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Mitochondria are required for ATM activation by extranuclear oxidative stress in cultured human hepatoblastoma cell line Hep G2 cells

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ABSTRACT

Ataxia–telangiectasia mutated (ATM) is a serine/threonine protein kinase that plays a central role in DNA damage response (DDR). A recent study reported that oxidized ATM can be active in the absence of DDR. However, the issue of where ATM is activated by oxidative stress remains unclear. Regarding the localization of ATM, two possible locations, namely, mitochondria and peroxisomes are possible. We report herein that ATM can be activated when exposed to hydrogen peroxide without inducing nuclear DDR in Hep G2 cells, and the oxidized cells could be subjected to subcellular fractionation. The first detergent-based fractionation experiment revealed that active, phosphorylated ATM was located in the second fraction, which also contained both mitochondria and peroxisomes. An alternative fractionation method involving homogenization and differential centrifugation, which permits the light membrane fraction containing peroxisomes to be produced, but not mitochondria, revealed that the light membrane fraction contained only traces of ATM. In contrast, the heavy membrane fraction, which mainly contained mitochondrial components, was enriched in ATM and active ATM, suggesting that the oxidative activation of ATM occurs in mitochondria and not in peroxisomes. In Rho 0-Hep G2 cells, which lack mitochondrial DNA and functional mitochondria, ATM failed to respond to hydrogen peroxide, indicating that mitochondria are required for the oxidative activation of ATM. These findings strongly suggest that ATM can be activated in response to oxidative stress in mitochondria and that this occurs in a DDR-independent manner.

1. Introduction

Ataxia–telangiectasia (A–T) is a highly radiosensitive autosomal recessive genetic disorder that causes progressive cerebeller ataxia, oculocutaneous telangiectasia, premature aging, a weakened immune system, and high incidences of lymphoma and leukemia [1,2]. The gene responsible for A–T is designated ataxia–telangiectasia mutated (*ATM*), and its product ATM, a serine/threonine protein kinase, a member of the phosphoinositide 3-kinase-related protein kinase (PIKK) family, is activated through autophosphorylation in response to DNA double-stranded breaks (DSBs), and plays a central role in the DNA damage response (DDR) [3].

The wide variety of the manifestations of A–T are thought to be mainly due to a genomic instability caused by defects in DDR such as checkpoint signaling. However, the defects in DDR alone are not sufficient to explain the complex symptoms associated with A–T. For instance, A–T cells are highly sensitive to oxidative stress other than DSBs, and appear to contain a decreased level of intracellular antioxidants [4]. In addition, the survival times of ATM-deficient mice were prolonged by the administration of antioxidants, by which the risk of carcinogenesis and immunodeficiency in the mice were decreased [5–8]. These data suggest that A–T symptoms can be attributed, at least in part, to an enhancement of the production of reactive oxygen species (ROS), and also suggest a strong relationship between oxidative stress and the ATM response. In fact, ATM is activated in response to changes in intracellular redox status [9–13].

A recent study proposed a mechanism in which ATM is activated by oxidative stress in the absence of DDR. In DDR, ATM is activated by cooperating with the Mre11-Rad50-Nbs1 (MRN) complex in nuclear, and thereby changes from an inactive non-covalent ATM homodimer to an active monomer. In contrast, oxidized ATM can be active and autophosphorylated via the formation of disulfide cross-links within the homodimer in the absence of DNA DSBs and the MRN complex [14]. ATM, when mutated in a cysteine residue that is critical for disulfide bond formation is unable to respond to oxidative stress [14].

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However, although the fact that the deletion of the last 10 amino acids of ATM, which has been suggested to be a peroxisome targeting signal [15], also results in an inability to respond to oxidative stress [14] suggests that the oxidative activation of ATM is related to its translocation activity to peroxisomes, the issue of where ATM is activated by oxidative stress remains unclear. With regard to the extranuclear localization of ATM, ATM is also known to be localized in mitochondria in addition to peroxisomes [15,16]. Very recently, Valentin-Vega et al. reported on the localization of ATM in mitochondria and that ATM is required for mitochondrial homeostasis [16]. The objective of this study was to clarify the specific location where ATM is activated by oxidative stress, using a hepatoblastoma cell line Hep G2 cells, which are frequently used as a material that is suitable for subcellular fractionation [17].

2. Materials and methods

2.1. Cell culture and treatment

The human hepatoblastoma cell line Hep G2 cells [18] were cultured in DMEM/F12 medium (Wako, Japan) supplemented with 10% fetal bovine serum (FBS; CCB, Nichirei Biosciences, Japan) and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin (Sigma)). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Cell density was determined with a cell counter (Z1 Cell and particle counter, Beckman Coulter). Exponentially growing cell cultures (2–3 $\times 10^6$ cells/ml) in tissue culture plates (Greiner Bio-one) were treated with hydrogen peroxide (H_2O_2; Wako) or etoposide (Wako), or irradiated with a ^{137}Cs γ-ray source (Gammacell 40 exactor, Nordion International) at a dose rate of 0.96 Gy/min. KU55933 was purchased from Synkinase. To generate Hep G2 cells depleted of mitochondrial DNA (mtDNA) (Rho 0-Hep G2 cells), Hep G2 cells were exposed to ethidium bromide (EtBr; Sigma), essentially following previously published procedures [19,20]. More precisely, the cells were cultured in two media: (1) high EtBr-medium, which is the DMEM/F12-10% FBS medium plus EtBr (100 ng/ml), Uridine (50 μg/ml), and Pyrubate (50 μg/ml). (2) low EtBr-medium, which is the DMEM/F12-10% FBS medium plus EtBr (30 ng/ml), Uridine (50 μg/ml), and Pyrubate (100 μg/ml). The cells were cultured in high-EtBr medium and passaged at 2- or 3-day intervals for 10 days, and then alternately cultured in low EtBr-medium or high EtBr-medium for intervals of 2–3 days for a period of 1–2 weeks. We used the cells as Rho 0-Hep G2 cells between 2 and 3 weeks after the start of the EtBr-treatment, since the mtDNA was almost depleted according to mtDNA analysis approximately from 2 weeks after the treatment started. The medium was changed to normal DMEM/F12-10% FBS medium when treated Rho 0-Hep G2 cells with H_2O_2 or etoposide. The protein concentrations of all the samples were determined using the BCA Protein Assay Reagent (Thermo Fisher Scientific) and equalized.

2.2. Immunoblotting analysis

Immunoblotting was performed essentially as described in a previous report [21]. For ATM analysis, SDS–PAGE was performed using a 5–20% gradient pre-cast gel (ATTO, Japan). We used the following antibodies as primary antibodies: ATM (NB100-104, NOVUS), pS1981-ATM (D6H9, Cell Signaling), Chk2 (2662, Cell Signaling), pT68-Chk2 (2661, Cell Signaling), H2AX (D17A3, Cell Signaling), γ-H2AX (JBW301, Millipore), p53 (DO-1, Santa Cruz Biotechnology), pS15-p53 (9284, Cell Signaling), KAP1 (ab10484, abcam), pS824-KAP1 (ab70369, abcam), Catalase (ab1877, abcam), COX IV (4850, Cell Signaling), HSP60 (4870, Cell Signaling), VDAC1 (ab15895, abcam), Apaf-1 (clone 24, BD Transduction Laboratories), or β-Actin (AC-15, Sigma).

2.3. Subcellular fractionation

Subcellular fractions were prepared by two kit methods: a detergent-based cell fractionation method (ab109719, Cell fractionation kit standard, abcam), which was also used by Valentin-Vega et al. [16], and a cell fractionation involving syringe homogenization and differential centrifugation (WSE-7422, EzSubcell Fraction, ATTO), in which the heavy membrane fraction was sedimented at 3000*g* for 5 min after removal of nuclei and unbroken cells by a 700*g* centrifugation for 10 min. The 3000*g* supernatant was further centrifuged at 100,000*g* for 60 min to yield the light membrane fraction. The supernatants of the 100,000*g* spin were used as the cytosolic fraction. For Electron microscopic analysis, a part of the heavy membrane fractions was fixed in 3% glutaraldehyde in physiological saline, further fixed in 1% OsO4 in 0.1 M phosphate-buffered saline (PBS), dehydrated through a graded series of alcohols, and embedded in Epon 812. Thin sections were cut with an ultra microtome and stained with 2% uranyl acetate and lead citrate. A part of the light membrane fractions was fixed in 3% glutaraldehyde in physiological saline and negatively stained with 2% uranyl acetate.

2.4. mtDNA analysis and measurement of mitochondrial membrane potential

Depletion of mtDNA was confirmed by semi-quantitative PCR analysis using a thermal cycler (PCR Thermal Cycler Dice, Takara) and specific primers for cytochrome *c* oxidase subunit I (MT-COI), which is encoded by mtDNA [22]. Cellular DNA was obtained by a phenol/chloroform extraction. The primer sequences were as follows: *MT-COI*, (forward) 5′-CGCATGAGCTGGAGTCCTA-3′, (reverse) 5′-TACAAATGCATGGGCTGTG-3′; *GAPDH* (for nuclear DNA quantitation), (forward) 5′-TACTAGCGGTTTTACGGGCG-3′, (reverse) 5′-TCGAACAGGAGGAGCAGAGAGCGA-3′. The percentage of cells losing their mitochondrial membrane potential ($\Delta\psi$m) was determined by JC-1 staining [23]. Cells were incubated in culture medium for 10 min at 37 °C with 5 μg/ml JC-1 (AdipoGen). The cells were then washed once, suspended in ice-cold PBS, and analyzed by flow cytometry (FACS Calibur, Becton Dickinson).

3. Results

3.1. ATM activation by H_2O_2 in the absence of DDR

In a previous study by Guo et al., only a subset of ATM targets was found to be phosphorylated by 250 μM H_2O_2 in human primary fibroblasts [14]. We speculated that the limited phosphorylation that occurred in the presence of H_2O_2 could be due to the intracellular localization of individual ATM targets, and could be explained by a limited activation of ATM at extranuclear sites. In the same report, two nuclear proteins, namely, H2AX and KAP1 were not phosphorylated as the result of exposure to the H_2O_2 level [14]. Since the phosphorylation of these two proteins were good indicators of DDR [24,25], it is possible that oxidative ATM activation, as mentioned in their paper [14], could occur in the absence of DDR.

Therefore, in order to investigate the oxidative ATM activation in the absence of DDR, and also to exclude the effect of ATM activation by DDR, we initially determined the concentration of H_2O_2 required for activating ATM without inducing nuclear DNA damage in Hep G2 cells. Fig. 1A shows dose–response data for H_2O_2 on the phosphorylation of ATM and its substrates, Chk2 and H2AX. At a

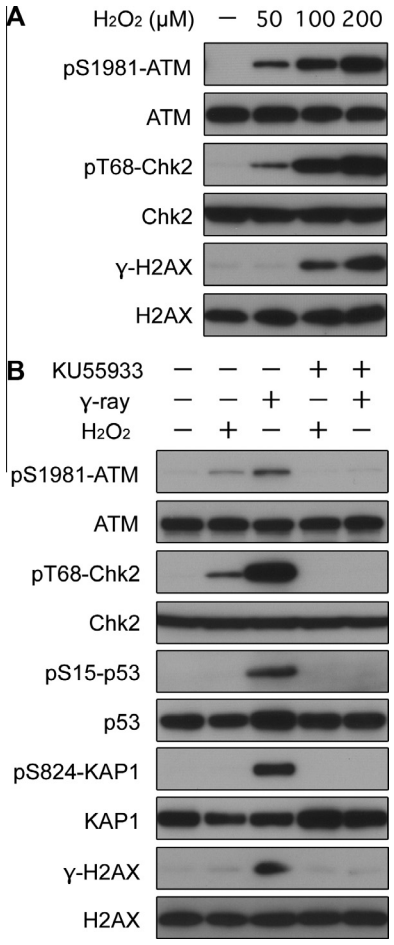

Fig. 1. Oxidative ATM activation in the absence of DDR. (A) Dose–response for H_2O_2 for the phosphorylation of ATM and its substrates, Chk2 and H2AX. Hep G2 cells were treated with the indicated concentrations of H_2O_2 for 30 min, and then harvested. (B) The effect of 50 μM H_2O_2 on the phosphorylation of ATM and its targets. Hep G2 cells were treated with 50 μM H_2O_2 for 30 min and harvested. In irradiation experiments, cells were harvested 30 min after 2 Gy-irradiation. The ATM kinase inhibitor KU55933 (20 μM) was added 30 min before stimulation.

concentration of 50 μM, H_2O_2 induced the phosphorylation of ATM and Chk2. In contrast, H2AX was not phosphorylated at 50 μM, and a higher concentration was required to produce the phosphorylated form, γ-H2AX. These data demonstrate that, at 50 μM, H_2O_2 does not damage nuclear DNAs in Hep G2 cells, suggesting that the H_2O_2 is adequately scavenged before it penetrates into the nucleus.

To further confirm this extranuclear oxidative damage, we examined the effect of 50 μM H_2O_2 on the phosphorylations of three nuclear proteins, compared with that of γ-ray as the controls. As expected, three nuclear proteins, p53, KAP1, and H2AX were phosphorylated only by γ-ray (2 Gy), and no response was detected for 50 μM H_2O_2 (Fig. 1B). On the other hand, ATM and Chk2 were phosphorylated by both stimuli, albeit at different levels. Further, all phosphorylations by both stimuli were inhibited by an ATM kinase inhibitor, KU55933 [26]. Collectively, these data indicate that, in response to oxidative stress, ATM and its substrate Chk2 can be phosphorylated without nuclear DDR in an ATM-dependent manner.

3.2. Oxidative ATM activation was observed in the fraction containing cytoplasmic membrane organelles

To reveal where ATM is activated by oxidative stress, we next performed a detergent-based subcellular fractionation, which was the same kit method as used by Valentin-Vega et al. [16]. As mentioned in the Introduction, Valentin-Vega et al. reported, as evidence for the mitochondrial localization of ATM, an examination of the mitochondrial fraction, which can be obtained by mild detergent extraction using this kit [16]. However, although the second fraction (Fraction 2), which Valentin-Vega et al. referred to as the "mitochondrial fraction," was, in fact, enriched in ATM, the fraction contained both mitochondria and peroxisomes, as assessed by mitochondrial marker proteins (COX IV, HSP60, and VDAC1) and by the presence of catalase, a peroxisomal marker protein (Fig. 2). In this fractionation, ATM was much more abundant in Fraction 2 than in Fraction 1 (the cytosolic fraction) or in Fraction 3 (the nuclear fraction), and ATM activation, which was assessed by phosphorylation of the autophosphorylation site of ATM (Ser 1981) [3], was observed exclusively in Fraction 2 in response to H_2O_2, and also observed in Fraction 3 from irradiated cells. These data indicate that the oxidative activation of ATM occurs in extranuclear, cytoplasmic membrane organelle(s). In addition, Chk2 phosphorylation was observed in both Fraction 1 and Fraction 2, suggesting that Chk2 is phosphorylated by ATM in organelle(s) that are located in Fraction 2, and thereafter released into the cytoplasm.

To determine whether mitochondria or peroxisomes are involved in the oxidative activation of ATM, we performed an alternative fractionation method that involved homogenization and differential centrifugation, which permits the light membrane fraction containing peroxisomes to be isolated, but not mitochondria. The light membrane fraction separated by this method contained a large proportion of catalase and only traces of ATM and active ATM (Fig. 3A). On the other hand, although the heavy membrane fraction contained a small proportion of catalase, it contained all of the mitochondrial proteins tested and the majority of both ATM and active ATM, suggesting that the oxidative activation of ATM occurs in mitochondria and not in peroxisomes. Electron microscopic observations revealed that the heavy membrane fractions predominantly contained mitochondria having typical cristae

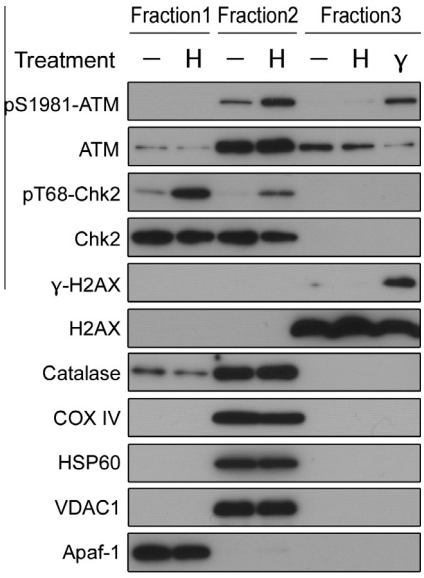

Fig. 2. ATM-enriched fraction produced by detergent-based subcellular fractionation contained not only mitochondria but also peroxisomes. Hep G2 cells were treated with 50 μM H_2O_2 for 30 min, or incubated 30 min after 2 Gy-irradiation. The cells were then fractionated, and subjected to immunoblotting analysis. COX IV, HSP60, and VDAC1 were used as mitochondrial markers. H2AX, catalase, and Apaf-1 were used as a nuclear marker, peroxisomal marker, and cytosolic marker, respectively. In the fractionation, the first, second, and third fractions (Fraction 1, 2, 3) mainly contained cytosolic components, mitochondrial and peroxisomal components, and nuclear components, respectively.

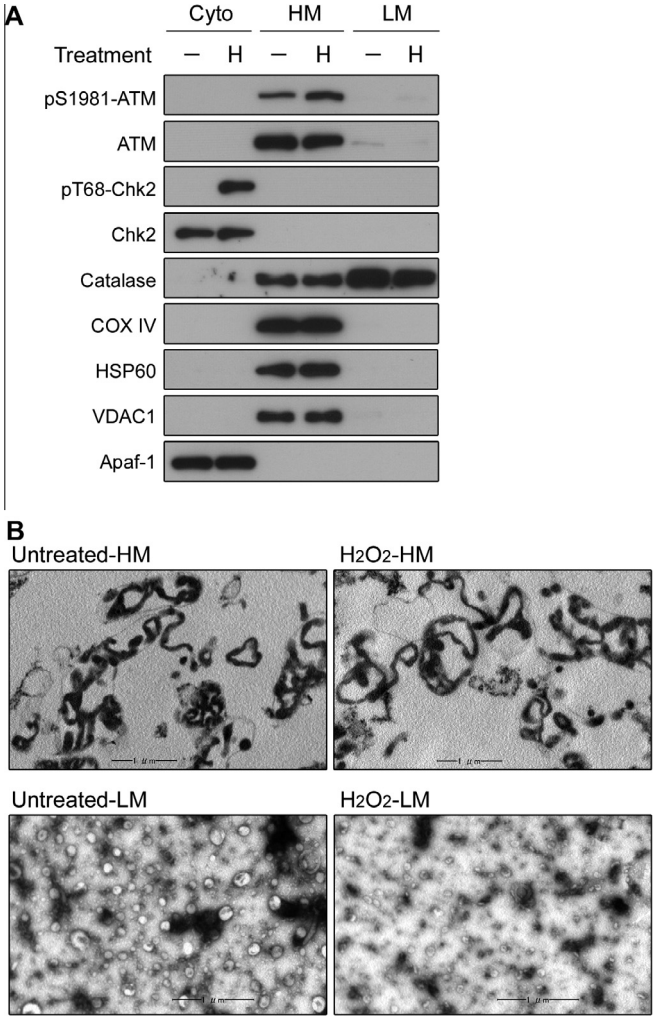

Fig. 3. The light membrane fraction separated by homogenization and differential centrifugation contained peroxisomes and only traces of ATM and active ATM. (A) Hep G2 cells were treated with 50 μM H_2O_2 for 30 min and harvested (H). The cells were then fractionated, and subjected to immunoblotting analysis. COX IV, HSP60, and VDAC1 were used as mitochondrial markers. Catalase and Apaf-1 were used as peroxisomal marker and cytosolic marker, respectively. Cyto, cytosolic fraction; HM, heavy membrane fraction; LM, light membrane fraction. (B) Electron microscopic analysis of each HM (20,000× magnification) and LM (25,000× magnification, negative staining). All scale bars indicate 1 μm.

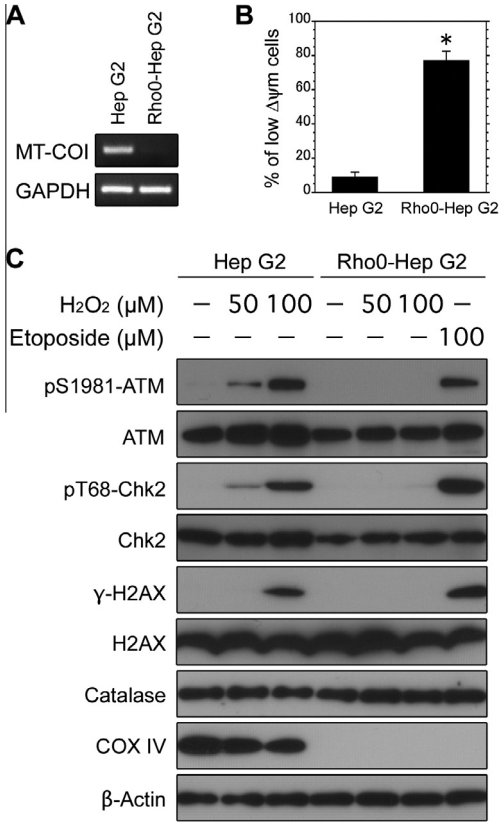

Fig. 4. ATM barely responded to H_2O_2 in Rho 0-Hep G2 cells. (A) mtDNA was detected by amplifying MT-COI by PCR. GAPDH was used as a control gene encoded by the nuclear genome. (B) The cytofluorimetric analysis of $\Delta\psi$m using JC-1 dye. The percentage of low $\Delta\psi$m cells was measured by flow cytometry. Data shown are the means ± SD from three independent experiments. Asterisk denotes significant difference from parental Hep G2 cells ($P < 0.05$) by Student's t-test. (C) Immunoblotting analysis of Hep G2 and Rho 0-Hep G2 cells. Cells were treated with the indicated concentrations of H_2O_2 or etoposide for 30 min, and then harvested.

structures, and that the light membrane fractions contained only microsomes, which are derived from the membranous structures such as peroxisome, endoplasmic reticulum, and plasma membrane [27], albeit the microsomes from H_2O_2-treated cells were smaller than those obtained from untreated cells (Fig. 3B).

3.3. Mitochondria are required for the oxidative activation of ATM

The above findings suggest the existence of a strong relationship between the oxidative activation of ATM and mitochondria. To investigate this further, we generated Rho 0 cells using EtBr to deplete the Hep G2 cells of mtDNA and functional mitochondria. The establishment of Rho 0-Hep G2 cells was confirmed by mtDNA analysis (Fig. 4A), the cytofluorimetric analysis of $\Delta\psi$m using JC-1 dye (Fig. 4B), and the lack of COX IV expression (Fig. 4C). In Rho 0-Hep G2 cells, the ATM failed to respond to H_2O_2, consistent with the conclusion that mitochondria are required for the oxidative activation of ATM (Fig. 4C). This unresponsiveness is not due to a decrease in intracellular ATP levels in the Rho 0 cells, because ATM and its targets were phosphorylated by etoposide-induced DDR (Fig. 4C).

4. Discussion

In this study, we determined the concentration of H_2O_2 needed to induce extranuclear oxidative stress and that required to activate ATM in the absence of DDR in Hep G2 cells. In detergent-based subcellular fractionation, the oxidative activation of ATM was observed exclusively in the second fraction containing mitochondria and peroxisomes. An alternative subcellular fractionation of the oxidized cells by homogenization and differential centrifugation revealed that the light membrane fraction contained peroxisomes and negligible levels of ATM, and that the heavy membrane fraction predominantly contained mitochondria and the majority of the ATM, including active ATM, although the heavy membrane fraction was contaminated with a small proportion of peroxisomes. Further study using Rho 0-Hep G2 cells demonstrated that the oxidative activation of ATM is dependent on functional mitochondria. Collectively, these findings strongly suggest that, in response to oxidative stress, ATM is activated in mitochondria in a DDR-independent manner.

The oxidative activation of ATM relies on its C-terminal cysteine (C2991), which is located in the FRAP/ATM/TRRAP (FATC) domain [14,28]. The cysteine residue is conserved in terrestrial vertebrates but not in marine animals, suggesting an evolutionary relationship between gaining this residue and the land invasion by vertebrates.

The evolved ATM might serve to protect the terrestrial vertebrates from oxidative stress [28]. Furthermore, oxidative ATM activation is also considered to be an emergent response for cells exposed to ionizing radiation in situations in which oxidized cells cannot correctly evoke MRN/DSBs-dependent ATM activation immediately after exposure, because ionizing radiation can generate ROS by ionizing H_2O, and MRN complexes can be inactivated by oxidative stress [28].

On the other hand, mitochondria are reported to be maintained by ATM. For example, ATM-null thymocytes show elevated mitochondrial numbers, increased mitochondrial ROS production, and decreased mitophagy [16]. ATM is also required for the maintenance of the copy number of mtDNA molecules by regulating certain types of ribonucleotide reductases and AMP-activated protein kinase (AMPK) [29–32]. The oxidative activation of ATM could be associated with these ATM functions regarding mitochondrial homeostasis. One possible role for ATM in mitochondria might be to function as a sensor for protecting mitochondrial components such as mtDNA from oxidative stress. In addition, the fact that peroxisomes in Hep G2 cells store some scavenging enzymes such as catalase and Cu, Zn-superoxide dismutase [33], and that mitochondria themselves are sources of ROS when their ROS removal capacity is insufficient [34] might make the sensitivity of ATM to H_2O_2 in mitochondria higher than that in peroxisomes in Hep G2 cells.

ATM has been reported to contain a putative peroxisome targeting signal [15]. Although there is no report of the existence of a mitochondria targeting signal for ATM, an unknown mitochondria targeting signal might be embedded in the ATM long polypeptide and could be discovered in the future. We also confirmed that ATM is localized in mitochondria, albeit low-levels, by immunofluorescence staining, but autophosphorylated ATM could not be detected *in situ*, since no antibody to autophosphorylated ATM that is suitable for immunostaining is currently available (data not shown). However, this is the first study to demonstrate a strong relationship between the oxidative activation of ATM and mitochondria. Further studies will be needed in order to completely elucidate the mechanisms that underlie this relationship.

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