finding that HMGA2 interferes with the basal H2AX phosphorylation mechanism,[17] but additional studies are needed to further address this issue.

In an attempt to gain insight into the physiological role of the HMGA1 and ATM interaction, we decided to investigate whether IR-induced cell cycle checkpoint activation was somehow impaired in cells lacking the *Hmga1* gene. Following the DNA damage, ATM triggers a complex regulatory pathway aimed to allow for DNA repair before proceeding through the cell cycle, and many proteins involved in this pathway show defects in IR-induced checkpoints, MDC1,[38] H2AX and 53BP1,[37] CHK1[39] and CHK2.[40,41] However, following IR treatment, both *Hmga1* –/– ES cells and MEFs did not show any defects in cell cycle checkpoint activation relative to wild-type cells under the conditions used in this study. One possible explanation may lie in that both ES cells and MEFs express the HMGA2 member of the HMGA family, which may compensate for HMGA1 loss. As a result, we are generating *Hmga1*/*Hmga2* double knock-out alleles in mice that will attempt to address this issue. As an alternative means to investigate this question, we used the human MCF-7 tumour-derived cell line that does not express either HMGA family members and analysed the effect of ectopic HMGA1b overexpression following IR-induced DNA damage. Interestingly, clonogenic survival following IR treatment was decreased in the HMGA1b expressing MCF-7 clones. Consistently, the same effect has been described in response to the radiomimetic antibiotic bleomycin,[12] suggesting that HMGA1 proteins play a role in the cellular response to DSBs-causing agents. It is not yet clear whether the ATM signalling pathway regulates HMGA1 in this context, since ATM inhibition by caffeine does not rescue survival of HMGA1b expressing cells.

In conclusion, we report a novel interaction between the HMGA1 and the ATM kinase and, whilst the physiological role of this interaction remains to be assessed, we propose that HMGA1 should be regarded as a potential ATM-downstream target. These findings are likely to contribute to our understanding of HMGA1 function in the mechanism of tumourigenesis and reveal new insights into potential therapeutic strategies. In fact, HMGA1 expression enhances sensitivity of breast cancer cells to ionising radiation (as reported here) or to other DNA-damaging agents such as bleomycin, cisplatin and UV irradiation.[12,16] Therefore, patients bearing HMGA1 expressing tumours might benefit from adjuvant therapy based on such DNA-damaging agents.

Conflict of interest statement

All the authors disclose any actual or potential conflict of interest including any financial, personal or other relation-

ships with other people or organisations that could inappropriately influence their work.

Acknowledgements

We are very grateful to Dr. M.B. Kastan for providing the FLAG-ATM wild-type and kinase-dead constructs. We thank Dr. G. Viglietto and Dr. F. Carlomagno for technical hints and valuable suggestions. This work was supported by grants from the Associazione Italiana Ricerca sul Cancro (AIRC), Progetto Strategico Oncologia, Consiglio Nazionale delle Ricerche, the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MIUR). This work was supported from NOGEC-Naples Oncogenomic Center. We thank the Associazione Partenopea per le Ricerche Oncologiche (APRO) for its support.

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