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Constitutive phosphorylation of ATM in lymphoblastoid cell lines from patients with ICF syndrome without downstream kinase activity

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ABSTRACT

Double strand DNA breaks in the genome lead to the activation of the ataxia-telangiectasia mutated (ATM) kinase in a process that requires ATM autophosphorylation at serine-1981. ATM autophosphorylation only occurs if ATM is previously acetylated by Tip60. The activated ATM kinase phosphorylates proteins involved in arresting the cell cycle, including p53, and in repairing the DNA breaks. Chloroquine treatment and other manipulations that produce chromatin defects in the absence of detectable double strand breaks also trigger ATM phosphorylation and the phosphorylation of p53 in primary human fibroblasts, while other downstream substrates of ATM that are involved in the repair of DNA double strand breaks remain unphosphorylated. This raises the issue of whether ATM is constitutively activated in patients with genetic diseases that display chromatin defects. We examined lymphoblastoid cell lines (LCLs) generated from patients with different types of chromatin disorders: Immunodeficiency, Centromeric instability, Facial anomalies (ICF) syndrome, Coffin Lowry syndrome, Rubinstein Taybi syndrome and Fascioscapulohumeral Muscular Dystrophy. We show that ATM is phosphorylated on serine-1981 in LCLs derived from ICF patients but not from the other syndromes. The phosphorylated ATM in ICF cells did not phosphorylate the downstream targets NBS1, SMC1 and H2AX, all of which require the presence of double strand breaks. We demonstrate that ICF cells respond normally to ionizing radiation, ruling out the possibility that genetic deficiency in ICF cells renders activated ATM incapable of phosphorylating its downstream substrates. Surprisingly, p53 was also not phosphorylated in ICF cells or in chloroquine-treated wild type LCLs. In this regard the response to chromatin-altering agents differs between primary fibroblasts and LCLs. Our findings indicate that although phosphorylation at serine-1981 is essential in the activation of the ATM kinase, serine-1981 phosphorylation is insufficient to render ATM an active kinase towards downstream substrates, including p53.

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1. Introduction

Surveillance systems in the cell detect DNA damage of many forms, including double strand breaks (DSBs) leading to the activation of checkpoints that arrest the cell cycle to allow time for DNA repair [1,2]. In mammalian cells, checkpoint activation by DSBs requires the 370 kDa protein kinase ataxia-telangiectasia mutated (ATM) [2]. ATM is lacking in patients with ataxia-telangiectasia (A-T) [3]. This disease is a rare autosomal-recessive inherited disorder characterized by radiation sensitivity, cell cycle abnormalities and genomic instability [3,4]. In wild type cells, Tip60 responds to DSBs by acetylating kinase inactive ATM [5]. ATM then autophosphorylates at serine-1981 to produce the kinase active ATM-s1981(P) that, in turn, phosphorylates several proteins [6]. Evidence that autophosphorylation at serine-1981 plays a role in ATM activation in vivo, was obtained by mutating the serine-1981 residue to an alanine. This mutation disrupted γ-irradiation (IR)-induced ATM autophosphorylation as well as the phosphorylation by ATM of downstream substrates [6].

DSB-activated ATM-s1981(P) phosphorylates a series of proteins that function in cell cycle arrest and in DNA repair [7–9]. Phosphorylation of p53 at serine-15 (p53-s15(P)) [10–12] either signals for cell cycle arrest or for apoptosis (for review see Ref. [13]). ATM is also recruited to the DSBs [14] in a process that requires the MRE11/Rad50/NBS1 (MRN) complex [15,16], which binds directly to the DSBs [17,18] and processes the damaged DNA ends [17,19]. Interaction with the MRN complex stimulates ATM autophosphorylation and downstream kinase activity [16,20–22]. There is additional evidence that ATM is also activated by a parallel pathway involving 53BP1 [21] that binds methylated lysine-79 of histone H3 at DSBs [23]. The localization of ATM to DSBs correlates with the phosphorylation of several additional proteins by ATM-s1981(P) that are involved in DNA repair and/or cell cycle checkpoints, including NBS1 at serine-343 (NBS1-s343(P)) [24–27] and SMC1 at serine-957 or serine-966 (SMC1-s966(P)) [28–30], and the histone variant H2AX at serine-139 to produce γ-H2AX [31,32]. γ-H2AX accumulates at the double strand breaks in megabase-sized regions that can be visualized as foci using immunofluorescence [33].

It was reported that ATM serine-1981 autophosphorylation occurs in human primary fibroblasts in response to conditions that alter chromatin but do not cause detectable double strand breaks [6]. The conditions were exposure of the cells to the topoisomerase inhibitor chloroquine, the histone deacetylase inhibitor trichostatin A or mild hypotonic conditions. These treatments also caused p53 phosphorylation at serine-15 [28]. None of the other ATM substrates examined (H2AX, NBS1 and SMC1) were phosphorylated under these conditions [6,28]. To reconcile this ATM activation with activation by DSBs, it was proposed that DSBs lead to a structural change in chromatin that signals ATM to be autophosphorylated and activated as a kinase [6]. It was further proposed that the ATM-s1981(P) kinase activated by chromatin-altering agents only phosphorylates p53 and ATM itself because these two proteins do not require the presence of DSBs to be phosphorylation substrates, whereas H2AX, NBS1 and SMC1 require recruitment to DSBs in order to be phosphorylated [6,28].

The finding that ATM is phosphorylated in response to chromatin-altering treatments raised the issue of whether ATM is constitutively in the kinase active ATM-s1981(P) state in cells from patients with mutations that cause chromatin defects. We chose to examine lymphoblastoid cell lines (LCLs) generated from patients with different types of chromatin disorders: Immunodeficiency, Centromeric instability, Facial anomalies (ICF) syndrome, Coffin Lowry syndrome (CLS), Rubinstein Taybi syndrome (RSTS) and Facioscapulohumeral Muscular Dystrophy (FSHD) (for detailed reviews see Refs. [34,35]). ICF syndrome is a rare autosomal recessive disease, caused by mutations in the de novo DNA methyltransferase 3b (DNMT3B) gene [36–38]. This mutation leads to DNA hypomethylation of a subset of repetitive sequences including the satellite regions in chromosomes 1, 9 and 16 [38] and the LINE-1 transposon sequences on the inactive X-chromosome [39]. Since *Dnmt3b−/−* mice die during embryogenesis [37], ICF patients are thought to be hypomorphs [36,40–42]. Cytologically, certain cell types, particularly primary lymphocytes, from ICF patients exhibit elongation of pericentromeric heterochromatin, primarily on chromosomes 1, 9 and 16, leading to genomic instability in these regions [43–47]. ICF cells have also been reported to display increased sensitivity to ionizing radiation, despite intact cell cycle checkpoints [48].

RSTS is a rare autosomal dominant disorder derived from a mutation of the CREB-binding protein, a histone acetyltransferase [49,50]. CLS is a rare, X linked disorder with a mutation in the gene encoding RSK-2, part of a family of growth factor-regulated serine/threonine kinases in the mitogen-activated protein kinase (MAPK) pathway [51,52]. Activated RSK-2 phosphorylates histone H3 and may also phosphorylate and activate CREB-binding protein [53,54]. Lastly, FSHD is an autosomal dominant disorder caused by deletions of integral copies of the tandemly repeated heterochromatic D4Z4 repeat unit [55] on chromosome 4. In normal individuals, this repeat unit varies between 11 and 150 copies, while patients exhibit a reduction of 1–10 copies [56]. Although the mechanism underlying FSHD is not clear, studies have shown that the normally methylated D4Z4 repeats [57] are hypomethylated in FSHD patients [58].

Herein, we report that ATM was constitutively phosphorylated at serine-1981 in non-irradiated cells from ICF patients but displayed little or no phosphorylation in the cells of patients with the other chromatin defects. ATM-s1981(P) in ICF cells was not associated with corresponding levels of double strand breaks and did not lead to phosphorylation of checkpoint and DNA repair proteins, including p53, which are downstream targets of the ATM kinase. Furthermore, we confirm that ICF cells have intact cell cycle checkpoints; however, in contrast to a recent report [48], we provide evidence that ICF cells respond normally to ionizing radiation. Our findings suggest that although ATM phosphorylation at serine-1981 plays an essential role in the activation of the kinase, event(s) in addition to this phosphorylation are required to render p53 and other downstream targets as phosphorylation substrates.

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2. Materials and methods

2.1. Cells and cell culture

Epstein Barr virus (EBV-) immortalized lymphoblastoid cell lines were purchased from Coriell Cell Repository (Camden, NJ): ICF patient GM08714A (female proband, ICF-1), her mother GM08728 (N-1) and father GM08729A (N-2). Unrelated ICF male LCLs, ICF-2 and ICF-3, were previously described as P5 [40] and P1[a] [36], respectively. LCLs from *ATM*−/− (A-T) cells previously described as AT203LA [59], while unrelated normal control LCL (N-3) is line Nat9 was from the collection of Richard A. Gatti. Other LCLs purchased from the Coriell Cell Repository were GM03317 (CLS) and GM13164 (RSTS-1). RSTS LCLs RT-2644 (RSTS-2) and RT-1271 (RSTS-3) were previously described [60]. The FHSD-1 and FHSD-2 LCLs were a generous donation by Dr. Sara T. Winokur (UC Irvine, CA). In some experiments, ICF-3 was not used due to the difficulty of growing this cell line. N-3 and A-T cells were used as positive and negative controls, respectively, for each experiment.

2.2. Western blot and antibodies

Nuclear protein lysates from each cell line were subjected to sodium dodecyl sulfate (SDS) polyacrylimide gel electrophoresis (PAGE), transferred to a charged PDVF membrane and immunoblotted with the appropriate antibody recognizing either native ATM (Novus, Littleton, CO), ATM-s1981(P) (Rockland, Gilbersville, PA), native SMC1 (Novus), SMC1-s966(P) (Novus), NBS1-s343(P) (Novus), Rad-50 (Novus), p53-s15(P) (Novus) or β-tubulin (Upstate, NY). Quantification of Western blots was done on with Typhoon 9410 (Amersham Biosciences) using ImageQuant 5.2 (Molecular Dynamic) software. Quantification of phosphorylated protein signals was determined by measuring the sum of pixels generated by each band and normalizing each band to the normal non-irradiated sample. Therefore, each graph depicts the fold phosphorylation signal over 1.0, the value given to the normal non-irradiated sample. Error bars represent the standard error of three independent repetitions of each experiment.

2.3. Chemicals used

Wortmannin (Calbiochem, CAS 19545-26-7) was resuspended in DMSO in a 10 mM stock concentration. The appropriate concentration was added to the cell culture for 1 h prior to irradiation. Chloroquine (Sigma, CAS 54-05-7) treatment was performed for 6 h as described [6,28].

2.4. Immunofluorescence microscopy

LCLs were harvested, washed once in PBS and dropped onto poly-D-lysine coated coverslips, and fixed with 4% paraformaldehyde, permeabilized using 0.5% Triton X-100 in PBS, then blocked with 10% fetal bovine serum in PBS. The LCLs were then treated with anti-H2AX-s139(P) (JBW301; Upstate) and with secondary antibody (donkey anti-mouse FTTC, Jackson ImmunoResearch). DAPI (1 μg/ml) in mounting media (Vectashield) was used as a DNA counter satin. Nuclei were visualized at 100× and 400× magnification using a Leica DM RXA automated microscope equipped with Photometrix SenSyn camera and Quips mFISH software (Vysis).

2.5. Colony survival assay (CSA)

This assay was conducted according to a protocol described by Sun et al. [61]. Briefly, LCLs were plated, in duplicate, in 96-well plates at 50, 100 or 200 cells per well. One plate was exposed to 1.0 Gy radiation, whereas the other was left non-irradiated. The cells were incubated for 10–13 days, at which time they were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye (tetrazolium-based colorimetric assay, Sigma). Each well was examined under the microscope; viable cells stained dark blue. The presence of a colony of >32 cells was scored as a positive well, and survival fractions (proportions of positive wells) were calculated. N-3 and A-T LCLs were included as positive and negative controls, respectively.

2.6. Radioresistant DNA synthesis (RDS)

RDS was performed as previously described [62]. LCLs were incubated with medium containing 0.04 μCi/ml ^{14}C thymidine (Perkin-Elmer; 1 Ci = 37 GBq) for 24 h. The medium was replaced with fresh media, and the cells were exposed to various doses of gamma rays. The cells were returned to the incubator for 60 min and pulse-labeled in medium containing 4 μCi/ml ^{3}H thymidine (Perkin-Elmer) for an additional 60 min. The samples were then harvested and counted in a Packard 2900TR scintillation counter. The ratio of incorporated ^{3}H to ^{14}C was used for quantification to standardize the variation in DNA recovery. Triplet replicates of each LCL were used to minimize the standard error of measurements.

3. Results

3.1. Chloroquine treatment triggers ATM phosphorylation in lymphoblastoid cell lines

It was previously reported that exposure of normal human primary fibroblasts to the chromatin-altering agent chloroquine triggers ATM phosphorylation at serine-1981 in the absence of detectable double strand breaks [6,28]. Fig. 1 reveals that chloroquine treatment of human LCLs similarly activated ATM phosphorylation. As in primary fibroblasts [28], the induction of ATM-s1981(P) by chloroquine was not accompanied by a corresponding increase in NBS1 phosphorylation, an indicator of double strand breaks (Fig. 1). Exposure of LCLs to high chloroquine concentrations expected to produce some DNA damage (100–200 μg/ml), resulted in ATM-s1981(P) levels that exceeded ATM-s1981(P) levels produced by 0.5 Gy of DNA damage-inducing IR. In contrast, the NBS1-s343(P) levels remained below the levels elicited by the IR (Fig. 1). We also examined p53 phosphorylation because in human primary fibroblasts 32–40 μg/ml chloroquine has been shown to elicit robust levels of p53-s15(P) that resemble the levels of p53-s15(P) generated by 0.5 Gy IR [6,28]. Surprisingly, 40 μg/ml of chloroquine brought forth little or no increase in p53 phosphorylation

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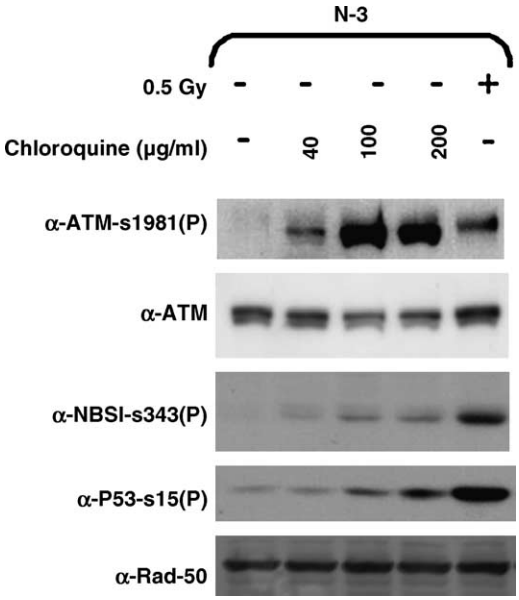

Fig. 1 – ATM is phosphorylated at serine-1981 in a normal human lymphoblastoid cell line (LCL) in response to chloroquine treatment. Normal (N-3) LCLs were either left untreated, subjected to increasing concentrations of chloroquine, or irradiated at 0.5 Gy. Nuclear extracts were resolved by SDS-PAGE and immunoblotted for ATM phosphorylated at serine-1981 (α-ATM-s1981(P)), total ATM (α-ATM), NBS1 phosphorylated at serine-343 (α-NBS1-s343(P)), p53 phosphorylated at serine-15 (α-p53-s15(P)) and Rad-50 (α-Rad-50) that served as a loading control. Low concentrations of chloroquine (40 μg/ml) induced ATM-s1981(P) at a level comparable to IR-treated LCLs. However, the ATM-s1981(P) arising in response to chloroquine was not accompanied by the corressponding increases in p53-s15(P) or NBS1-s343(P) displayed by the IR-treated samples. Higher concentrations of chloroquine (100 and 200 μg/ml) caused low levels of p53-s15(P) and NBS1-s343(P), indicating that these concentrations lead to DNA damage.

in LCLs (Fig. 1). Exposure of LCLs to 100 μg/ml chloroquine induced relatively low levels of p53-s15(P) that appeared to be roughly proportional to the levels of NBS1-s343(P) (Fig. 1). Consequently, the p53-s15(P):ATM-s1981(P) ratio was much higher in IR-treated samples than even the samples subjected to high chloroquine concentrations. We conclude first that chloroquine activates ATM phosphorylation in LCLs as it does in primary fibroblasts. Second, LCLs are not equivalent to primary fibroblasts in their response to chloroquine. Third, ATM phosphorylation at serine-1981, although essential in the activation of the ATM kinase [6], is insufficient to render ATM an active kinase towards p53, at least in LCLs.

3.2. *ATM is constitutively phosphorylated in non-irradiated ICF LCLs and this phosphorylation is inhibited by Wortmannin*

The observation that ATM is autophosphorylated at serine-1981 in response to the chromatin-altering agent chloroquine

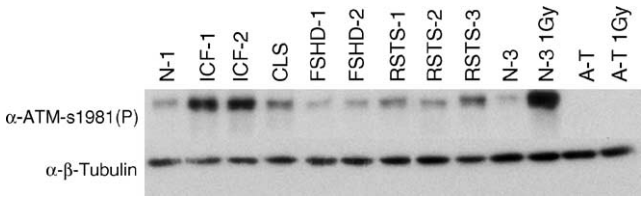

Fig. 2 – ATM is markedly phosphorylated at serine-1981 in non-irradiated LCLs from unrelated ICF patients (ICF-1 and ICF-2), but not from patients with RSTS, CLS, or FSHD, A-T, or normal cells (N-1 and N-3). Nuclear lysates from cells were immunoblotted for phosphorylation of ATM on serine-1981 (α-ATM-s1981(P)) and reprobed using an antibody recognizing β-tubulin that served as a loading control. N-1 is from the unaffected mother of ICF-1. An unrelated normal LCL (N-3) was tested before and after irradiation (1.0 Gy). Cells from an A-T patient were similarly tested.

raised the issue of whether ATM phosphorylation is constitutively activated in cells bearing mutations that alter chromatin. LCLs from patients with different types of chromatin abnormalities were obtained: ICF syndrome, CLS, FSHD and RSTS. Two of the three RSTS samples had confirmed mutations in CREB-binding protein [60]. Nuclear extracts from these LCLs were immunoblotted for ATM-s1981(P). Fig. 2 demonstrates

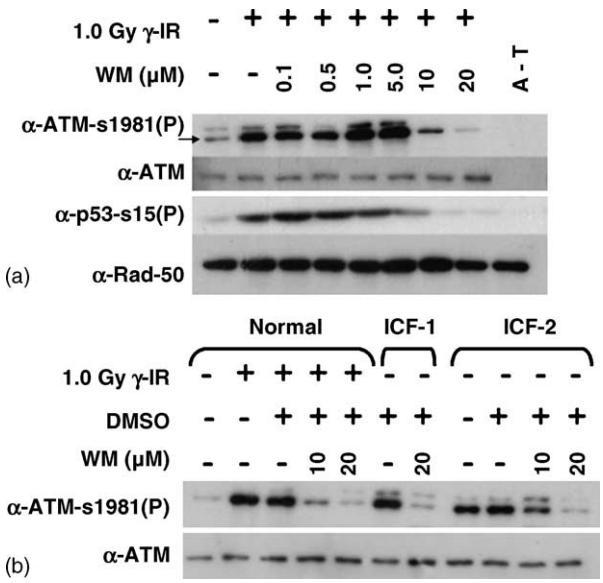

Fig. 3 – Wortmannin (WM) inhibited ATM phosphorylation at serine-1981 in ICF LCLs. LCLs that were either untreated, treated with DMSO or treated with the indicated concentrations of WM (dissolved in DMSO) for 1 h were irradiated with 1.0 Gy IR, and nuclear lysates were immunoblotted using the indicated antibodies. (a) Dose–response curve of ATM-s1981(P) (arrow) and p53-s15(P) vs. WM concentration in IR-treated normal (N-3) LCLs. WM partially inhibited ATM-s1981(P) at 10 μM and strongly inhibited phosphorylation at 20 μM. (b) ATM-s1981(P) was partially inhibited by 10 μM and strongly inhibited 20 μM WM in the ICF samples.

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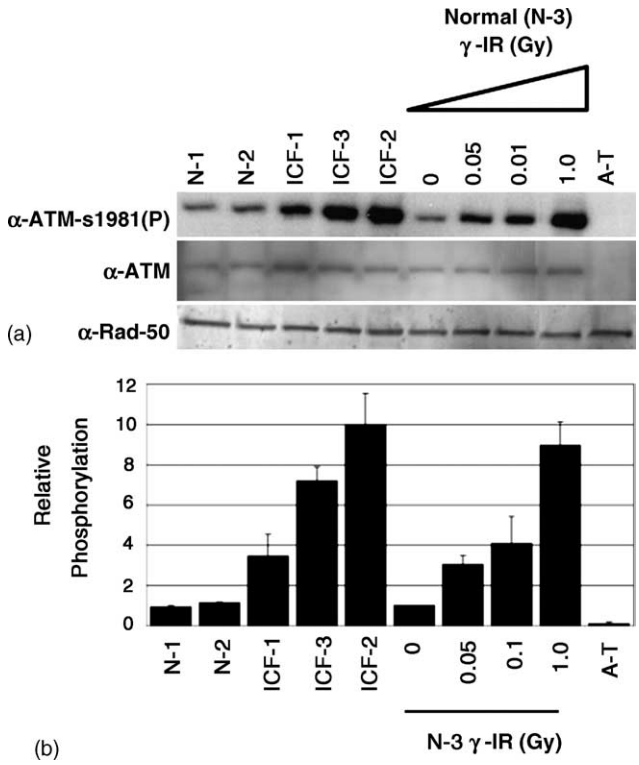

Fig. 4 – Comparison of ATM phosphorylation on serine-1981 in three unrelated ICF LCLs to the phosphorylation in γ-irradiated normal (N-3) LCLs. Nuclear extracts from normal maternal (N-1) and paternal (N-2) controls for ICF-1, three unrelated ICF patients (ICF-1, ICF-3 and ICF-2), an unrelated normal control (N-3) subjected to various IR doses were immunoblotted using α-ATM-s1981(P) and two loading controls: α-ATM and α-Rad50. (a) Western blots from one experiment and (b) quantification of the signal obtained for ATM serine-1981 phosphorylation obtained from three independent experiments. Phosphorylation signals were normalized to the non-irradiated control N-3 that was assigned a value of one. Error bars represent the standard error of three independent experiments. ATM phosphorylation at serine-1981 was significantly higher in non-irradiated ICF cells compared to the non-irradiated N-3 control, using a paired T-test of an average of three independent experiments: ICF-1, $p = 0.0229$; ICF-3, $p = 0.0038$ and ICF-2, $p = 0.0024$.

that non-irradiated LCLs from ICF patients displayed markedly increased levels of ATM-s1981(P) that resembled irradiated (1.0 Gy) normal cells (N-3). In contrast to the ICF LCLs, samples from two FSHD patients displayed low phosphorylation levels that resembled the non-irradiated control samples N-1 and N-3. Samples from three RSTS patients and a patient with CLS also displayed low phosphorylation levels that were slightly higher than the control samples, an effect that was reproducible (not shown). LCLs from an *ATM*−/− patient (A-T) failed to show ATM-s1981(P) even after IR, as previously reported [62].

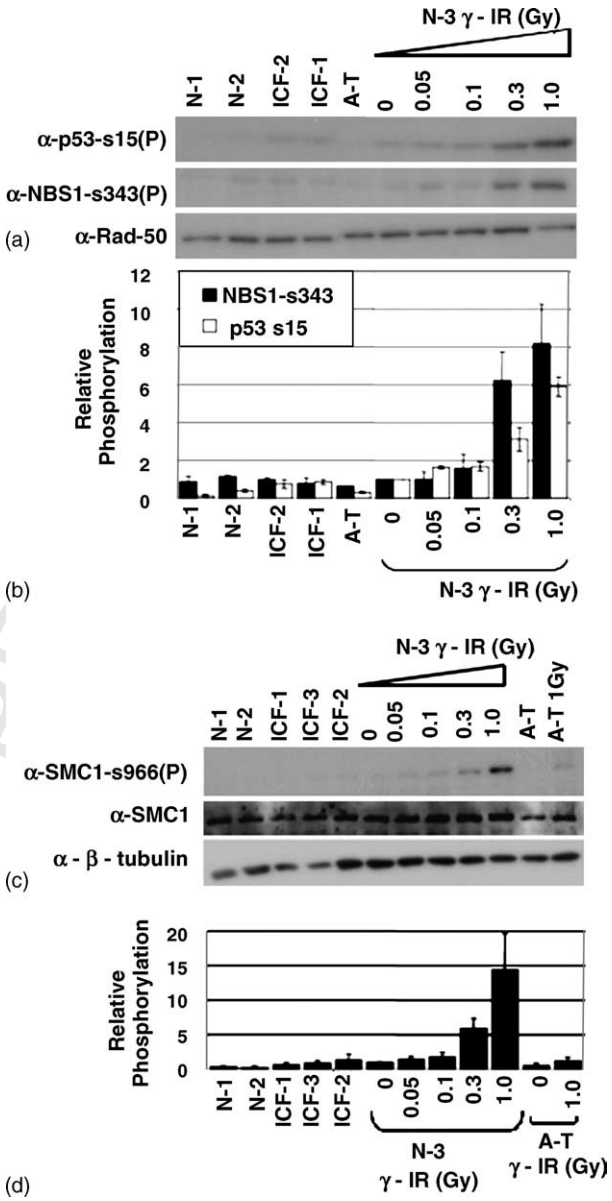

Fig. 5 – The ATM targets p53, NBS1 and SMC1 are hypophosphorylated in non-irradiated ICF LCLs. Nuclear extracts were prepared from the indicated cells and subjected to immunoblots for the phosphorylated forms of ATM targets: (a) Western blot for NBS1-s343(P), p53-s15(P) and for the loading control Rad-50. (b) Quantification of the p53-s15 phosphorylation and NBS1-s343 phosphorylation signals obtained from three independent repetitions of the experiment in panel (a). (c) Immunoblot for SMC1-s966(P) and the loading controls SMC1 and β-tubulin. (d) Quantification of the SMC1-s966(P) signals displayed in panel (c) and from two additional independent experiments. Values represent phosphorylation levels in relation to the non-irradiated normal (N-3) in each experiment. Error bars represent the standard error of three independent experiments.

The robust ATM-s1981(P) signal in the ICF samples prompted us to further examine these LCLs. We first addressed whether ATM-s1981(P) in ICF cells is inhibited by Wortmannin (WM). Fig. 3a shows a dose–response curve in which normal (N-3) LCLs were treated with increasing concentrations of WM for 1 h prior to exposure to 1.0 Gy IR. Nuclear extracts immunoblotted for ATM-s1981(P), revealed partial inhibition of phosphorylation at 10 μM and strong inhibition at 20 μM to below the background level of non-irradiated samples, but above the level of the *ATM*−/− control. Phosphorylation of p53 at serine-15 was also inhibited at these WM concentrations (Fig. 3a). To determine the sensitivity of ATM-s1981(P) in ICF cells, samples were treated with WM or with DMSO, which had been used to dissolve the WM. As in the IR-treated LCLs, WM partially inhibited ATM-s1981(P) in ICF LCLs at 10 μM and strongly inhibited ATM-s1981(P) at 20 μM, while treatment with DMSO alone had no effect (Fig. 3b).

In order to assess the extent of ATM-s1981(P) in non-irradiated ICF cells, we compared the level of ATM-s1981(P) from three ICF LCLs to an IR dose–response curve of ATM-s1981(P) in normal (N-3) cells. ICF-1 cells exhibited levels of ATM-s1981(P) similar to that of 0.1 Gy, while ICF-2 and ICF-3 resembled exposure of IR to about 1.0 Gy (Fig. 4). All three non-irradiated ICF LCLs showed significantly higher levels of ATM-s1981(P) than cells from the mother (N-1) or father (N-2) of ICF-1, which were normal. Since ICF patients are hypomorphs, we suspect that the reduction in DNMT3B function is less severe in ICF-1 than in ICF-2 and ICF-3.

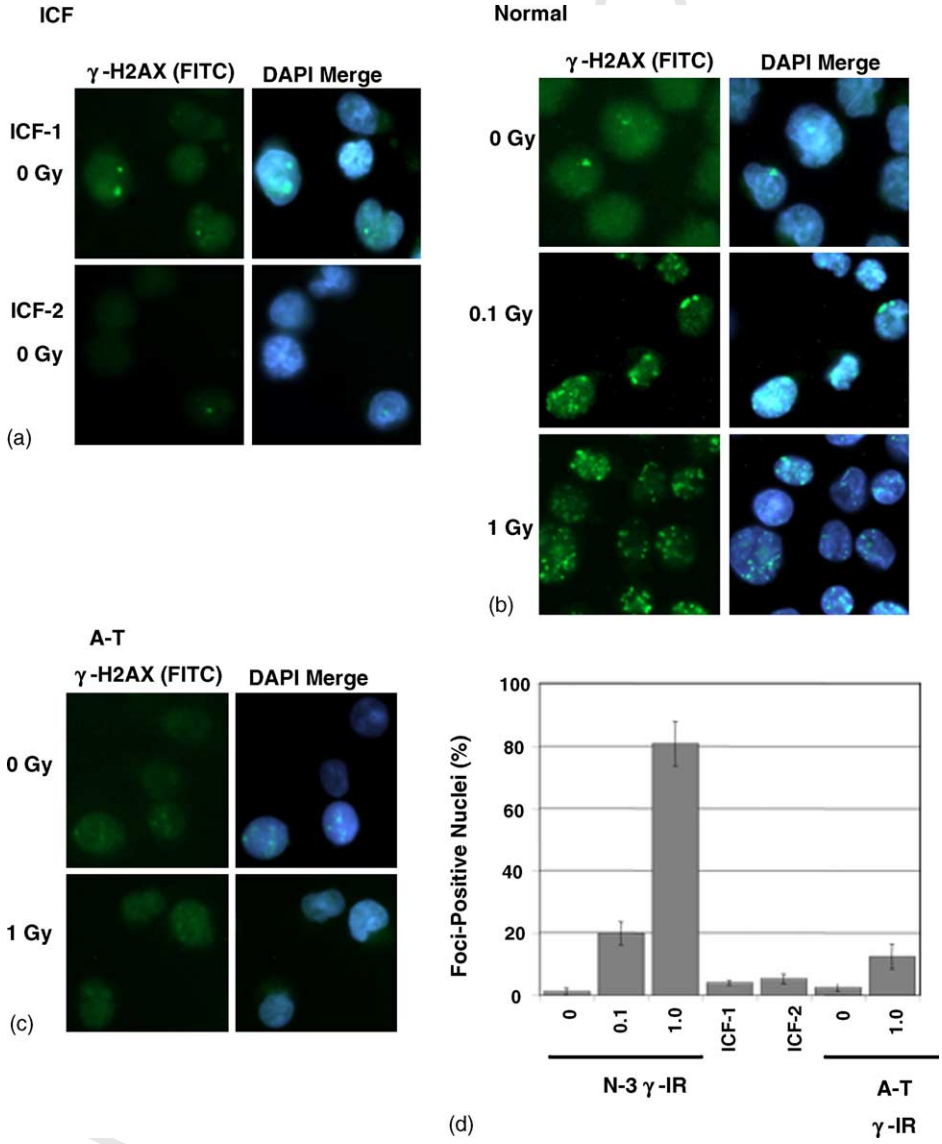

Fig. 6 – Assessment of DNA double strand breaks (DSBs) in non-irradiated ICF LCLs as determined by γ-H2AX nuclear foci formation. Cells were irradiated at the indicated level and γ-H2AX nuclear foci were identified by immunofluorescence (FITC). Nuclei were counterstained with a DNA dye (DAPI). (a) ICF-1 and ICF-2 non-irradiated. (b) Normal (N-3) non-irradiated, irradiated at 0.1 and 1.0 Gy. (c) A-T cells non-irradiated and irradiated at 1.0 Gy. (d) Proportion of nuclei displaying γ-H2AX foci. Nuclei displaying four or more γ-H2AX foci were scored as "foci-positive nuclei". The percent foci-positive nuclei were tabulated in three independent experiments, each counting at least 100 nuclei. Error bars represent the standard error of the three independent experiments.

3.3. ICF LCLs do not display significant endogenous phosphorylation of p53 at serine-15

As shown in Fig. 1, human LCLs subjected to chromatin-altering treatment do not display significant p53-s15(P), in contrast to reports in human fibroblasts [6,28]. To determine whether p53, a downstream target of ATM, is phosphorylated in non-irradiated ICF LCLs, nuclear extracts were immunoblotted for p53-s15(P). Non-irradiated ICF LCLs did not display p53-s15(P) that reflected the ATM-s1981(P) in the same cells (Fig. 5a). Although ATM was phosphorylated at serine-1981 in ICF LCLs, this phosphorylation was insufficient to render p53 a substrate.

3.4. ICF LCLs do not have significant endogenous DNA double strand breaks

The absence of elevated p53-s15(P) in ICF LCLs argued against the presence of DSBs in these cells [10,11]. However, ICF LCLs have been reported to display genomic instability and

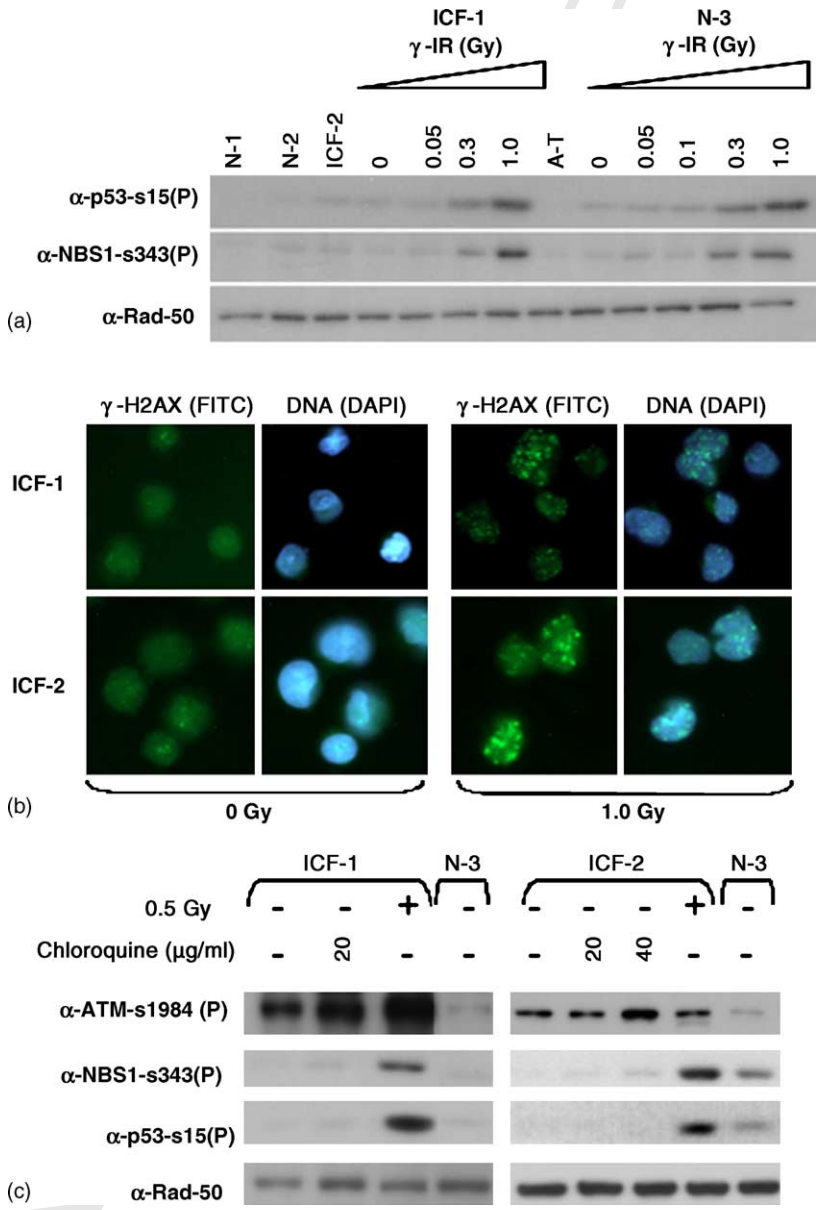

Fig. 7 – Evidence that ICF LCLs respond normally to γ-irradiation and chloroquine. (a) p53 and NBS1 phosphorylation in response to γ-irradiation are similar in samples from ICF and normal (N-3) cells. Nuclear extracts from ICF-1 and N-3 LCLs subjected to the indicated doses of γ-irradiation were immunobloted for p53-s15(P), NBS1-s343(P) and the loading control Rad-50. (b) ICF-1 and ICF-2 LCLs form irradiation-induced γ-H2AX nuclear foci in response to IR (1.0 Gy). γ-H2AX foci were identified by immunofluorescence (FITC) and cells were counterstained with a DNA dye (DAPI). (c) The combination of chloroquine treatment and Dnmt3b-deficiency does not result in a synergistic increase in ATM-s1981(P) signal nor lead to p53-s15(P) or NBS1-s343(P). ICF cells were incubated with the indicated doses of chloroquine for 6 h. Nuclear lysates were immunoblotted for ATM-s1981(P), NBS1-s343(P), p53-s15(P) and Rad-50.

chromosome aberrations [43–46], raising the possibility that the DSBs in ICF cells triggered the phosphorylation of ATM-s1981(P) but not p53-s15(P). In response to DSBs, ATM-s1981(P) phosphorylates a number of proteins involved in DNA repair, including NBS1 at serine-343 (NBS1-s343(P)) [25] and SMC1 at serine-966 (SMC1-s966(P) [28,29]. ICF cells did not display NBS1-s343(P) (Fig. 5a and b) or SMC1-s966(P) (Fig. 5c and d) above the background level of normal cells.

DSBs also cause H2AX phosphorylation by ATM at serine-139 to yield γ-H2AX, which accumulates in megabase-sized domains around DSB [31–33]. This allows individual DSBs to be visualized as nuclear foci using immunofluorescence for γ-H2AX [33]. It has been reported that non-irradiated human fibroblasts display an average of six γ-H2AX nuclear foci [63] that arise endogenously during processes such as DNA replication [64]. Fluorescent immunostaining revealed that LCLs derived from ICF patients displayed low numbers of γ-H2AX foci (Fig. 6a) and resembled non-irradiated normal N-3 (Fig. 6b) and phosphorylation-deficient *ATM*−/− (Fig. 6c) LCLs. In contrast, considerably more foci were observed in N-3 cells subjected to 0.1 or 1.0 Gy IR (Fig. 6b). At least 100 nuclei were considered for each condition for each cell line and any nucleus displaying four or more nuclear foci was scored positive for γ-H2AX nuclear foci. This revealed that no more than 5% of nuclei in either ICF-1 nor ICF-2 were positive for γ-H2AX foci, which was only marginally higher than the 1–3% for non-irradiated N-3 and *ATM*−/− cells, and well below the 20 and 80% of normal N-3 cells that scored positive after being subjected to 0.1 and 1.0 Gy, respectively (Fig. 6d). *ATM*−/− cells treated with 1.0 Gy of IR exhibited 12% positive foci (Fig. 6c and d). Most likely, ataxia-telangiectasia mutated and Rad3 related kinase [65] and DNA-dependent protein kinase (DNA-PK) produce this background level of γ-H2AX [32,66]. Therefore, although the ATM-s1981(P) levels of non-irradiated in ICF cells are comparable to those of normal cells treated with 0.1 and 1.0 Gy (Fig. 4), the numbers of γ-H2AX nuclear foci are much lower in ICF cells than in irradiated normal cells (Fig. 6a). Responses of ICF cells to IR are further evaluated below.

3.5. *ICF LCLs respond normally to ionizing radiation (IR) and chromatin-altering agents*

The observation that non-irradiated ICF LCLs display elevated levels of ATM-s1981(P) without corresponding phosphorylation of the downstream substrates, p53, NBS1, SMC1 and H2AX, raised the possibility that a genetic defect(s) in ICF LCLs may impair the ability of ATM-s1981(P) to phosphorylate these downstream substrates. Consistent with this notion, it has been reported that ICF LCLs are hypersensitive to ionizing radiation [48]. Western blots on nuclear extracts from ICF cells revealed that p53 and NBS1 became phosphorylated at levels comparable to normal LCLs irradiated at the same doses (Fig. 7a). Moreover, fluorescent immunostaining revealed IR-induced γ-H2AX nuclear foci in ICF LCLs at levels similar to those of IR-treated normal cells (compare Fig. 7b to Fig. 6b). These data indicate that in ICF LCLs, ATM is appropriately sensing IR-induced DNA damage and phosphorylating downstream substrates.

We also examined how ICF cells responded to chloroquine treatment. ICF LCLs were incubated in chloroquine at levels shown not to induce detectable DNA damage (Fig. 1) and nuclear lysates were immunoblotted for ATM-s1981(P), NBS1-s343(P), p53-s15(P) and Rad-50. Fig. 7c demonstrates that despite chromatin abnormalities stemming from two distinct sources, *DNMT3b*-deficiency and chloroquine treatment, p53 and NBS1 remained unphosphorylated in ICF LCLs. ICF cells displayed only a moderate increase in ATM-s1981(P) signal in response to chloroquine treatment (Fig. 7c). The absence of NBS-s343(P) suggests that ICF cells are not hypersensitive to DNA damage by chloroquine treatment and we conclude that combining the two sources of chromatin defects did not synergistically increase the levels of ATM-s1981(P).

3.6. *ICF LCLs have intact cell cycle checkpoints and do not display radiosensitivity*

In normal cells, IR activates cellular signaling pathways that either lead to cell cycle arrest or apoptotic cell death [67]. The

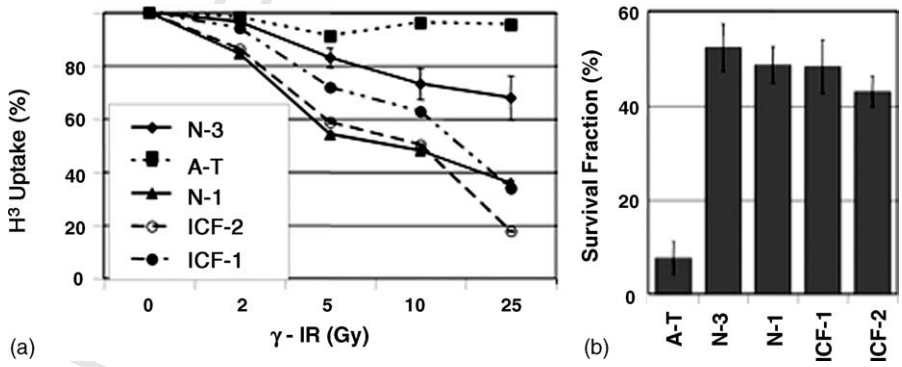

Fig. 8 – Evidence that ICF LCLs display intact cell cycle checkpoints and are not radiosensitive: (a) ICF cells display normal response in an assay for radioresistant DNA synthesis. The radioresistant DNA synthesis assay [68] measured the amount of H^3 uptake (DNA replication) in cells subjected to the indicated levels of γ-irradiation. In contrast to normal and ICF cells, A-T cells displayed radioresistant DNA synthesis, as expected [4]. (b) Normal response of ICF cells to the colony survival assay [61]. The cell lines indicated were plated on a 96-well plate, subjected to 1.0 Gy irradiation, allowed to recover for 2 weeks, and the survival fraction was calculated. Cells that are radiosensitive exhibit <21% survival fraction, while cells that are not radiosensitive will display greater than 36% survival fraction.

integrity of cell cycle checkpoints can be ascertained using a radioresistant DNA synthesis assay that tests the ability of cells to inhibit DNA synthesis, as measured by tritium (H^3) uptake in response to a dose–response curve of IR [62,68]. Fig. 8a demonstrates that ICF LCLs reduced the amount of H^3 uptake in a manner that was indistinguishable from normal cells. In contrast, *ATM*−/− LCLs which have a defective S-phase checkpoint continued to synthesize DNA even when exposed to high doses of irradiation, in accordance with previous reports [69,70]. These results indicated that ICF LCLs have a normal S-phase checkpoint. Consistent with these results, it was previously reported that ICF LCLs showed normal radiation-responsive cell cycle arrest when examined using flow cytometry [48].

ICF cells have been reported to be radiosensitive, using an assay that measured ICF cell viability 24–96 h after IR with trypan-blue exclusion [48]. The observation that ATM substrates were phosphorylated normally in response to IR prompted us to re-examine the radiosensitivity of ICF cells by using the colony survival assay [61]. This assay is often used to diagnose radiosensitivity in cells from suspected *ATM*−/− patients; it measures the colony forming ability of lymphoblastoid cell lines 10–13 days after exposure to 1.0 Gy IR [61]. *ATM*−/− LCLs display a survival fraction of <21%, while cells with greater than 36% survival fraction are considered non-radiosensitive [61]. ICF-1 and ICF-2 exhibited survival fractions of 48.3 and 40.3%, respectively, similar to control cells N-3 and N-1; thus, ICF cells were not radiosensitive in this assay (Fig. 8b). It is important to note that the ICF-1 and maternal N-1 LCLs used in our CSA experiments, are the same cell lines previously used to report that ICF LCLs were radiosensitive by a trypan-blue exclusion assay [48].

4. Discussion

Since experimental treatments that cause chromatin defects in the absence of detectable DNA breaks activate the ATM kinase [6,28], we investigated whether ATM is constitutively activated in LCLs from patients with chromatin disorders by examining ATM phosphorylation at serine-1981. It was found that ATM displays little phosphorylation at serine-1981 in LCLs from three RSTS patients, a CLS patient and two patients with FSHD. In contrast, LCLs from three ICF patients displayed increased levels of ATM-s1981(P) that resembled the ATM-s1981(P) levels of normal LCLs after irradiation. Moreover, ATM-s1981(P) in ICF cells was inhibited by the PI-3 kinase inhibitor Wortmannin at concentrations that produced similar levels of inhibition of ATM-s1981(P) in normal cells exposed to IR. The elevated ATM-s1981(P) levels in the ICF cell lines were not accompanied by an increase in the ATM-phosphorylated forms of NBS1 and SMC1 and did not lead to corresponding levels of γ-H2AX foci. This suggests that the ATM-s1981(P) arose in the ICF cells independently of DNA DSBs, and that its downstream kinase activity towards these substrates failed to be activated. In addition, we found that, in contrast to the robust p53 phosphorylation reported to be produced by chromatin-altering agents in primary human fibroblasts [6,28], neither chloroquine treatment nor *DNMT3b*-deficiency elicited significant p53-s15(P) in LCLs. This suggests that the response to chromatin-altering agents is not equivalent between primary fibroblasts and LCLs. Our findings indicate that although phosphorylation at serine-1981 is essential for ATM kinase activation [6], serine-1981 phosphorylation in LCLs is insufficient to render ATM an active kinase towards downstream substrates, including p53.

Although chromatin has been implicated in the DSB damage response [71–74], lack of substrate phosphorylation by ATM-s1981(P) in ICF LCLs was not due to an impaired ability to activate ATM in these cells. ICF cells subjected to IR produced normal numbers of γ-H2AX nuclear foci and normal levels of p53 and NBS1 phosphorylation. IR also caused DNA synthesis to be inhibited at normal levels indicating the presence of a normal S-phase cell cycle checkpoint in response to DNA damage, in agreement with previous results [48]. Finally, IR of ICF LCLs resulted in normal levels of cell survival using an established colony survival assay [61]. This finding was surprising because it had previously been reported that ICF cells are radiosensitive [48]. One ICF LCL was used in both studies [48], yet displayed different results, suggesting that the discrepancy between the two studies stems from differences in the methods employed for testing radiosensitivity. The earlier report had used trypan-blue exclusion to assess cell survival 24, 48, 72 and 96 h post IR. Our study used the vital dye, MTT, to evaluate cell survival 10–13 days post IR. This discrepancy can be reconciled if 1.0 Gy of IR causes ICF cells to die more quickly than wild type cells but that similar proportions of cells survive after 10–13 days.

Why did we observe strong ATM-s1981(P) signals in only the ICF cells and not mutant cell lines with other chromatin defects? One possibility is that RSTS, CLS and FSHD LCLs have insufficient abnormal chromatin to elicit a strong response from the putative chromatin surveillance system involving ATM. Consistent with this possibility, a slight but reproducible increase in ATM-s1981(P) signal was observed in CLS and RSTS samples, while a much stronger signal was observed in ICF syndrome where large pericentromeric regions display abnormal heterochromatin. A second possibility is that ATM-s1981(P) in ICF LCLs arises in response to chromosomal DNA instabilities reported in ICF LCLs [43–46], rather than from the primary chromatin defects caused by *DNMT3B*-deficiency. If so, then the genomes of the LCLs from RSTS, CLS and FSHD patients may be too stable to elicit such a response. This explanation would require DNA defects other than DSBs to elicit a qualitatively distinct response that involves the appearance of ATM-s1981(P) that is incapable of phosphorylating p53, NBS1, SMC1 or H2AX. A third possibility is that certain chromatin abnormalities are detected by ATM while qualitatively distinct chromatin defects escape this detection.

The failure of ATM-s1981(P) to phosphorylate p53 in LCLs bearing chromatin defects revealed that although serine-1981 phosphorylation is essential for ATM kinase activity [6], it is insufficient to activate ATM kinase with respect to the p53 substrate. ATM autophosphorylation (and activation) requires protein phosphatase 5 (PP5) activity [75], the histone acetyltransferase MOF [72], and acetylation of ATM via the protein acetylase Tip60 [5]. All three of these proteins bind ATM. Furthermore, phosphatase 2A (PP2A) binds ATM and a PP2A inhibitor leads to ATM activation [63]. In ICF LCLs or normal LCLs treated with chloroquine, ATM-s1981(P) may arise by an

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alternative or altered pathway that does not involve one or more of these activities, and this form of ATM-s1981(P) is inactive towards p53 and other downstream substrates. Another explanation for the failure of p53 to be phosphorylated in LCLs is that in primary fibroblasts chromatin-altering agents cause p53 to be phosphorylated at serine-15 by a protein other than ATM. For example, chromatin-altering treatments may create stress that activates a pathway in which ATR phosphorylates p53 but not NBS1, SMC1 or H2AX. Such a pathway may be non-functional in LCLs due to differences in cell type or because it was disabled during the immortalization of the cells.

One protein that may cause ATM to be phosphorylated in response to chromatin defects is 53BP1. This protein binds histone H3 methylated at lysine-79 at DSBs [23] and has been shown to stimulate ATM autophosphorylation [21]. It has been proposed that chromatin defects expose methylated lysine-79 in a manner that allows 53BP1 to bind and activate ATM [8]. In support of this model, cells treated with mild hypotonic conditions displayed reduced mobility of 53BP1-GFP fusion protein, suggesting that the fusion protein has associated with chromatin [23]. Efforts to apply this model to our observations in ICF cells raised two issues. The first issue is the location in the ICF genome where methylated lysine-79 is exposed leading to ATM-s1981(P). Lysine-79 methylation is a feature of transcriptionally active chromatin [76] and has been shown to be excluded from transcriptionally silent heterochromatin [77]. Although the pericentromeric regions of chromosomes 1, 9 and 16 have abnormal chromatin structures, these regions are still heterochromatic: they retain H3 lysine-9 methylation and HP1, and show no signs of transcription or histone acetylation (a euchromatic feature) [78]. Although the inactive X-chromosome in ICF cells is hypomethylated [39,79] and displays gene reactivation [79], male ICF cells that lack the inactive X-chromosome displayed a strong ATM-s1981(P) signal. The genes most likely to expose lysine-79 are several genes of the immune system which show abnormal expression levels in ICF LCLs [80]. The second issue raised by our study is that the 53BP1 model, as it stands, involves a single signal and fails to explain why p53 is phosphorylated at serine-15 in primary fibroblasts treated with various chromatin-altering agents, but not in chloroquine-treated or DNMT3B-deficient LCLs. Therefore, the response to chromatin abnormalities may be more complicated than proposed by the 53BP1–H3–lysine-79 model.

In sum, our study indicates that ATM is constitutively phosphorylatd at serine-1981 in cells from patients with ICF syndrome, but that this occurs without kinase activity towards downstream substrates, including p53. Although the mechanism(s) that lead to kinase activity in response to chromatin are yet to be determined, further studies using cells with defined chromatin defects should characterize the pathway(s) that link ATM kinase and chromatin.

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