Purkinje Cell Degeneration in pcd Mice Reveals Large Scale Chromatin Reorganization and Gene Silencing Linked to Defective DNA Repair*

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DNA repair protects neurons against spontaneous or disease-associated DNA damage. Dysfunctions of this mechanism underlie a growing list of neurodegenerative disorders. The Purkinje cell (PC) degeneration mutation causes the loss of nna1 expression and is associated with the postnatal degeneration of PCs. This PC degeneration dramatically affects nuclear architecture and provides an excellent model to elucidate the nuclear mechanisms involved in a whole array of neurodegenerative disorders. We used immunocytochemistry for histone variants and mechanisms involved in a whole array of neurodegenerative disorders. We used immunocytochemistry for histone variants and components of the DNA damage response, an *in situ* transcription assay, and *in situ* hybridization for telomeres to analyze changes in chromatin architecture and function. We demonstrated that the phosphorylation of H2AX, a DNA damage signal, and the trimethylation of the histone H4K20, a repressive mark, in extensive domains of genome are epigenetic hallmarks of chromatin in degenerating PCs. These histone modifications are associated with a large scale reorganization of chromatin, telomere clustering, and heterochromatin-induced gene silencing, all of them key factors in PC degeneration. Furthermore, ataxia telangiectasia mutated and 53BP1, two components of the DNA repair pathway, fail to be concentrated in the damaged chromatin compartments, even though the expression levels of their coding genes were slightly up-regulated. Although the mechanism by which Nna1 loss of function leads to PC neurodegeneration is undefined, the progressive accumulation of DNA damage in chromosome territories irreversibly compromises global gene transcription and seems to trigger PC degeneration and death.

The cell nucleus is highly organized in nuclear compartments mainly involved in transcription and RNA processing. The first level of nuclear compartmentalization includes chromosome territories and the interchromatin compartment. Each interphase chromosome occupies a distinct nuclear sub-volume, the chromosome territory, which includes active euchromatin and inactive heterochromatin domains (1). The nonrandom organization of chromosome territories is an essential component of nuclear architecture and function (1, 2).

In the case of the PC nucleus, active euchromatin is distributed in extensive nuclear domains, whereas inactive chromatin is mainly confined to the centromeric and telomeric heterochromatin masses located around the nucleolus and at the nuclear periphery (3–6). The predominant euchromatic nuclear architecture and the high density of nuclear pores in PCs (3, 7) correlate with their high transcriptional and metabolic rate, which is required to sustain the enormous synaptic activity of these neurons (3). However, the euchromatic open configuration of chromatin and high oxidative phosphorylation activity render PCs particularly vulnerable to oxidative stress and genotoxic agents that can induce DNA damage during the life span and in neurodegenerative disorders (8, 9).

The autosomal recessive mouse mutation known as PC degeneration (pcd) was first reported by Mullen et al. (10). The spontaneous specific neuronal degeneration of PCs starts around postnatal day 20 (P20) and progresses rapidly over a 2-week period, resulting in a massive loss of PCs, together with neurological manifestations of cerebellar ataxia (11, 12). In addition, retinal photoreceptors, mitral cells in the olfactory bulb, and a discrete population of thalamic neurons undergo late degeneration (13–16). In pcd mice, only the nna1 gene is affected (17, 18). This gene encodes a protein, Nna1, with a putative zinc carboxypeptidase domain, which is essential for PC survival (12, 18). Nna1 contains nuclear localization signals and is localized in both the nucleus and cytoplasm (19). The primary function of Nna1 in nuclear processes remains elusive, although it has been suggested that it could play a role in chro-
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Chromatin remodeling, in particular during the motoneuron response to axotomy (19).

Although the molecular pathways through which the loss of Nna1 function leads to PC degeneration and death in pcd mice remain undefined, recent studies on pcd mice have proposed the participation of several cellular mechanisms in neurodegeneration. They include endoplasmic reticulum stress (20), decreased peptide turnover downstream from the proteasome (21), mitochondrial dysfunction and altered proteolytic processing of Nna1-interacting proteins (22), a dysfunction of microtubule stability (23), and disruption of nucleoli and Cajal bodies (24). Furthermore, our previous studies have demonstrated that the pcd mutation induces the formation of nuclear foci of DNA damage in both mitral and Purkinje cells (16, 24). Moreover, defects in DNA repair pathways have recently emerged as a new fundamental pathology linked to several spinocerebellar ataxias, as well as other neurodegenerative disorders (9, 25–27). Because of their post-mitotic nature and long life span, neurons are particularly prone to the progressive accumulation of unrepaired DNA lesions, which lead to cell death (28).

PC degeneration in pcd mice provides a relevant neuronal system for investigating the nuclear mechanisms involved in neurodegeneration. In this study, we analyze the chromatin remodeling that accompanies PC degeneration in pcd mice. In particular, we investigate the epigenetic marks of nucleosomal histones, with their associated changes in transcriptional activity, the structural and spatial reorganization of chromatin and telomeres, and the participation of a DNA damage response (DDR) in the mechanism of PC death. Our results demonstrate that the expression of repressive and DNA damage histone marks is associated with a profound large scale reorganization of chromatin and telomeres. The propagation of DNA damage to extensive domains of the genome irreversibly compromises gene transcription and seems to trigger PC death.

EXPERIMENTAL PROCEDURES

Mouse Genotyping—C57BL/6J male mice heterozygous for the pcd1J mutated gene were purchased from The Jackson Laboratory (Bar Harbor, ME). Because the exact location of the mutation remains unknown, the pcd allele must be associated with a known genetic background. Therefore, male pcd11 mice were mated with DBA/2J females without the pcd mutation. The pcd11 allele was secluded with the genetic background of the C57BL/6J strain, whereas the normal allele was associated with the genetic background of the DBA/2J strain. This differential segregation of both pcd and normal alleles allowed the control and heterozygous mice to be differentiated to expand the colony and genetically typify the experimental and control animals from the same litters. Male pcd mice are infertile (11), and hence heterozygous mice were crossed to obtain mutant mice under Mendelian inheritance. To identify and avoid the backcross phenomena, we characterized two different microsatellite regions that are located close to the mutated region. DNA was extracted from the tail of the mice, and PCR was performed using the primers proposed for the markers D13Mit250 and D13Mit283 in the web resource of The Jackson Laboratory. The primers used were as follows: D13Mit250, forward, 5′-AGGTTCTCAATCTCACAGTGG-3′; D13Mit283, reverse, 5′-GGAGACAGCTTGCCTGCCT-3′, and reverse, 5′-GAGAGGTGCCACATAGGTT-3′. The PCR products of the microsatellite regions D13Mit250 and D13Mit283, which are of different sizes in both the C57BL/6J and DBA/2J strains, were resolved by electrophoresis in a 3% agarose gel. The animals were kept, handled, and sacrificed in accordance with the directives of Council of the European Communities and current Spanish legislation, and the experiments were approved by the Bioethical Committee of the University of Salamanca.

Microarray Analyses—To check the gene expression changes in pcd mice, we compared the gene expression profiles of control and pcd mice. Three control and three 20-day-old pcd mice were used. The mice were decapitated, and the vermis of the cerebellum was rapidly removed. Total RNA from the vermis was extracted with TRIzol and was purified using the RNase-free DNase I digestion. Once the samples of total RNA had been obtained, its integrity and purity were determined using the Agilent 2100 Bioanalyzer (Agilent Technologies). The messengers (mRNA) were retrotranscribed to afford cDNA using a commercial kit from Invitrogen (Superscript Choice System for cDNA synthesis). Then the cDNA was placed under in vitro transcription in the presence of biotin-labeled nucleotides to generate cRNA using the IVT kit (Affymetrix, Charleroi, Belgium). cRNAs were degraded by alkaline digestion and were used for hybridizations with commercial chips, but only after having been subjected to a second quality control with an Affymetrix biochip test (TestArray 3). The hybridizations were performed with the GeneChip Mouse Genome 430 2.0 array from Affymetrix. The signal level was calculated using the Robust Microarray Analysis algorithm (29), and differential expression was calculated using the significance analysis of microarrays, which includes an estimation of the error by means of the false discovery rate.

Rota-rod Test—The Rota-rod test was used to assess the motor coordination of both P20 control (n = 10) and P20 pcd male mice (n = 10). The experiment was always performed under the same conditions, at 9.00 a.m., and in silence to avoid the startle reflex. The animals were initially habituated to the experimental room for 10 min. They were then placed on a rota-rod (Rota-rod LE 8200, Letica Scientific Instruments, Barcelona, Spain), at initial speed of 4 rpm and at an acceleration of 0.6 rpm/s (from 4 to 40 rpm in 10 min). The speed and the time taken to fall were annotated. This procedure was repeated seven times for each animal, with an interval of 20 min each. Values are means ± S.D. One-way analysis of variance followed by Student’s t test were used to compare both groups. Significant differences were considered at *** p < 0.001.

X-ray Irradiation—Exogenous DNA damage was induced by x-ray irradiation using an x-ray generator system (MaxiShot-d, Yxlon International) equipped with an x-ray tube working at 200 kV and 4.5 mA. The animals, deeply anesthetized, were placed at 25 cm away from the x-ray source that generated an x-ray beam with an absorbed dose rate of ~0.9 Gy/min. The body of the animal was protected with a lead tube, exposing only the head, and the beam was focused on the head to avoid adverse effects on the bone marrow, spinal cord, and any other
tissues, caused by global animal radiation. The animals were exposed to ionizing radiation for 4 min and 20 s to administer a sublethal dose of 4 Gy, which is a reference dose in DNA damage/repair experiments. For this work, we used wild-type and pcd mice at 3 h post-irradiation (n = 3 for each group). After this period, the animals were sacrificed, and the cerebellar cortex was processed for immunofluorescence studies.

**Tissue Preparation**—After deep anesthesia with a mixture of xylazine (Rompum, Bayer, Kiel, Germany) and ketamine hydrochloride (Imalgene, Merial, Lyon, France), mice were perfused with heparinized saline for 1 min and fixative solution containing 4% paraformaldehyde and 0.2% saturated picric acid in 0.1 M phosphate buffer, pH 7.4 (PB), for 15 min. After perfusion, the vermis of the cerebellum was dissected out and post-fixed in the same solution for 2 h at room temperature. The tissue blocks were washed in PB and cryoprotected with 30% sucrose over-night at 4 °C until they sank. Thirty-μm-thick sagittal sections were cut using a freezing-sliding microtome (Leica Frigomobil, Jung SM 2000, Nussloch, Germany), and the slices were collected in PB and stored at −20 °C in a freezing mixture containing 30% glycerol and 30% polyethylene glycol in PB, until their use.

The number of PCs per mm of the PC layer was estimated in sagittal sections of the vermis by means of confocal images of P15, P20, and P25 control and pcd mice (n = 3 animals for each group). The *nna1* mutation causes a reduction in the volume of the cerebellum (11, 16), and hence the effect of size reduction was considered, and the densities were estimated for this “shrinkage effect” in all comparisons, as described previously (16). The data are expressed according to “corrected” conditions.

The percentage of PCs showing DNA damage (calbindin- and γH2AX-positive) was estimated in comparable sagittal sections of the vermis from P20 control (n = 3) and P20 pcd mice (n = 3). A minimum of 300 PCs for each wild-type and pcd animal group was examined. Statistical analyses were carried out using one-way analysis of variance followed by Student’s t test to compare both groups. Significant differences were considered at *, p < 0.05, and **, p < 0.01. Data are expressed as means ± S.D.

**Immunohistochemistry**—Cryo-cut sections from the vermis of control and mutant mice of 15, 20, and 25 days of age were treated with 1% NaBH₄ in PB for 20 min at room temperature as described previously (30), rinsed in PB (three times for 10 min), and incubated for 1 h in blocking serum (5% goat serum and 0.1% Triton X-100). The primary rabbit polyclonal anti-calbindin antibody (1:7,000; Swant, Switzerland) was incubated in the same solution overnight at 4 °C. Cy3-conjugated secondary anti-rabbit antibody (1:500; The Jackson Laboratory) was then applied for 2 h at room temperature. Finally, the sections were washed in phosphate-buffered saline (PBS), pH 7.4, mounted, and coverslipped with antifade solution. The resulting material was examined with a confocal microscope (Zeiss LSM 510).

**Squash Preparations and Immunofluorescence**—Squash preparations of PCs from fragments of the cerebellum were obtained as described below. This technique provides an excellent preservation of neuronal perikarya and allows the examination of the spatial organization of neuronal compartments in the whole neuronal nucleus by direct examination of samples or with confocal microscopy (31).

The animals were perfused with 3.7% paraformaldehyde in PBS for 10 min. The vermis was removed and post-fixed in the same solution for 20 min at room temperature. The small blocks containing the PC layer were isolated and cut into small fragments. Each cerebellar portion was transferred to a drop of PBS on a siliconized slide (SuperFrostPlus, Menzel-Gläser, Germany). A coverslip was then applied to the top of the slide, and the tissue was squashed by mechanical pressure with a histological needle to dissociate neuronal perikarya. The preparation was then frozen in dry ice, and the coverslip was removed using a razor blade. With this procedure, most Purkinje neurons remained adhered to the slide. Cell samples were then sequentially processed in 96% ethanol at 4 °C for 10 min and PBS at 4 °C. Finally, the samples were stored at 4 °C until use.

To perform immunocytochemical studies of the dissociated PCs, the samples were sequentially treated with 0.1 M glycine in PBS for 15 min and 0.5% Triton X-100 in PBS for 45 min. Then they were incubated with the primary antibody overnight at 4 °C, washed with 0.05% Tween 20 in PBS, incubated for 45 min in the specific secondary antibody conjugated with FITC or Texas Red (Jackson Immunoresearch, West Grove, PA), rinsed in PBS, and counterstained with propidium iodide (1:2,000) for 15 min. Double immunofluorescence experiments were also carried out. Finally, the samples were mounted with the Vectashield antifade medium (Vector Laboratories, Burlingame, CA). They were examined with a laser confocal microscope (Zeiss LSM 510) using a 63× plan-apochromatic objective (1.4 NA) and argon ion (488 nm) and HeNe (543 nm) lasers.

The following primary antibodies were used: rabbit polyclonal antibodies anti-53BP1 (Novus Biologicals, Littleton, CO); anti-ATM pS1981 (Rockland, PA); anti-lamin A/C (Santa Cruz Biotechnology); anti-histone H4k20me3 (Upstate); and mouse monoclonal antibody anti-γH2AX (Upstate).

**Run-on Transcription Assays In Situ**—Active transcription sites were labeled by the incorporation of 5'-FU into nascent RNA, as reported previously (35). Briefly, under anesthesia (pentobarbital 60 mg/kg) mice were given an intraperitoneal injection of 5'-FU (Sigma) at doses of 5 μl/g of a stock solution of 0.4 M 5'-FU in 0.9% saline. The mice were killed at 60 min post-injection. The animals were fixed by perfusion with 3.7% paraformaldehyde in HPEM buffer (2× HPEM: 60 mM Hepes, 130 mM Pipes, 20 mM EGTA, and 4 mM MgCl₂), containing 0.5% Triton X-100 for 10 min. The vermis were removed, post-fixed in the same solution for 20 min, washed in HPEM buffer containing 0.5% Triton X-100 for 10 min, and cut into small fragments. Mechanical PC dissociation was performed as described previously. Then the samples were sequentially treated with protease K (0.25 μg/ml in 1 M Tris buffer, pH 8) for 1 min at 25 °C, 0.1 M glycine in PBS containing 1% bovine serum albumin (BSA) for 15 min, and 0.01% Tween 20 in PBS for 5 min. The incorporation of 5'-FU into nascent RNA was detected with a mouse monoclonal (clone BU-33) anti-BrdU antibody (Sigma) diluted 1:50 in PBS (overnight at 4 °C). Then the samples were washed with 0.01% Tween 20 in PBS, incubated for 45 min with an anti-mouse FITC-conjugated secondary antibody (Jackson Immunoresearch), washed in PBS, and
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mounted with the Vectashield antifade medium. Some samples were either counterstained with propidium iodide or processed for a double labeling with immunofluorescence.

Terminal Deoxynucleotidyltransferase-mediated Fluorescein dUTP Nick End-labeling (TUNEL) Staining—An in situ cell death detection kit (Roche Diagnostics) was employed to detect apoptotic bodies. Dissociated Purkinje neurons were obtained as described previously. Then they were rinsed in PBS (three times for 10 min) and permeabilized at room temperature for 30 min with 0.5% Triton X-100 diluted in PBS. Dissociated PCs were incubated at 37 °C with the TUNEL reaction mixture containing Cy3-conjugated terminal deoxynucleotidyltransferase and nucleotide mixture for 45 min. The samples were then washed with PBS and mounted with antifade solution. TUNEL staining was also conjugated with the immunocytochemistry technique. In this case, the best resolution was obtained by performing the immunocytochemistry before the TUNEL assay. The percentage of apoptotic PCs (calbindin- and TUNEL-positive) was determined in squash preparations of the vermis in both control \((n = 3)\) and pcd P20 mice \((n = 3)\). At least 100 PCs per animal were counted. Values are means ± S.D.

Electron Microscopy—Three controls and three pcd 15- and 20-day-old mice were used to analyze the ultrastructure of PCs. Mice were perfused with 3% glutaraldehyde in 0.1 m phosphate buffer, pH 7.4. The cerebellum was removed, and the vermis was isolated. 500-μm-thick sagittal sections were obtained using a vibratome (Leica). Then the sections were rinsed in 0.1 m phosphate buffer, post-fixed in 2% osmium tetroxide in double-strength buffer (containing 3.5% dextrose in 0.2 m phosphate buffer, pH 7.4), dehydrated in acetone, and embedded in Araldite (Durecopan, Fluka, Switzerland). Ultrathin sections stained with uranyl acetate and lead citrate were examined with a Philips EM-208 electron microscope operated at 60 kV.

Immunoelectron Microscopy—For immunoelectron microscopy, the animals were perfused with 3.7% paraformaldehyde in 0.1 m cacodylate buffer for 10 min at room temperature. Small tissue fragments of the vermis were washed in 0.1 m cacodylate buffer, dehydrated in increasing concentrations of methanol at –20 °C, embedded in Lowicryl K4M at –20 °C, and polymerized with ultraviolet irradiation. Ultrathin sections were mounted on nickel grids and sequentially incubated with 0.1 m glycine in PBS for 15 min, 5% BSA in PBS for 30 min, and the primary mouse monoclonal anti-γH2AX antibody (1:25) and rabbit polyclonal anti-H4K20me3 (1:50), both diluted in 50 mm Tris-HCl, pH 7.6, containing 1% BSA and 0.1 m glycine for 2 h at 37 °C. After washing, the sections were incubated with the goat anti-mouse IgG or goat anti-rabbit IgG antibody coupled to 10-nm gold particles (BioCell, UK; diluted 1:50 in PBS containing 1% BSA) for 1 h at room temperature. Following immunogold labeling, the grids were stained with lead citrate and uranyl acetate and examined with a Philips EM208 electron microscope operated at 60 kV. As controls, ultrathin sections were treated as described above but with the primary antibodies omitted.

In Situ Hybridization of Telomeric DNA—Samples of dissociated PCs were processed for in situ hybridization of telomeric DNA using the Cy3-labeled telomere-specific peptide nucleic acid probe (DakoCytomation, Denmark). Slides were placed in deionized water (3 min) and 70, 96, and 100% ethanol for 1 min each and then air-dried. Ten microliters of a fluorescent Cy3-labeled telomere-specific probe was applied to each sample, which was then covered with an 18 × 18-mm coverslip, and denatured on a heat block at 80 °C for 3 min. The slides were then moved to a dark humidity chamber at room temperature for 2 h. Following this, the coverslips were carefully removed in Tris-buffered saline with Tween 20 (TBST), and the slides were washed with 70% formamide in TBST. They were then dehydrated in an alcohol series, air-dried, and mounted with Vectashield.

For quantitative analysis of telomeres, squash preparations of PCs from both P20 control \((n = 50)\) and mutant mice \((n = 50)\) were double-stained for telomeric DNA and DAPI. Serial sections of whole neuronal nuclei, with an interval of 0.3 μm between optical sections, were captured with a laser confocal microscope (Leica TCS SP, Germany). The number of telomeric spots and their diameter were estimated using ImageJ (Version 1.38x, Java-based image processing program developed at the National Institutes of Health). Values are means ± S.D. Statistical analyses were carried out using Student’s t test to compare both experimental groups.

Immunoblotting—For the preparation of nuclear extracts from the cerebellar vermis of wild-type and pcd mice, tissue samples were lysed using a pellet-pestle motor (Sigma) on ice in cold extraction buffer RSB (10 mm Tris, pH 7.5, 10 mm NaCl, 3 mm MgCl2, 1% Nonidet P-40) supplemented with a protease and phosphatase inhibitor mixture (Halt™ protease and phosphatase inhibitor single use mixture, Thermo Scientific) and incubated for 20 min on ice. After centrifugation (5 min at 1,000 rpm), the pellets were washed with RSB buffer (without Nonidet P-40) and centrifuged at 2,000 rpm for 5 min at 4 °C; the supernatant was discarded. The pellet was then resuspended with NB buffer (50 mm Tris, pH 7.5, 0.4 mm NaCl, 1 mm EDTA) supplemented with a protease and phosphatase inhibitor mixture (Halt™ Protease and Phosphatase inhibitor single use mixture, Thermo Scientific) and incubated for 15 min at 4 °C. Samples were sonicated with the Bioruptor UCD-200 (Diagenode) at 4 °C and centrifuged at 11,000 rpm for 5 min at 4 °C. The supernatant was taken as the nuclear fraction. Nuclear fraction proteins were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes using standard procedures. Protein bands were detected and quantified with the Odyssey™ infrared imaging system (Li-Cor Biosciences) according to the Odyssey™ Western blotting protocol. The following primary antibodies were used for immunoblotting: rabbit polyclonal anti-ATM (Cell Signaling, Beverly); mouse monoclonal anti-ATM pS1981 (Rockland); mouse monoclonal anti-GAPDH (Abcam, Cambridge, UK); mouse monoclonal anti-γH2AX (Millipore-Upstate, MA); rabbit polyclonal anti-RAD50 (Millipore); rabbit polyclonal anti-53BP1 (Novus Biologicals); rabbit polyclonal anti-NBS1 (Novus Biologicals); rabbit polyclonal anti-histone H4K20me3 (Upstate, MA); and rabbit polyclonal anti-fibrillarin (Abcam, MA). Immunoblots were developed with anti-mouse IRDye800DX or anti-rabbit IRDye700DX (Rockland) secondary antibodies.
RESULTS

The time course of the loss of PCs during the postnatal development of the cerebellar vermis in pcd mice was studied in sagittal sections of the vermis immunostained for calbindin, a molecular marker of PCs. In the control mice, calbindin immunoreactivity labeled the molecular and PC layers of all lobules completely (Fig. 1A). A similar immunostaining pattern was observed in the pcd mice at P15 (Fig. 1B). In agreement with the findings of Landis and Mullen (11), PCs in pcd mutant mice degenerated asynchronously along a postnatal time window that approximately started at P18 and ended at P30, when a massive neuronal loss occurred (Fig. 1, C and D). Quantitative analysis of PC density in the PC layer confirmed the dramatic loss of PCs from P15 to P25 in the pcd mice (Fig. 1E). Furthermore, this postnatal neuronal loss was correlated with the severe deficit in rotor-rod performance recorded in P20 pcd male mice (Fig. 1F). This indicates a dysfunction in motor coordination that results in ataxic behavior. Given that in the cerebellar cortex of pcd mice at P20 both healthy PCs and PCs undergoing different stages of neuronal degeneration coexist, we selected this postnatal day to study the reorganization of the nuclear architecture that is associated with this type of neurodegeneration. At this postnatal stage, the granule cell layer was well preserved, and no cytological signs of granule cell degeneration were observed, as described previously (18).

Purkinje Cell Degeneration in pcd Mice Produces Ultrastructural Changes in Chromatin Organization—The chromatin configuration in control PCs was characterized by the presence of extensive euchromatin domains throughout the nucleus (Fig. 2A), as reported previously (3). The first pre-degenerative structural mark of chromatin in pcd mice consisted of the formation of numerous small foci of chromatin condensation scattered throughout the nucleus, in addition to isolated larger masses of heterochromatin (Fig. 2, B and D). These chromatin changes were accompanied by a moderate increase in the electron-density of both the nucleoplasm and the cytosol (Fig. 2B). As PC degeneration proceeded, the heterochromatin clumps tended to coalesce into a few large rounded masses (Fig. 2C). They frequently appeared detached from the nuclear envelope and exhibited a fine granular texture (Fig. 2E), corresponding to the cross-section of closely packed chromatin fibers (32).

pcd Mutation Induces Transcriptional Repression Associated with the Accumulation of DNA Damage in Purkinje Cells—It is well known that transcriptional repression at heterochromatin

FIGURE 1. pcd mutation causes a rapid Purkinje cell degeneration. A–D, confocal microscopy images of sagittal sections of the vermis of P25 control (A) and P15 (B), P25 (C), and P30 (D) mutant mice immunolabeled for calbindin D-28K. Note the dramatic loss of PCs at P30, only those located in lobule X remain (arrow in D). Scale bar, 1 mm. E, quantitative analyses of the relative PC density from P15 to P25 in control and pcd mice. Note the severe and rapid decrease of PC in the cerebellum of pcd mice. F, rota-rod test for P20 control and pcd male mice. Note the significant dramatic decrease in the latency to fall of the P20 mutants.
domains is associated with specific epigenetic marks of nucleosomal histones, such as the trimethylation of H3K9 and H4K20 (33, 34). In this study, we used double immunolabeling for H4K20me3 as a marker of silent heterochromatin domains and for γH2A as a sensor of DNA damage sites (35). In control PCs, H4K20me3-positive heterochromatin was restricted to a few masses associated with the nucleolus and nuclear envelope (Fig. 3, A and C), mainly corresponding to the previously described constitutive centromeric heterochromatin (4, 5, 36). In the pcd mice, numerous PCs exhibited large domains of the genome enriched in the repressive mark H4K20me3. They appeared as large nuclear foci immunoreactive for H4K20me3 (Fig. 3, D, F, and G), which tended to fuse into larger rounded domains as PC degeneration proceeded (Fig. 3J). As expected, immunogold electron microscopy for H4K20me3 revealed that the ultrastructural counterparts of these nuclear domains were heterochromatin clumps of varying size and granular texture (Fig. 4A).

To investigate whether the heterochromatin-associated silencing of the genome was correlated with DNA damage, we studied the expression patterns of γH2AX at different stages of neuronal degeneration. In control PCs, no γH2AX immunoreactivity was detected, with the exception of a very few neurons bearing isolated foci of spontaneous DNA damage (Fig. 3B). At an initial stage of PC degeneration, nuclear microfoci of γH2AX immunoreactivity were detected, especially at the periphery of the H4K20me3-positive heterochromatin domains (Fig. 3, E and F). By immunogold electron microscopy for γH2AX, we characterized the microfoci of DNA damage as small nuclear aggregates of amorphous material distributed in euchromatin domains and decorated with a cluster of immunogold particles (Fig. 4B).

As PC degeneration progressed, an increase in the γH2AX-positive nuclear domains was observed by immunofluorescence, as reported previously (24). The signal of this histone mark colocalized with the H4K20me3-positive heterochromatin domains (Fig. 3, G–J). Interestingly, the colocalization of these two histone epigenetic marks was preserved in the largest heterochromatin domains observed in more advanced stages of PC neurodegeneration (Fig. 3, J–L). Immunogold electron microscopy confirmed the presence of numerous γH2AX-positive sites of DNA damage signal within large heterochromatin masses (Fig. 4C). The increase in the expression levels of both H4K20me3 and γH2AX in the cerebellar vermis of pcd mice was confirmed by Western blotting (Fig. 3P). Furthermore, quantitative analysis revealed that ∼15% of PCs showed the nuclear DNA damage signal (γH2AX foci) in P20 mutant mice (14.56 ± 2.21; means ± S.D.). It may be concluded that the H4K20me3-dependent heterochromatin silencing of the genome in the PCs of pcd mice is correlated with the formation of nuclear foci of DNA damage. Next, we investigated whether the pcd mutation induced the activation of the MRN (MRE11-Rad50-NBS1) complex involved in the DDR to double strand breaks (DSBs). Western blot analyses revealed a significant increase in the protein levels of both NBS1 and Rad50 in the pcd cerebellum in comparison with the wild-type one (Fig. 3Q).

Having established that PC degeneration changes chromatin structure, we then wished to analyze whether this chromat reorganization occurs at the nuclear periphery, a domain of the genome enriched in repressive marks (2, 37). Double immunolabeling for lamin A/C, as a marker of nuclear lamina, and γH2AX revealed the typical organization of the nuclear envelope in control PCs in the absence of DNA damage (Fig. 3M). In the pcd mice, the formation of round heterochromatin masses immunolabeled for the γH2AX histone mark mainly occurred in the nuclear interior at varying distances from the nuclear envelope (Fig. 3N). As these heterochromatin masses coalesced, the resulting larger masses tended to become localized at the nuclear periphery (Fig. 3O). Interestingly, the expression of lamin A/C was preserved throughout PC degeneration (Fig. 3, N and O).

To further investigate the relationships between DNA damage and chromatin silencing, we performed a run on transcription assay in situ based on the incorporation of the RNA precursor 5′-FU into nascent RNA (38). After a 60-min pulse of intraperitoneal administration of 5′-FU, nascent RNA was distributed in the nucleolus, in numerous extranucleolar transcription foci and diffusely throughout the nucleoplasm in control PCs free of DNA damage signal (Fig. 5, A–C). In pre-degenerating PCs with γH2AX-positive nuclear microfoci, a dramatic reduction in the nucleoplasmic signal of 5′-FU incorporation into nascent RNA was observed (Fig. 5, D–F), reflect
ing a severe inhibition of the transcription rate. In advanced stages of PC degeneration, no signal of 5’-FU incorporation was detected, indicating the complete silencing of gene expression (Fig. 5, G–I).

pcd Mutation Causes Long Range Chromatin Repositioning in Purkinje Cells Mediated by Telomeres—It is well known that telomeres play an important role in the dynamic architecture of chromosome territories within the nuclear space (2, 37, 39). Furthermore, a telomeric dysfunction associated with DNA damage has been implicated in the pathogenesis of several neurodegenerative disorders, including Alzheimer disease (40). In light of this, we performed *in situ* hybridization for telomeric DNA to determine whether the chromatin reorganization observed in the PCs of pcd mice was correlated with changes in

![FIGURE 3. pcd mutation induces DNA damage and H4K20me3 expression in extensive domains of the Purkinje cells genome. A–L, confocal microscopy images from squash preparations of PC from P20 control and pcd mice, costained for H4K20me3 (red) and γH2AX (green). A–C, two H4K20me3-positive foci appeared at the perinucleolar region of a control PC, whereas no nuclear signal of DNA damage was detected with the anti-γH2AX antibody. D–F, at the onset of PC degeneration, numerous H4K20me3-positive heterochromatin domains were observed (D), concomitantly with the appearance of γH2AX-positive nuclear foci of DNA damage (arrowheads in E). G–I, heterochromatin domains tended to aggregate as degeneration proceeded (G), simultaneously with the spread of the γH2AX signal into heterochromatin domains. J–L, colocalization of H4K20me3 and γH2AX was preserved in the largest heterochromatin domains observed in advanced stages of PC degeneration. M–O, double labeling for lamin A/C (red) and γH2AX (green) in control (M) and mutant PCs (N and O). Note the preservation of the nuclear lamina in the affected PCs of pcd mice. Scale bar, 10 μm. P, Western blot analysis of phosphorylated histone H2AX at Ser-139 (γH2AX) and H4K20me3 in nuclear extracts from wild-type and pcd mouse cerebellar verm. Phosphorylation of this histone H2AX variant is activated in the pcd cerebellum, in addition to an increased expression of H4K20me3. The expression of fibrillarin band was used as a protein loading control. Quantification of the protein levels of γH2AX and H4K20me3 was normalized to fibrillarin and the fold increase estimated. Q, proteins levels of NBS1 and Rad50 were analyzed by Western blot of total lysates from the cerebellar vermis from wild-type and pcd mice. Quantification of the protein levels of NBS1 and Rad50 were normalized to GAPDH, and the fold increase was estimated.
and brightest spots would represent nuclear microdomains in which several telomeres are clustered together. Interestingly, the PCs of pcd mice had a lower mean number of telomeric spots, 26 ± 8.47 (means ± S.D.), ranging from 0.2 to 1.1 μm in diameter (Fig. 6, B–D). This suggests that some telomeres undergo a loss of telomeric DNA, producing weak and undetectable fluorescent signals. The reduction in the number of telomeric spots also supports the notion that telomere clustering is increased in degenerating PCs of pcd mice. Furthermore, whereas the telomeric spots appeared scattered throughout the nucleus in control PCs, they progressively tended to concentrate in several discrete nuclear domains as PC degeneration proceeded (Fig. 6, B and C). In advanced stages of neuronal degeneration, the PCs tended to show a very few large spots of telomeric DNA, reflecting the clustering of numerous neuronal telomeres in nuclear microdomains (Fig. 6D).

We next investigated the possible relationships between telomere clustering and DNA damage. In an early stage of PC degeneration, double labeling experiments for telomeric DNA and γH2AX revealed the spatial association and partial colocalization of some DNA damage microfoci with telomeric spots (Fig. 6, E–G). As PC degeneration proceeded, most telomeres were concentrated in a few nuclear domains of DNA-damaged heterochromatin (Fig. 6, H–M). Indeed, quantitative analysis of the spatial distribution of telomeric spots revealed that 86.32 ± 10.51% (means ± S.D.) was localized to DNA-damaged heterochromatin clumps, the rest appearing free in the nucleoplasm. In advanced stages of PC degeneration, the progressive physical proximity of telomeric signals led to the appearance of large telomeric spots immunoreactive for the histone mark γH2AX (Fig. 6, N–P). In conclusion, telomeric DNA damage and telomere clustering are essential cellular events in the reorganization of the nuclear architecture of mutant PCs.

**Sensor and Mediator Proteins of DDR, pATM, and 53BP1 Are Not Recruited in Sites of DNA Damage in Purkinje Cells from pcd Mice**—ATM kinase is an essential transducer of the DNA damage signaling pathway (35, 41, 42). Its deficiency may lead to delayed DDR and neurodegeneration (43, 44). We investigated the response of active autophosphorylated ATM on Ser-1987 (pATM) to endogenous DNA lesions detected in degenerating PCs. In control PCs, pATM appeared diffusely distributed throughout the nucleus, with the exception of the nucleolus (Fig. 7, A–C). Importantly, double immunostaining for γH2AX and pATM in the PCs of pcd mice revealed the nucleoplasmic localization of pATM, but the signal for this kinase was undetectable or very weak in both nuclear microfoci and heterochromatin domains of DNA damage immunostained for γH2AX (Fig. 7, D–I). We next investigated whether the deficiency in the recruitment of pATM to sites of DNA damage affected downstream mediators of the signal transduction pathway, in particular 53BP1, a key factor of the DDR (45). In healthy PCs, 53BP1 exhibited a homogeneous nuclear staining pattern with the exception of the nucleolus (Fig. 7, J–L). In PCs with DNA damage, 53BP1 was concentrated in the nucleoplasm, but the majority of γH2AX nuclear microfoci did not colocalize with 53BP1 (Fig. 7, M–O). Similarly, 53BP1 was not recruited in the heterochromatin domains labeled with the anti-γH2AX antibody (Fig. 7, P–R). Taken together, these
results support the view that a deficiency in DNA repair is also involved in the induction of PC death in pcd mice.

Microarray analysis of cerebellar mRNA expression revealed a modest up-regulation of several genes involved in the DNA repair signaling pathway in pcd mice as compared with control wild-type mice, although others remained unmodified (Tables 1 and 2). The transcripts that increased by 2-fold or more in pcd mice compared with wild-type included those encoding 53BP1, Artemis, a repair protein of the nonhomologous DNA end joining pathway (46), and RAD50 interactor 1, a factor induced by DNA damage that is required for the control of telomere length (47). Western blot analysis confirmed the increase in the protein levels of 53BP1 in cerebellar vermis lysates of pcd mice, whereas no significant variations in either pATM or total ATM protein levels of 53BP1 in cerebellar vermis lysates of pcd mice were detected in the cerebella of mutant mice in comparison with the wild-type mice (Fig. 7, A–F). The transcripts that increased by 2-fold or more in pcd mice as compared with control included those encoding 53BP1, Artemis, a repair protein of the nonhomologous DNA end joining pathway (46), and RAD50 interactor 1, a factor induced by DNA damage that is required for the control of telomere length (47).

To verify the deficient recruitment of pATM and 53BP1 to sites of DNA damage. However, it should be noted that these microarray and Western blot data refer to the whole cerebellum, not only to PC. Therefore, as a response to PC degeneration, the involvement of other cerebellar cell populations cannot be excluded.

To verify the deficient recruitment of pATM and 53BP1 in DNA-damaged heterochromatin of pcd mice, we exposed cerebella from wild-type and pcd mice to 4 Gy of ionizing radiation with x-rays and examined the PC response to DSBs at 3 h post-irradiation. As shown in Fig. 8, A–F, in response to DSBs control PCs exhibited numerous typical γH2AX-positive foci of irradiation in which very high levels of pATM or 53BP1 colocalized with this histone variant. In contrast, exposure to ionizing radiation did not induce the recruitment of either pATM or 53BP1 to small γH2AX-positive nuclear foci or large heterochromatin masses in the PCs of pcd mice (Fig. 8, G–L).

Accumulation of Unrepaired DNA in Heterochromatin Domains Leads to Internucleosomal DNA Fragmentation—To determine whether the accumulation of DNA damage in the PCs of pcd mice triggers a neuronal death mechanism mediated by internucleosomal DNA fragmentation, a marker of cell death by apoptosis (48), we combined the TUNEL assay for DNA fragmentation and immunolabeling for γH2AX. Interestingly, at an initial stage of DNA lesions, typical microfoci of γH2AX immunoreactivity lacked a TUNEL signal (Fig. 9, A–C). At an intermediate stage, heterochromatin domains containing the epigenetic signal of DNA damage (γH2AX) also lacked a TUNEL signal, although some TUNEL-positive nuclear microfoci appeared scattered throughout the nucleus (Fig. 9, D–F). In more advanced stages of PC degeneration, the majority of DNA-damaged heterochromatin domains were TUNEL-positive, although they showed different levels of signal intensity (Fig. 9, G–O). Quantitative analysis revealed that ~10% of PCs exhibited TUNEL-positive foci in the P20 mutant mice (10.83 ± 3.21; means ± S.D.). In conclusion, the accumulation of unrepaired DNA at heterochromatin DNA damage sites finally leads to internucleosomal DNA fragmentation and cell death.

**DISCUSSION**

The PCs of the pcd mouse provide an excellent neuronal model to analyze the nuclear mechanisms involved in DNA damage-dependent neurodegeneration. Although the pcd...
mutation is a model of ataxic mice, it shares clinical and neuropathological features with inherited human spinocerebellar ataxias (12, 49). The identification of mutations in DNA repair genes in some human spinocerebellar ataxias with PC degeneration phenotypes (50–52) clearly links DNA damage and defective DNA repair to neurodegeneration. Moreover, bioinformatics analyses of the protein-protein interaction network for human and mouse ataxic disorders, including the pcd mouse, have shown that these disorders share molecular pathways that account for their phenotypic similarities (26). Collectively, these data highlight the usefulness of the pcd mutant mouse as a model of PC degeneration and cerebellar ataxia. The molecular mechanisms by which Nna1 loss of function lead to progressive accumulation of DNA damage and neurodegeneration in pcd mice are undefined. However, a recent study (53) demonstrated that Nna1 loss of function results in altered expression of oxidative phosphorylation enzymes and disrupted bioenergetics function. It is tempting to speculate that the energy reduction-induced oxidative stress (54) may cause oxidative DNA damage in PCs of pcd mice.

In a previous study (24), we have demonstrated that the pcd mutation causes disruption of the nucleolus in PCs, with transcriptional inhibition of ribosomal genes and consequent deficit in the biogenesis of ribosomes. Our present results indicate that the pcd mutation also induces the repression of protein-coding genes in extensive domains of the genome of PCs. This transcriptional repression involves a profound reorganization of the nuclear architecture of chromatin, which is associated with a deficient DDR that leads to the accumulation of unrepairred DNA and, ultimately, to neuronal cell death. A schematic drawing of the sequence of events that occur during large scale chromatin...
Reorganization is shown in Fig. 10. It includes the following:

(i) the distribution of euchromatic chromosome territories and telomeres in healthy PCs (Fig. 10A); (ii) the initial formation of small H4K20me3-positive heterochromatin domains with associated γH2AX nuclear foci in chromosome territories of PCs from pcd mice (Fig. 10B); (iii) the propagation of H4K20me3-dependent heterochromatinization along the chromosome territories (Fig. 10C); (iv) the spreading of the γH2AX signal of DNA damage within heterochromatin domains (Fig. 10C); (v) the large scale repositioning of telomeres within heterochromatin domains (Fig. 10, C and D); and (vi) the spatial grouping of DNA-damaged sites.

**FIGURE 7.** pATM and 53BP1 are not recruited in DNA damage sites in mutant Purkinje cells. A–R, confocal microscopy images from squash preparations of PC from P20 control (A–C and J–L) and pcd mice (D–I and M–R). A–L, double immunolabeling for γH2AX (green) and pATM (red). A–C, note the absence of DNA damage signal and the diffuse distribution of pATM throughout the nucleus, excluding the nucleolus in the control PC. D–I, in PCs from the mutant mice, pATM was not colocalized with either nuclear foci (arrowheads in D) or large heterochromatin domains (G) immunolabeled for γH2AX. J–R, double immunolabeling for γH2AX (green) and 53BP1 (red). J–L, in a control PC, 53BP1 showed a diffusive nuclear distribution, whereas the γH2AX signal of DNA damage was absent. M–R, although 53BP1 was expressed in the nucleoplasm in degenerating PCs, it did not colocalize with either γH2AX-positive microfoci (arrowheads in O) or heterochromatin domains. Scale bar, 10 μm. S, Western blot of total lysates of the cerebellar vermis from wild-type and pcd mice to detect the protein levels of pATM and 53BP1. The pATM level was normalized to total ATM, and the fold variation was estimated. The protein level of 53BP1 was normalized to GAPDH, and the fold increase was estimated.
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### TABLE 1
Differentially expressed transcripts involved in the DNA damage response and apoptosis in vermis of pcd mutants compared with wild type mice

| Symbol      | Gene                                  | Fold   | p value |
|-------------|---------------------------------------|--------|---------|
| Aifm2       | Apoptosis-inducing factor, mitochondrion-associated 2 | +1.58  | 0.025   |
| Apaf1       | Apoptotic peptidase-activating factor 1 | +1.85  | 0.016   |
| Atm         | Ataxia telangectasia mutated homolog (human) | +1.41  | 0.020   |
| Becl1       | Beclin 1 (coiled-coil, myosin-like BCL2-interacting protein) | +2.08  | 0.037   |
| Bcl211      | BCL2-like 11 (apoptosis facilitator)   | +1.84  | 0.025   |
| Brcal       | Breast cancer 1                        | +1.51  | 0.020   |
| Cideb       | Cell death-inducing DNA fragmentation factor, a subunit-like effector B | +2.85  | 0.030   |
| Dclre1a     | DNA cross-link repair 1A, Artemis      | +4.60  | 0.045   |
| Ddit3       | DNA-damage inducible transcript 3     | +1.83  | 0.013   |
| Fas         | Fas (TNF receptor superfamily member)  | +2.65  | 0.005   |
| Hrk         | Harakiri, BCL2-interacting protein (contains only BH3 domain) | +1.95  | 0.013   |
| Mre11a      | Meiotic recombination 11 homolog A (Sacccharomyces cerevisiae) | +1.80  | 0.006   |
| Nupr1       | Nuclear protein 1                     | +1.85  | 0.025   |
| Parc        | p53-associated Parkin-like cytoplasmic protein | +1.87  | 0.013   |
| Rnt1        | RAD50 interactor 1                    | +2.03  | 0.001   |
| Trp53bp1    | Transformation related protein 53-binding protein 1 | +2.43  | 0.002   |
| Xrc3        | X-ray repair complementing defective repair in Chinese hamster cells 3 | +1.90  | 0.045   |

### TABLE 2
Unmodified transcripts involved in the DNA damage response and apoptosis in vermis of pcd mutants compared with wild type mice

| Symbol | Gene                                 |
|--------|--------------------------------------|
| Bax    | Bcl2-associated X protein             |
| Casp9  | Caspase 9                            |
| Diabo  | Diabo homolog (Drosophila)            |
| EndoG  | Endonuclease G                       |
| H2aqf  | H2A histone family, member X         |
| GzmB   | Granzyme B                           |
| Lig4   | Ligase IV, DNA, ATP-dependent        |
| Mdc1   | Mediator of DNA damage checkpoint 1   |
| Nbn    | Nbn (Nijmegen breakage syndrome protein) 1 |
| Parg1  | Poly(ADP-ribose) polymerase family, member 1 |
| Pkrdc  | Protein kinase, DNA-activated, catalytic polypeptide |
| Rad50  | RAD50 homolog (S. cerevisiae)         |
| Xrc5c  | X-ray repair complementing defective repair in Chinese hamster cells 4 |
| Xrc5e  | X-ray repair complementing defective repair in Chinese hamster cells 5 (Ku70) |
| Xrc6e  | X-ray repair complementing defective repair in Chinese hamster cells 6 (Ku80) |

heterochromatin domains with their associated telomeres into a large heterochromatin clump (Fig. 10D).

Under physiological conditions, H4K20me3 is a repressive mark mainly restricted to centromeric heterochromatin attached to the nucleolus (present results and see Ref. 5). In degenerating PCs from pcd mice, this epigenetic mark propagates throughout the extensive euchromatin domains of PCs, resulting in a H4K20me3-dependent global heterochromatinization of chromosome territories and gene repression. Chromatin domains enriched in H4K20me3 have the structural characteristics of heterochromatin, as revealed by their fine granular structure observed with electron microscopy, which corresponds to the cross-section of 30-nm chromatin fibers (35, 55). The existence of H4K20me3-associated gene silencing is consistent with the striking reduction in 5′-FU incorporation into nascent RNA detected with an in situ transcription assay in degenerating PCs of pcd mice. Initially, H4K20me3-dependent chromatin compaction could be a protective cellular response to safeguard genome domains against cellular insult. However, the progressive heterochromatin-induced gene silencing may deprive PCs of vital transcripts and may contribute toward triggering cell death. The dramatic decrease of 5′-FU incorporation signal in PCs of pcd mice supports the hypothesis that neuronal transcription is very sensitive to neurodegeneration-associated DNA damage (56).

Neurons are post-mitotic cells in G0 phase. In the absence of a template sister chromatid for DNA repair by homologous recombination, neuronal DSBs are repaired by the nonhomologous end-joining pathway (42, 46). In this pathway, ATM and DNA-PK phosphorylate H2AX in response to DSBs (41). Moreover, the increased protein levels of Rad50 and NBS1 detected with Western blot analysis in the pcd cerebellum support the participation of the MRN complex in the DNA damage signaling pathway. It is well established that DNA repair is induced by a signal transduction pathway, the DDR, which involves ATM signaling and several mediator proteins, including 53BP1 (46, 60, 62). Our finding that two essential components of the DDR, the active kinase pATM and the repair protein 53BP1 (42, 63, 64), are not concentrated in DNA-damaged heterochromatin masses clearly supports the existence of a deficient DNA repair in degenerating PCs of pcd mice. Indeed, whereas normal PCs recruited pATM and 53BP1 to DNA damage sites induced by exposure to ionizing radiation, the DNA-damaged chromatin domains in mutant PCs failed to recruit these repair factors upon irradiation treatment. Importantly, although our microarray analysis of the relative expression of Atm and Trp53bp1 (encodes 53BP1) revealed a modest up-regulation in the expression of both genes in the pcd cerebellum, only the protein levels of 53BP1 were increased in the mutant cerebella, and no significant variations in the total and phosphorylated forms of ATM were detected by Western blot analysis. Taken together, these results suggest that the failure in the recruitment of pATM and 53BP1 to DNA-damaged heterochromatin domains in mutant PCs does not result from a reduced expression of Atm and Trp53bp1 genes but may be related to a deficient ATM activation in response to the pcd mutation-induced DNA damage.

In agreement with the immunofluorescence colocalization of histone H4K20me3, a heterochromatin marker, and γH2AX in large nuclear domains of pcd mice PCs, our immunogold electron microscopy study with the anti-γH2AX antibody clearly identified the fine structure of these nuclear domains as heterochromatin clumps containing DNA damage sites. The persistence in heterochromatic regions of γH2AX immunolabeling suggests that DNA lesions in heterochromatin are refractory to repair in PCs of pcd mice. Although the compact structure of heterochromatin prevents repair proteins from gaining adequate access to sites of DNA lesions (46, 57), previous studies have shown that heterochromatin undergoes a transient ATM-dependent decondensation in normal cells that facilitates DNA repair in highly compacted chromatin domains (57, 61). More specifically, recent studies have shown that ATM phosphorylates the transcriptional corepressor KAP-1 (KRAB-associated protein 1), which produces a transient heterochromatin relaxation (61). This exposes the otherwise hidden methyl...
H4K20 residues, allowing their interaction with the ATM signaling mediator 53BP1 (64–66) and the subsequent DNA repair. In the pcd mice, however, PCs are able to induce the γH2AX signaling response to DNA damage, but there is a progressive heterochromatinization of DNA-damaged chromatin and deficient repair activity, as indicated by the striking accumulation of γH2AX during the neurodegeneration process. We suggest that in the PCs of pcd mice, the DNA-damaged heterochromatin is not modified by remodeling factors that transiently open chromatin in response to DNA damage, resulting in a deficient recruitment of essential proteins of the DNA repair pathway such as pATM and 53BP1. In fact, our electron microscopy analysis indicates that the DNA-damaged heterochromatin domains of variable size immunoreactive for γH2AX failed to accumulate either pATM or 53BP1 at 3 h post-ionizing radiation. Scale bar, 5 μm.

What is noteworthy is the reorganization of telomeres in the PCs of pcd mice, in particular the spatial repositioning, the dramatic reduction in the number of detectable telomeric spots, and the increased clustering in larger and brighter spots. Whereas telomeres are randomly distributed throughout the nuclei in normal PCs, as occurs in other mammalian cells (39, 67), they are largely repositioned and clustered in DNA-damaged heterochromatin domains in the PCs of pcd mice. This relocalization may require a large scale motion of telomeres in parallel with a progressive reorganization of chromosome territories within a few heterochromatic domains. In normal interphase cells, long range chromatin motions occur only during a short time window at early G1 phase, when the cell achieves a stable differentiated state (1, 2, 68). However, long range chromatin motion associated with heterochromatinization is a hallmark in the nuclear response of degenerating PCs. Although a cause-effect relationship is unclear, we propose that the spatial repositioning of telomeres would contribute directly to chromatin motion in the PCs of pcd mice.

Another important finding in degenerating PCs of pcd mice is the reduction in the number of telomeric signals, accompanied by the appearance of some larger telomeric spots. The reduction in telomere signals may be partially due to the existence of telomeric DNA loss induced by DNA damage, which produces undetectable fluorescent signals with confocal microscopy.

**FIGURE 8.** Ionizing radiation induces recruitment of both pATM and 53BP1 in control PCs but not in those of mutants. Double immunolabeling for γH2AX and 53BP1 in dissociated PCs was from wild-type (A–F) and pcd (G–L) mice exposed to ionizing radiation (4 Gy). A–F, in wild-type PCs, strong γH2AX immunofluorescent areas colocalized with bright nuclear domains of pATM and 53BP1 in numerous ionizing radiation-induced foci at 3 h post-irradiation. G–L, in pcd PCs, DNA-damaged chromatin domains of variable size immunoreactive for γH2AX failed to accumulate either pATM or 53BP1 at 3 h post-ionizing radiation. Scale bar, 5 μm.
microscopy. This interpretation is consistent with our finding of numerous telomeres immunolabeled with \( \gamma H2AX \) in degenerating PCs. Additionally, telomeric DNA damage may promote telomere-telomere interactions, resulting in increased telomere clustering. In keeping with this, previous studies have shown that dysfunctional telomeres are generated by telomeric DNA damage or by alterations of the nucleoprotein cap that prevents chromosome ends from being recognized as DNA damage sites (69). The cellular response to telomere dysfunction is similar to that of intra-chromosomal DNA damage, involving the ATM-dependent DDR and activation of the non-homologous DNA end joining repair pathway (70). The sustained presence of \( \gamma H2AX \) in telomeric spots of PCs in \( pcd \) mice indicates that unrepaired DNA is also accumulated at the chromosome ends. It also suggests that a deficient repair of telomeric DNA produces telomere instability with an increased motion of chromosome ends that facilitates telomere-telomere interactions and telomere clustering. This interpretation is consistent with previous experimental evidence in cancer cell lines showing that the loss of the protective cap at telomeres strongly increases telomeric motions (71). The participation of telomere dysfunction in PC degeneration supports emerging findings suggesting that telomere damage/dysfunction plays a role in other neurodegenerative disorders such as Alzheimer disease (40).

Regarding the classical mechanisms of neuronal cell death, apoptosis, necrosis, and autophagy (49), our findings in the PCs of the \( pcd \) cerebellum, in particular chromatin condensation, DNA fragmentation, and up-regulation of the \( Cideb \) gene (this encodes a cell death-inducing DNA fragmentation factor), are consistent with previous studies indicating that PC death in this mutant occurs by apoptosis (20, 49, 72, 73). Interestingly, we failed to observe typical apoptotic bodies with a very dense and homogeneous texture, indicating a severe disruption of nucleosomal organization of 30-nm chromatin fibers (35). In contrast, the heterochromatin of the PCs in the \( pcd \) cerebellum preserves the typical granular configuration of heterochromatin observed in healthy PCs from wild-type mice (3, 55). Similarly, the
expression of lamin A/C, which is cleaved during apoptotic cell death (74), and the nuclear envelope are relatively well preserved in advanced stages of PC degeneration in pcd mice. This suggests that a nonconventional apoptotic cell death occurs. Indeed, alternative, apoptosis-like, death pathways have been implicated in neurodegeneration (75). In the case of PC degeneration in pcd mice, nonconventional apoptosis seems to involve a lesser degree of degradation of nuclear lamins and nucleosomal histones than in classical apoptosis, resulting in the preservation of the nuclear envelope and nucleosomal structure of chromatin.

In conclusion, in PCs the pcd mutation induces a profound reorganization of chromosome territories and telomere repositioning. These two cellular events are associated with two epigenetic modifications of nucleosomal histones, the trimethylation of H4K20 and the phosphorylation of H2AX, involved in gene silencing and DNA damage signaling, respectively. We propose that the deprivation of essential mRNA transcripts for neuronal survival and the accumulation of unrepaired DNA lesions are essential nuclear mechanisms for triggering the rapid PC degeneration during the 3rd and 4th postnatal weeks in pcd mice.

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