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# Activation of AMP-activated protein kinase in cerebella of *Atm*−/− mice is attributable to accumulation of reactive oxygen species

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ABSTRACT

Ataxia telangiectasia (A-T) is an inherited disease, the most prominent feature of which is ataxia caused by degeneration of cerebellar neurons and synapses. The mechanisms underlying A-T neurodegeneration are still unclear, and many factors are likely to be involved. AMP-activated protein kinase (AMPK) is a sensor of energy balance, and research on its function in neural cells has gained momentum in the last decade. The dual roles of AMPK in neuroprotection and neurodegeneration are complex, and they need to be identified and characterized. Using an *Atm* (ataxia telangiectasia mutated) gene deficient mouse model, we showed here that: (a) upregulation of AMPK phosphorylation and elevation of reactive oxygen species (ROS) coordinately occur in the cerebella of *Atm*−/− mice; (b) hydrogen peroxide induces AMPK phosphorylation in primary mouse cerebellar astrocytes in an *Atm*-independent manner; (c) administration of the novel antioxidant monosodium luminol (MSL) to *Atm*−/− mice attenuates the upregulation of both phosphorylated-AMPK (p-AMPK) and ROS, and corrects the neuromotor deficits in these animals. Together, our results suggest that oxidative activation of AMPK in the cerebellum may contribute to the neurodegeneration in *Atm*−/− mice, and that ROS and AMPK signaling pathways are promising therapeutic targets for treatment of A-T and other neurodegenerative diseases.



## 1. Introduction

A Ataxia-telangiectasia (A-T) is an autosomal recessive genetic disease caused by mutations of the *Atm* (ataxia-telangiectasia mutated) gene [1,2]. Humans with A-T display multiple phenotypes, of which, the most prominent is progressive neurodegeneration. The *Atm* gene product is the ATM protein kinase. When activated, ATM plays a critical role in regulation of cell cycling, DNA repair, and cellular redox status [2,3]. In the last of these functions, ATM participates in upregulation of antioxidants, such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase [4]. ATM might also regulate cellular ROS levels by increasing production of reductive precursors and decreasing energy consumption and ROS production by mitochondria [2]. In the brain of ATM-deficient mice, levels of ROS are intrinsically upregulated in a number of cell types [5–8]. Persistent oxidative stress in the ATM-deficient brain disturbs intracellular antioxidant defense systems and redox homeostasis, thereby activating downstream signaling pathways, including those involving p38 [8] and ERK1/2 [6,7]. Lack of ATM expression also causes mitochondria dysfunction with decreased membrane potential and energy balance [2].

AMP activated protein kinase (AMPK) is an evolutionarily conserved serine/threonine protein kinase that is expressed in most mammalian tissues, including the brain [9]. It is a master metabolic switch that is involved in many regulatory pathways [10]. In peripheral tissues, rising AMP/ATP ratios results in AMPK phosphorylation and its activation restores energy balance [11]. In the central nervous system (CNS), AMPK participates both in neuroprotection and in neurodegeneration [12]. Of the several cell types in mammalian brains, neurons require the largest amount of energy, and they consume more than 50% of the total available blood glucose [13]. However, neurons themselves generate and store glucose poorly, making them sensitive to fluctuations in blood glucose levels. Therefore, it is not surprising that AMPK is expressed throughout the CNS [14,15]. It has been shown that neurodegeneration induced by β-amyloid through AMPK activation can be attenuated by treatment of melatonin and resveratrol [16]. In a mouse model for cerebral ischemia, AMPK phosphorylation was

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persistently elevated after middle cerebral artery occlusion [17]. Furthermore, in a mouse model for stroke, administration of a modulator of neuronal AMPK, diminishes stroke damage. As noted above, phosphorylation of AMPK also plays positive roles in the brain. For example, AMPK activation by metabolic stress or ischemia increases $GABA_B$ activation, which improves neuronal survival [18]. Similarly, AMPK activation increases expression of brain-derived neurotrophic factor, which is neuroprotective [19]. In the brain, AMPK activation is linked to the energy status in hypothalamic neurons to the regulation of food intake and energy expenditure. This AMPK activation can be suppressed by α-lipoic acid [20].

It has been shown that AMPKα is activated in an ATM dependent manner in response to IGF-1 stimulation [21]. AMPK activation was also observed in HeLa cells treated with 5-aminoimidazole-4-carboxamide-1-β-4-ribofuranoside, but this was found to be inhibited by an ATM specific inhibitor, KU-55933 [22]. Both of these results suggest that ATM is essential for AMPK activation. In other words, AMPK activation is likely to be defective or abnormal in ATM deficiency.

In this study, we compared the levels of p-AMPKα, the catalytic subunit of AMPK, in *Atm+/+* and *Atm−/−* mouse cerebella. Unexpectedly, we found that p-AMPKα levels were higher in *Atm−/−* cerebella than in *Atm+/+* cerebella. Because *Atm−/−* cerebella suffer from oxidative stress [23] that in turn causes AMPK activation [24], we proposed that activation of AMPK in *Atm−/−* cerebella may be attributable to ROS accumulation in the tissues. The results presented in the paper confirm that oxidative stress marker MDA (malondialdehyde) is increased in *Atm−/−* cerebella, compared to its levels in *Atm+/+* cerebella. In addition, in primary cultured cerebellar astrocytes, hydrogen peroxide induces AMPK activation in an *Atm*-independent manner. Moreover, in *Atm−/−* mice, administration of the novel antioxidant monosodium luminol (MSL) reduces the abnormally high levels of ROS and of p-AMPKα in their cerebellar tissues, substantiating the notion that upregulation of ROS and AMPK activation are linked. Finally, MSL treatment also corrects the neuromotor deficits that are otherwise characteristic of adult *Atm−/−* mice.

## 2. Materials and methods

### 2.1. Animals and animal treatments

*Atm−/−* mice used in this study were originated by Barlow et al. [3]. Heterozygous *Atm+/−* mice were purchased from the Jackson Laboratory, mated, and kept in the Animal Center at The University of Texas, MD, Anderson Cancer Center, Department of Molecular Carcinogenesis as described previously [25]. Genotyping was carried out by polymerase chain reaction, as described [26]. All studies were reviewed and approved by the Institutional Animal Care and Use Committee.

Monosodium luminol, or MSL, treatment started immediately after weaning (at 21 days after birth). *Atm−/−* mice received MSL (1 g/L) at 150 mg/kg/day via drinking water and control *Atm−/−* mice received water alone. The solution was changed every other day. The treatment lasted until indicated time when the treated and control animals were used for different experiments. Another antioxidant, NAC-amide (AD4), was also used in one group of mice. After intraperitoneal injection, AD4 efficiently enters brain cells [27]. In this group, *Atm−/−* mice were treated with AD4 at 200 mg/kg/day, via ip injection, for comparison of its effects with those of MSL.

### 2.2. Immunohistochemistry

Paraffin section immunohistochemistry was performed as described previously [7]. Briefly, *Atm−/−* ($n = 5$) and *Atm+/+* ($n = 5$) mice were anesthetized at 3 months old by intraperitoneal injection of pentobarbital (150 mg/kg), and then transcardially perfused with 10% buffered formalin. After 12 h of fixation, each brain was dissected, and the cerebellar segments separated for further processing. For localization of p-AMPKα, paraffin-embedded sections were cut (6 μm), and then were deparaffinized and washed with Tris-buffered saline. The sections were then subjected to an antigen retrieval protocol, and then incubated overnight at 4 °C with rabbit anti-p-AMPKα antibody (Cell signaling). After three 5-min washes in TBS, the sections were incubated with biotin-conjugated anti-rabbit or anti-goat immunoglobulin G (IgG) and then treated with reagents from a Vecta-Elite streptavidin peroxidase kit with a benzidine substrate for color development. The sections were counterstained with diluted hematoxylin.

Alternatively, cerebella tissues removed from *Atm−/−* and *Atm+/+* animals were frozen in Tissue-Tek OCT embedding medium (Electron Microscopy Sciences, Hatfield, PA) in liquid nitrogen, and cut as 5 μm sections. The sections were then stained as described previously [28]. The primary antibodies include rabbit anti-mouse GFAP and goat anti-MDA (GeneTex). Anti-goat or anti-rabbit IgG conjugated either with fluorescein or Texas Red (Jackson Immunoresearch, West Grove, PA) are used as secondary antibodies.

### 2.3. Western blotting

Preparation of protein lysates from cerebella tissue and primary cultured cerebellar astrocytes and Western blot analyses were performed as described previously [7]. The antibodies used here included anti-AMPKα, p-AMPKα (Cell signaling), and anti-MDA.

### 2.4. Scoring of neuromotor deficiencies

We have devised a sensitive test for the neuromotor deficiencies. The apparatus combines walking across and balancing on a beam to test the motor coordination of the mice. Under conditions of minimal distraction the mice were placed on a thin hexagonal wood beam (7 mm diameter, 30 cm long) that was spun at a speed of 24 rpm, controlled by a rotator. The time the mouse holds on to the beam while walking across it is observed. Motor coordination and balance were scored by measuring the time (in seconds) the mouse stay on the beam without falling off. Prior to the test, mice had trained on the rotating beam for 3 days starting at 3 weeks of age for 1 week. Each mouse was tested 3 times/test, 1 test/week. Testing began at 4 weeks of age, and was continued up to 3 months of age), which is part of the *Atm−/−* syndrome. Each treatment group contained at least 10 animals (*Atm +/+* untreated, $n = 10$; *Atm−/−* untreated, $n = 11$; *Atm−/−* MSL treated, $n = 10$).

### 2.5. Statistic analysis

Data are presented as means ± standard error (SEM). Statistical significance of the results was determined by analysis of variance (ANOVA) or Student's *t*-test. *P* values of $<0.05$ were considered statistically significant.

## 3. Results

### 3.1. p-AMPKα levels increased in cerebella of Atm−/− mice

ATM kinase has been reported to be essential for AMPK activation [21,22]. To assess AMPK activation in *Atm−/−* cerebella, we compared p-AMPKα levels in the tissues from 3-month old *Atm+/+* vs. *Atm−/−* mice using Western blot analysis. Fig. 1A shows that p-AMPKα levels in *Atm−/−* cerebella are increased, rather than decreased (as we expected), compared to those in *Atm+/+* cerebella.

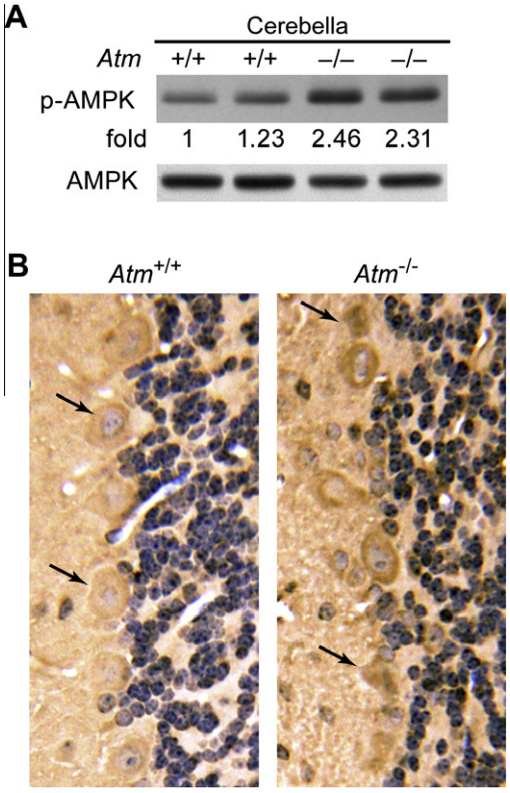


**Fig. 1.** Increased levels of p-AMPK in *Atm*−/− cerebella. (A) Western blot analysis for p-AMPK and total AMPK levels. Proteins extracted from whole cerebellar tissues of 3-month old *Atm*+/+ and *Atm*−/− mice were immunoblotted after electrophoretic separation. (B) Immunohistochemistry staining for p-AMPK in paraffin-embedded cerebellar sections from 3-month old *Atm*+/+ and *Atm*−/− mice. Arrows indicate the Purkinje cells.

To confirm the elevation of p-AMPKα in *Atm*−/− cerebella, and to visualize the cells in which this occurs, we used immunohistochemistry analysis. Fig. 1B shows that the cytoarchitecture of the Purkinje cells is disorganized in cerebella from the *Atm*−/− mice, compared to the normal cerebella from *Atm*+/+ animals. In addition, immunostaining of p-AMPKα is more intense in the *Atm*−/− sections than in the *Atm*+/+ sections, confirming that more p-AMPKα is present.

### 3.2. The oxidative stress marker MDA is increased in Atm−/− cerebella

Oxidative stress is the hallmark of *Atm* deficiency in all of the tissues affected by this mutation [1,29,30]. Typically, increased levels of ROS are present in the cerebella of adult *Atm*−/− mice [23] as shown by significant alterations in the activity of thioredoxin, catalase, and manganese superoxide dismutase. In this study, we extended this picture further by immunostaining for the lipid peroxidation marker malondialdehyde (MDA) in *Atm*−/− vs. *Atm*+/+ cerebellar tissues. Oxidation of membrane lipids produces a reactive product MDA, which can then form adducts with normal cellular proteins. Production of MDA is a late manifestation of extreme oxidant stress in cells. Fig. 2 shows that Purkinje cell bodies in normal *Atm*+/+ cerebella are only lightly stained for MDA (green staining), and that these cells are closely associated with Bergmann astrocytes (red GFAP staining), which also show anti-MDA staining (merged). In these normal cerebellar tissues, the GFAP-positive Bergmann astrocytes surround the Purkinje cell bodies with normal foot processes, and their longitudinal processes extend horizontally into the molecular layer. The situation is quite different in cerebellar sections from *Atm*−/− mice. In these abnormal brains, *Atm*−/− Purkinje cell bodies and Bergmann astrocytes show significant staining with anti-MDA. In addition, the linear arrangement of the MDA-stained *Atm*−/− Purkinje cells is disorganized, compared to the precise and orderly cytoarchitecture of these cells in the *Atm*+/+ cerebellum. These data confirm that extreme oxidative damage, caused by membrane lipid peroxidation reactions, occurs in both neurons and astrocytes of *Atm*−/− cerebella.

### 3.3. AMPK is activated in response to hydrogen peroxide in both Atm+/+ and Atm−/− cerebellar neural cells in vitro

Because elevated ROS levels can induce AMPK activation, which occurs coordinately with oxidative stress in *Atm*−/− cerebella [24], we hypothesized that the accumulation of ROS is responsible for AMPK activation in *Atm*−/− cerebella. To test this idea, we prepared primary cultures of cerebellar astrocytes from *Atm*+/+ and *Atm*−/− newborn mice, and compared their levels of p-AMPKα after exposure of the cells to hydrogen peroxide. Fig. 3A shows that hydrogen peroxide treatment induces AMPKα phosphorylation in cerebellar astrocytes from *Atm*+/+ and *Atm*−/− mice alike, and that *N*-acetylcysteine (NAC) and MSL, both of which are antioxidants, can decrease AMPKα phosphorylation induced by hydrogen peroxide. This observation confirms that oxidative stress can cause AMPK activation in cultured cerebellar astrocytes, even when ATM is absent. Consistent with the major characteristic of progressive neurodegeneration in A-T, there is no difference in p-AMPKα levels between *Atm*−/− and *Atm*+/+ cerebellar astrocytes in newborn mice (Fig. 3A bands 1 and 4 of top line). However, when treated with hydrogen peroxide alone p-AMPKα level is higher in *Atm*−/− cerebellar astrocytes than in *Atm*+/+ ones (Fig. 3A bands 2 and 5 of top line). These results show that in cerebellar astrocytes AMPK activation is ATM-independent, and that *Atm*−/− cerebellar astrocytes is more sensitive to hydrogen peroxide in activation of AMPK than *Atm*+/+ cells.

### 3.4. Administration of MSL prevents the elevation of ROS, thereby suppressing AMPK activation in Atm−/− cerebella

We have shown that the MSL prevents oxidative neurodegeneration and immunodegeneration induced by the retrovirus *ts*1 [28]. The data shown here establish that MSL blocks hydrogen peroxide induced AMPKα phosphorylation in cerebellar astrocytes (Fig. 3B). Together, these results prompted us to perform experiments to test the effects of MSL on ROS production and AMPK activation in *Atm*−/− cerebella, and to determine whether the characteristic neurobehavioral deficits of *Atm*−/− mice could be reversed. Fig. 4 shows that both MDA levels (Fig. 4A) and p-AMPKα levels (Fig. 4B) are reduced in the cerebella of MSL-treated *Atm*−/− mice, compared with those in untreated *Atm*−/− mice. The antioxidant AD4 was similarly effective in reducing p-AMPKα in *Atm*−/− cerebella (Fig. 4B).

### 3.5. The neuromotor deficit in Atm−/− mice is corrected by MSL treatment

We recognized that the neuromotor deficits of *Atm*−/− mice do not reach the level of severity observed in A-T humans, but histopathological evidence of cerebellar neurodegeneration is present in *Atm*−/− mice. For more sensitive detection of subtle *Atm*−/− neuromotor deficit, we have devised an apparatus as described in Section 2. We then compared the neurobehavioral performances of MSL-treated vs. untreated *Atm*−/− mice. The recorded units for each mouse tested were seconds during which the animal could remain on the beam without falling off. Fig. 4C shows that MSL treatment significantly improves neuromotor performance in *Atm*−/− mice, allowing them to remain on the beam significantly longer than do untreated *Atm*−/− animals.

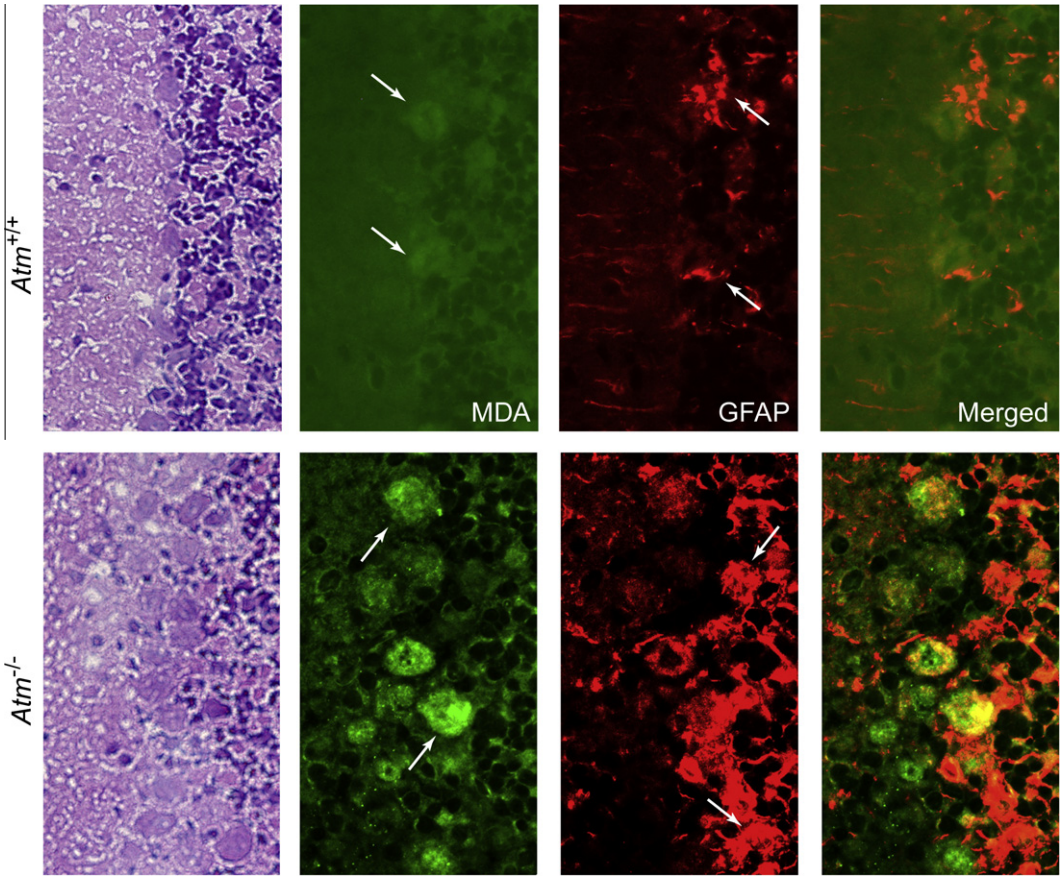


**Fig. 2.** Increased MDA levels in *Atm*−/− cerebella. The panels on top and bottom are the same fields in frozen sections of *Atm*+/+ and *Atm*−/− cerebella, respectively. The granular layer of the cerebella is at the right in each section, and the molecular layer is at the left. (1) Hematoxylin and Eosin staining; (2) MDA staining (green) with arrows indicating the Purkinje cells; (3) GFAP (astrocyte marker) staining (Red) with arrows indicating the Bergmann astrocytes; (4) 2 and 3 merged (yellow). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

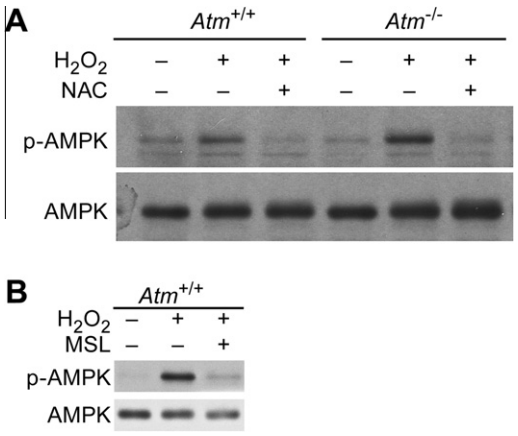


**Fig. 3.** Hydrogen peroxide ($H_2O_2$) induces AMPK phosphorylation in primary cultured cerebellar astrocytes. (A) Western blot analysis for p-AMPK in *Atm*+/+ and *Atm*−/− cerebellar astrocytes treated or untreated with hydrogen peroxide (200 μM) and/or NAC (2 mM) for 2 h. (B) Western blot analysis for p-AMPK in *Atm*+/+ cerebellar astrocytes treated or untreated with hydrogen peroxide (200 μM) and/or MSL (2 mM) for 2 h.

## 4. Discussion

In both mice and humans, the hallmark of A-T is progressive neurodegeneration, manifested as cerebellar ataxia [3]. The brains and the immune system of *Atm*−/− mice are sites of oxidant stress [5–7,29–31], and that this condition is critically involved in neuroimmunodegeneration. In the brain, a major target of *Atm*−/− oxidative damage is cerebellar Purkinje neurons (PNs) and subventricular zone neural stem cells (NSCs) [5,6]. Our previous work has shown that chronic oxidative stress is responsible for damage of brain cells including astrocytes and NSCs in *Atm*−/− mice with activation of the ERK1/2 and the MAPK signaling pathways, respectively [6–8]. In this study, we also detected oxidative stress marker in PN. However, we showed here that oxidative stress is responsible for activation of AMPKα in *Atm*−/− cerebella in an ATM-independent manner.

The ATM protein kinase participates in maintenance of cellular redox homeostasis and mitochondrial function. Accordingly, ATM-deficient lymphoblastoid cells exhibit an intrinsic upregulation of ROS and mitochondrial dysfunction [32]. Using transcriptomics, proteomics, and metabolomics, Cheema et al. have reported that ATM regulates purine, pyrimidine, and urea cycle pathways, by way of its control of oxidative stress and activation of AMPK [33]. As noted above, other recent studies have shown that AMPK phosphorylation by IGF-1 and AICAR is ATM-dependent activity [21,22]. However, whether ATM directly phosphorylates AMPK in the CNS is unclear. Our data, presented here show conclusively that AMPK phosphorylation in response to hydrogen peroxide can occur in the absence of ATM. This means that not all events that induce AMPK phosphorylation depend on ATM activity.

Emerging studies indicate that AMPK signaling is implicated in oxidative stress in Alzheimer's disease (AD) pathogenesis [34,35]. It is well known that the amyloid-β peptide (Aβ) is a major participant in the pathogenesis of AD [36] and abnormalities in

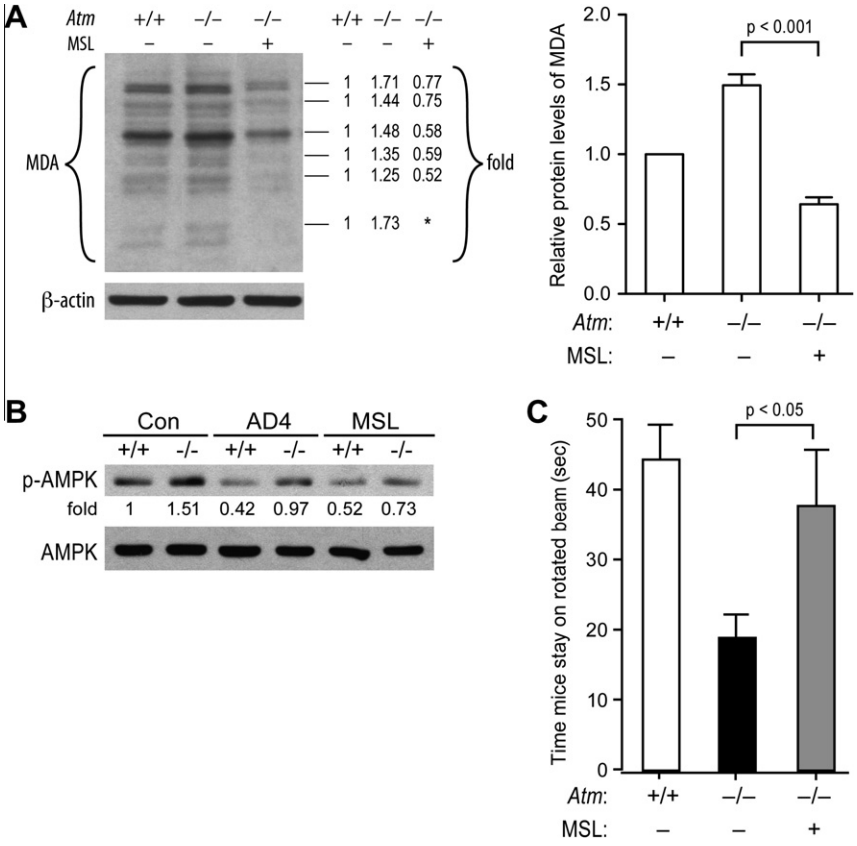


**Fig. 4.** MSL treatment in vivo reduces levels of MDA and p-AMPK, and corrects neurobehavioral deficits in *Atm*−/− mice. (A) MDA protein addicts detected by Western blot using cerebellar lysates isolated from *Atm*−/− or *Atm*+/+ mice untreated vs. treated with MSL. *Not detectable. Relative protein levels of MDA (upper right panel) were analyzed based on fold changes when compared with *Atm*+/+ untreated mice. (B) Levels of p-AMPK in cerebellar lysates isolated from *Atm*−/− or *Atm*+/+ mice untreated vs. treated with AD4 and MSL. (C) Neurobehavioral performance in *Atm*−/− or *Atm*+/+ mice untreated vs. treated with MSL.

neuronal energy metabolism [34]. These include low glucose uptake, mitochondrial dysfunctions and defects in cholesterol metabolism. When human neuronal cells are treated with Aβ1–42 for 24 h, signs of oxidative stress as well as p-AMPK levels are increased and neuronal cell death is induced [16]. Treatment of these cells with antioxidants melatonin or resveratrol attenuates the upregulation of Aβ1–42-induced oxidative stress and reduces AMPK phosphorylation [16,35,37–39].

AMPK activation induces phosphorylation of p53 on serine 15, and this phosphorylation is regulated to initiate AMPK-dependent cell cycle arrest [40]. Interestingly, ATM activation also phosphorylates p53 on serine 15 and initiate ATM-dependent cell cycle arrest. One wonders whether these kinases have similar effects (redundant, but they are independent to each other). When ATM is absent, AMPK is upregulated to increase its effect on the cells.

We have previously reported that MSL is a scavenger of free radicals and prevent upregulation of ROS induced by the neuropathogenic mouse retrovirus *ts*1 in cultured murine astrocytes, and prevents the neurodegeneration that is an end-stage manifestation of *ts*1 infection [28]. In addition to its direct antioxidant and redox buffering properties, we have shown that MSL upregulates and stabilizes intracellular levels of the nuclear transcription factor NF-E2-related factor 2 (Nrf2) [41], a regulator of the intracellular antioxidant defense systems [42]. In this study, we showed for the first time that MSL prevents oxidative stress mediated AMPK activation in *Atm*−/− cerebella. Because ROS accumulation activate AMPK protein, and both AMPK and ROS are pathogenically important in a number of neurodegenerative diseases, MSL could be clinically beneficial for neurodegenerative diseases such as A-T that involve underlying oxidative stress and AMPK activation.

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