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Studying the cerebellar DNA damage response in the tissue culture dish

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ABSTRACT

The cerebellum is exquisitely sensitive to deficiencies in the cellular response to specific DNA lesions. Genetic disorders caused by such deficiencies involve relentless, progressive cerebellar atrophy with striking loss of Purkinje and granule neurons. The reason for the extreme sensitivity of these cells to defective response to certain DNA lesions is unclear. This is particularly true for ataxia-telangiectasia (A-T) – a genomic instability syndrome whose major symptom is cerebellar atrophy. It is important to understand whether the DNA damage response in the cerebellum, particularly in Purkinje neurons, has special characteristics that stem from the unique features of these cells. Murine cerebellar organotypic cultures provide a valuable experimental system for this purpose since they retain the tissue organization for several weeks in culture and appear to provide the delicate Purkinje neurons with a physiological environment close to that *in vivo*. We have optimized this system and are using it to examine the Atm-mediated DNA damage response (DDR) in the cerebellum, with special emphasis on Purkinje cells. Our results to date, which indicate special chromatin organization in Purkinje cells that affects certain pathways of the DDR, demonstrate the usefulness of cerebellar organotypic cultures for addressing the above questions.

1. Introduction

Genetic defects that reduce the efficacy of specific branches in the DNA damage response (DDR) lead to genomic instability syndromes, which typically involve degeneration in specific tissues, cancer predisposition and sensitivity to DNA damaging agents (Ciccia and Elledge, 2010; Jeppesen et al., 2011; O'Driscoll, 2012). The nervous system becomes severely damaged in some of these disorders, with the cerebellum exhibiting marked vulnerability. Striking cerebellar atrophy leading to progressive ataxia is a hallmark of DDR deficiencies such as ataxia with oculomotor apraxia-1 (AOA1), AOA2, spinocerebellar ataxia with axonal neuropathy-1 (SCAN1), ataxia-telangiectasia (A-T) and A-T-like disease (A-TLD) (Jeppesen et al., 2011; O'Driscoll, 2012; Perlman et al., 2012). Understanding the role and mode of action of the DDR in the cerebellum is essential for understanding these diseases and for selecting appropriate readouts for high-throughput drug screens aimed at identifying potential treatment. However, most investigations in the DDR field are carried out using the workhorses of cell biology – commonly used cancer cell lines and cell lines derived from patients with genomic instability syndromes. Indeed, these cell lines have been used successfully to decipher the intricate DDR networks and DNA repair mechanisms, but they differ from post-mitotic neurons in numerous respects. Furthermore, Purkinje cells, which are critical for cerebellar function and are typically lost in the DDR-associated ataxias, are unique in their structure among neurons, and thus deserve special attention regarding their DDR mechanisms.

A common tissue culture proxy for neuronal cells are neuron-like cells (NLCs) obtained by induced differentiation of human neuroblastomas or stem cell lines (Edsjo et al., 2007; Paquet-Durand and Bicker, 2007; Robertson et al., 2008; Schwartz et al., 2008; Wei et al., 2010). Cells representing different neuronal lineages can also be obtained from induced pluripotent stem (iPS)

Abbreviations: 53BP1, p53 binding protein 1; AOA1/2, ataxia with oculomotor apraxia type 1; A-T, ataxia-telangiectasia; A-TLD, ataxia-telangiectasia-like disorder/disease; ATM, ataxia-telangiectasia, mutated; ATR, ATM- and Rad3-related protein kinase; CaBP, calbindin protein (calbindin-D28K); DDR, DNA damage response; DNA-PKcs, DNA dependent protein kinase, catalytic subunit; DSB, double strand break; GFAP, glial fibrillary acidic protein; H2AX, histone 2AX; γH2AX, phosphorylated histone 2AX; H3K9Me3, trimethylated lysine 9 of histone 3; HDR, homology-directed repair; HP1α, heterochromatin protein 1alpha; HR, homologues recombination; iPS cells, induced pluripotent stem cells; Mre11, meiotic recombination 11; MRN complex, Mre11/Rad50/Nbs1 complex; NBS, Nijmegen breakage syndrome; NeuN, neuronal nucleus; NLC, neuron-like cell; NHEJ, nonhomologues end-joining; PI3K, phosphatidylinositol 3- kinase; PIK-related kinases, phosphatidyl inositol kinase-related protein kinases; SCAN1, spinocerebellar ataxia with axonal neuropathy.

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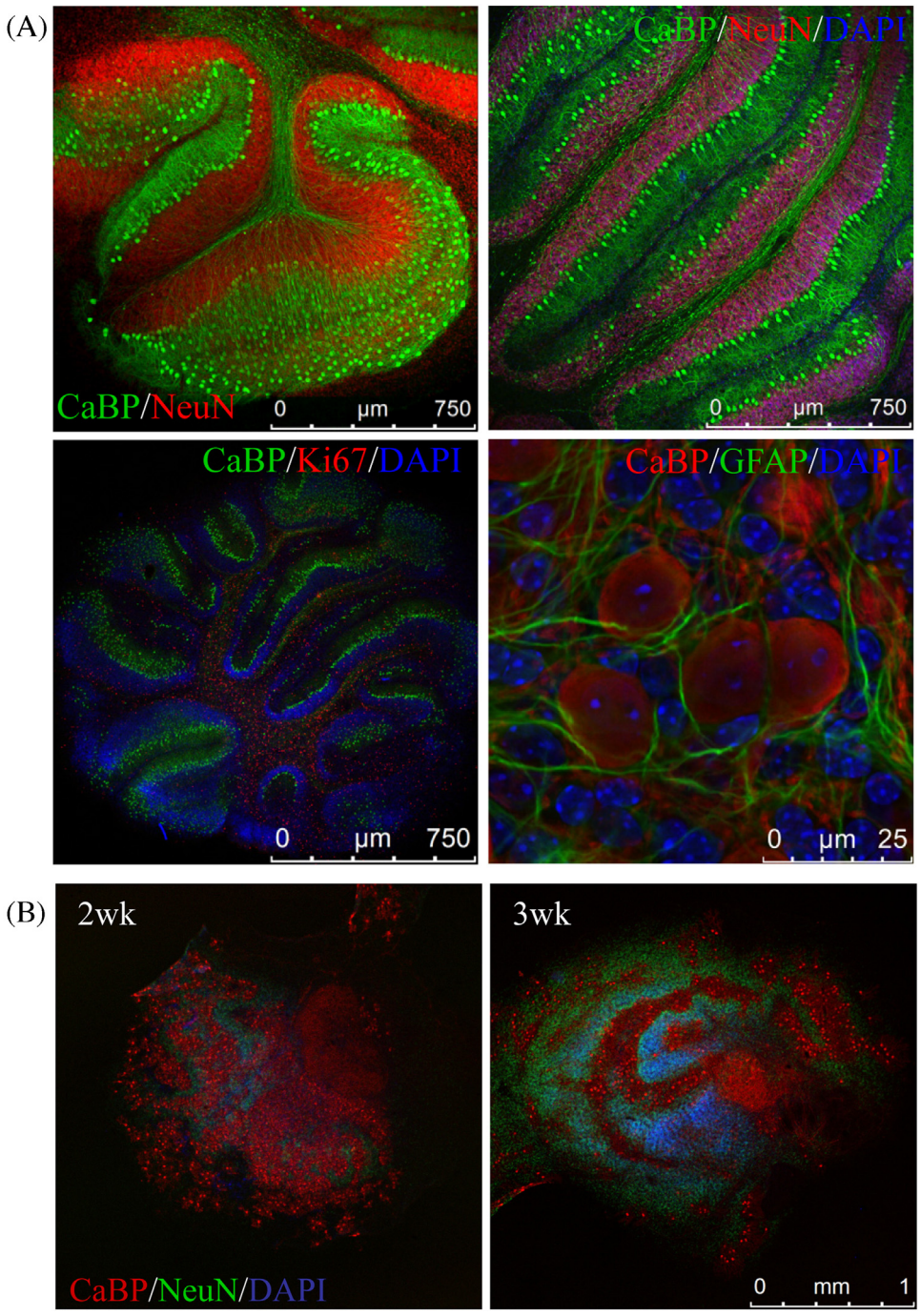

Fig. 1. Overview of cerebellar organotypic cultures. (A) Cultures established from P12 mice, 12 days after their establishment. CaBP: calbindin D28k – a Purkinje cell marker; NeuN – a neuronal nucleus marker, which does not stain Purkinje nuclei; Ki67 – a cell proliferation marker. DAPI – 4′,6-diamidino-2-phenylindole, a fluorescent stain of DNA; GFAP – glial fibrillary acidic protein, an astrocyte marker. The bottom right image provides a close look at Purkinje cells. Note the scanty DAPI staining in Purkinje cells in contrast to the prominent DAPI-stained chromatin blocks in the surrounding cells. (B) Overview of an organotypic cerebellar culture established from a P0 mouse at different time points after its establishment. Note the progressive reorganization of the cultured tissue.

cells (Chipman et al., 2012; Xia et al., 2012). Alternatively, primary cultures enriched for different nervous system cell types can be obtained directly from the corresponding tissues following their dissociation (Aschner, 2011; Boukhtouche et al., 2010; de Luca et al., 2009; Giordano et al., 2011; Melli and Hoke, 2009; Oldreive and Doherty, 2010). Such cultures likely do not recapitulate the natural *in vivo* environment of these cells, particularly with regards to highly specialized neurons such as Purkinje cells, which occupy a highly structured layer in the cerebellum. Consequently, physiological conditions in these cells are presumably different when in culture and *in vivo*. Organotypic slice cultures provide a culture-based system that is much closer to a tissue and yet is amenable to most of the manipulations that can be applied to dissociated cell cultures (Dupont et al., 2006; Hurtado de Mendoza et al., 2011; Julien et al., 2008; Kapfhammer and Gugger, 2012; Kessler et al., 2008; Lebrun et al., 2012; Lonchamp et al., 2006; Lu et al., 2011; Padmanabhan et al., 2007; Zanjani et al., 2012).

Our focus is on A-T, an autosomal recessive genomic instability disorder (Lavin, 2008; Perlman et al., 2012), caused by null mutations in the *ATM* gene, which encodes the ATM protein

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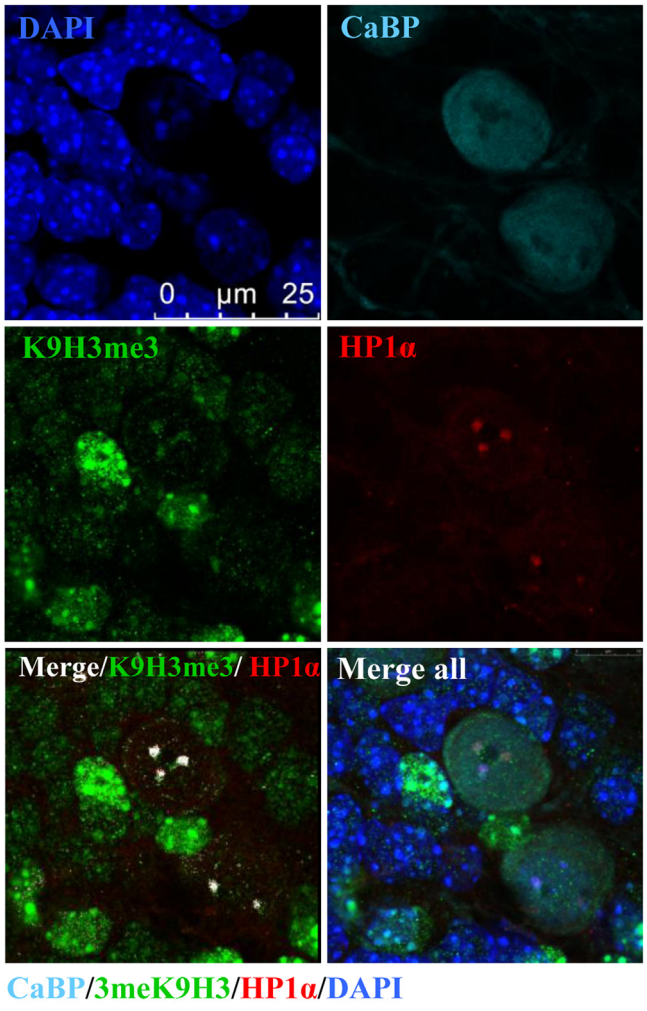

Fig. 2. Purkinje cells exhibit a small proportion of heterochromatin. DAPI- and immunostaining with heterochromatin markers of cerebellar organotypic cultures show few heterochromatin blocks occupying a small volume of the large Purkinje cell nucleus, in contrast to the predominant heterochromatic blocks in surrounding cells. Green: trimethyl lysine 9 of histone H3 (H3K9me3); red: heterochromatin protein 1α (HP1α); cyan: calbindin D28k; blue: DAPI. All images are of the same field. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Savitsky et al., 1995a,b). The disease is characterized by progressive neurodegeneration that mainly affects the cerebellum and develops into severe neuromotor dysfunction, telangiectasias (dilation of blood vessels observed primarily in the eyes), immunodeficiency that spans the B- and T-cell lineages, thymic and gonadal atrophy, marked predisposition to malignancies (primarily lymphoreticular), elevated serum levels of alpha-fetoprotein and carcinoembryonic antigen, acute sensitivity to ionizing radiation, and, occasionally, growth retardation, premature aging and insulin resistance. Cultured cells from A-T patients exhibit increased chromosomal breakage and sensitivity to DNA damaging agents, which is most evident when the cells are treated with physical or chemical agents that induce DNA double-strand breaks (DSBs). This sensitivity represents a marked defect in the activation of the cellular response to DSBs, whose chief mobilizer is the ATM protein kinase which is missing in these patients (reviewed by Bhatti et al., 2011; McKinnon, 2012).

DSBs can be generated by DNA damaging agents, or following the collapse of stalled replication forks (Branzei and Foiani, 2010; Errico and Costanzo, 2012; Jones and Petermann, 2012; Nam and Cortez, 2011) or in response to uncapped telomeres (O'Sullivan and Karlseder, 2010; Peuscher and Jacobs, 2012). DSBs are also inevitable intermediates in meiotic recombination (Inagaki et al., 2010) and in the assembly of the antigen receptor genes during lymphocyte maturation, through V(D)J and class switch recombination (Helmink and Sleckman, 2012). Unrepaired DSBs can severely disrupt DNA replication in proliferating cells, usually leading to cell death, or leaving chromosomal aberrations that may initiate a vicious cycle of further aberrations, setting the stage for cancer formation (Hartlerode and Scully, 2009; Hiom, 2010; Kasparek and Humphrey, 2011). The cellular response to DSBs comprises a vast signaling system, which calls repair mechanisms to action, activates special cell cycle checkpoints, affects gene expression on a large scale, moderates protein synthesis, activity and turnover, and affects many aspects of cellular metabolism (Bhatti et al., 2011; Ciccia and Elledge, 2010; Hartlerode and Scully, 2009; Hiom, 2010; Jeppesen et al., 2011; Roos and Kaina, 2012; Thompson, 2012).

The early phase of the DSB response includes massive build-up of the rapidly expanding, multi-protein foci at DSB sites, which is highly structured in space and time (Chapman et al., 2012; Lukas et al., 2011). The recruited proteins, dubbed "sensors" or "mediators", carry out chromatin reorganization and initial processing of the DSB ends, setting the stage for DSB repair and the activation of the transducers of the DSB alarm, most notably, ATM (Hartlerode and Scully, 2009; Hiom, 2010; Kasparek and Humphrey, 2011; Shiloh and Ziv, 2013). Actual DSB repair is mediated by nonhomologous end-joining (NHEJ) or homologous recombination repair (HRR) (also called homology-directed repair–HDR) (Amunugama and Fishel, 2012; Lieber, 2010). Several specialized variations on these two themes have recently been described (Mladenov and Iliakis, 2011). These processes are accompanied by extensive post-translational modification of DDR players as well as of core histones at the vicinity of the breaks, with subsequent alteration in the epigenetic landscape in that region (Lukas et al., 2011; Miller and Jackson, 2012; Polo and Jackson, 2011; Psakhye and Jentsch, 2012; Ramaekers and Wouters, 2011). A notable example is phosphorylation of the tail of histone H2AX. Phosphorylated H2AX (γH2AX) anchors some of the initiating proteins in the DSB-associated focus (Lukas et al., 2011; Polo and Jackson, 2011; Yuan et al., 2010). In parallel, the transducers are activated. These are protein kinases such as ATM, which relay a strong, wide-spread signal to numerous downstream effectors that control processes throughout the cell. An important determinant of the broad span of the DSB response is the extensive range of substrates activated by the main transducer of this response–the ATM protein kinase (Bensimon et al., 2010; Matsuoka et al., 2007; Mu et al., 2007; Shiloh and Ziv, 2013).

ATM is a relatively large protein with a carboxy-terminal active site, which contains a phosphatidylinositol 3-kinase (PI3K) signature despite its protein kinase activity. This domain places ATM within a family of PI3K-like protein kinases (PIKKs), most of which are involved in cellular responses to various stresses (Lempiainen and Halazonetis, 2009; Lovejoy and Cortez, 2009). Two other, prominent members of this group that are directly involved in the cellular response to genotoxic stress are ATR (ataxia-telangiectasia and Rad3-related) and DNA-PKcs (DNA-dependent protein kinase catalytic subunit). ATR has a major role in coordinating DNA replication origin firing and guarding replication fork stability, and it is activated following replication fork stalling (Errico and Costanzo, 2012; Nam and Cortez, 2011). DNA-PKcs best known for its central role in the NHEJ pathway of DSB repair (Hill and Lee, 2010; Neal and Meek, 2011).

A key player in the early stage of the DSB response is the trimolecular MRN (MRE11-RAD50-NBS1) complex, which is essential for ATM activation. MRN is one of the first complexes to be recruited to DSB sites, where it acts as a damage sensor that

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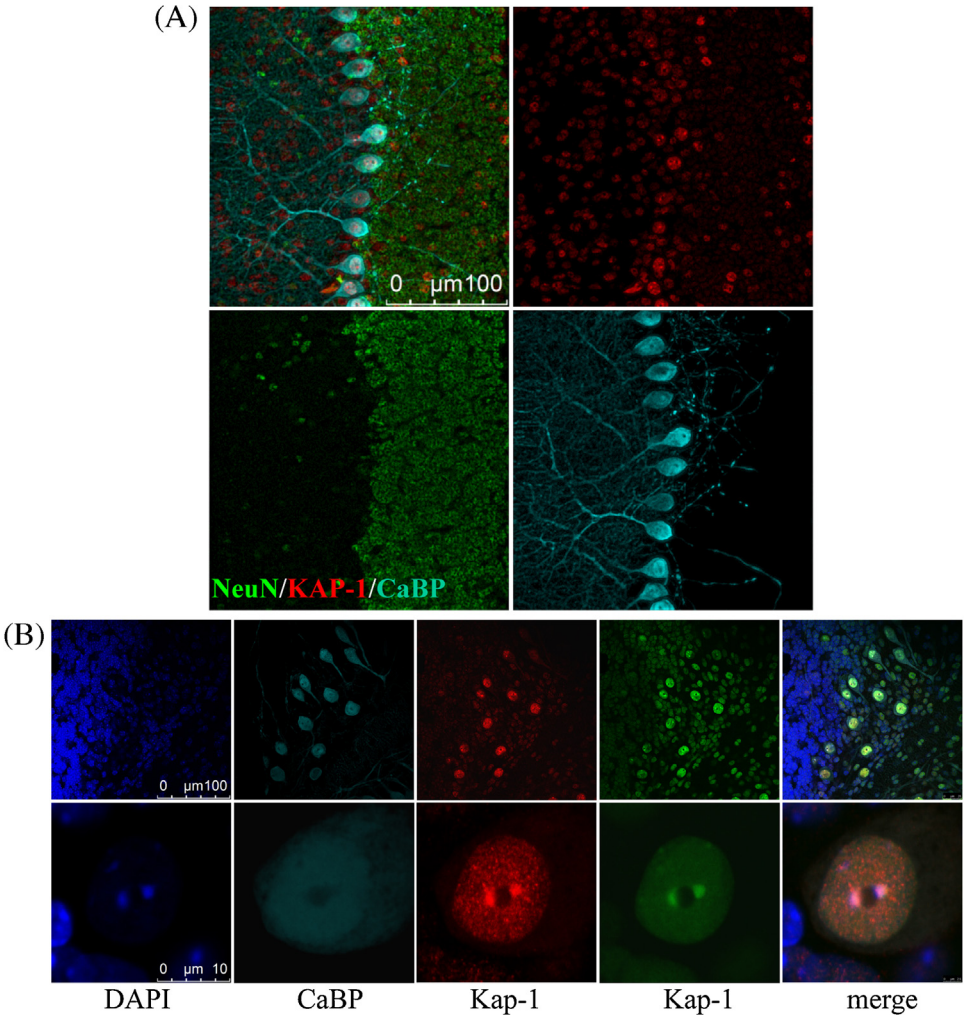

Fig. 3. High levels of Kap-1, but low level of its DNA damage induced phosphorylation, in mouse Purkinje cells. (A) Differential staining of neurons (NeuN, green), Purkinje cells (CaBP, cyan), and Kap-1 (red). Note the high levels of Kap-1 in Purkinje cells compared with the other cell types in this tissue. (B) A closer look at Kap-1 in Purkinje cells, showing higher concentrations of Kap-1 around heterochromatin. Green: polyclonal antibody against Kap-1. Red: a monoclonal antibody against this protein. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

can also form a physical bridge over the DSB ends (Stracker and Petrini, 2011). It is required for timely repair by both NHEJ and HRR, and by virtue of its nuclease component, MRE11, it takes part in DSB end resection, which is essential for HRR. Another genomic instability disorder, A-T-like disease (A-TLD), is similar to mild A-T, with later age of onset of the cerebellar attrition and slower progression of this symptom compared to classical A-T (Taylor et al., 2004). A-TLD is caused by hypomorphic mutations in the *MRE11* gene, encoding the MRE11 nuclease (Stewart et al., 1999).

Understanding A-T means first and foremost understanding the physiological and molecular basis of its cardinal symptom – the relentless neurodegeneration affecting primarily the cerebellum. Tying this major component of the A-T phenotype to ATM's role as the chief mobilizer of the DSB response network has been the subject of ongoing debate. While ATM's most documented function is its role as an apical activator of the DSB response, recent data suggest that ATM is also involved in the regulation of oxidative stress (Ditch and Paull, 2012; Guo et al., 2010; Shiloh and Ziv, 2013). Furthermore, evidence is accumulating that ATM's broad capacity as a protein kinase is continuously exploited in other signaling pathways that respond to various stimuli or physiological situations. Some of these pathways are not nuclear and touch upon various metabolic processes (Ditch and Paull, 2012; Li et al., 2012; Yang et al., 2011). The attempts to understand which of ATM's functions are those whose loss is most critical in the cerebellar tissue are continuing. Several considerations lead us to believe that, similarly to the other symptoms of this disease, the loss of ATM's cardinal role in the DDR is a major cause of this symptom. A full discussion of the rationale behind this claim is not within the scope of this article, but it suffices to mention the striking similarity between the cerebellar atrophy seen in A-T on the one hand, and AOA1, AOA2, SCAN-1 and A-TLD, in which the missing proteins are DNA processing enzymes (El-Khamisy, 2011; Jeppesen et al., 2011). Presumably, however, the ATM-mediated DDR in cerebellar tissues, including Purkinje cells, is structured differently from that of actively proliferating cancer cell lines, in which it is studied most of the time. We therefore set forth to revisit this system in wild-type and ATM-deficient neurons.

We have previously studied the ATM-dependent DDR in human NLCs, finding ATM to be largely nuclear in these cells, with basic readouts of the ATM-mediated DDR comparable to those in proliferating cells (Biton et al., 2006, 2007). Similar results were obtained in such cells by (Carlessi et al., 2009). Notably, ATM-deficient neuron-like cells were recently obtained using differentiated iPS cells from A-T patients (Nayler et al., 2012). We then went on to obtain similar results in murine cerebellar organotypic

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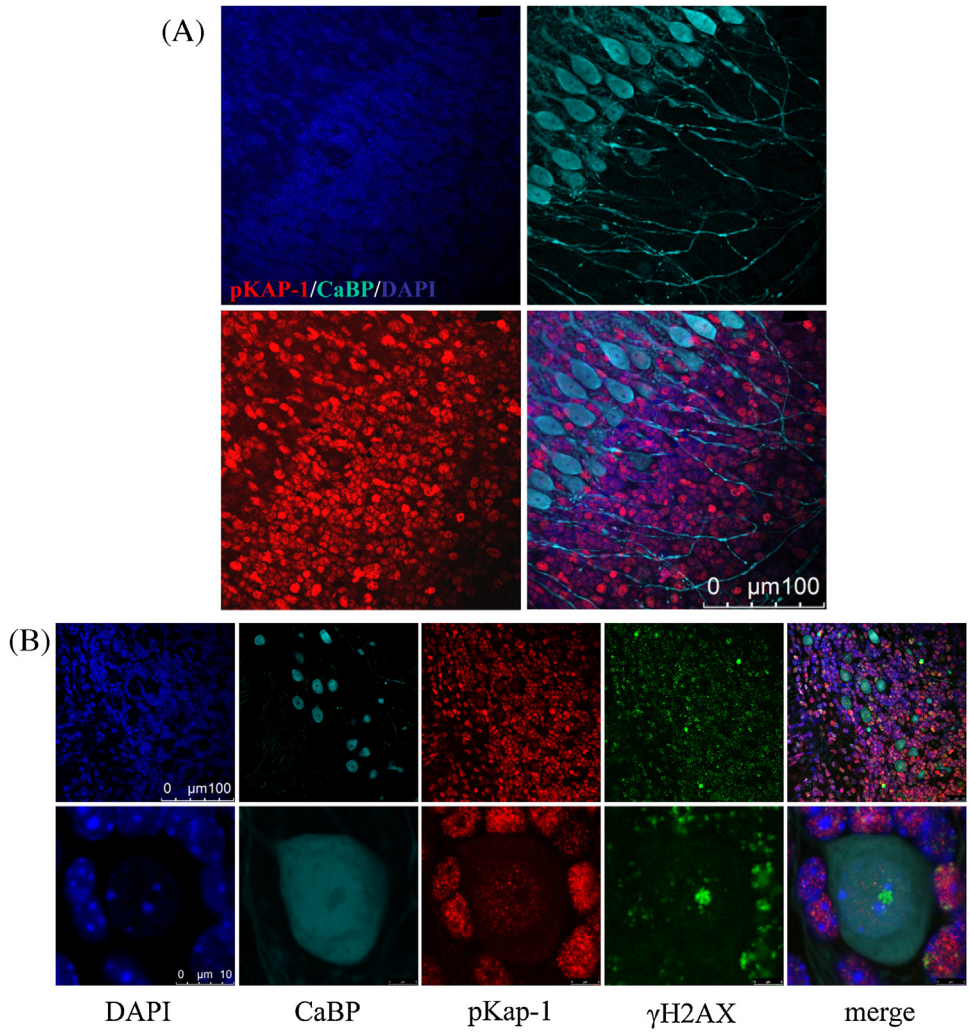

Fig. 4. Kap-1 phosphorylation 30 min following irradiation of cerebellar organotypic cultures with 2 Gy of IR. (A) An overview of the culture. (B) A close look at a Purkinje cell. The antibody against the phosphorylated form of Kap-1 (red) is highly specific for this phosphorylation (Ziv et al., 2006). Note the marked difference between the amounts of phosphorylated Kap-1 in Purkinje cells and the surrounding cells and the difference in the size of γH2AX foci between these cells. Cyan: calbindin D28k; green: γH2AX; blue: DAPI. The nuclear region that is strongly stained by the anti-γH2AX antibody overlaps the nucleolus; the significance of this staining is unclear. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cultures, with special emphasis on Purkinje cells. The ATM-dependent DDR seemed robust in these cells (Dar et al., 2006, 2011).

Using the murine cerebellum to study the ATM-mediated DDR raises a special issue, since Atm-deficient mice obtained using several knockout strategies show a minute or no effect of Atm loss on cerebellar function, contrary to the striking cerebellar degeneration typical of A-T patients (Barlow et al., 1996; Borghesani et al., 2000; Elson et al., 1996; Xu et al., 1996). This difference between the human and murine phenotypes may stem from the short life-span of mice, which does not allow time for the cerebellar phenotype to develop (in humans this phenotype develops gradually during the first decade of life). Another possible reason for this difference might be that different thresholds need to be crossed in the two organisms regarding the extent of DDR deficiency, in order for cerebellar attrition to occur (Biton et al., 2008). Indeed, deficiency of the Nbs1 component of the MRN complex in the murine nervous system does in fact lead to severe cerebellar degeneration and ataxia (Frappart et al., 2005) and a similar phenotype is obtained by combining Atm deficiency with loss of other DDR proteins (P. McKinnon, personal communication). Our basic assumption is that the functions of human ATM and mouse Atm are similar in the different tissues of the two organisms.

We regard the cerebellar organotypic cultures as a valuable tool for studying ATM's functions in the cerebellum. Recently, we optimized the protocol for establishing these cultures, allowing us to obtain higher resolution of DDR analysis in these cultures, with emphasis on Purkinje cells, which exhibit unique chromatin organization.

2. Materials and methods

2.1. Mice

Wild-type and $Atm^{-/-}$ mice that were used in this study and the corresponding genotyping protocols have been previously described in (Dar et al., 2006, 2011). All mice in this study have a 129/sv genetic background.

2.2. Antibodies

The following commercial antibodies were used: monoclonal mouse anti-calbindin D28K (diluted 1/2500; Sigma–Aldrich, St. Louis, MO), polyclonal rabbit anti-calbindin D28K (1/1200; Chemicon, Temecula, CA), polyclonal goat anti-calbindin D28K (1/250; Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal mouse anti-γH2AX (1/350; Upstate Biotechnology, Lake Placid, NY), monoclonal mouse anti-Neuronal Nuclei (NeuN) (1/4000; Millipore, Billerica, MA), polyclonal

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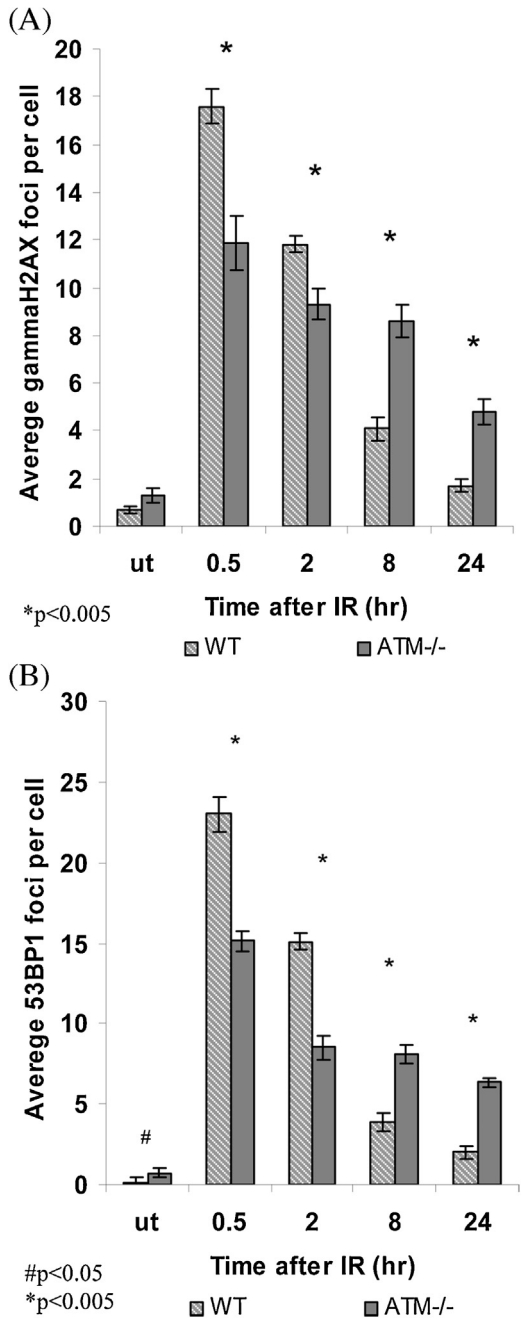

Fig. 5. Loss of Atm affects the kinetics of DSB repair in Purkinje cells. Cerebellar organotypic cultures from wild-type and *Atm*−/− mice were irradiated with 2 Gy of IR, and γH2AX (A) and 53BP1 (B) foci were quantified in Purkinje cells at different time points after irradiation. At least 30 cells were analyzed in triplicates for each time point. Average values and standard errors are presented. The significance of the differences was examined using two-tail homoscedastic *t*-test. Note the reduction in the amount of both markers in Atm-deficient Purkinje cells at the early time point (30 min), and the reversion of this situation at later time points, with a higher fraction of unrepaired DSBs in *Atm*−/− cells.

rabbit anti-glial fibrillary acidic protein (GFAP) (1/400; DAKO, Glostrup, Denmark), monoclonal mouse anti-KAP-1 (1/1000; BD Transduction Laboratories, San Jose, CA), polyclonal rabbit anti-KAP-1 (1/500; Cell Signaling Technology, Beverly, MA), polyclonal rabbit anti-pS824/KAP-1 (1/25000; Bethyl Laboratories, Montgomery, TX), polyclonal rabbit anti-ki67 (1/200; Neomarkers, Fremont, CA), monoclonal mouse anti-heterochromatin protein α (HP1α) (1/500; Chemicon). Monoclonal mouse anti-53BP1 (1/20) and polyclonal rabbit anti-trimetylated lysine 9 of histone 3 (3meK9H3, 1/100) were generous gifts from Thanos Halazonetis and Thomas Jenuwein, respectively.

2.3. X-irradiation

Cultures were irradiated using an X-ray machine (160HF, Philips, Germany) at a dose rate of 1 Gy/min, 12 days after culture establishment.

2.4. Cerebellar organotypic cultures

Brains of mice at age P0 or P12 were dissected in cold Hank's based salts solution (HBSS) (Life Technologies, Gibco products, Invitrogen Corporation, Grand Island, NY, USA) supplemented with 5 mg/ml glucose, and the meninges were removed. Parasagittal cerebellar slices 400 μm thick were cut using a McIlwain tissue chopper and transferred onto 25 mm Millipore membrane culture inserts (0.4 μm pore size, Millicell CM; PICMORG50, Millipore). Slices were maintained for 12 days at 37 °C and 5% CO_2, in 6-well plates containing 1 ml/well medium made of 50% basal medium with Earl's salts (BME) (Life Technologies, Gibco Products), 25% HBSS, 25% horse serum (Biological Industries, Beit Haemek, Israel), 1 mM L-glutamine and 5 mg/ml glucose, with fresh medium replaced every 2–3 days (Dusart et al., 1997). 450 μm thick P0 cultures were maintained in medium made of 95% BME, 5 mg/ml glucose, 4 mM L-glutamine, 0.5 mg/ml BSA and supplements including 5 μg/ml insulin, 5 μg/ml transferrin and 5 μg/ml sodium selenite (Sigma–Aldrich I-1884) at 37 °C with 5% CO_2 for 7–28 days, with fresh medium replaced every 2–3 days (Dusart et al., 1997; Lebrun et al., 2012).

2.5. Immunocytochemistry

Cultures were fixed with 4% paraformaldehyde in PBS for 1 h at RT and then washed with a wash solution (PBS containing 0.25% Triton X-100, PBST). The slices were then incubated for 1 h in blocking solution (PBS containing 2 gr/l gelatin, 0.1 M lysine, 1 gr/l sodium azide, 0.25% Triton X-100), and were subsequently incubated at 4 °C O/N with shaking in primary antibody solution in PBS supplemented with 2 gr/l gelatin, 1 gr/l sodium azide and 0.25% Triton X-100. Slices were then washed 4 times using PBST and incubated for 2 h on a shaker at RT with secondary antibodies in PBS containing 2 gr/l gelatin and 0.05% Triton X-100. They were then washed 4 times, 10 min each, using PBST and incubated for 10 min with 0.1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) in PBS, washed once using wash solution and mounted with aqueous mounting medium containing anti-fading agents (Biomeda, Foster City, CA). Immunofluorescence was visualized using a Leica TCS SP2 confocal microscope (Leica Microsystems GmbH, Germany). Nuclear γH2AX and 53BP1 foci were quantitated using Image-Pro software (Media Cybernetics, Inc.).

3. Results and discussion

3.1. Optimization of murine cerebellar organotypic cultures

Our protocol for establishing cerebellar organotypic cultures from mice (see Section 2) maintains the typical organization of the tissue slices from P12 mice (Fig. 1A) for several weeks, and allows a certain degree of *ex vivo* development of the tissue. Because the development of the murine cerebellum is almost completed about two weeks postnatally (Sudarov and Joyner, 2007; van Welie et al., 2011), young cultures from P0 animals do not show the highly structured, layered organization typical of the mature cerebellar cortex (Fig. 1B). However, during 3 weeks in culture, marked changes towards further organization are observed (Fig. 1B). Although the tissue organization does not reach that of P12 cultures (Fig. 1A), the occurrence of this process in culture indicates that its physiological milieu allows it to proceed to a certain extent.

3.2. Unique heterochromatin content in Purkinje cells

The cellular response to DNA damage takes place in the chromatin. Following the induction of DNA damage, several DDR arms oversee extensive chromatin reorganization at the damage sites and throughout the genome. This process includes relaxation of the chromatin, from its higher order down to the 30 nm fiber, and nucleosome organization mediated *via* spatial dynamics of histones accompanied by post-translational modifications of the histones and many chromatin-associated proteins (Gospodinov and Herceg, 2012; Lukas et al., 2011; Miller and Jackson, 2012; Papamichos-Chronakis and Peterson, 2012; Shi and Oberdoerffer, 2012; Soria et al., 2012). In the DSB response, this process is largely regulated by ATM *via* phosphorylation of effectors that modulate

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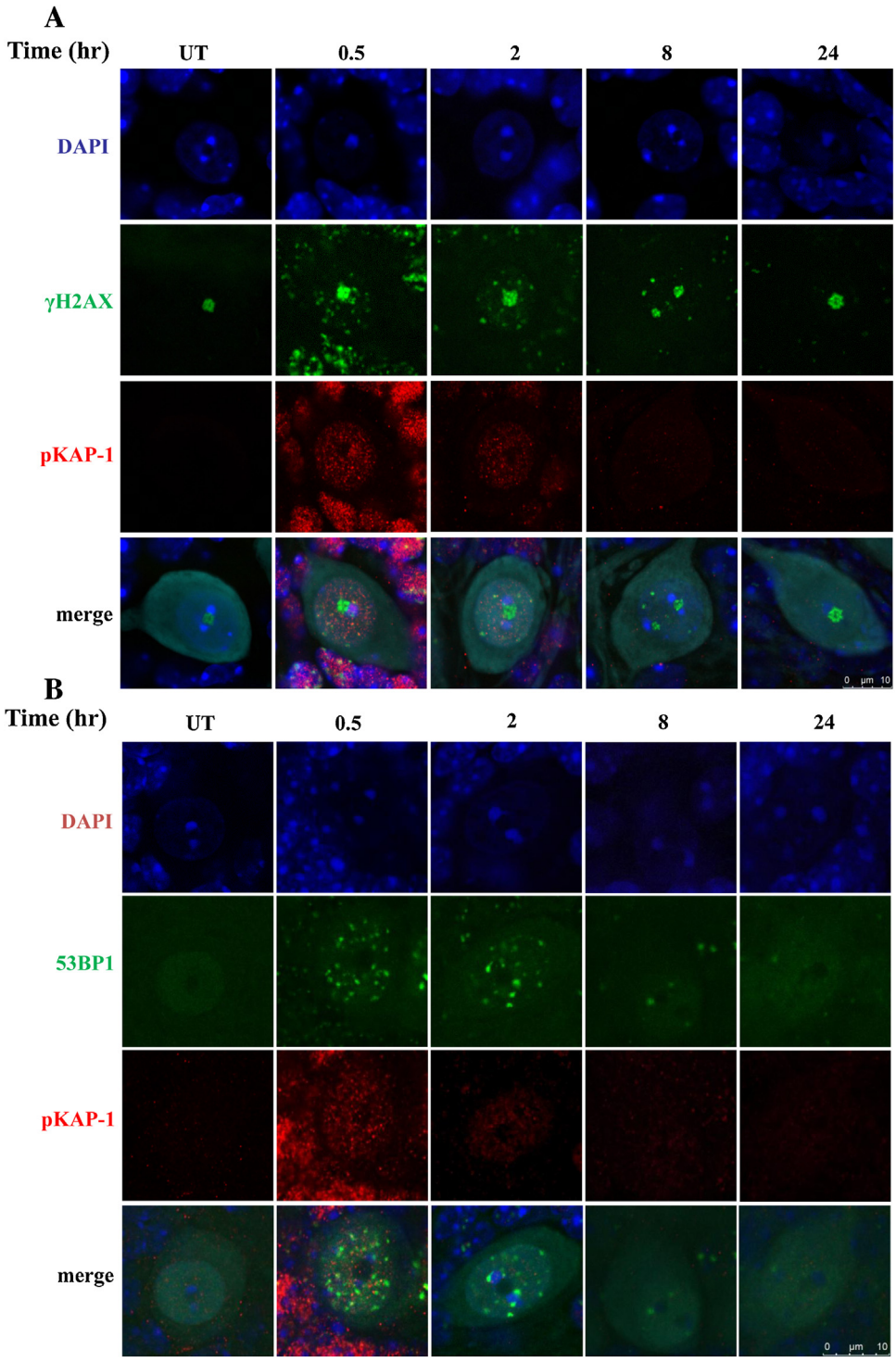

Fig. 6. Phosphorylated Kap-1 in Purkinje cells does not co-localize with DSB sites. Cerebellar organotypic cultures were irradiated with 2 Gy of IR and the images were captured at the indicated time points. Phosphorylated Kap-1 appears in a speckled pattern, which does not coincide with the nuclear foci of γH2AX (A) or 53BP1 (B).

chromatin compaction (Goodarzi et al., 2009; Moyal et al., 2011; Nakamura et al., 2011; Shiloh et al., 2011; Ziv et al., 2006). One of these effectors is the KAP-1 (TRIM28) protein. KAP-1 is best known as a transcription co-repressor, but following its ATM-mediated phosphorylation it mediates chromatin relaxation, which is essential for timely DSB repair (Goodarzi et al., 2010, 2008; Ziv et al., 2006). It has recently been shown that in proliferating cell lines, KAP-1 phosphorylation is particularly important for ATM-dependent DSB repair in the vicinity of heterochromatin (Goodarzi et al., 2008, 2011).

The extra compaction of heterochromatin plausibly poses a special challenge to chromatin relaxation, calling for special treatment by the DDR. Therefore, varying heterochromatin content may determine differences in DDR function in different cell types. The Purkinje cell is characterized by a large cell body and nucleus as well as extensive transcriptional activity, presumably keeping large portions of its chromatin decondensed. Indeed, special heterochromatin organization has been noticed in Purkinje cells (Solovei et al., 2004; Vadakkan et al., 2006). We visualized

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microscopically the heterochromatin of Purkinje cells in our cultures using several staining protocols. Mere DAPI staining indicated strikingly different heterochromatin content in Purkinje cells and the surrounding cells (Fig. 1A, bottom right image). In most murine cells the large blocks of pericentric heterochromatin are evident as prominent DAPI-stained nuclear bodies. In the large Purkinje cell nuclei, only a handful of such DAPI-stained bodies were noticed, much more limited comparing the conspicuous DAPI bodies in the surrounding cells (Fig. 1A, bottom right image). The smaller fraction of heterochromatin in Purkinje cell chromatin was further demonstrated by immunofluorescent staining with antibodies against two heterochromatin markers: heterochromatin protein 1 α (HP1α) and trimethylated lysine 9 of histone 3 (H3K9me3), whose staining coincided with that of the DAPI nuclear bodies (Fig. 2). Interestingly, dense HP1α staining characterized the few heterochromatic blocks in Purkinje cells, but not the much more prominent blocks of the surrounding cells (Fig. 2), suggesting that the organization of the small heterochromatic fraction of the chromatin in Purkinje cells may be packed differently than that of other cell types.

3.3. Analyzing the DDR in Purkinje cells using cerebellar organotypic cultures

Using the cerebellar organotypic cultures, we are comparing the DDR in Purkinje cells with the well-documented network in laboratory cell lines. While these cultures provide Purkinje cells with close to their natural environment, they carry an inherent experimental limitation: analysis focused on specific cell types in these cultures must be based on microscopic visualization and not bulk assays. Currently most such analyses are based on immunostaining and require the availability of antibodies that detect in immunostaining the corresponding mouse proteins in a specific manner. The results below show such experiments, which lead to new insights on the DDR in Purkinje cells.

In view of the special heterochromatin organization in Purkinje cells, and the role of KAP-1 in ATM-mediated chromatin decondensation and DSB repair near heterochromatin, we examined the amount, distribution and DNA damage-induced phosphorylation of murine Kap-1 in these cells. Interestingly, murine Purkinje cells exhibited considerably higher Kap-1 levels compared with the surrounding cells (Fig. 3A). A closer look at these cells revealed that areas of Kap-1 concentration coincided with areas of dense DAPI staining, presumably representing heterochromatin (Fig. 3B). However, when Kap-1 phosphorylation following irradiation was examined using an antibody that is highly specific for this phosphorylation (Ziv et al., 2006), a contrasting image was obtained: while marked Kap-1 phosphorylation was observed in most of the cells in this tissue, phosphorylation of this protein in Purkinje cells was much less prominent (Fig. 4A and B). Importantly, it was previously shown that in both humans and mice, ATM levels in Purkinje cells are similar to those in other cell types in the cerebellum (Dar et al., 2006; Gorodetsky et al., 2007). The low Kap-1 phosphorylation is understood in view of the constitutive relaxation of much of the chromatin in these cells and their low heterochromatin fraction, which make Kap-1's roles in general chromatin decondensation (Ziv et al., 2006), and specifically in DSB repair near heterochromatin (Goodarzi et al., 2008, 2010), less critical in Purkinje cells compared with other cell types. On the other hand, the disparity between Kap-1's high level in Purkinje cells and its low DNA damage-induced phosphorylation is in stark contrast to the situation in laboratory cell lines, in which the entire cellular content of this abundant protein is rapidly and robustly phosphorylated in response to DSBs (Ziv et al., 2006). This observation suggests that, in specific cell types, certain branches of the DDR can be adapted to unique characteristics of these cells, such as special chromatin organization. It is interesting to note that Purkinje cell nuclei are characterized by both high Kap-1 levels and largely open, relaxed chromatin. In these cells, the high amount of Kap-1 may be required primarily for gene expression control rather than for its transient roles in the DDR.

Nuclear foci of phosphorylated histone H2AX (γH2AX) are thought to mark the sites of unrepaired DSBs (Yuan et al., 2010). Fewer and smaller γH2AX foci were observed in irradiated Purkinje nuclei compared with the surrounding cells (Fig. 4B). The small size of these foci may reflect the relaxed state of the chromatin in these cells, which keeps phosphorylated H2AX molecules further apart compared to their proximity in more condensed chromatin. As expected, the disappearance of these foci in Atm-deficient cells was slower (Dar et al., 2006) (Fig. 5A), demonstrating the well documented, partial deficiency of DSB repair in the absence of ATM in these cells (Riballo et al., 2004). Similarly, the decay of nuclear foci of the 53BP1 protein was slower in Atm-/- compared with wild-type cells (Fig. 5B). 53BP1 is one of the major players in the early phase of the DSB response, and its recruitment to DSB sites is important for timely assembly of the protein conglomerates spanning the damaged sites, as well as for regulation of chromatin reorganization and damage repair at these sites (Noon and Goodarzi, 2011). Collectively, γH2AX and 53BP1 dynamics indicated that the Atm-dependent arm of DSB repair is important in Purkinje cells. In view of the small heterochromatin fraction in murine Purkinje cells, the Atm-mediated component of DSB repair is probably not associated particularly with DSBs close to heterochromatin as it is in proliferating cells (Goodarzi et al., 2010), again demonstrating a difference in certain aspects of the DDR between Purkinje cells and the commonly used cell lines.

A closer look at Kap-1 phosphorylation in irradiated Purkinje cells revealed a speckled pattern at the time this phosphorylation peaked (Figs. 4B and 6A and B), compared with the dense, pan-nuclear pattern observed in proliferating cell lines (Ziv et al., 2006). This pattern is similar to that of total Kap-1 (Fig. 3B). Importantly, most of the pKap-1 speckles did not coincide with γH2AX or 53BP1 foci (Fig. 6A and B). Plausibly, the low, partial phosphorylation of Kap-1 in these cells is required for relaxation of chromatin in areas that are not transcribed. In such regions, the chromatin may retain its regular compactness and thus require the documented function of phosphorylated Kap-1 in general chromatin relaxation throughout the nucleus (Ziv et al., 2006). Importantly, pKAp-1 speckles were not concentrated around the few heterochromatin blocks in Purkinje nuclei, suggesting that in these cells pKap-1 is not required specifically for DSB repair in these regions. This observation further suggests that this arm of the DSB response has adapted to the unique chromatin organization in Purkinje cells.

The susceptibility of the cerebellum to defects in specific DNA damage responses (El-Khamisy, 2011; Jeppesen et al., 2011; McKinnon, 2012) is not clearly understood. This is particularly true for A-T, where the role of ATM deficiency in the neurological symptoms of this disease is debated (Biton et al., 2008; Ditch and Paull, 2012; Li et al., 2012; Yang et al., 2011). We believe that the ATM function whose loss is most critical in the cerebellum is the one associated with the cellular response to genotoxic stress. In order to reach a firm conclusion in this direction, the unique aspects of the DDR in this organ must be better understood, particularly with regard to its unique neurons–Purkinje cells. Cerebellar organotypic cultures are a valuable tool in this research. Our results to date with this experimental system demonstrate some of the unique characteristics of the DDR in Purkinje cells and hold promise for progress in addressing a central question in A-T research.

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References

Amunugama, R., Fishel, R., 2012. Homologous recombination in eukaryotes. Progress in Molecular Biology and Translational Science 110, 155–206.
Aschner, M., 2011. Volume measurements in cultured primary astrocytes. Methods in Molecular Biology 758, 391–402.
Barlow, C., Hirotsune, S., Paylor, R., Liyanage, M., Eckhaus, M., Collins, F., Shiloh, Y., Crawley, J.N., Ried, T., Tagle, D., Wynshaw-Boris, A., 1996. Atm-deficient mice: a paradigm of ataxia telangiectasia. Cell 86, 159–171.
Bensimon, A., Schmidt, A., Ziv, Y., Elkon, R., Wang, S.Y., Chen, D.J., Aebersold, R., Shiloh, Y., 2010. ATM-dependent and -independent dynamics of the nuclear phosphoproteome after DNA damage. Science Signaling 3, rs3.
Bhatti, S., Kozlov, S., Farooqi, A.A., Naqi, A., Lavin, M., Khanna, K.K., 2011. ATM protein kinase: the linchpin of cellular defenses to stress. Cellular and Molecular Life Sciences 68, 2977–3006.
Biton, S., Barzilai, A., Shiloh, Y., 2008. The neurological phenotype of ataxia-telangiectasia: solving a persistent puzzle. DNA Repair (Amst) 7, 1028–1038.
Biton, S., Dar, I., Mittelman, L., Pereg, Y., Barzilai, A., Shiloh, Y., 2006. Nuclear ataxia-telangiectasia mutated (ATM) mediates the cellular response to DNA double strand breaks in human neuron-like cells. The Journal of Biological Chemistry 281, 17482–17491.
Biton, S., Gropp, M., Itsykson, P., Pereg, Y., Mittelman, L., Johe, K., Reubinoff, B., Shiloh, Y., 2007. ATM-mediated response to DNA double strand breaks in human neurons derived from stem cells. DNA Repair (Amst) 6, 128–134.
Borghesani, P.R., Alt, F.W., Bottaro, A., Davidson, L., Aksoy, S., Rathbun, G.A., Roberts, T.M., Swat, W., Segal, R.A., Gu, Y., 2000. Abnormal development of Purkinje cells and lymphocytes in Atm mutant mice. Proceedings of the National Academy of Sciences 97, 3336–3341.
Boukhtouche, F., Brugg, B., Wehrle, R., Bois-Joyeux, B., Danan, J.L., Dusart, I., Mariani, J., 2010. Induction of early Purkinje cell dendritic differentiation by thyroid hormone requires RORalpha. Neural Development 5, 18.
Branzei, D., Foiani, M., 2010. Maintaining genome stability at the replication fork. Nature Reviews Molecular Cell Biology 11, 208–219.
Carlessi, L., De Filippis, L., Lecis, D., Vescovi, A., Delia, D., 2009. DNA-damage response, survival and differentiation in vitro of a human neural stem cell line in relation to ATM expression. Cell Death and Differentiation 16, 795–806.
Chapman, J.R., Sossick, A.J., Boulton, S.J., Jackson, S.P., 2012. BRCA1-associated exclusion of 53BP1 from DNA damage sites underlies temporal control of DNA repair. Journal of Cell Science 125, 3529–3534.
Chipman, P.H., Toma, J.S., Rafuse, V.F., 2012. Generation of motor neurons from pluripotent stem cells. Progress in Brain Research 201, 313–331.
Ciccia, A., Elledge, S.J., 2010. The DNA damage response: making it safe to play with knives. Molecular Cell 40, 179–204.
Dar, I., Biton, S., Shiloh, Y., Barzilai, A., 2006. Analysis of the ataxia telangiectasia mutated-mediated DNA damage response in murine cerebellar neurons. The Journal of Neuroscience 26, 7767–7774.
Dar, I., Yosha, G., Elfassy, R., Galron, R., Wang, Z.Q., Shiloh, Y., Barzilai, A., 2011. Investigation of the functional link between ATM and NBS1 in the DNA damage response in the mouse cerebellum. The Journal of Biological Chemistry 286, 15361–15376.
de Luca, A., Vassallo, S., Benitez-Temino, B., Menichetti, G., Rossi, F., Buffo, A., 2009. Distinct modes of neuritic growth in purkinje neurons at different developmental stages: axonal morphogenesis and cellular regulatory mechanisms. PLoS ONE 4, e6848.
Ditch, S., Paull, T.T., 2012. The ATM protein kinase and cellular redox signaling: beyond the DNA damage response. Trends in Biochemical Sciences 37, 15–22.
Dupont, J.L., Fourcaudot, E., Beekenkamp, H., Poulain, B., Bossu, J.L., 2006. Synaptic organization of the mouse cerebellar cortex in organotypic slice cultures. Cerebellum 5, 243–256.
Dusart, I., Airaksinen, M.S., Sotelo, C., 1997. Purkinje cell survival and axonal regeneration are age dependent: an in vitro study. The Journal of Neuroscience 17, 3710–3726.
Edsjo, A., Holmquist, L., Pahlman, S., 2007. Neuroblastoma as an experimental model for neuronal differentiation and hypoxia-induced tumor cell dedifferentiation. Seminars in Cancer Biology 17, 248–256.
El-Khamisy, S.F., 2011. To live or to die: a matter of processing damaged DNA termini in neurons. EMBO Molecular Medicine 3, 78–88.
Elson, A., Wang, Y., Daugherty, C.J., Morton, C.C., Zhou, F., Campos-Torres, J., Leder, P., 1996. Pleiotropic defects in ataxia-telangiectasia protein-deficient mice. Proceedings of the National Academy of Sciences 93, 13084–13089.
Errico, A., Costanzo, V., 2012. Mechanisms of replication fork protection: a safeguard for genome stability. Critical Reviews in Biochemistry and Molecular Biology 47, 222–235.
Frappart, P.O., Tong, W.M., Demuth, I., Radovanovic, I., Herceg, Z., Aguzzi, A., Digweed, M., Wang, Z.Q., 2005. An essential function for NBS1 in the prevention of ataxia and cerebellar defects. Nature Medicine 11, 538–544.
Giordano, G., Hong, S., Faustman, E.M., Costa, L.G., 2011. Measurements of cell death in neuronal and glial cells. Methods in Molecular Biology 758, 171–178.
Goodarzi, A.A., Jeggo, P., Lobrich, M., 2010. The influence of heterochromatin on DNA double strand break repair: getting the strong, silent type to relax. DNA Repair (Amst) 9, 1273–1282.
Goodarzi, A.A., Kurka, T., Jeggo, P.A., 2011. KAP-1 phosphorylation regulates CHD3 nucleosome remodeling during the DNA double-strand break response. Nature Structural & Molecular Biology 18, 831–839.
Goodarzi, A.A., Noon, A.T., Deckbar, D., Ziv, Y., Shiloh, Y., Lobrich, M., Jeggo, P.A., 2008. ATM signaling facilitates repair of DNA double-strand breaks associated with heterochromatin. Molecular Cell 31, 167–177.
Goodarzi, A.A., Noon, A.T., Jeggo, P.A., 2009. The impact of heterochromatin on DSB repair. Biochemical Society Transactions 37, 569–576.
Gorodetsky, E., Calkins, S., Ahn, J., Brooks, P.J., 2007. ATM, the Mre11/Rad50/Nbs1 complex, and topoisomerase I are concentrated in the nucleus of Purkinje neurons in the juvenile human brain. DNA Repair (Amst) 6, 1698–1707.
Gospodinov, A., Herceg, Z., 2012. Shaping chromatin for repair. Mutation Research 752, 45–60.
Guo, Z., Kozlov, S., Lavin, M.F., Person, M.D., Paull, T.T., 2010. ATM activation by oxidative stress. Science 330, 517–521.
Hartlerode, A.J., Scully, R., 2009. Mechanisms of double-strand break repair in somatic mammalian cells. Biochemical Journal 423, 157–168.
Helmink, B.A., Sleckman, B.P., 2012. The response to and repair of RAG-mediated DNA double-strand breaks. Annual Review of Immunology 30, 175–202.
Hill, R., Lee, P.W., 2010. The DNA-dependent protein kinase (DNA-PK): more than just a case of making ends meet? Cell Cycle 9, 3460–3469.
Hiom, K., 2010. Coping with DNA double strand breaks. DNA Repair (Amst) 9, 1256–1263.
Hurtado de Mendoza, T., Balana, B., Slesinger, P.A., Verma, I.M., 2011. Organotypic cerebellar cultures: apoptotic challenges and detection. Journal of Visualized Experiments.
Inagaki, A., Schoenmakers, S., Baarends, W.M., 2010. DNA double strand break repair, chromosome synapsis and transcriptional silencing in meiosis. Epigenetics 5, 255–266.
Jeppesen, D.K., Bohr, V.A., Stevnsner, T., 2011. DNA repair deficiency in neurodegeneration. Progress in Neurobiology 94, 166–200.
Jones, R.M., Petermann, E., 2012. Replication fork dynamics and the DNA damage response. Biochemical Journal 443, 13–26.
Julien, S., Schnichels, S., Teng, H., Tassew, N., Henke-Fahle, S., Mueller, B.K., Monnier, P.P., 2008. Purkinje cell survival in organotypic cultures: implication of Rho and its downstream effector ROCK. Journal of Neuroscience Research 86, 531–536.
Kapfhammer, J.P., Gugger, O.S., 2012. The analysis of purkinje cell dendritic morphology in organotypic slice cultures. Journal of Visualized Experiments.
Kasparek, T.R., Humphrey, T.C., 2011. DNA double-strand break repair pathways, chromosomal rearrangements and cancer. Seminars in Cell & Developmental Biology 22, 886–897.
Kessler, M., Kiliman, B., Humes, C., Arai, A.C., 2008. Spontaneous activity in Purkinje cells: multi-electrode recording from organotypic cerebellar slice cultures. Brain Research 1218, 54–69.
Lavin, M.F., 2008. Ataxia-telangiectasia: from a rare disorder to a paradigm for cell signalling and cancer. Nature Reviews Molecular Cell Biology 9, 759–769.
Lebrun, C., Avci, H.X., Wehrle, R., Doulazmi, M., Jaudon, F., Morel, M.P., Rivals, I., Ema, M., Schmidt, S., Sotelo, C., Vodjdani, G., Dusart, I., 2012. Klf9 is necessary and sufficient for Purkinje cell survival in organotypic culture. Molecular and Cellular Neuroscience 54, 9–21.
Lempiainen, H., Halazonetis, T.D., 2009. Emerging common themes in regulation of PIKKs and PI3Ks. EMBO Journal 28, 3067–3073.
Li, J., Chen, J., Ricupero, C.L., Hart, R.P., Schwartz, M.S., Kusnecov, A., Herrup, K., 2012. Nuclear accumulation of HDAC4 in ATM deficiency promotes neurodegeneration in ataxia telangiectasia. Nature Medicine 18, 783–790.
Lieber, M.R., 2010. The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. Annual Review of Biochemistry 79, 181–211.
Lonchamp, E., Dupont, J.L., Beekenkamp, H., Poulain, B., Bossu, J.L., 2006. The mouse cerebellar cortex in organotypic slice cultures: an in vitro model to analyze the consequences of mutations and pathologies on neuronal survival, development, and function. Critical Reviews in Neurobiology 18, 179–186.
Lovejoy, C.A., Cortez, D., 2009. Common mechanisms of PIKK regulation. DNA Repair (Amst) 8, 1004–1008.
Lu, H.X., Levis, H., Liu, Y., Parker, T., 2011. Organotypic slices culture model for cerebellar ataxia: potential use to study Purkinje cell induction from neural stem cells. Brain Research Bulletin 84, 169–173.
Lukas, J., Lukas, C., Bartek, J., 2011. More than just a focus: the chromatin response to DNA damage and its role in genome integrity maintenance. Nature Cell Biology 13, 1161–1169.
Matsuoka, S., Ballif, B.A., Smogorzewska, A., McDonald 3rd, E.R., Hurov, K.E., Luo, J., Bakalarski, C.E., Zhao, Z., Solimini, N., Lerenthal, Y., Shiloh, Y., Gygi, S.P., Elledge, S.J., 2007. ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. Science 316, 1160–1166.

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McKinnon, P.J., 2012. ATM and the molecular pathogenesis of ataxia telangiectasia. Annual Review of Pathology 7, 303–321.
Melli, G., Hoke, A., 2009. Dorsal root ganglia sensory neuronal cultures: a tool for drug discovery for peripheral neuropathies. Expert Opinion on Drug Discovery 4, 1035–1045.
Miller, K.M., Jackson, S.P., 2012. Histone marks: repairing DNA breaks within the context of chromatin. Biochemical Society Transactions 40, 370–376.
Mladenov, E., Iliakis, G., 2011. Induction and repair of DNA double strand breaks: the increasing spectrum of non-homologous end joining pathways. Mutation Research 711, 61–72.
Moyal, L., Lerenthal, Y., Gana-Weisz, M., Mass, G., So, S., Wang, S.Y., Eppink, B., Chung, Y.M., Shalev, G., Shema, E., Shkedy, D., Smorodinsky, N.I., van Vliet, N., Kuster, B., Mann, M., Ciechanover, A., Dahm-Daphi, J., Kanaar, R., Hu, M.C., Chen, D.J., Oren, M., Shiloh, Y., 2011. Requirement of ATM-dependent monoubiquitylation of histone H2B for timely repair of DNA double-strand breaks. Molecular Cell 41, 529–542.
Mu, J.J., Wang, Y., Luo, H., Leng, M., Zhang, J., Yang, T., Besusso, D., Jung, S.Y., Qin, J., 2007. A proteomic analysis of ataxia telangiectasia-mutated (ATM)/ATM-Rad3-related (ATR) substrates identifies the ubiquitin-proteasome system as a regulator for DNA damage checkpoints. The Journal of Biological Chemistry 282, 17330–17334.
Nakamura, K., Kato, A., Kobayashi, J., Yanagihara, H., Sakamoto, S., Oliveira, D.V., Shimada, M., Tauchi, H., Suzuki, H., Tashiro, S., Zou, L., Komatsu, K., 2011. Regulation of homologous recombination by RNF20-dependent H2B ubiquitination. Molecular Cell 41, 515–528.
Nam, E.A., Cortez, D., 2011. ATR signalling: more than meeting at the fork. Biochemical Journal 436, 527–536.
Nayler, S., Gatei, M., Kozlov, S., Gatti, R., Mar, J.C., Wells, C.A., Lavin, M., Wolvetang, E., 2012. Induced pluripotent stem cells from ataxia-telangiectasia recapitulate the cellular phenotype. Stem Cells Translational Medicine 1, 523–535.
Neal, J.A., Meek, K., 2011. Choosing the right path: does DNA-PK help make the decision? Mutation Research 711, 73–86.
Noon, A.T., Goodarzi, A.A., 2011. 53BP1-mediated DNA double strand break repair: insert bad pun here. DNA Repair (Amst) 10, 1071–1076.
O'Driscoll, M., 2012. Diseases associated with defective responses to DNA damage. Cold Spring Harbor Perspectives in Biology 4 .
O'Sullivan, R.J., Karlseder, J., 2010. Telomeres: protecting chromosomes against genome instability. Nature Reviews Molecular Cell Biology 11, 171–181.
Oldreive, C.E., Doherty, G.H., 2010. Effects of tumour necrosis factor-alpha on developing cerebellar granule and Purkinje neurons in vitro. Journal of Molecular Neuroscience 42, 44–52.
Padmanabhan, J., Brown, K., Shelanski, M.L., 2007. Cell cycle inhibition and retinoblastoma protein overexpression prevent Purkinje cell death in organotypic slice cultures. Developmental Neurobiology 67, 818–826.
Papamichos-Chronakis, M., Peterson, C.L., 2012. Chromatin and the genome integrity network. Nature Reviews Genetics 14, 62–75.
Paquet-Durand, F., Bicker, G., 2007. Human model neurons in studies of brain cell damage and neural repair. Current Molecular Medicine 7, 541–554.
Perlman, S.L., Boder, E., Sedgewick, R.P., Gatti, R.A., 2012. Ataxia-telangiectasia. Handbook of Clinical Neurology 103, 307–332.
Peuscher, M.H., Jacobs, J.J., 2012. Posttranslational control of telomere maintenance and the telomere damage response. Cell Cycle 11, 1524–1534.
Polo, S.E., Jackson, S.P., 2011. Dynamics of DNA damage response proteins at DNA breaks: a focus on protein modifications. Genes and Development 25, 409–433.
Psakhye, I., Jentsch, S., 2012. Protein group modification and synergy in the SUMO Pathway as exemplified in DNA repair. Cell 151, 807–820.
Ramaekers, C.H., Wouters, B.G., 2011. Regulatory functions of ubiquitin in diverse DNA damage responses. Current Molecular Medicine 11, 152–169.
Riballo, E., Kuhne, M., Rief, N., Doherty, A., Smith, G.C., Recio, M.J., Reis, C., Dahm, K., Fricke, A., Krempler, A., Parker, A.R., Jackson, S.P., Gennery, A., Jeggo, P.A., Lobrich, M., 2004. A pathway of double-strand break rejoining dependent upon ATM, Artemis, and proteins locating to gamma-H2AX foci. Molecular Cell 16, 715–724.
Robertson, M.J., Gip, P., Schaffer, D.V., 2008. Neural stem cell engineering: directed differentiation of adult and embryonic stem cells into neurons. Frontiers in Bioscience 13, 21–50.
Roos, W.P., Kaina, B., 2012. DNA damage-induced apoptosis: from specific DNA lesions to the DNA damage response and apoptosis. Cancer Letters 332, 237–248.
Savitsky, K., Bar-Shira, A., Gilad, S., Rotman, G., Ziv, Y., Vanagaite, L., Tagle, D.A., Smith, S., Uziel, T., Sfez, S., Ashkenazi, M., Pecker, I., Frydman, M., Harnik, R., Patanjali, S.R., Simmons, A., Clines, G.A., Sartiel, A., Gatti, R.A., Chessa, L., Sanal, O., Lavin, M.F., Jaspers, N.G., Taylor, A.M., Arlett, C.F., Miki, T., Weissman, S.M., Lovett, M., Collins, F.S., Shiloh, Y., 1995a. A single ataxia telangiectasia gene with a product similar to PI-3 kinase. Science 268, 1749–1753.
Savitsky, K., Sfez, S., Tagle, D.A., Ziv, Y., Sartiel, A., Collins, F.S., Shiloh, Y., Rotman, G., 1995b. The complete sequence of the coding region of the ATM gene reveals similarity to cell cycle regulators in different species. Human Molecular Genetics 4, 2025–2032.
Schwartz, P.H., Brick, D.J., Stover, A.E., Loring, J.F., Muller, F.J., 2008. Differentiation of neural lineage cells from human pluripotent stem cells. Methods 45, 142–158.
Shi, L., Oberdoerffer, P., 2012. Chromatin dynamics in DNA double-strand break repair. Biochimica et Biophysica Acta 1819, 811–819.
Shiloh, Y., Shema, E., Moyal, L., Oren, M., 2011. RNF20-, RNF40: a ubiquitin-driven link between gene expression and the DNA damage response. FEBS Letters 585, 2795–2802.
Shiloh, Y., Ziv, Y., 2013. The ATM protein kinase: regulating the cellular response to genotoxic stress, and more. Nature Reviews Molecular Cell Biology 14, 197–210.
Solovei, I., Schermelleh, L., During, K., Engelhardt, A., Stein, S., Cremer, C., Cremer, T., 2004. Differences in centromere positioning of cycling and postmitotic human cell types. Chromosoma 112, 410–423.
Soria, G., Polo, S.E., Almouzni, G., 2012. Prime, repair, restore: the active role of chromatin in the DNA damage response. Molecular Cell 46, 722–734.
Stewart, G.S., Maser, R.S., Stankovic, T., Bressan, D.A., Kaplan, M.I., Jaspers, N.G., Raams, A., Byrd, P.J., Petrini, J.H., Taylor, A.M., 1999. The DNA double-strand break repair gene hMRE11 is mutated in individuals with an ataxia-telangiectasia-like disorder. Cell 99, 577–587.
Stracker, T.H., Petrini, J.H., 2011. The MRE11 complex: starting from the ends. Nature Reviews Molecular Cell Biology 12, 90–103.
Sudarov, A., Joyner, A.L., 2007. Cerebellum morphogenesis: the foliation pattern is orchestrated by multi-cellular anchoring centers. Neural Development 2, 26.
Taylor, A.M., Groom, A., Byrd, P.J., 2004. Ataxia-telangiectasia-like disorder (ATLD)-its clinical presentation and molecular basis. DNA Repair (Amst) 3, 1219–1225.
Thompson, L.H., 2012. Recognition, signaling, and repair of DNA double-strand breaks produced by ionizing radiation in mammalian cells: the molecular choreography. Mutation Research 751, 158–246.
Vadakkan, K.I., Li, B., De Boni, U., 2006. Cell-type specific proximity of centromeric domains of one homologue each of chromosomes 2 and 11 in nuclei of cerebellar Purkinje neurons. Chromosoma 115, 395–402.
van Welie, I., Smith, I.T., Watt, A.J., 2011. The metamorphosis of the developing cerebellar microcircuit. Current Opinion in Neurobiology 21, 245–253.
Wei, F., Xie, Y., Tao, L., Tang, D., 2010. Both ERK1 and ERK2 kinases promote G2/M arrest in etoposide-treated MCF7 cells by facilitating ATM activation. Cellular Signalling 22, 1783–1789.
Xia, G., Santostefano, K., Hamazaki, T., Liu, J., Subramony, S.H., Terada, N., Ashizawa, T., 2012. Generation of human-induced pluripotent stem cells to model spinocerebellar ataxia type 2 In vitro. Cell Reprogram 15, 166–177.
Xu, Y., Ashley, T., Brainerd, E.E., Bronson, R.T., Meyn, M.S., Baltimore, D., 1996. Targeted disruption of ATM leads to growth retardation, chromosomal fragmentation during meiosis, immune defects, and thymic lymphoma. Genes and Development 10, 2411–2422.
Yang, D.Q., Halaby, M.J., Li, Y., Hibma, J.C., Burn, P., 2011. Cytoplasmic ATM protein kinase: an emerging therapeutic target for diabetes, cancer and neuronal degeneration. Drug Discovery Today 16, 332–338.
Yuan, J., Adamski, R., Chen, J., 2010. Focus on histone variant H2AX: to be or not to be. FEBS Letters 584, 3717–3724.
Zanjani, H.S., Lohof, A.M., McFarland, R., Vogel, M.W., Mariani, J., 2012. Enhanced survival of wild-type and lurcher Purkinje cells in vitro following inhibition of conventional PKCs or stress-activated MAP kinase pathways. Cerebellum (Epub ahead of print).
Ziv, Y., Bielopolski, D., Galanty, Y., Lukas, C., Taya, Y., Schultz, D.C., Lukas, J., Bekker-Jensen, S., Bartek, J., Shiloh, Y., 2006. Chromatin relaxation in response to DNA double-strand breaks is modulated by a novel ATM- and KAP-1 dependent pathway. Nature Cell Biology 8, 870–876.

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