Chapter 30

Monitoring the ATM-Mediated DNA Damage Response in the Cerebellum Using Organotypic Cultures

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Abstract

The *ATM* gene and its protein product, the ATM protein kinase, were identified as a result of attempts to understand the molecular basis of the genetic disorder, ataxia-telangiectasia (A-T). The cardinal symptom of A-T is neurodegeneration expressed primarily as progressive cerebellar atrophy. A major tool in the investigation of ATM functions in the cerebellum is cerebellar organotypic cultures, which allow cerebellar slices to live in culture for several weeks without losing their viability and organization. These cultures are amenable to various treatments and manipulations and provide a close look at Purkinje cells in their almost natural environment. We optimized the protocol for establishing and maintaining these cultures and provide here examples of readouts of the DNA damage response in cerebellar organotypic cultures treated with a DNA-damaging agent.

Key words Ataxia-telangiectasia (A-T), ATM kinase, DNA damage response (DDR), Cerebellum, Organotypic cultures

1 Introduction: Ataxia-Telangiectasia and ATM: Focus on the Cerebellum

Ataxia-telangiectasia (A-T; OMIN#208900) is a highly pleiotropic, autosomal-recessive human disorder [1–8] caused by loss of the ATM protein function. The responsible mutations create null alleles at the *ATM* (*A-T*, mutated) gene, which encodes the ATM protein kinase [9–11]. The prominent symptom in the typical form of A-T is progressive cerebellar ataxia that develops into general motor dysfunction, eventually confining most patients to a wheelchair toward the end of their first decade. The main underlying pathology is progressive cerebellar cortical degeneration that involves primarily Purkinje and granule neurons, but also affects basket cells. Peripheral neuropathy develops in many A-T patients during the second decade of life. Additional symptoms are oculocutaneous telangiectasias (dilated blood vessels), which usually appear in the eyes, marked immunodeficiency, and cancer predisposition, with most

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malignancies being lymphoreticular. A-T patients show a striking sensitivity to the cytotoxic effect of ionizing radiation (IR), and cells derived from these patients exhibit marked chromosomal instability and sensitivity to IR and radiomimetic chemicals. This sensitivity results from a profound defect in the cellular response to DNA double strand breaks (DSBs), whose chief mobilizer is the ATM protein kinase [12–14]. Many A-T symptoms can be attributed to the abrogation of the cellular response to physiological DSBs and to DSBs induced by endogenous reactive oxygen species [13]. However, the cause of the most devastating manifestation of this disease—progressive neurodegeneration, particularly cerebellar atrophy—is still being debated [5, 15–17]. In our attempts to understand the critical role of ATM in the cerebellum, a major experimental tool is cerebellar organotypic cultures established from mice with different *Atm* genotypes [18].

Organotypic slice cultures provide a culture-based system that is much closer to a tissue compared to dissociated cultures or neuron-like cells obtained by induced differentiation, and yet are amenable to most of the manipulations that can be applied to cultured cells [19–30]. We consider cerebellar organotypic cultures a valuable tool for studying ATM's functions in the cerebellum, and therefore recently optimized the protocol for their establishment [18]. When generated from P12 mice, our cultures maintain the typical organization of the tissue for several weeks (Fig. 1), and also allow a certain degree of ex vivo development of the tissue [18]. This system increases the resolution at which we can analyze the DNA damage response (DDR) in cerebellar cells, with emphasis

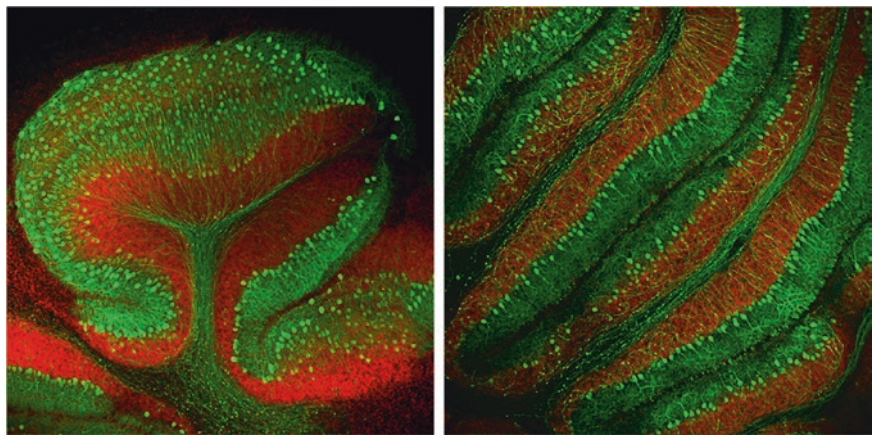

Fig. 1 Organotypic cultures established from P12 wild-type mice, 12 days after their establishment, demonstrate normal tissue organization. *Green*: Calbindin-DK28, a Purkinje cell marker; *red*: NeuN, a neuronal nucleus marker, which does not stain Purkinje nuclei; *blue*: DAPI, a fluorescent stain of DNA (from ref. [18]). Reprinted with permission from Tzur-Gilat et al. Studying the cerebellar DNA damage response in the tissue culture dish, Mechanisms of Ageing and Development, 2013, v134 (10):496–505. Copyright © 2013 Elsevier Ireland Ltd. All rights reserved

on Purkinje cells, which exhibit a unique chromatin organization that is mainly euchromatic [18]. Importantly, the ATM-dependent DDR appears to be robust in Purkinje cells [18, 31, 32]. We should point out, however, that while these cultures provide Purkinje cells close to their natural environment, they carry an inherent experimental limitation: specific cell types in these cultures must be analyzed by microscopic visualization and not bulk assays. Currently, most such analyses are based on immunostaining and antibodies that specifically detect the corresponding mouse proteins.

2 Materials

All solutions must be stored at 4 °C, unless indicated otherwise.

2.1 Organotypic Culture Reagents and Equipment

All solutions must be prepared and stored under sterile conditions.

1. 50% D-Glucose solution: In a glass beaker dissolve 50 g of D-glucose in 100 ml double distilled water (*see* **Note 1**). In a sterile environment, filter the solution through a 0.22 μm filter. Divide the solution into 2 ml aliquots and store at −20 °C.
2. Dissection medium: HBSS containing 0.5% D-glucose. Add 0.5 ml of 50% D-Glucose solution to 50 ml Hank's balanced salt solution (HBSS) buffer without Mg^{2+} and Ca^{2+}.
3. BME medium: Mix 100 ml of Basal Medium Eagle (BME) with phenol red and without L-glutamine and HEPES, with 50 ml of HBSS with phenol red, 50 ml heat-inactivated horse serum (*see* **Note 2**), 2 ml of 50% D-glucose and 1 ml of 200 mM L-glutamine. Mix and filter through a 0.22 μm filter (*see* **Note 3**). Store at 4 °C.
4. Sterile cell culture inserts of 0.4 μm, 30 mm diameter (Millicell, Merck Millipore, Germany).
5. Six-well cell culture plates, flat-bottom with lid.
6. Three-cm tissue culture plates.
7. Mcllwain tissue chopper (Cavey Laboratory Engineering Co. Ltd., UK).
8. Binocular microscope.
9. Light source for binocular microscope.
10. X-ray machine (suitable for cell cultures plates).
11. Etoposide. 50 mM in DMSO.
12. 70% ethanol.
13. Whatman paper.
14. Tweezers.

15. Surgical tools: mouse decapitation device, standard forceps slightly curved, surgical scissors, smooth-tip forceps, jeweler forceps, and a blunt-end spatula.

2.2 Immunostaining Reagents

All solutions must be stored at 4 °C.

1. 0.1 M Phosphate buffer: Prepare solution A by dissolving 12 g of NaH_2PO_4 monobasic anhydrous in 500 ml of double distilled water (ddw). Prepare solution B by dissolving 14.2 g of Na_2HPO_4 dibasic anhydrous in 500 ml of ddw. Prepare 0.2 M Phosphate buffer by mixing 115 ml of buffer A and 385 ml of buffer B. Before use, dilute the 0.2 M phosphate buffer 1:1 with ddw to a final concentration of 0.1 M.
2. 4% paraformaldehyde (PFA) fixative: Use 16% PFA stock solution (*see* **Note 4**). Add 10 ml of 16% PFA and 10 ml of ddw to 20 ml of 0.2 M Phosphate buffer (*see* **Note 5**).
3. PBS: Dulbecco's phosphate buffer without Mg^{2+} and Ca^{2+}.
4. Wash solution: Dissolve 1.25 ml of Triton X-100 in 500 ml of PBS (*see* **Note 6**).
5. 1 M L-lysine: Dissolve 9.13 g of L-lysine in 50 ml of PBS.
6. Blocking solution: Dissolve in a glass bottle 1 g of gelatin in 100 ml of PBS (*see* **Note 7**). Add 0.5 g of sodium azide and 1.25 ml of Titon X-100 (*see* **Note 6**). Complete volume to 500 ml with PBS. Keep 140 ml in a separate bottle for later use. Add 40 ml of 1 M L-lysine to the remaining 360 ml of solution.
7. Primary antibody dilution solution: Use the blocking solution (without the L-lysine) (*see* Subheading 2.2, **item 6**).
8. Secondary antibody dilution solution: Dissolve 0.1 g of gelatin in 50 ml of PBS (*see* **Note 7**). Add 25 μl of Triton X-100 (*see* **Note 6**).
9. Polyclonal IgG goat Anti-calbindin D28K antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Working dilution: 1:250.
10. Monoclonal mouse anti-Neuronal Nuclei (NeuN) antibody (Merck Millipore, MA, USA). Working dilution 1:4000.
11. Monoclonal mouse IgG antibody against phosphorylated serine 139 of histone H2AX (γH2AX) (Merck Millipore, MA, USA). Working dilution 1:1000.
12. Polyclonal rabbit anti-53BP1 antibody (Novus, CO, USA). Working dilution 1:500.
13. Polyclonal rabbit anti-pS824 KAP-1 (Bethyl Laboratories, TX, USA). Working dilution 1:1000.
14. Alexa Fluor 488 donkey anti-goat IgG antibody (Molecular Probes, Netherlands).

15. Alexa Fluor 568 donkey anti-mouse IgG antibody (Molecular Probes, Netherlands).
16. Alexa Fluor 633 donkey anti-mouse IgG antibody (Molecular Probes, Netherlands).
17. Alexa Fluor 568 donkey anti-rabbit IgG antibody (Molecular Probes, Netherlands).
18. 4′,6-Diamidino-2-phenylindole (DAPI) 20 mg/ml.
19. Fluorescent mounting medium aqueous (ready to use).
20. Scalpel.
21. 48-well cell culture plate.
22. Microscope cover glasses, thickness #1.0, 0.13–0.17 mm.
23. SuperFrost Plus Slides (Fisher Scientific, PA, USA).
24. Confocal microscope.

3 Methods

3.1 Preparation of Organotypic Culture

Carry out all procedures in a sterile environment.

1. Use 6-well plates to prepare cultures from mice with different genotypes (e.g., wild-type $Atm^{+/+}$ and $Atm^{-/-}$). Add 1 ml of BME medium to each of six wells (*see* **Note 8**). Using sterile forceps, place one cell culture insert in each well (*see* **Note 9**). Put the plate in an incubator at 37 °C, 5% CO_2, for at least 2 h prior to dissection.
2. Prepare a surgery area: In a biological hood place the Mcllwain tissue chopper, mouse decapitation device, binocular microscope, and light source (*see* **Note 10**). Place all surgical tools in a cup filled with 70% ethanol (*see* **Note 11**). Fill 3-cm culture plates with cold dissection medium and place on ice (one plate per cerebellum).
3. Using the mouse decapitation device and the surgical tools, separate the cerebellum from a P10–P12 mouse and place it in the cold dissection medium in the 3-cm plate prepared in advance (*see* **Note 12**). Repeat with additional animals according to the experimental plan.
4. Adjust the Mcllwain tissue chopper to cut slices 400 μm thick. Place the cerebellum on the cutting platform vertical to the cutting angle and serially cut sagittal sections (*see* **Note 13**).
5. Transfer the tissue slices back to the cold dissection buffer (*see* **Note 14**). Under the binocular microscope, use two spatulas to gently separate the slices.
6. Transfer the separated tissue slices from the dissection buffer onto the cell culture inserts prepared in advance in the 6-well

plates. Up to six tissue slices can be placed on one insert (*see* **Note 15**). Repeat **steps 3–6** for the next cerebellum. Incubate the tissue cultures at 37 °C, 5% CO_2.

7. Grow the cultures for 12 days with medium change every 2–3 days. Change the medium by adding 1 ml of BME medium to each of the wells in a new 6-well plate. Using a sterile forceps, transfer the culture insert to the new plate.

3.2 Monitoring Tissue Organization Using Immunostaining

1. Wash the cultures by aspirating the medium and replacing it with 1 ml of PBS.
2. Immediately fix the tissue by replacing the PBS with 4% PFA. Apply 1 ml of PFA underneath the insert and 1 ml above the insert and leave at room temperature for 1 h (*see* **Note 16**).
3. Remove the PFA and wash the inserts by adding 1 ml of wash solution underneath the insert and 1 ml above it. Place the plate on a shaker at room temperature for 5 min. Repeat washes four times.
4. Replace the wash solution with blocking solution, 1 ml underneath the culture insert and 1 ml above it. Place the plate on a shaker and shake gently at room temperature for 1 h.
5. Separate the tissue slices from the cell culture insert: using a scalpel, cut the membrane of the cell culture insert in small circles around each individual cerebellar slice (*see* **Note 17**). Place each portion in a separate well of a 48-well plate. Add 50 μl of 0.1 M phosphate buffer to prevent drying.
6. For monitoring tissue organization, use antibodies against the Purkinje cell marker, calbindin D28k, and the neuronal marker, NeuN. Dilute the anti-calbindin antibody 1:250 and the NeuN antibody 1:4000 in the primary antibody dilution buffer. Final primary antibody volume = (100 μl) × (the number of tissue slices to be stained).
7. Remove the phosphate buffer and apply 100 μl of primary antibody mix to each well (*see* **Note 18**). Incubate with gentle shaking overnight at 4 °C.
8. From this step on, minimize the light exposure.
9. Prepare the secondary antibody solution. Dilute the donkey anti-mouse 568 antibody 1:500, and the donkey anti-goat 488 antibody in the secondary antibody dilution solution. Final secondary antibody volume = (100 μl) × (the number of tissue slices to be stained).
10. Wash the primary antibody from the samples with the wash solution: four times for 5 min with gentle shaking (*see* **Note 18**).
11. Replace the wash solution with 100 μl of secondary antibody solution. Incubate with gentle shaking for 2 h at room temperature in the dark.

12. Prepare DAPI staining: Dilute DAPI 1:20,000 in PBS (*see* **Note 19**). The volume of the DAPI solution = (100 μl) × (the number of tissue slices to be stained).
13. Wash the secondary antibody from the samples with the wash solution: four times for 5 min with gentle shaking (*see* **Note 18**).
14. Replace the wash solution with DAPI staining solution (*see* **step 12** of Subheading 3.2) by applying 100 μl of DAPI to each well. Incubate for 20 min at room temperature in the dark.
15. Place the tissues on microscope glass slides, membrane facing down and tissue facing the cover glass. Mount and cover with a cover glass. Place the slides on a flat surface and let them dry overnight in the dark.
16. Store the slides at 4 °C in a light-proof box.
17. Capture images using a confocal microscope. You should be able to identify the typical cerebellar folia and clearly identify the Purkinje cells (Fig. 1).

3.3 Observation of the DNA Damage Response in Cerebellar Organotypic Cultures

DNA damage can be induced by irradiation or chemical agents: X-irradiation and treatment with the topoisomerase II inhibitor, etoposide. DDR readouts are then monitored using immunostaining (Fig. 2).

1. Prepare fresh organotypic cultures by repeating all steps in Subheading 3.1.
2. Divide the cell culture inserts from different genotypes into separate 6-well plates according to the experimental plan. When applying X-ray irradiation prepare separate 6-well plates for the treated and untreated inserts.
3. Etoposide treatment: Prepare etoposide solution in BME medium at the desired concentration. We describe here treatment with 10 μM etoposide. Prepare 1:50 intermediate dilution in BME medium, dilute it 1:100 in BME medium to obtain a final concentration of 10 μM. Remove culture medium and replace with etoposide solution. Replace the medium in control samples with fresh BME medium. Place organotypic cultures in an incubator for 1 h. Aspirate the medium and wash with 1 ml of PBS. Immediately fix the tissue as described in **step 2** of Subheading 3.2.
4. Ionizing radiation treatment: Irradiate one plate of each genotype at the desired dose using an X-irradiator and place in the incubator for various periods of time. We refer here to the response to 5 Gy of X-rays. Place cultures in an incubator for 30 min.

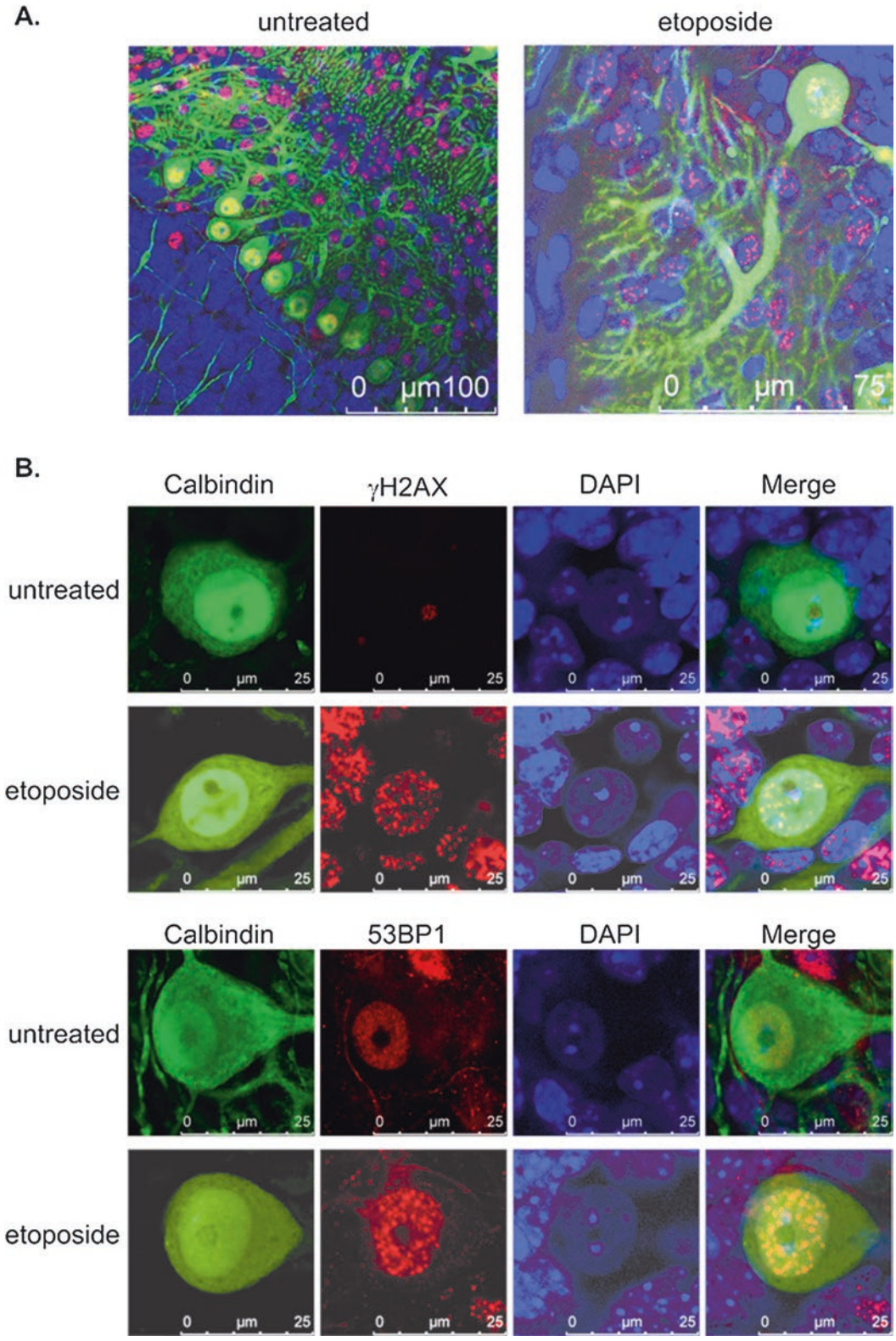

Fig. 2 Demonstration of nuclear foci formed at DSB sites 1 h after treatment of cerebellar organotypic cultures with 10 μM etoposide. (**a**) *Red*: 53BP1; *Green*: calbindin-DK28; *blue*: DAPI. (**b**) A close look at a Purkinje cell in etoposide-treated culture. The nuclear region that is strongly stained by the anti-γH2AX and anti-53BP1 antibody overlaps the nucleolus; the significance of this staining is unclear

5. Wash the cultures by aspirating the medium and replacing it with 1 ml of PBS and immediately fix the tissue, as described in **step 2** of Subheading 3.2.
6. Repeat **steps 3–5** of the protocol in Subheading 3.2.
7. For monitoring the DSB response in Purkinje cells, use primary antibodies that reveal the DSB markers, γH2AX and 53BP1 nuclear foci, and Kap-1 phosphorylation. Stain the cultures with mixtures that each contains primary antibodies diluted in primary antibody dilution buffer: (a) Anti-calbindin antibody (1:250) and anti-γH2AX antibody (1:1000). (b) Anti-calbindin antibody (1:250) and anti-53BP1 antibody (1:500) (*see* **Note 20**). (c) Anti-calbindin antibody (1:250), anti-pKAP-1 antibody (1:1000), and anti-γH2AX antibody (1:1000). The volume of each solution = (100 μl) × (the number of tissue slices to be stained).
8. Remove the buffer and add 100 μl of primary antibody mix per well—add only one primary antibody mix in to each well. Stain at least one cerebellum tissue from each genotype and treatment with each antibody mix. Incubate overnight at 4 °C with gentle shaking.
9. Prepare secondary antibody solution: (a) Donkey anti-mouse 568 antibody (1:500) and donkey anti-goat 488 antibody (1:500). (b) Donkey anti-rabbit 568 (1:500) and donkey anti-goat 488 antibody (1:500). (c) Donkey anti-rabbit 568 antibody (1:500), donkey anti-goat 488 antibody (1:500), and donkey anti-mouse 633 antibody (1:500) (*see* **Note 21**). The volume of each solution = (100 μl) × (the number of tissue slices to be stained).
10. After incubation with primary antibodies, wash the cultures with the wash solution four times for 5 min with gentle shaking.
11. Replace the wash solution with 100 μl of secondary antibody solution: cultures incubated with primary mix a, b, or c should be incubated with the matching secondary antibody mix a, b, or c. Incubate with gentle shaking for 2 h at room temperature in the dark.
12. Repeat **steps 12–17** of the protocol in Subheading 3.2.

4 Notes

1. The glucose can be easily dissolved by heating the solution to 70 °C. Let it cool down before continuing.
2. Horse serum should be stored at −20 °C. Freeze in 50 ml aliquots to minimize freezing-thawing cycles.
3. The medium is good for up to 1 month when stored at 4 °C.

4. 16% PFA can be purchased commercially. Dilute in a chemical hood.
5. Prepare fresh 4% PFA at room temperature. Although it can be stored up to 1 month in the dark at 4°C, freshly prepared solution is recommended.
6. Use a 1000 μl plastic tip with blunt end to add the Triton detergent. When ejecting the Triton into the PBS, leave the tip in the bottle to allow complete dissolution of the entire amount of the detergent. Shake gently until Triton dissolves completely.
7. Heat the PBS and gelatin solution for 1 min in a microwave oven and stir. Make sure the gelatin is completely dissolved; if not, heat it again but do not allow it to boil. Let the solution cool to room temperature.
8. Plan your experiment such that you have separate inserts for each treatment, time point, and genotype.
9. Float the membrane in the medium, with the bottom side submerged and the top side exposed to the air. Make sure no bubbles are left between the membrane and the medium.
10. Keep a sterile environment and wipe all instruments in 70% ethanol. The surgery can be carried out comfortably in a laminar flow hood.
11. Make sure all tools are accessible to avoid unnecessary movements and contamination.
12. Sacrificing the animals and separating the cerebellum should be performed by a professional investigator, in accordance with local ethics regulations.
13. To prevent the tissues from sticking to the cutting knife, soak up extra buffer around the cerebellum with a piece of Whatman paper. Be careful not to touch the tissue with the paper.
14. Using a spatula, transfer the sliced cerebellum from the cutting platform to the dissection buffer as a whole; slide the spatula underneath the cerebellum and lift the entire tissue together. To avoid damaging the tissues, separate the slices only when in the dissection buffer.
15. It is possible to produce up to 12, 400 μM slices from one cerebellum. Divide the cerebellar slices between the inserts in one 6-well plate. Place slices from one cerebellum on each insert. When placing two or more slices on an insert, make sure some space is left between them.
16. It is possible to stop at this step and proceed with the immunostaining at a later time. To do so, wash the tissues with 0.1 M phosphate buffer four times for 5 min with gentle shaking. Tissues can be stored submerged in the buffer for up to 2 weeks at 4 °C.

17. Do not separate the tissue from the membrane; cut only the membrane around the tissue. The membrane protects the tissue from being damaged and will not interfere with the staining procedure. Make sure to keep the slices wet at all times.
18. When aspirating the buffer be careful not to damage the tissue. If vacuum suction is used, use a fine tip.
19. Prepare an intermediate dilution of 1:2000 in PBS and dilute it further to 1:10 in PBS.
20. It is possible to costain with anti γH2AX and anti 53BP1 antibodies, but this may result in heavy background staining.
21. Make sure your antibodies are tagged with fluorophores with different excitation and emission wavelengths.

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