Inactive Atm abrogates DSB repair in mouse cerebellum more than does Atm loss, without causing a neurological phenotype

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1. Introduction

The DNA damage response (DDR) is a main axis of genome stability [1,2]. Severe DDR defects lead to genome instability syndromes, which usually combine tissue degeneration, cancer predisposition, segmental premature aging, and sensitivity to DNA damaging agents [3–7]. The corresponding mouse models are important for understanding the link between impaired DDR and the resultant phenotypes [8,1–10].

A prototype genome instability disorder is ataxia-telangiectasia (A-T), a highly pleiotropic, autosomal recessive human disorder [11] caused by mutations in the ATM (A-T, mutated) gene [12,13], which encodes the ATM protein [14,15]. ATM is a homeostatic protein kinase with many physiological functions [15,16–22]. A widely documented one is its role as the chief mobilizer of the cellular response to DNA double-strand breaks (DSBs), a complex, tightly regulated signaling network that modulates numerous branches of cellular physiology in response to DSB induction [2,15,23,24]. ATM activates and regulates this network by phosphorylating a multitude of substrates in its various branches [15,17,18,25,26]. ATM belongs to a family of PI-3 kinase-like protein kinases (PIKKs), which includes, among others, the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) and ATR, both of which are also involved in responding to genotoxic and other stresses [27].

Most A-T mutations are null alleles that truncate the ATM protein. Since truncated ATM is usually unstable, most A-T patients are typically devoid of ATM [11,28]. The prominent symptom is progressive cerebellar ataxia that develops into severe motor dysfunction [11,29]. The main underlying pathology is progressive cerebellar degeneration that involves primarily Purkinje cells (PCs) and granule neurons. Peripheral neuropathy may develop during the second decade of life. Oculocutaneous telangiectasia (dilated blood vessels) appear variably in the eyes and facial skin. Marked immunodeficiency is manifested by reduction...
of various immunoglobulin isotypes, and diminished B and T lymphocyte counts. Lung infections often result from food aspiration combined with the immunodeficiency. Another hallmark is gonadal and thymic dysgenesis. Cancer predisposition is manifested as increased tendency to develop lymphoreticular malignancies, and various carcinomas appear in older patients. Growth retardation and occasional endocrine abnormalities are also seen, among them insulin-resistant diabetes. An important, emerging aspect of A-T is premature aging [17], evidenced in part by the markedly accelerated senescence found in primary fibroblasts derived from A-T patients [30]. Major laboratory findings are elevated serum levels of alpha-fetoprotein and carcinoembryonic antigen. A-T patients show a striking sensitivity to the cytotoxic effect of ionizing radiation (IR), and cultured A-T cells exhibit marked chromosomal instability, sensitivity to IR and radiomimetic chemicals, and reduced telomere length. IR sensitivity results from a profound defect in initiating the ATM-dependent response to DSBs.

Many A-T symptoms can be attributed to the aberration of the cellular response to DSBs, both physiological ones and those induced by endogenous reactive oxygen species. However, the cause of the most devastating symptom – the progressive cerebellar atrophy – is still being debated, in view of the many physiological functions of ATM in addition to its role in the DSB response [15–17,19–22,31–50]. An important research tool in the attempts to understand this component of the A-T phenotype is mouse models of A-T.

Most mouse models of A-T are based on truncating or frameshift mutations in the murine Atm gene, similar to the null Atm mutations that cause A-T in humans. Atm-deficient mice were found to recapitulate major A-T symptoms, including the profound cancer predisposition, acute radiation sensitivity and sterility, but were largely spared the progressive cerebellar atrophy [51–54]. Several studies noted, however, morphological and functional abnormalities in the cerebellar cortex of Atm-deficient mice, such as ectopic and abnormally differentiated Purkinje cells [54], decreased duration of calcium currents and firing in these cells [55], and degenerative changes in several types of neurons, identified using electron microscopy [56]. Further abnormalities were observed in tissue organization and various physiological and molecular circuits of the murine Atm-deficient nervous system [39,51–70]. It appears, therefore, that Atm loss in the mouse can cause physiological damage in various tissues, similar to what is seen in A-T patients, but unlike the human cerebellum, the murine cerebellum can largely tolerate Atm loss and maintain its neuromotor functions. Furthermore, daily monitoring of Atm-deficient mice in our colonies turned up no behavioral abnormalities in animals up to 2 years of age. One possible approach to obtaining a mouse model of A-T that will exhibit cerebellar atrophy is to induce Atm mutations in this organism that will produce a harsher effect than that caused by the null alleles that eliminate the Atm protein. Such are mutations that produce catalytic inactive (‘kinase-dead’) Atm.

Yamamoto et al. [71] and Daniel et al. [72] showed that expression of physiological levels of kinase-dead in mice Atm leads to early embryonic lethality. Furthermore, cultured cells expressing inactive Atm exhibited greater genome instability compared to Atm-deficient cells [71,72]. Detailed mechanistic explanations for this observation are lacking, but it is assumed that the presence of inactive Atm interferes with the cellular response to genotoxic stress, such as the cleavage of trapped protein adducts in replicating cells [73], and perhaps other ATM-dependent pathways more than does the absence of Atm [74]. We asked therefore whether conditional expression of kinase-dead Atm in the nervous system of the mouse will exert a more profound cerebellar effect than Atm loss.

2. Materials and methods

2.1. Mouse strains

Animals were held in a specific pathogen-free facility. All experimental procedures complied with the standards as defined by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Tel Aviv University Institutional Animal Care and Use Committee.

The study was carried out using mice with a mixed C57BL/129 Sv background. The following Atm alleles were used: an Atm-knockout allele (Atm<sup>−/−</sup>) [51] (B6.129S6-Atm<sup>tm1Awb</sup>/J in the Jackson Labs Repository); a ‘floxed’ Atm allele (Atm<sup>fl</sup>) containing loxP sites flanking exons 57–58 of the gene [75,76] (strain 129-Atm<sup> targeted1fl/J in Jackson Labs; and a kinase-dead Atm allele (Atm<sup>KD</sup>), which produces an ATM protein with two inactivating amino acid substitutions (D2880 A/ N2885K) in its catalytic domain [71]. Cre recombine was expressed from a transgene driven by the Nestin promoter (strain B6.Cg-Tg(Nes-cre) 1Kln/J in the JAX Repository) was used to inactivate floxed alleles in the central nervous systems [77]. The L7 (Pcp2) promoter was used to express Cre in PCs [78] (JAX strain Tg(Pcp2-cre)1Amc/J).

2.2. Antibodies

The following antibodies were used: polyclonal goat anti-Calbindin D28 K (Santa Cruz Biotechnology Inc., Santa Cruz, CA), monoclonal mouse anti-Neuronal Nuclei (NeuN) (Millipore, Billerica, MA), polyclonal rabbit anti-GABA(A) receptor alpha 6 subunit receptor (Millipore, Temecula, CA), monoclonal mouse anti-pS139-H2AX (Merck Millipore, Darmstadt, Germany), polyclonal rabbit anti-p53BP1 (Novus Biologicals, Littleton CO), monoclonal rabbit anti-ATM D2E2 (Cell Signaling, Danvers, MA), polyclonal rabbit anti-pS824- KAP-1 (Bethyl Laboratories, Montgomery, TX), monoclonal mouse anti-KAP-1 (BD Transduction Laboratories, San Jose, CA), monoclonal mouse anti-HSC70 (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

2.3. Inhibitors

DNA-PK inhibitor: NU7441 (Tocris Bioscience, Bristol, UK). ATR inhibitor: AZ220 (Tocris).

2.4. X-irradiation

Mice underwent total body irradiation using an X-irradiator (160 H F, Philips, Germany) at a dose rate of 1 Gy/min. Cultures were irradiated using an X-irradiator (Faxitron, Tucson, AZ) at a dose rate of 0.5 Gy/min.

2.5. Immunoblotting of mouse tissues

Irradiated mice were sacrificed 30 min after irradiation and organs were immediately harvested and snap-frozen in liquid nitrogen. Protein extraction and immunoblotting were performed as previously described [79,80].

2.6. Histological analysis

Mice were anesthetized with 1% Ketamine and 0.1% Xylazine solution (1 ml per 10 g of body weight) and sacrificed by PBS perfusion. Cerebellar tissues were fixed in 4% paraformaldehyde and dehydrated in graded ethanol series using the Leica TP1020 Tissue Processor. The tissues were cut into 10 µm parasagittal sections, deparaffinized in two 5 min incubations in xylene and rehydrated in a graded series of ethanol. The sections were then stained using hematoxylin and 1% eosin solution (Kaltec, Padova, Italy). For immunostaining, the sections were treated with antigen unmasking solutions (Vector Laboratories, Burlingame, CA), washed with PBS, and blocked with PBSTg (0.2% Tween, 0.2% gelatin in PBS). The tissue sections were then incubated with the primary antibody overnight, washed with PBS and PBSTg and incubated with the secondary antibody in PBS for 2 h. The sections were then washed and stained with 0.1 mg/ml 4,6- diamidino-2-
phenylindole (DAPI), and mounted with aqueous mounting medium containing anti-fading agents (Biomeda Corp, Foster City, CA). Immunofluorescent images were captured using a Leica TCS SP2 confocal microscope (Leica Microsystems GmbH, Germany).

2.7. Cerebellar organotypic cultures

Cerebellar organotypic cultures were established and stained as previously described [81,82]. Cultures were X-irradiated 12 days after their establishment.

2.8. Behavioral analysis

Initial testing for possible behavioral changes reflecting cerebellar defects was based on the scoring scheme described by Guyenet et al. [83]. Mice were tested once every 1–3 months from 1 to 24 months of age. Tests included hind limb clasping, ledge test, gait and appearance of kyphosis [83]. Animals were also examined at 12 and 20 month using the computerized CatWalk gait analysis system (Noldus, Wageningen, Netherlands, which recorded and subsequently analyzed the animals’ footprints as they went along a glass runway 3 times. Mice were also tested for general locomotor activity and willingness to explore using an open field test performed in a 50 X 50 cm arena for 20 min. Their motion was recorded using a GigE color 1/2" Basler acA1300-60gc camera (Basler, Ahrensburg, Germany) and analyzed using EthoVision XT 11.5 software (Noldus). The CatWalk and Open Field tests were carried out in the Myers Neuro-Behavioral Core Facility of Tel Aviv University.

3. Results

3.1. Generation of mice expressing no Atm or kinase-dead Atm in the CNS or in PCs only

Mice in which Atm is conditionally ablated in the CNS (Atm-Δ-CNS) or specifically in PCs (Atm-Δ-PC) were obtained by combining an Atm<sup>floxed</sup> allele with expression of Nes-Cre or Pcp2-Cre, respectively. The use of a floxed Atm allele against a null allele was intended to increase the chance of eliminating the Atm protein in the target cells by reducing the occurrence of excision of one out of two floxed alleles). Lifespan and cage behavior of both the Atm-Δ-CNS and Atm-Δ-PC animals were normal. Mice expressing Atm<sup>KD</sup> in the CNS or specifically in PCs (Atm-KD-CNS and Atm-KD-PC, respectively) were obtained by combining the Nes-Cre or Pcp2-Cre transgenes with an Atm<sup>KD-floxed</sup> genotype (Fig. 1). In these animals, the Atm<sup>fl</sup> allele was expected to be inactivated by the Cre recombinase, leaving Atm<sup>KD</sup> as the only viable Atm allele in Cre-expressing cell types. Of note, the Atm<sup>KD</sup> genotype itself (which is common to all body cells) does not cause any discernible phenotype [71]. Both the Atm-KD-CNS and Atm-KD-PC mice were viable and completed a normal lifespan. Notably, the body weight of the mice expressing the Nestin-Cre was reduced compared to non-Nestin-Cre expressing animals (Supplementary Fig. 1). This was previously reported and attributed to metabolic effects of nestin promoter-driven expression of the Cre recombinase [84].

3.2. Atm level and activity readout in tissues of mice with different Atm genotypes

Immunoblotting analysis of tissue extracts from the Atm-Δ-CNS mice indicated that Atm protein level was below detection in the cerebrum and cerebellum of these animals (Fig. 2a). As expected, the Atm signal obtained from CNS tissues of the Atm-KD-CNS animals was lower than that of WT tissues (Fig. 2a), since it represented only one allele that expresses the mutant protein. We also examined a well-documented readout of ATM activity after DSB induction: the robust phosphorylation of its substrate, Kap-1 on Ser824, which is detected using a highly specific anti-phospho antibody [85]. Indeed, 30 min following treatment of the animals with 10 Gy of IR, Kap-1 phosphorylation in the Atm-Δ-CNS and Atm-KD-CNS animals was considerably reduced in the CNS but not in the spleen, which served as the control tissue (Fig. 2a). This result indicated that Atm<sup>KD</sup> was indeed catalytically inactive in the Atm-KD-CNS animals. The residual phosphorylation of Kap-1 that is occasionally detected in the absence of Atm activity in the brain (Fig. 2a) is attributed primarily to DNA-PK [76] (and our unpublished data). Similar results were obtained in cerebellar organotypic (slice) cultures, which we routinely use to analyze the cerebellar DDR [81,82]. These cultures maintain the organization of the cerebellar tissue for several weeks in culture and are amenable to treatment with DNA damaging agents [65,81,82]. The cultures were irradiated, and phosphorylations of histone H2ax and Kap-1 were detected using immunostaining. Both phosphorylations were profoundly down-regulated in Atm-Δ-CNS and Atm-KD-CNS cultures (Fig. 2b). Notably, this result suggests that in this tissue Atm has a major role in H2ax and Kap-1 phosphorylation in response to DSB induction. Importantly, a small, distinct group of cells exhibited vigorous Kap-1 phosphorylation in both mutant genotypes (Fig. 2b), presumably representing cells in which the Nestin promoter had not been activated during development or cells in which the floxed Atm allele had escaped Cre-mediated excision.

We used inhibitors against DNA-PK and ATR to assess the share of each of the three PIKKs in these phosphorylations in the murine cerebellum. Inhibition of Dna-pk or Atr in Atm-proficient, Atm<sup>fl</sup> cells reduced the two phosphorylations considerably (Fig. 2b), pointing to the
importance of concerted action of these kinases in these damage-induced phosphorylations in this organ.

Fig. 2c presents similar analysis of cerebellar organotypic cultures derived from Atm-Δ-PC and Atm-KD-PC mice demonstrating the lack of Atm activity specifically in PCs of both genotypes.
3.3. Expression of catalytically inactive Atm in the CNS impairs DSB repair in PCs, more than Atm absence

In order to follow DSB repair in PCs with the various Atm genotypes, we used cerebellar organotypic cultures treated with IR. We monitored in PCs the dynamics of two immunofluorescent DSB readouts: nuclear foci of phosphorylated histone H2ax (γH2ax) and the DDR protein, 53bp1 [86]. In the early time points after irradiation, the response in Atm-deficient cells was reduced compared to Atm-proficient cells, but later on, the decline in foci number over time, which is considered to represent DSB repair, was delayed in PCs from Atm-Δ-CNS compared to those lacking Atm altogether [71,72]. Interestingly, however, when Atm loss or replacement by a kinase-dead protein was confined to PCs, the increased impact on DSB repair of inactive Atm compared to Atm absence was more pronounced (Fig. 3c, d). Importantly, the delay in DSB repair was greater in PCs in Atm-Δ-CNS cultures than in those of Atm-Δ-CNS mice (Fig. 3c, d). This result suggests that the presence of kinase-dead Atm in the CNS exerts a more pronounced effect on DSB repair than Atm absence. This result is in accord with previous findings showing greater genome instability in murine embryonic stem cells and lymphocytes expressing kinase-dead Atm compared to those lacking Atm altogether [71,72]. Interestingly, however, when Atm loss or replacement by a kinase-dead protein was confined to PCs, the increased impact on DSB repair of inactive Atm compared to Atm loss was not observed (Fig. 3e, f). It should be stressed in this regard that, in the Atm-Δ-PC expression of kinase-dead Atm was confined to PCs while the surrounding cells contained normal levels of WT Atm, as evidenced by the Atm activity assay (Fig. 2c).

3.4. Conditional expression of inactive atm in the CNS or just in PCs does not lead to a significant neurological phenotype

The animals expressing kinase-dead Atm in the CNS or in PCs were tested every 1–3 months from 1 to 24 months using the Gait Test and Ledge Test that measure coordination of movements, and the Hind Limb Clasping Test, that can highlights severe cerebellar abnormalities. Special attention was paid to the appearance of kyphosis, which may be caused by the loss of muscle tone in the spinal muscles secondary to neurodegeneration [83]. No abnormalities were observed in these parameters in the two mutant genotypes (data not shown) during the 24 months. When the mice were evaluated at 20 months using the Open Field and CatWalk systems Atm-Δ-CNS, but not Atm-Δ-PC mice demonstrated changes in their footprints suggesting alterations in stepping pattern and coordination (Fig. 4e). However, collectively all other parameters did not detect behavioral pattern suggesting cerebellar dysfunction (Fig. 4). Similar to the normal neurological phenotype observed in mice expressing kinase-dead Atm in the CNS, Atm-Δ-CNS mice expressing no Atm protein in the CNS also displayed no significant phenotype. Some behavioral changes were observed in the Atm-Δ-CNS and Nestin-Cre-expressing mice which might due to the Cre expression pattern and coordination (Fig. 4e). However, collectively all other parameters did not detect behavioral pattern suggesting cerebellar dysfunction (Fig. 4). Similar to the normal neurological phenotype observed in mice expressing kinase-dead Atm in the CNS, Atm-Δ-CNS mice expressing no Atm protein in the CNS also displayed no significant phenotype. Some behavioral changes were observed in the Atm-Δ-CNS and Nestin-Cre-expressing mice which might due to the Cre expression in the tissue (Supplementary Fig. 2). We concluded that conditional expression of Atm in the CNS or PCs did not lead to changes in behavior indicative of motor deficits.

The cerebellar structure and organization of the animal were examined every 3 months from 3 weeks to 24 months using hematoxylin-eosin staining and immunohistochemical staining of cell type markers. No gross organizational abnormalities in the cerebella of the Atm-Δ-CNS and Atm-Δ-PC animals were observed during this period (Fig. 5).

4. Discussion and conclusions

The conditional ablation of the Atm gene in the Atm-Δ-CNS mouse allows circumventing the extreme cancer predisposition of the Atm Δ/−.
The comparison between Atm-KD-PC and Atm-KD-CNS animals highlighted a difference in the effect of AtmKD on DSB repair in PCs surrounded by AtmKD-expressing cells or PCs with AtmKD surrounded by WT cells. This result may point to the importance of the surrounding cells in determining the functional outcome of PCs’ own Atm genotype. Support for this concept comes from a recent study with mixed cell cultures, in which the functionality of Atm-deficient murine granule cells depended on the Atm genotype of astrocytes that interacted with them [90]. Thus, conditional expression of Atm genotypes in a specific cell type may yield different physiological results depending on the genotype of surrounding cells. Our results therefore surface certain limitations of the conclusions that can be drawn when the Cre-lox technology is used for tissue-specific gene manipulation. An overarching conclusion concerning A-T is emerging from studies based on CNS-specific ablation of the Atm gene in mice: the cerebellar atrophy in A-T may be influenced not only by Atm loss in specific cerebellar cell types, but also by lack of Atm in other tissues that neighbor or connect with the cerebellum.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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**Fig. 5. Normal cerebellar morphology in mice expressing AtmKD in the CNS or in PCs.** Histological analysis of the cerebellum was performed between ages 1–24 months. Representative images are shown. a. Hematoxylin-eosin staining of cerebellar paraffin sections produced from 6-month old mice with different genotypes. b. Immunostaining of different cell populations in cerebellar sections obtained from 6-month old mice. Calbindin D28k (cyan); NeuN (red) highlighting neurons; GABA (green), which marks GABAergic cells; DAPI (blue).

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