Dysmyelination not demyelination causes neurological symptoms in preweaned mice in a murine model of Cockayne syndrome

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Cockayne syndrome (CS) is a rare autosomal recessive neurodegenerative disease that is associated with mutations in either of two transcription-coupled DNA repair genes, *CSA* or *CSB*. Mice with a targeted mutation in the *Csb* gene (Cs-b$^{m/m}$) exhibit a milder phenotype compared with human patients with mutations in the orthologous *CSB* gene. Mice mutated in *Csb* were crossed with mice lacking *Xpc* (Xp-c$^{-/-}$), the global genome repair gene, to enhance the pathological symptoms. These Cs-b$^{m/m}$.Xp-c$^{-/-}$ mice were normal at birth but exhibited progressive failure to thrive, whole-body wasting, and ataxia and died at approximately postnatal day 21. Characterization of Cs-b$^{m/m}$.Xp-c$^{-/-}$ brains at postnatal stages demonstrated widespread reduction of myelin basic protein (MBP) and myelin in the sensorimotor cortex, the stratum radiatum, the corpus callosum, and the anterior commissure. Quantification of individual axons by electron microscopy showed a reduction in both the number of myelinated axons and the average diameter of myelin surrounding the axons. There were no significant differences in proliferation or oligodendrocyte differentiation between Cs-b$^{m/m}$.Xp-c$^{-/-}$ and Cs-b$^{m/+}$.Xp-c$^{-/-}$ mice. Rather, Cs-b$^{m/m}$.Xp-c$^{-/-}$ oligodendrocytes were unable to generate sufficient MBP or to maintain the proper myelination during early development. Csb is a multifunctional protein regulating both repair and the transcriptional response to reactive oxygen through its interaction with histone acetylase p300 and the hypoxia-inducible factor (HIF)1 pathway. On the basis of our results, combined with that of others, we suggest that in Csb the transcriptional response predominates during early development, whereas a neurodegenerative response associated with repair deficits predominates in later life.

transcription-coupled repair | behavior | rotenone | γH2Ax

Cockayne syndrome (CS) is a rare autosomal recessive neurodegenerative disease, with a wide range of severity, including cachectic dwarfism, retinopathy, optic nerve atrophy, microcephaly, deafness with cochlea loss, neural defects, ganglial calcification, and retardation of growth and development after birth; patients with CS have a lifespan that varies widely, with an average of 12.5 y (1–3). In humans and mice the cerebellum shows distinctive atrophy with loss of Purkinje cells that are associated with regulating balance and walking (4, 5). The brains of CS patients and mice have shown evidence for accumulation of unrepaired damage (6, 7). The brain shows reduced numbers of oligodendrocytes and less myelination, but it remains unclear whether the disease is primarily one of neurodegeneration with neuronal and myelin loss (demyelination) or a failure of oligodendrocyte differentiation and myelin synthesis (dysmyelination) (4).

DNA nucleotide excision repair (NER) has two main branches: global genome repair (GGR), which is associated with repair of nontranscribed regions of the genome; and transcription-coupled repair (TCR), which is associate with enhanced repair of the transcribed strand of active genes. CS is associated with mutations in two genes dedicated to TCR, *CSA* and *CSB*. These are cofactors for RNA polymerase II that facilitate increased rates of repair of the transcribed strand and continuous transcription through sites of DNA damage (3, 8). Evidence from the analogous system in *Escherichia coli*, the mfd protein, suggests that one function is to displace the transcription apparatus during head-on collisions between replication and transcription (9). Consequently, CS cells show reduced recovery of RNA and DNA synthesis after UV damage (10). Patients have also been reported with mild photosensitivity and no reported neurological symptoms, named UV^s (11, 12), and with a very severe disorder known as cerebro-oculo-facio-skeletal syndrome (COFS) (13). These extremes of CS are associated with mutations in *CSA*, *CSB*, and at least one other presently uncharacterized gene but have similar cellular defects in UV sensitivity and TCR to other CS patients (14–17).

Despite extensive knowledge about the molecular functions of the CSA and CSB proteins and their role in TCR and chromatin remodeling (3, 8, 18–21), the mechanisms by which these proteins are involved in neuropathology is still largely speculative. Suggestions have been made that CS caused by faulty responses to endogenously generated oxidative damage through additional deficiencies in base excision repair (18), mitochondrial activity (22), and coordination of a transcriptional response (4, 23). However, although CSA and CSB patients have indistinguishable clinical disease, only CSB and not CSA or UV^s cells are sensitive to oxidative damage (14, 17, 24).

We have analyzed a mouse model of CS in which both TCR (*Csb*) and GGR (*Xpc*) were removed by gene targeting (the Cs-b$^{m/m}$.Xp-c$^{-/-}$mouse strain). The Csb mutation (Cs-b$^{m/m}$) mimics a truncation found in a human CS patient. Neither human nor mouse mutated in *XPC* show any neuropathology (3). Previously, we demonstrated that major neurological deficiencies occurred in the cerebellum, with the granule cells demonstrating high TUNEL staining indicative of apoptosis (25). Purkinje cells, identified by the marker calbindin, were severely depleted and, although not TUNEL-positive, displayed strong immunoreactivity for p53, indicating cellular stress.

Here, we report that the neural phenotype in Cs-b$^{m/m}$.Xp-c$^{-/-}$ mice is attributable primarily to widespread dysmyelination resulting from the inability of oligodendrocytes to produce or maintain normal levels of myelination after terminal differentiation. This observation contrasts with the idea that premature reentry of differentiated neural cells into cycle could trigger apoptosis (26). Our results also indicate that not all of the symptoms of NER diseases necessarily represent premature aging and degenerative disorders (5, 27). Because Csb is a multifunctional protein regulating both repair and the transcriptional response to reactive oxygen species (ROS) (4, 28) we suggest that defective transcriptional regulation may predominate during early development to generate the dysmyelination phenotype, whereas

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repair deficiencies resulting in a neurodegenerative disorder may predominate in later life.

Results

Body Weight, Brain Weight, and Survival. The Cs-b$^{m/m}$.Xp-c$^{-/-}$ mice were normal at birth but failed to thrive and steadily lagged behind their littermates in both body weight and brain weight (Fig. S1) and exhibited whole-body wasting and ataxia and died around postnatal day (P)21, with an ~50% reduction in body weight (25). Heterozygosity in the *Csb* gene on the Xpc$^{-/-}$ background produced a viable strain with no apparent neurological symptoms (Fig. S1). A similar but more severe phenotype has been reported in the double mutant strain Cs-b$^{m/m}$.Xp-a$^{-/-}$ (29). In our studies, we compared strains homozygous and heterozygous for *Csb*, on the *Xpc*-negative background, to identify those features of the pathology dictated by complete loss of Csb functions. The Xp-c strain exhibits no reported neurological dysfunction but, like XP-C patients, develops skin cancer after exposure to UV light (30). The mild phenotype of the Csb$^{m/m}$ strain alone and the very severe phenotype of the double mutant are reminiscent of the human extremes of CS symptoms that present as the UV^{s} and the COFS disorders.

Behavioral Deficits in Double Mutant Mice. The abnormal clasping reflex and complete inactivity upon tail suspension observed in the double mutant (Fig. 1*A*) is typical of mouse models of neurodegeneration (31). Similarly, in a rotarod test, the double mutant mice were unable to stay on the rotating beam (Fig. 1*B*), whereas the strain heterozygous for *Csb* held for extended periods, similar to those seen in completely wild-type mice. The double mutant mice also showed deficits in the catwalk gait analysis test (Fig. 1*C*). Similar behavior is observed in other DNA repair-deficient mouse models (32, 33). These behaviors correspond to human patients who show difficulty walking and eventually become wheel chair bound (1).

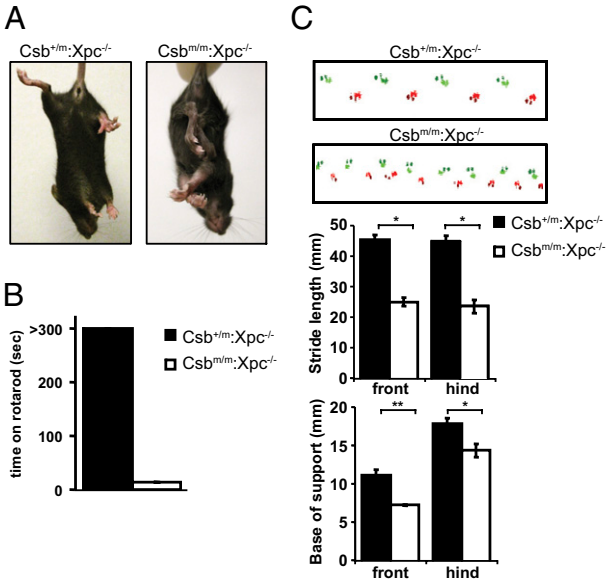

Fig. 1. Neurological defects in P17 Cs-b$^{m/m}$.Xp-c$^{-/-}$ mice compared with Cs-b$^{+/m}$.Xp-c$^{-/-}$ littermates. (*A*) Limb-clasping reflex in mice suspended by the tail. (*B*) Rotarod analysis measures the ability to run on a rotating cylinder. Any mouse that stayed on the rod for the full 30 s was allocated a maximum value of 300. (*C*) Catwalk analysis. Light green depicts the right front paws, dark green the right hind paws, light red the left front paws, and dark red the left hind paws. Stride length and base of support measured in millimeters for front and hind paws. Each assay point represents at least five mice. A significant difference was found according to Student *t* test (*P = 0.02; **P = 0.04).

General Brain Architecture: Normal Except for Loss of MBP. We previously reported that the Cs-b$^{m/m}$.Xp-c$^{-/-}$ mice showed extensive cerebellar pathology and loss of Purkinje cells, similar to human patients (1, 5, 34). The general architecture of the brain stained by hematoxylin/eosin (H&E), NeuN, and glial fibrillary acidic protein (GFAP) appeared normal at p7 and p17 (p17 only shown in Fig. S2). In the cerebral cortex, anterior commissure, corpus callosum, and dentate gyrus, however, we observed a decrease in the myelin basic protein (MBP) in Cs-b$^{m/m}$.Xp-c$^{-/-}$ mice compared with Cs-b heterozygotes (Cs-b$^{+/m}$.Xp-c$^{-/-}$) (Fig. 2 *A* and *B* and Fig. S2). Most notably, the sagittal sections show a clear reduction in the number of axons stained with MBP in the hippocampus and anterior commissure of Cs-b$^{m/m}$.Xp-c$^{-/-}$ mice at p13 (Fig. 2 *A* and *B*).

MBP Axon Numbers and Thickness. To determine whether the reduced MBP staining (Fig. S2) translated in a compromised myelin density, we analyzed sagittal sections of the anterior commissure by electron microscopy. This showed a severe reduction in the numbers of axons in both the anterior commissure and the corpus callosum (Fig. S3), and the membranes of individual axons were hypomyelinated (Fig. 2 *C*–*E*).

Oligodendrocyte Maturation: Double Labeling with Olig2 and BrdU. The severe reduction in MBP and axon numbers suggests that the oligodendrocytes may be unable to differentiate and become mature MBP-synthesizing cells. We tested this possibility by labeling the mice with BrdU to identify proliferating cells and killed mice at P7 and P18 and stained with olig2 and BrdU antibodies (Fig. 3). There was, however, no significant difference between heterozygous and homozygous mice either at P7 or P18. Both genotypes showed a similar reduction in oligodendrocyte numbers and reduction of BrdU labeling of oligodendrocytes, which suggests that oligodendrocytes differentiate and exit the cell cycle normally but do not mature into MBP-synthesizing cells. The subsequent deficits in MBP are then sufficient to cause neuronal dysfunction with pathological and behavioral consequences. This phenotype is more clearly related to dysmyelination than to a degradation of preexisting myelin. The failure of differentiated cells to synthesize large quantities of specific gene product is reminiscent of the phenotype of trichothiodystrophy (TTD) in which mutations in *XPD*, part of the TFIIH transcription factor, result in terminally differentiated cells being unable to synthesize myelin, keratin and other specific products (35–37). Because the *CSB* gene is a cofactor to RNA Pol II, we speculate that high levels of transcription necessary for the production of MBP cannot be generated in the double mutant.

Histone Deacetylase p300 in the Response to DNA Damage. Csb plays a role in the responses to endogenously generated oxidative damage through additional deficiencies in base excision repair (18), mitochondrial activity (22), and coordination of a transcriptional response (4, 23). As part of the transcription response, Csb displaces the histone acetylase p300 from p53, resulting in activation of the hypoxic hypoxia-inducible factor (HIF)1α response (23). We, therefore, questioned whether abrogation of p300 itself would affect either the UV or the ROS response. Mouse knockouts of p300 are not viable, and their fibroblasts senesce prematurely, and only SV40-transformed mouse embryo fibroblasts are available (38). Csb$^{m/m}$ cells were sensitive to both rotenone and UV damage (Fig. 4 *A* and *B*). p300$^{-/-}$ cells, however, were more sensitive than wild-type cells to killing by endogenous ROS generated by rotenone (Fig. 4*D*) (39, 40) but were not UV-sensitive (Fig. 4*C*). The DNA damage marker γH2Ax only showed an increase following UV exposure, not rotenone (Fig. 4*E*), suggesting limited DNA damage from endogenous ROS. These observations suggest that loss of Csb can cause UV sensitivity through its role as a cofactor for RNA Pol II and TCR, and independently cause

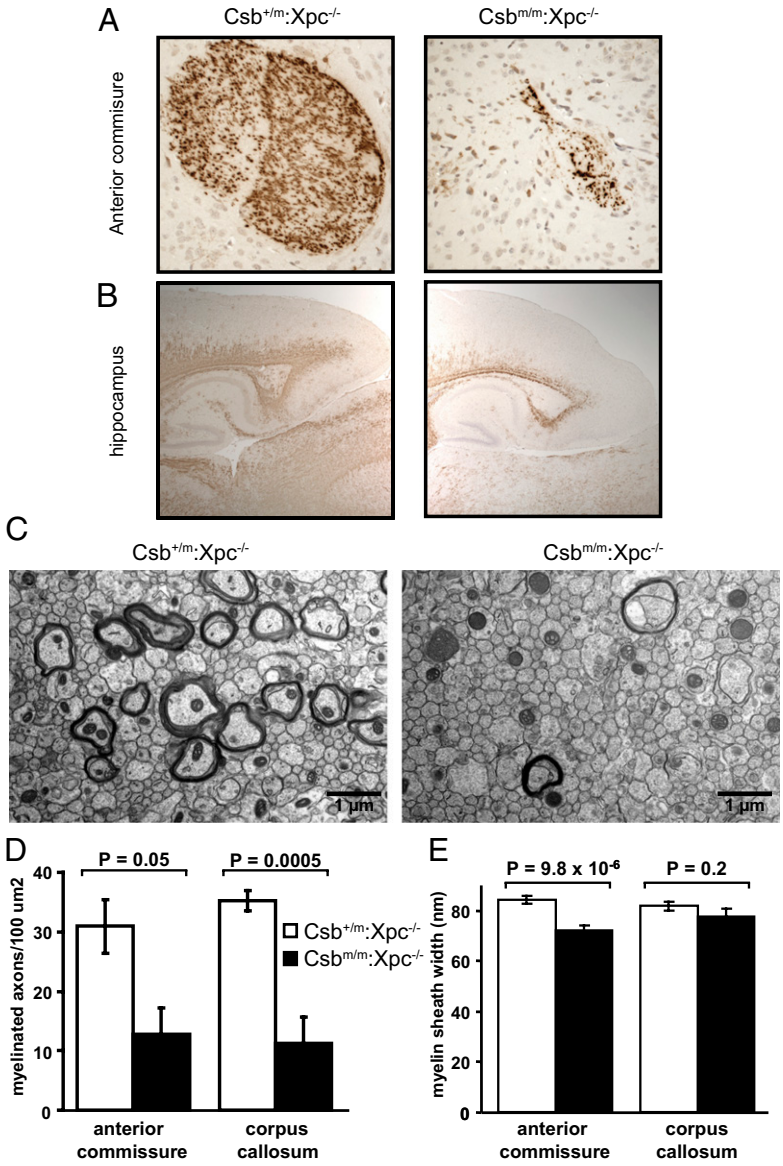

Fig. 2. Hypomyelination in Cs-b$^{m/m}$.Xp-c$^{-/-}$mice. Sagittal brain sections of P13 Cs-b$^{m/m}$.Xp-c$^{-/-}$and Cs-b$^{+/m}$.Xp-c$^{-/-}$ depicting the anterior commissure (*A*) and hippocampus (*B*) stained with MBP showing a reduction in Cs-b$^{m/m}$.Xp-c$^{-/-}$ mice. (*C*) Electron micrographs of the anterior commissure of P17 Cs-b$^{m/m}$.Xp-c$^{-/-}$deficient mice compared with Cs-b$^{+/m}$.Xp-c$^{-/-}$ littermates. (*D* and *E*) Number of myelinated axons per 100 μm^2 (*D*) and myelin sheath width in, respectively, anterior commissure and corpus callosum of Cs-b$^{m/m}$.Xp-c$^{-/-}$ mice compared with Cs-b$^{+/m}$.Xp-c$^{-/-}$(*E*).

sensitivity to ROS, although loss of transcriptional regulation, via p300 interaction. The failure of differentiated oligodendrocytes to synthesize MBP could then be attributable to the deficiency in transcriptional response to endogenous ROS and not because of a deficient repair of DNA lesions by TCR (4, 37).

Discussion

There remains a major challenge in relating the wide range of functions of the CSB protein to the similarly wide range of clinical symptoms in human and mouse (18, 41). The CSB protein has been reported to be involved in the TCR of large DNA adducts, base excision repair, recovery of RNA synthesis as a cofactor of RNA pol II, chromatin remodeling, the transcriptional response to ROS, and, most recently, in mitochondrial function (3, 4, 8, 18–22, 28, 37). Humans and mice show growth failure, pathological aging, hypomyelination, and neurodegeneration (1, 2, 42). No correlation has yet been made between sites of mutation in the CSB gene and the severity of clinical symptoms (18, 41); patients with no detectable CSB protein may be affected either severely (13, 43, 44) or mildly (45). Most relevant to our study, patients show reduced cerebellar function with hypomyelination and white matter loss (1). Mutant mice show similar features, although with lower penetrance in single Csb$^{m/m}$ mutants. Double mutant mice, either Cs-b$^{m/m}$.Xp-a$^{-/-}$ or, as described here, the Cs-b$^{m/m}$.Xp-c$^{-/-}$ strain, have reduced body weight and progressive cachexia, photoreceptor loss, cochlea hair cell loss, and motor dysfunction and are sensitive to ionizing radiation and oxidative damage (2, 42).

Our gross functional analysis recapitulates features already described in Cs-b$^{m/m}$.Xp-c$^{-/-}$ mice (5), but we have now concentrated on the hypomyelination distinctive for human patients, especially COFS patients, who have a similar extremely shortened lifespan to these mice (13). The preweaning period is one of cell proliferation and differentiation in the brain, leading to the development of MBP, which coats developing neurons. We observed a striking

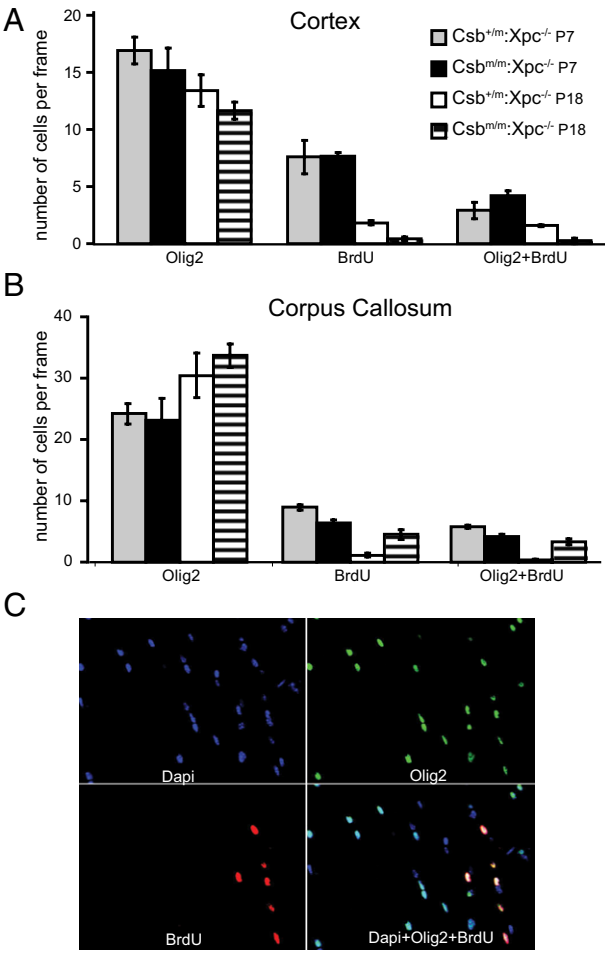

Fig. 3. (*A* and *B*) BrdU and Olig2 staining of P7 and P18 Cs-b$^{m/m}$.Xp-c$^{-/-}$ mice compared with Cs-b$^{+/m}$.Xp-c$^{-/-}$littermates in the cortex (*A*) or corpus callosum (*B*). (*C*) Corpus callosum staining of P7 Cs-b$^{+/m}$.Xp-c$^{-/-}$mice with, respectively, DAPI in blue, olig2 in green, and BrdU in red.

reduction in the synthesis of MBP and corresponding reduction in the numbers of axons and their membrane thickness. This was particularly evident in the cortex and by the atrophy of the anterior commissure. Previous analysis of human CS-B and mouse Cs-b concentrated on the Purkinje layer in the cerebellum that appears to be a major site of degeneration (5, 46). This layer has elevated p53 and shows accumulation of the DNA damage markers ATM and Mre11 (5, 46). We previously reported increased expression of MBP in the cerebellum of Csb$^{-/-}$ mice (5), as also reported for Ttd$^{-/-}$ mice that correspond to the human disorder TTD (37), showing that there is considerable regional variation in MBP expression. Our study now shows that hypomyelination is widespread in the brain, consistent with human symptomology, and was not associated with visible degenerative pathology. Myelination is required for efficient propagation of action potentials in neurons, so the abnormal myelination pattern in the Cs-b;Xp-c double mutants may result in defective functions in the generation, propagation, and maintenance of their action potentials. Defective action potentials would contribute to the behavioral, motor, and cognitive impairments of CS patients.

Our results allow us to consider possible mechanisms for hypomyelination. Two major interpretations of the NER disorders attribute the symptoms either to a DNA repair disorder or to a defective transcriptional response (4). In both interpretations, the ultimate cause may be endogenous ROS, but only the DNA repair hypothesis requires actual DNA damage. TTD is a disorder of DNA repair associated with mutations in components of the transcription factor TFIIH, which promotes the recruitment of thyroid hormone and vitamin D receptors to promoters involved in myelination, acting as a coactivator for MBP synthesis (37). CS, however, does not show abnormalities in nuclear hormone and vitamin D receptors, indicating that CS involves a different mechanism of dysmyelination to Ttd (47). In contrast, in conditional mutants of Cs-b$^{m/m}$.Xp-a$^{-/-}$ mice, elimination of GGR late in life sets up a demyelination phenotype (48), suggesting that dysmyelination predominates during early development associated with TCR defects, whereas a neurodegenerative response associated with GGR deficits predominates in later life.

Our results in p300 knockout cells suggests that loss of the HIF1α response pathway that regulates the response to reactive ROS can have significant effects on the response to endogenous damage but not UV. Alternatively, less specific mechanisms such as chronic inflammatory stress and microvasculature may be involved (4, 49). The arrest of transcription in CS cells is a strong apoptotic signal (50, 51), and we previously observed apoptotic cells in the cerebellum of Cs-b$^{m/m}$.Xp-c$^{-/-}$ mice (5). In the present study we did not observe widespread apoptosis, but observed an orderly reduction in BrdU labeled oligodendrocytes in cortex and anterior commissure of heterozygous and homozygous Csb$^{m/m}$ mice. Apparently, the differentiated oligodendrocytes are unable to support synthesis of major terminal products such as MBP, analogous to the situation observed in TTD. This failure may be due to accumulated ROS damage, and the loss of the Csb/p300 interaction that regulates the response to ROS (23).

CS, therefore, represents a developmental disorder of dysmyelination that is especially distinct in COFS, the severest form of CS with a very short lifespan similar to our double mutant mice (13). This pathology differs from chronic neurodegenerative diseases that exhibit demyelination, such as multiple sclerosis, or neuronal cell death attributable to protein aggregation such as Parkinson disease, Huntington disease, and amyotropic lateral sclerosis. Therapeutic intervention may, therefore, require attention to the sources of endogenous DNA damage and mechanisms of remyelination that could by-pass the DNA repair defect.

Materials and Methods

Mouse Breeding. A parental Cs-b$^{m/m}$.Xp-c$^{-/-}$ strain was established (5) from Cs-b$^{m/m}$ mice [a gift from G. T. van der Horst and J. H. Hoeijmakers, Erasmus University Medical Center, Rotterdam, The Netherlands (52)] and Xp-c$^{-/-}$ mice [purchased from Taconic (53)]. Genotypes were monitored by PCR (see *SI Materials and Methods*). When the parental strain was bred, we obtained three genotypes in Mendelian ratios, but the double homozygote failed to thrive and died before weaning at 14–21 d. Studies of the homozygote were, therefore, confined to the period up to 17 d. Behavioral analysis was performed at the University of California San Francisco (UCSF) Neurobehavioral Core for Rehabilitation Research (NCRR) using three main tests: clasping reflex, rotarod test, and catwalk (see *SI Materials and Methods*). All data were assembled from five or more mice per genotype and expressed as SEMs.

Histological Analysis. Mice were killed with an overdose of anesthetic (2,2,2-tribromoethanol) and perfused transcardially with PBS followed by the 4% paraformaldehyde fixative. For BrdU staining, mice were injected i.p. with BrdU (Roche Applied Science) dissolved in PBS (50 mg/kg) 24 h before they were killed. Brains were removed, postfixed in the same fixative overnight at 4 °C, and paraffin-embedded. Tissue was sectioned at 5-μm thickness sagittally or coronally and placed on precoated slides. The following primary antibodies were used for immunohistochemistry: NeuN (MAB377; Millipore), GFAP (3670; Cell Signaling), and MBP (Sc-13914; Santa Cruz Biotechnology). The olig2 antibody was kindly provided by David Rowitch (Department of Pediatrics and Howard Hughes Institute, University of California, San Francisco, CA). Immunocomplexes were visualized using appropriate, Alexa 488- or 568-conjugated (Invitrogen) or biotinylated (Vector Labs) secondary antibodies followed by mounting in Vectashield with DAPI (H-1200; Vector Labs) or staining with a Vectastain kit (PK-6100; Vector Labs), respectively, according to the protocol of the manufacturer.

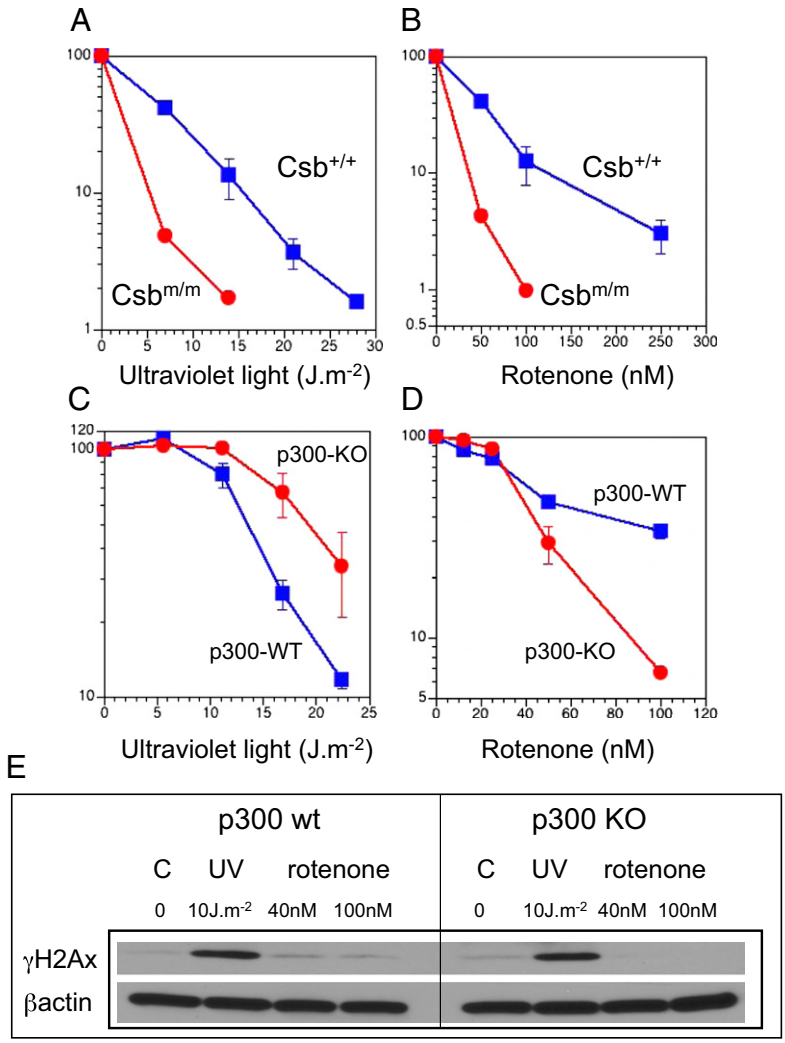

Fig. 4. DNA damage response in Csb$^{m/m}$, p300 knockout, and wild-type mouse embryo fibroblasts. (*A*) Survival of Csb$^{m/m}$ (red circles) and wild-type (blue squares) cells to UV light. (*B*) Survival of Csb$^{m/m}$ (red circles) and wild-type (blue squares) cells to ROS generated from continuous exposure to rotenone. (*C*) Survival of p300 knockout (red circles) and wild-type (blue squares) cells to UV light. (*D*) Survival of p300 knockout (red circles) and wild-type (blue squares) cells to ROS from continuous exposure to rotenone. (*E*) Induction of γH2Ax 4 h after UV irradiation or the start of exposure to rotenone in p300 knockout and wild-type cells. Note low level of γH2Ax in wild-type cells and complete absence from knockout cells. Error bars represent SD of six determinations.

Electron Microscopy. For electron microscopy, mice were killed with an overdose of anesthetic (Avertin) and perfused transcardially with PBS, followed by the 4% paraformaldehyde fixative. Brains were removed and postfixed in the same fixative overnight at 4 °C. For electron microscopy, the blocs of tissue were dehydrated with a graded series of ethanol solutions, cleared with propylene oxide, and embedded in polymerized Eponate 12 (Ted Pella). Ultrathin sections (1 μm) were cut with a microtome (Reichert-Jung) and collected on copper grids. Sections were examined with a JEOL 1200EX electron microscope.

Tissue Culture. Mouse embryonic fibroblasts (MEFs), Cs-b$^{m/m}$ and wild-type, were developed from embryos by standard techniques (donated by W. Bohr, National Institutes on Aging, Baltimore, MD). p300 knockout and wild-type SV40-transformed MEFs were donated by J. A. DeCaprio (Dana Farber Institute, Boston, MA) (38).

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