Intrinsic mitochondrial dysfunction in ATM-deficient lymphoblastoid cells

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ABSTRACT

One of the characteristic features of cells from patients with ataxia telangiectasia (A-T) is that they are in a state of continuous oxidative stress and exhibit constitutive activation of pathways that normally respond to oxidative damage. In this report, we investigated whether the oxidative stress phenotype of A-T cells might be a reflection of an intrinsic mitochondrial dysfunction. Mitotracker Red staining showed that the structural organization of mitochondria in A-T cells was abnormal compared to wild-type. Moreover, A-T cells harbored a much larger population of mitochondria with decreased membrane potential (ΔΨ) than control cells. In addition, the basal expression levels of several nuclear DNA-encoded oxidative damage responsive genes whose proteins are targeted to the mitochondria - polymerase gamma, mitochondrial topoisomerase I, peroxiredoxin 3, and manganese superoxide dismutase - are elevated in A-T cells. Consistent with these results, we found that overall mitochondrial respiratory activity was diminished in A-T compared to wild-type cells. Treating A-T cells with the antioxidant, alpha lipoic acid (ALA), restored mitochondrial respiration rates to levels approaching those of wild-type. When wild-type cells were transfected with ATM-targeted siRNA we observed a small but significant reduction in the respiration rates of mitochondria. Moreover, mitochondria in A-T cells induced to stably express full-length ATM, exhibited respiration rates approaching those of wild-type cells. Taken together, our results provide evidence for an intrinsic mitochondrial dysfunction in A-T cells, and implicate a requirement for ATM in the regulation of mitochondrial function.
INTRODUCTION

Patients diagnosed with the autosomal recessive disorder, ataxia telangiectasia (A-T), exhibit a broad range of disease phenotypes, including progressive neurodegeneration, ocular telangiectasias, immunodeficiency, increased chromosomal instability, premature aging, and are greatly predisposed to developing lymphoid malignancies (1-4). The A-T syndrome is caused by mutations in the ATM gene (ataxia telangiectasia mutated) that result in the loss of ATM protein (5). ATM is a high-molecular weight (350kDa) serine/threonine kinase that is closely related to DNA-PKcs (DNA-dependent protein kinase catalytic subunit), ATR (A-T and Rad3-related protein), mTOR/FRAP (mammalian target of rapamycin/FKBP-rapamycin-associated protein) and ATX/SMG1, all of which are involved in the DNA damage response (6-7). The kinase function of ATM is activated immediately following exposure to damaging agents like ionizing radiation (IR) and other radiomimetic compounds that cause double strand DNA breaks (DSBs). Once activated ATM coordinates the subsequent phosphorylation and transactivation of many downstream protein substrates and signaling responses involved in repair and G1-S, intra-S and G2/M cell cycle checkpoints (3, 8-10). A-T cells show signs of elevated chromosome breaks and accelerated telomere shortening, and display an extreme sensitivity to challenges by IR.

Amongst these intriguing characteristics of A-T cells is their overwhelming sensitivity to agents that generate reactive oxygen species (ROS) and cause oxidative DNA damage, such as, IR, $t$-butyl hydroperoxide ($t$-BOOH), chromium IV, and nitric oxide (11,12). In addition, the basal expression levels of several different oxidative-damage responsive pathways involving p53, p21, Gadd45, NFκB, heme-oxygenase (HO-
1) and manganese superoxide dismutase (MnSOD) are constitutively elevated in A-T cells and in certain tissues from Atm−/− mice (13-16). Levels of lipid peroxidation and oxidized DNA bases, such as 8-hyrdoxy-2-deoxyguanosine (8-OHdG), are elevated in A-T cells (17). Taken together, these data have been interpreted by investigators to suggest that ATM-deficient cells are in a chronic state of oxidative stress, and that the ATM protein kinase plays a role in regulating cellular redox homeostasis, and in regulating genes whose proteins have antioxidant functions (18). However, the precise source and/or cause of this oxidative stress, is not understood. Given that the basal expression levels of certain oxidative-damage responsive pathways are elevated in ATM-deficient cells in the absence of any known exposure to exogenous sources of ROS and oxidative damage, we hypothesized that the molecular trigger(s) inducing the constitutive expression of many of these oxidative damage defense systems might be generated primarily within the ATM-deficient cell themselves.

While there are numerous external sources of ROS, the great majority of ROS within eukaryotic cells is thought to be derived from the mitochondrion as by-products during the generation of adenosine triphosphate (ATP), through the process of oxidative phosphorylation (19). The human mitochondrial genome is a circular double-stranded DNA molecule of ~16, 600 base pairs. The mitochondrial DNA (mtDNA) encodes 37 genes: 13 genes coding for the protein subunits of the electron transport chain/oxidative phosphorylation system (ETC/OXPHOS system), while another 24 genes specify two ribosomal RNAs (rRNAs) and twenty-two transfer RNAs (tRNAs) that are required to synthesize the 13 ETC/OXPHOS subunits (19). However, mitochondria are only semiautonomous and the bulk of the proteins of the ETC/OXPHOS system, as well as
those required for mtDNA replication, transcription, repair and mitochondrial metabolism, are encoded by over 1,000 nuclear genes (nDNA) (20).

The ETC/OXPHOS system is composed of five complexes: complex I (NADH ubiquinone oxidoreductases), complex II (succinate ubiquinone oxidoreductases), complex III (ubiquinone-cytochrome c reductase), complex IV (cytochrome c oxidase) and complex V (ATP synthase), all located in the inner mitochondrial membrane (19, 20). The flux of protons at these sites is thought to create a proton electrochemical gradient across the inner mitochondrial membrane, and the eventual flow of electrons through complex V (i.e., ATP synthase) drives the condensation of bound adenosine diphosphate (ADP) and phosphate (Pi) to generate ATP in the mitochondrial matrix (20). However, this process is apparently inherently inefficient, and it is estimated that ~1-5% of electrons flowing through the ETC system are donated to molecular oxygen to the form superoxide (O$_2^-$), the primary mitochondrial free radical (21).

Experiments with specific ETC inhibitors demonstrate that any disruption to the flow of electrons through the various ETC subunits, in particular at complexes I-III, can cause electrons to be retained by these complexes and donated to molecular oxygen (22). While O$_2^-$ is inert, it can be removed by superoxide dismutase (MnSOD) to form hydrogen peroxide (H$_2$O$_2$) which is more stable and can readily diffuse into the cytosol and nucleus. H$_2$O$_2$, in turn, in the presence of transition metals like iron or copper, can undergo Fenton chemistry resulting in the production of highly reactive hydroxyl radicals (OH$^-$), a far more damaging ROS to the cell (21). In addition, O$_2^-$ can also react with nitric oxide (NO) to generate the cytotoxic peroxynitrite anions (ONOO$^-$) (21). While basal levels of ROS are believed to function as important signal transducers, excessive
production of ROS - that overwhelms the cellular DNA repair and ROS scavenging defense mechanisms leading to DNA, protein and lipid damage - results in a state of continuous oxidative stress (23). In fact, mitochondrial dysfunction is thought to underlie the aging process, and has been shown to be an important contributing factor to the disease phenotypes associated with many progressive neurodegenerative disorders, such as Alzheimer’s disease, Parkinson’s disease, Huntington disease, amyotrophic lateral sclerosis (ALS), and Friedreich ataxia (24).

Herein, we provide evidence that mitochondrial dysfunction contributes to the continuous oxidative stress of A-T cells as well.
RESULTS

Visualizing mitochondria and determining mitochondrial membrane potential:

We visualized the mitochondria of LCLs derived from A-T patients using fluorescence microscopy and Mitotracker Red. Mitotracker Red is a noncytotoxic mitochondrion specific dye that accumulates in mitochondria in a membrane potential-dependent manner. Staining of mitochondria in wild-type cells (WT) was homogeneous and the mitochondrial appeared evenly distributed around the cell, indicative of well-preserved and actively respiring mitochondria (Fig. 1). In striking contrast, mitochondria in A-T cells seemed almost absent exhibiting a polarized distribution at one end of the cell (Fig. 1). This could be best appreciated when viewed across many focal levels.

One possible explanation for the different mitochondrial staining patterns is that the overall mitochondrial content might be lower in A-T than WT cells. To test this possibility, we extracted total cellular DNA from WT and A-T cells and measured genomic and mitochondrial DNA (mtDNA) content by PCR amplification using primers designed to target 18s RNA and the mtDNA-encoded genes, NADH dehydrogenase (ND) and 16S RNA. After separating the PCR products on an agarose gel, the ratio of mtDNA:genomic DNA (ND2:18s RNA and 16s RNA: 18s RNA) was calculated by measuring the intensity of each band. The results depicted in Fig. 2, show that the ratios of mtDNA:genomic DNA were not significantly different in WT and A-T cells, indicating that mitochondrial content did not differ.

Given that the retention of the Mitotracker Red by mitochondria depends upon membrane potential, we suspected that certain subpopulations of mitochondria in A-T
cells might have decreased membrane potential ($\Delta \Psi$), and hence be depolarized. Indeed, a diffuse Mitotracker Red staining pattern throughout the cytosol could be indicative of mitochondria with low membrane potential. To test this idea, $\Delta \Psi$ was monitored using the JC-1 non-toxic fluorescence probe and flow cytometry. When JC-1 exists as a monomer it fluoresces green and sorts in the FL1 region; in well-preserved mitochondria it accumulates selectively to form so-called “JC” aggregates which fluoresce red and sort in the FL2 region as a consequence of intact $\Delta \Psi$. The electrochemical gradient is responsible for this aggregation, thus a cell sorting into the FL1 region would be indicative of it harboring mitochondria with decreased membrane potential. This can be readily appreciated when, as a positive control, cells are treated with the mitochondrial poison, CCCP; 97% of cells sort into the FL1 region as seen in representative experiments depicted in Fig. 3. We found that whereas WT (NAT2, NAT9) cells sorted mostly as a single population in the FL2 region, A-T cells (AT160LA, AT187LA, AT7LA) sorted more distinctly as two populations of cells; one with intact membrane potential (88% in FL2) and another with decreased mitochondrial membrane potential (12% in FL1). The percentage of mitochondria with decreased membrane potential was greater in A-T than WT cells (12% versus 3%).

The basal expression of mitochondrial DNA repair and ROS scavenging genes is up-regulated in ATM-deficient cells

Many investigators have reported that the expression of certain oxidative damage responsive pathways is constitutively elevated in ATM-deficient cells (13-16). This prompted us to test whether the basal expression levels of those genes whose proteins
normally have a DNA repair and/or ROS scavenging role in the mitochondria are up-regulated in A-T cells. Using a quantitative RT-PCR approach, we measured the transcript levels of: (i) polymerase gamma (POLG), the major mitochondrial DNA replication and repair polymerase (31-33); (ii) mitochondrial-targeted topoisomerase 1 (TOP1mt), which responds to reactive oxygen modified DNA (29); (iii) peroxiredoxin 3 (Prx3), the major mitochondrial peroxidase that regulates physiological levels of H2O2 (34); (iv) manganese superoxide dismutase (MnSOD); and (v) cytochrome b5 (CYB5B), an outer mitochondrial membrane bound hemoprotein which functions as an electron carrier for several membrane bound oxygenases and appears not to participate in repair of oxidative DNA damage. Interestingly, while all four DNA repair/ROS scavenging genes were significantly up-regulated in A-T cells compared to WT, the expression of CYB5B was significantly down-regulated (Fig. 4). The CYB5B results are compatible with the reduced membrane potential exhibited by the mitochondria of A-T cells.

Mitochondrial oxidation and respiratory function

We measured the reduction of resazurin an indicator of overall mitochondrial function. Resazurin is blue and non-fluorescent and is reduced by oxidoreductases in mitochondria to resorufin, which is pink and highly fluorescent (30). The reduction of resazurin in our experiments was near linear over a three-hour time period time (Fig. 5).

While both WT (NAT2) and A-T (AT7LA, AT160LA, AT187LA) cells are able to reduce resazurin, the rate of resazurin reduction exhibited by A-T cells was significantly diminished at each time point over the three-hour course (Fig. 5). In order to demonstrate that the changes in resazurin fluorescence were indeed reflective of
mitochondrial activity, we incubated WT cells in the presence of the mitochondrial inhibitor, amiodarone, for 60 min prior to performing the resazurin assay. Amiodarone is a potent mitochondrial toxin that uncouples oxidative phosphorylation and inhibits the activities of subunits of the respiratory chain complexes I, II, III and IV (36). As shown in Fig. 5, amiodarone significantly reduced the ability of WT cells to reduce resazurin. These data demonstrate that overall mitochondrial respiration and oxidation rates are greatly compromised in unperturbed A-T cells. In addition, in limited studies, we used the resazurin assay to test mitochondrial respiration rates in frozen and fresh peripheral blood lymphocytes (PBLs) derived from A-T patients (AT2LA, AT143LA, AT220LA) and have obtained similar results (Supplemental Fig1a, b).

**Effects of ATM on mitochondrial function**

To test whether the mitochondrial dysfunction in A-T cells reflects a basic requirement the ATM protein, we modified ATM levels by either siRNA knockdown or by cadmium-chloride (CdCl₂) induction. We transfected WT cells with ATM-targeted siRNA and used the resazurin assay to measure overall mitochondrial respiration rates. We found that ATM- siRNA transfected WT cells showed diminished respiration, as compared to non-transfected- and control-siRNA transfected-WT cells (Fig. 6a). However, knockdown of ATM protein levels was greater than the reduction of mitochondrial respiration rates, suggesting that the latter is not solely dependent on ATM. We also tested whether stably transducing full-length ATM cDNA into A-T cells might increase mitochondrial respiration. A-T (AT7LA) cells were transfected with a pMAT1 construct containing the full-length ATM cDNA under the control of a CdCl₂-responsive promoter. The results show that mitochondria in A-T cells induced to express the ATM protein had
significantly higher rates of respiration than those in non-transduced A-T cells (Fig. 6b).

Taken together, these results indicate that mitochondrial function partially requires ATM.

**Localization of ATM to the microsomal fraction**

We tested whether ATM protein localizes to mitochondria, pre- and post-irradiation. We prepared nuclear, cytoplasmic and mitochondrial fractions of WT and A-T cells treated with and without IR (5G\textsubscript{\gamma}). As shown in Fig. 7, ATM was detected in both nuclear and mitochondrial fractions of WT (NAT9) but not A-T (AT160LA) cells. A slight increase in ATM was detected in the mitochondrial fraction after irradiation. These fractions were shown to be well separated by control immunoblots by using the anti-ki67 for the nucleus, anti-HSP90 for the cytoplasm, and anti-HSP70 antibody for the mitochondria (Fig. 7). Our results suggest that ATM localizes to the microsomal fraction (containing mitochondria), and are consistent with previously published reports (43, 45).

**Effects of alpha-lipoic acid (ALA) on mitochondrial respiration**

Based on the evidence presented above, we anticipated that treating A-T cells with an antioxidant that specifically targets mitochondria might reduce overall oxidative stress, which in turn would be reflected in the restoration of mitochondrial respiration rates to A-T cells but not to WT cells. ALA is a multifunctional antioxidant that eliminates many different reactive oxygen species (ROS), prevents the generation of ROS, and also increases the levels of native cellular antioxidants (37, 38). Previous studies have demonstrated that the constitutive activation of certain oxidative damage pathways in A-T cells can be greatly suppressed by treating these cells with appropriate concentrations of ALA for extended periods of time (39).
The results in Fig. 8a show that ALA significantly enhanced the respiration rates of A-T (AT7LA, AT187LA) cells to levels approaching those of WT (NAT8, NAT9) cells. At the same time, ALA seemed to have little effect on the respiration rates of WT (Fig. 8b). These data strongly suggest an involvement of increased reactive oxygen generation in diminishing the mitochondrial respiration rates in A-T cells, and provide further support for the notion that some of the oxidative stress experienced by A-T cells can be attributed to an intrinsic mitochondrial dysfunction.

**DISCUSSION**

ATM protein is predominantly a nuclear protein that initiates cell cycle checkpoint arrest and DNA repair in response to DNA double-strand break-inducing agents. However, the role(s) of the small fraction of ATM that exists outside of the nucleus remains largely unexplored. It has been reported that ATM appears to be present in, or even restricted to, the cytoplasm of Purkinje cells in humans and mice (40, 41). Cytoplasmic ATM has been shown to bind to the protein, β-adaptin, a component of the AP-2 adaptor complex that is involved in clathrin-mediated endocytosis (42), and localizes to microsomal fractions which include both endosomes and peroxisomes (43-45). Furthermore, it has been demonstrated that lysosomal proliferation is dysregulated in ATM-deficient mice (41). The reason(s) for the localization of ATM to these cellular compartments, or whether it plays an important role(s) in regulating their function, is not known at present, but might involve a hitherto unknown functional role of ATM in addition to its already well-known role as a serine/threonine kinase in the DNA damage response.
In this report, we investigated whether the function of mitochondria, the major source of intracellular ROS, is intrinsically impaired in lymphoblastoid cell lines derived from A-T patients. We note previous indirect experimental evidence for some dysfunction and dysregulation of certain mitochondrial-targeted proteins in ATM-deficient cells. For example, SOD2 activity is increased in the cerebella of Atm\(^{(-/-)}\) mice (46). However, to our knowledge, there are very few published studies directly addressing mitochondrial dysfunction in A-T cells.

To directly address mitochondrial function in A-T cells, we first compared the structural organization of mitochondria in WT and A-T cells using the mitochondrial specific stain, Mitrotracker Red; we found marked differences. Mitochondria in A-T cells stained poorly and tended to polarize at one end of the cell, phenomena usually exhibited by lymphoblastoid cells derived from patients suffering from known mitochondrial diseases (47). We suspected that the different mitochondrial staining observed in WT and A-T cells might result from a decrease in the overall numbers of mitochondria. In contrast, we found that the overall numbers of mitochondrial were very similar. Furthermore, the results of the Mitotracker experiments were more consistent with our JC-1 flow cytometry data showing that A-T cells harbor subpopulations of mitochondria with greatly reduced membrane potential (~4-fold) compared to WT. Consistent with these results, subpopulations of mitochondria with reduced membrane potential were also noted in thymocytes of Atm\(^{(-/-)}\) mice (48).

Another possible explanation for the observed variation in mitochondrial staining between WT and A-T cells could be that some A-T cells are undergoing apoptosis. Indeed, changes in mitochondrial membrane potential are an early marker of apoptosis.
However, when we formally tested this hypothesis, we found no clear indicators of apoptosis in A-T cells. Instead, we found that the overall numbers of mitochondria in WT and A-T cells were similar. In subcellular fractionation experiments with A-T cells, followed by immunoblotting for the apoptosis-inducing factor, AIF, which is usually released into the cytoplasm upon the initiation of apoptosis from mitochondria, we found that AIF was still fully contained in the mitochondrial-fraction and was absent from the cytosol and nuclear fractions (unpublished). Lastly, ultrastructural studies on A-T and WT cells confirmed that there was no difference in the numbers of mitochondria, and no signs of gross mitochondrial aberrations (unpublished).

A characteristic feature of A-T cells is their constitutive expression of signaling pathways that respond to ROS and oxidative DNA damage (13-16, 18). In the present study, we wanted to determine whether the expression levels of several mitochondrial-targeted transcripts might also be similarly up-regulated in A-T cells. Consistent with previous reports on ATM-deficient cells (46), we found a significant increase in the basal mRNA expression levels of SOD2 in the A-T cells tested. In addition, we found that the expression levels of certain mitochondrial-targeted DNA repair proteins, namely, POLG and TOP1mt, together with another ROS scavenging enzyme, Prx3, are significantly elevated in A-T compared to WT cells. In contrast, the expression level of an integral outer mitochondrial membrane electron carrier, CYB5B, is significantly down-regulated and appears to be compatible with the reduced mitochondrial membrane potential demonstrated by the A-T cells in the JC-1 assay. The elevated expression of the mitochondrial-targeted DNA repair proteins could reflect a compensatory mechanism to deal with mitochondrial DNA damage resulting from the mitochondrial dysfunction in A-
T cells. In this regard, it has been demonstrated that the close proximity of mtDNA to the electron transport chain predisposes mtDNA to damage from ROS species resulting from mitochondrial respiratory dysfunction (51). Furthermore, the elevated SOD2 and Prx3 transcript levels might also reflect a response to elevated cellular ROS concentrations, $O_2^-$ and $H_2O_2$, respectively (21, 22, 34). $H_2O_2$ is relatively stable and can readily diffuse into the cytoplasm and nucleus, where it can undergo Fenton chemistry producing the hydroxyl radical (OH') (20-23). On this line, it was previously reported that labile iron levels are increased in ATM-deficient cells, and that incubating these cells with iron-chelators can dramatically reduce DNA damage in these cells (52). On the basis of our data, it is tempting to speculate that A-T cells appear to be experiencing mitochondrial-derived oxidative stress, which combined with their dysregulation in iron homeostasis might only serve to exacerbate their constitutive state of oxidative stress.

The abnormal structural distribution of mitochondria in A-T cells, together with their reduced membrane potential and elevated levels of oxidative-damage responsive repair systems could result from an underlying mitochondrial respiratory dysfunction. Consistent with this idea, we found that the overall respiration rates of mitochondria in A-T cells were significantly diminished in all of the A-T cells lines tested. Further, this defect could be alleviated with the multifunctional anti-oxidant, alpha lipoic acid (ALA). Given that ALA is an essential co-factor for two mitochondrial enzyme complexes, namely, pyruvate dehydrogenase and alpha-ketoglutarate dehydrogenase (37, 38), it is difficult to tease apart the exact role of alpha lipoic acid in restoring mitochondrial function in A-T cells. However, the negligible affect of ALA on mitochondrial
respiration rates in WT cells seems to suggest that ALA is more likely restoring mitochondrial function in A-T cells through its anti-oxidant capacities.

These data, and the fact that ATM has been shown to localize to a number of different cytoplasmic organelles, prompted us to examine whether ATM plays a role in maintaining normal mitochondrial respiratory function, and hence mitochondrial stability. We tested this idea by confirming that ATM localizes to microsomes. In addition, we found that reducing ATM protein levels in WT cells using ATM-targeted siRNA led to a significant reduction in mitochondrial respiration rates. Furthermore, induced expression of ATM in stably transfected A-T cells expressing full-length ATM cDNA restored mitochondrial respiration to levels approaching WT. How ATM might be regulating mitochondrial respiration is not yet known. Efforts to physically localize ATM to mitochondria by electron microscope (EM) immunocytochemistry produced only equivocal results (unpublished). Our data suggest that ATM could be modulating mitochondrial respiratory rates directly or indirectly, or perhaps in some DNA repair role. In this regard, a number of ATM-dependent substrates, cyclic AMP response element-binding protein (CREB), p53 and BRCA1, and ATM-related kinases (for example, mTOR) have been shown to translocate to the mitochondria and to be required for proper mitochondrial function (53-57). Another possibility is that ATM might be regulating mitochondrial function in a DNA-repair-independent, and hence metabolic capacity. In either case, our data implicate a need for functional ATM protein in maintaining proper mitochondrial function.

In summary, our results suggest that A-T is similar to other progressive neurological disorders that are characterized by oxidative stress and intrinsic
mitochondrial dysfunction (19). This may be of translational relevance, in that clinical investigations for A-T patients should target the correction of mitochondrial function, such as with antioxidant compounds that specifically act to reduce the oxidative stress that results from mitochondrial dysfunction.

MATERIALS AND METHODS

Cell culture and cell lines

Wild-type (WT) and A-T lymphoblastoid cells (LCLs) were cultured with 5% CO₂ at 37°C in Roswell Park Memorial Institute (RPMI) growth medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin-gentamicin. A-T LCLs transfected with the pMAT1 construct, which contains the full-length ATM cDNA under the control of the inducible metallothionein II promoter, were cultured in the presence of hygromycin B (200µg/ml).

Subcellular Fractionation

Nuclear and cytoplasmic extracts were prepared using the NE-PER™ Nuclear and Cytoplasmic Reagent’s kit (Pierce Biotechnology) and following the manufacturer’s instructions. Mitochondrial isolation was achieved by using the Mitochondrial Isolation Kit (Pierce Biotechnology) and by following the manufacturer’s instructions outlined for the reagent-based method. Mitochondrial pellets were lysed in a buffer containing 20mM Tris-Cl pH 7.4, 300mM NaCl, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100, 14mM 2-mercaptoethanol and a protease cocktail. Protein concentration was determined with the Bradford assay, with bovine serum albumin as a standard.

Immunoblot Analysis
Unless otherwise stated, a total of 100µg of nuclear, cytoplasmic and mitochondrial lysates was denatured by boiling for 5 min in SDS sample buffer and subjected to SDS-polyacrylamide gel electrophoresis. Separated proteins were transferred to a polyvinylidene difluoride membrane at 4°C overnight, after which time the membrane was incubated with 5%(w/v) non-fat dry milk prepared in 1X phosphate-buffered saline supplemented (PBS) with 0.1%(v/v) Tween-20 for 1 h at room temperature. After blocking, the membrane was incubated with the relevant primary antibody at 4°C overnight. Immune complexes were detected with a horseradish peroxidase-conjugated anti- rabbit or anti-mouse secondary antibody and by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Antibodies

The following antibodies were used in these experiments: β-actin (1:3000, Sigma), and Ki67 (1:1000), ATM (1:1000), mitochondrial HSP70 (1:1000) and HSP90 (1:30,000) were from Novus.

PCR and real-time quantitative PCR

To measure the mitochondrial DNA content (mtDNA) in WT and A-T cells, total DNA was extracted. Primer sets for two mtDNA-encoded genes, ND2 and 16s RNA, were used for PCR analysis, together with a primer set for the nuclear DNA (nDNA)-encoded gene, 18s RNA. PCR products were electrophoresed on a 1% (w/v) agarose gel, stained with 0.5 µg/ml ethidium bromide, and visualized with an UV transilluminator. mtDNA content was assessed by determining the ratio of ND2:18s RNA and 16s RNA:18s RNA by scanning and comparing the intensities. The sequences of the primers used in this study were: 18s RNA, 5’-tagagggacaagtgcggttc-3’, and 5’-cgtgagccgctgtcgtgt-3’ (25); for
16s RNA, 5’-ccaattaagaagctcaag-3’, and 5’-catgcctgtgtgggtgaca-3’; and for ND2, 5’-
cctagccccccatctcaatcata-3’, and 5’-gaatgcggtagtagttaggat-3’ (26, 27). The PCR consisted of
a 3 min step at 94°C and 25 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 1 min, and a
final step of 72°C for 8min.

For QPCR analysis, RNA was isolated from three WT and three A-T cells by using
RNeasy isolation kit (Qiagen). One microgram of total RNA was reversed transcribed by
using random primers and Superscript –III reverse transcriptase (Invitrogen).

Quantitative RT-PCR was performed by using an ABI Prism 7700 (Applied
Biosystems, Foster City, CA) and SYBR Green detection (SYBR Green Taq ready mix;
Sigma). The primers used for QPCR analysis were: Prx3, 5’-ggagtcatcaagcatttgagg-3’
and 5’-gtaggagaatccccgttca-3’ (28); for TOP1mt, 5’-ctacaaccgagcctgagcag-3’ and 5’-
acccatgaaccgctgacca-3’ (29); and for SOD2, 5’-tggtaagccccagagctcctca-3’ and 5’-
cagttgatggttccagcaact-3’ (Integrated DNA Technologies, Inc). POLG
(Hs_POLG_1_SG Quantitect Primer assay) and CYB5B (Hs_CYB5B_1_SG Quantitect
Primer assay) were from Qiagen. The PCR consisted of a 2 min step at 94°C and 40
cycles of 94°C for 15 s, 60°C for 1 min, 72°C for 1 min, and ending with a slow heating
step from 55°C to 95°C to generate the melting curve data. Serial dilutions of the pooled
samples were used to construct the standard curve and for determining the real-time PCR
efficiency for each primer pair by using the ABI Prism 7700 software. Each individual
sample cDNA was analyzed separately and corrected for the β2M expression. The final
data are expressed as relative mRNA expression.

siRNA-mediated down-regulation of ATM
For siRNA experiments, cells were transfected with for 96 h with 50nM siRNA in medium containing 10% (v/v) FBS using HiPerfect reagent (Qiagen) according to the manufacturer’s protocol for cells in suspension. Hs_ATM_12 HP Validated siRNA was from Qiagen.

Confocal microscopy using MitoTracker Red

Mitochondrial were viewed in LCLs by staining live-cells and using the Mitotracker Red CMXROS kit (Molecular probes). Briefly, 1x10⁶ cells/ml cells were pelleted and resuspended in pre-warmed RPMI growth medium containing the Mitotracker Red probe (50nM). Cells were incubated for 45 min under growth conditions. After staining, cells were pelleted by centrifugation and resuspended in pre-warmed growth medium containing 3.7% (v/v) formaldehyde and incubated at 37°C for 15 min, after which time the cells were rinsed several times with 1x PBS and mitochondria visualized at 63x (oil) magnification using a Leica TCS-SP automated confocal microscope equipped with Leica software.

Flow cytometry and JC-1 assay

For each sample, 1x10⁶ cells were suspended in 1ml of warm 1x PBS and stained with 2µM JC-1 (5,5’-6,6’-tetrachloro-1,1’,3,3’-tetracyethylbenzimidazol-carbocyanine iodide)(Molecular Probes) and incubated under growth conditions for 30min. To confirm that the JC-1 probe was responding to changes in mitochondrial membrane potential, cells were incubated with 50µM CCCP (supplied with the kit) simultaneously with JC-1. Cells were then pelleted by centrifugation and resuspended in 500µL 1xPBS and immediately analyzed using a BD FACS Calibur flow cytometer.
Mitochondrial respiration assay.

Mitochondrial respiration in whole cells was measured using the established resazurin (also known as Alamar Blue) assay (30). WT and A-T cells (1x10^5 cells/well) were incubated with resazurin (3µM) in a 96-well microtitre plate format under growth conditions. Each experimental condition was repeated in 6 different wells. WT cells were also pre-treated with the mitochondrial inhibitor amiodarone (200 µM), for 60 min prior to performing the resazurin assay. Wells containing only resazurin and medium served as controls. Resazurin fluorescence intensity was measured (530nm excitation and 590nm emission) every 60 min over a 3 h time course using the FLX-800 microplate fluorescence reader (Bio-Tek Instruments, Inc).

Amiodarone and alpha lipoic acid (ALA)

Amiodarone and ALA were from Sigma. When required, WT and A-T cells were treated with ALA (250µg/ml) for 72 h prior to performing the resazurin assay.
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Conflict of Interest statement. None declared
REFERENCES


**LEGENDS to FIGURES**

**Figure 1.** Mitotracker Red CMXROS staining reveals abnormal mitochondrial staining and distribution in A-T cells. Live-cells (1x10^6 cells/ml) were incubated with Mitotracker Red (50nM) for 45 min under growth conditions, followed by fixation with 3.7% (v/v) formaldehyde. Mitochondria were visualized via confocal microscopy (x63[oil] objective). The left column indicates the nuclei, the middle column indicates Mitotracker Red staining, and the right column indicates their merged images.

**Figure 2.** mtDNA content is similar in WT and A-T cells. Total cellular DNA was extracted from WT (NAT9) and A-T (A-T1 is AT160LA; A-T2 is AT187LA) cells and genomic and mtDNA content assessed by PCR using primers targeting 18s RNA and the mitochondrial-encoded genes, ND2 and 16s RNA. PCR products were separated on an 1% (w/v) agarose gel. The ratio of ND2:18s RNA and 16s RNA:18s RNA was calculated by measuring the intensity of each band (bar graphs).

**Figure 3.** Membrane potential of mitochondria in WT (NAT9) and A-T cells (AT187LA). Live WT and A-T cells were incubated with JC-1 (2µM) alone, or JC-1 plus the mitochondrial membrane potential-disruptor, CCCP (50µM), for 15 min at 37°C. Cells were pelleted by centrifugation and resuspended in PBS, and mitochondrial membrane potential assessed via flow cytometry. Loss of mitochondrial membrane potential (Δψ) is indicated by a decrease in FL2/FL1 fluorescence intensity ratio. The results are representative of four independent experiments.

**Figure 4.** The basal expression of four mitochondrial DNA repair and ROS scavenging genes is up-regulated in ATM-deficient cells, while the expression of an outer mitochondrial membrane electron carrier is down-regulated. Total RNA was extracted
from three WT (NAT5, NAT8, NAT9) and three A-T (AT7LA, AT160LA, AT220LA) cells. The expression of TOP1mt, Prx3, SOD2, POLG and CYB5B were assessed by quantitative RT-PCR. Expression was standardized to β2M control expression.

**Figure 5.** Mitochondrial function is impaired in A-T cells. WT (NAT2) and A-T (AT-1 is AT160LA; A-T2 is AT7LA; and AT-3 is AT187LA) cells were incubated with resazurin (3µM) in a 96-well microtitre plate format under growth conditions, in replicates of six for each condition. As an additional control, WT cells were pre-treated with the mitochondrial inhibitor, amiodarone (200µM), for 60 min prior to performing the assay. Wells containing only resazurin and medium served as controls. Resazurin fluorescence intensity was measured (530nm excitation and 590nm emission) every 60 min over a 3 h time course using the FLX-800 microplate fluorescence reader. N=6. Mean±SD.

**Figure 6.** Effects of ATM on mitochondrial function. (a) WT (NAT9) cells were not-transfected or transfected with double stranded small interfering oligos (siRNA, 50nM) specific for ATM or with non-targeting (NT) oligos as control. After 96 h of incubation under growth conditions, mitochondrial function was assessed in control, siRNA-transfected, and A-T (AT221LA) cells using the resazurin assay. ATM-knockdown was confirmed by immunoblotting. A total of 25µg of nuclear protein per lane was separated on a 6% SDS-polyacrylamide gel. The immunoblot was probed with a polyclonal anti-ATM antibody, and a monoclonal anti-β-actin antibody as a loading control. (b) A-T (AT7LA) cells were stably transfected with the ATM cDNA expression vector, pMAT1. Expression of ATM was achieved by induction of transfected cells with 5 µM CdCl₂ over a 72 h period. Mitochondrial function was assessed in control (NAT5), mock- and CdCl₂-
treated A-T cells using the resazurin assay. ATM-induction was confirmed by immunoblotting. A total of 50µg of nuclear protein per lane was separated on a 6% SDS-polyacrylamide gel. The immunoblot was probed with a polyclonal anti-ATM antibody, and a monoclonal anti-β-actin antibody as a loading control.

**Figure 7.** Localization of ATM to the microsomal fraction of WT LCLs pre- and post-irradiation. Nuclear (NF), cytosolic (CytF) and microsomal fractions (MitF) were prepared from WT (NAT9) and A-T (AT160LA) treated with and without ionizing radiation (5Gγ). A total of 100µg of nuclear, cytoplasmic and microsomal protein per lane was separated on a 6% SDS-polyacrylamide gel. The immunoblot was probed with polyclonal anti-ATM, anti-ki67 (nuclear-marker), anti-HSP90 (cytoplasmic-marker) and anti-mtHSP70 (mitochondrial-marker) antibodies.

**Figure 8.** The multifunctional antioxidant, alpha lipoic acid (ALA), restores mitochondrial function in A-T cells. (a) A-T (A-T1 is AT187LA, A-T2 is AT7LA) cells were treated with ALA (250 µM) for 72 h under growth conditions after which time mitochondrial function was assessed using the resazurin assay. The WT is NAT9. (b) Effects of ALA on mitochondrial function in WT (WT1 is NAT9; WT2 is NAT8) cells was negligible, as compared to A-T (AT222LA) cells.
Fig. 2

WT  A-T1  A-T2
ND2 16s RNA  ND2 16s RNA  ND2 16s RNA
16s RNA

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<td>Ratio of 16s RNA</td>
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Fig. 3

WT

Not-treated

FL2

10^2

10^4

10^6

10^8

FL1

97%

3%

FL1

10^2

10^4

10^6

10^8

FL1

97%

3%

FL1

A-T

FL2

10^2

10^4

10^6

10^8

FL1

88%

12%

FL1

10^2

10^4

10^6

10^8

FL1

97%

3%

FL1

JC-1 ratio FL2/FL1

WT

A-T

0

5

10

15

20

25

30

35

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Fig 4

![Graph showing gene expression levels](image-url)

- TOP1mt: p = 0.03
- Prx3: p = 0.34
- SOD2: p = 0.06
- PolG: p = 0.12
- CYBSB: p = 0.03
Fig. 5

[Graph showing fluorescence over time for different conditions: WT, A-T1, A-T2, A-T3, WT + amiodarone, medium + resazurin.]
Fig. 6

**A**

![Graph A]

**B**

![Graph B]
Fig. 7

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