Histone H2AX promotes neuronal health by controlling mitochondrial homeostasis

Urbaın Weyemi, Bindu D. Paul, Deeya Bhattacharya, Adarsha P. Malla, Myriem Boufraçech, Maged M. Harraz, William M. Bonner, and Solomon H. Snyder

DNA repair by phosphorylating the histone variant H2AX following double-stranded DNA breaks (16). This process is substantiated by the well-established role of H2AX in DNA damage repair (5). Similarities between ATM and H2AX are evident in the phenotypes of their knockout mouse models. In both instances, males are sterile, and there is increased genomic instability evidenced by abnormalities in chromosome structure, immunodeficiency, and enhanced radiosensitivity (12, 17). These similarities may reflect well their common roles in DNA repair. We recently provided evidence that H2AX facilitates redox homeostasis by controlling an Nrf2-regulated antioxidant response pathway (18). Mitochondrial defects are a major source of impaired redox homeostasis and are often associated with age-related neurological diseases (19, 20). A major risk factor for several of these diseases is the concomitant dysfunction of mitochondrial activity leading to increased ROS and decreased DNA repair (21). Here we show that H2AX loss leads to substantially diminished mitochondrial activity associated with disturbed mitochondrial shape. These defects reflect repression of the mitochondrial biogenesis gene peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α) and the oxidative phosphorylation subunits. Ectopic expression of PGC-1α partially rescued cells from mitochondrial defects and restored toxicity associated with mitochondrial damage. We also report that H2AX mutant mice were uniquely vulnerable to mitochondrial damage in the brain. These findings identify histone H2AX as a key regulator of mitochondrial homeostasis, which promotes neuroprotection and clarifies links between genomic instability and redox homeostasis in neurodegeneration and aging.

Results and Discussion
Histone H2AX Deficiency Leads to Repression of Mitochondrial Biogenesis Genes and Impairment of Oxidative Phosphorylation. Deficits in DNA repair genes are associated with diverse forms of neurodegeneration (1). Neurologic disorders arising from deficient DNA repair involve oxidative stress as well as the inability to repair oxidative DNA damage. Histone H2AX elicits proper DNA repair through its phosphorylation by ataxia-telangiectasia mutated (ATM) kinase. While ATM senses reactive oxygen species, the role of H2AX in the maintenance of redox homeostasis remains unknown. Here we establish that H2AX deletion leads to impairment of mitochondrial function and repression of the mitochondrial biogenesis gene PGC-1α. Restoring PGC-1α abrogates mitochondrial deficits and mitigates cell death. This study unveils a role for H2AX in mitochondrial homeostasis associated with neuroprotection.

Significance

Histone H2AX elicits proper DNA repair through its phosphorylation by ataxia-telangiectasia mutated (ATM) kinase. While ATM senses reactive oxygen species, the role of H2AX in the maintenance of redox homeostasis remains unknown. Here we establish that H2AX deletion leads to impairment of mitochondrial function and repression of the mitochondrial biogenesis gene PGC-1α. Restoring PGC-1α abrogates mitochondrial deficits and mitigates cell death. This study unveils a role for H2AX in mitochondrial homeostasis associated with neuroprotection.

Author contributions: U.W. and S.H.S. designed research; U.W., B.D.P., D.B., A.P.M., M.B., and M.M.H. performed research; W.M.B. contributed new reagents/analytic tools; U.W. and S.H.S. analyzed data; and U.W. and S.H.S. wrote the paper.

Reviewers: O.A.M., Peter MacCallum Cancer Centre; and R.S., University of Maryland School of Medicine.

Conflict of interest statement: U.W. and O.A.M. are coauthors on a 2016 article; they did not collaborate directly on the paper. The authors declare no competing financial interests.

Published under the PNAS license.

1To whom correspondence should be addressed. Email: snyder@jhmi.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1820245116/-/DCSupplemental.

Published online March 25, 2019.
Fig. 1. Histone H2AX depletion elicits pronounced diminution of mitochondrial biogenesis genes and reduced expression of OXPHOS complexes. (A–D) H2AX deletion reduces PGC-1α, a major mitochondrial biogenesis gene. Protein lysates from wild-type and H2AX knockout MEFs, as well as lysates from the striatum, were processed for immunoblot detection of H2AX and PGC-1α. Actin was used as a loading control. (A) Representative image of PGC-1α expression in MEFs and (B) quantification. (C) Representative image of PGC-1α expression in the striatum and (D) quantification. Data are means ± SEM (n = 3). Statistical significance was determined by a two-tailed, unpaired Student’s t test. (E) Analysis of POLRMT, TFB2M, TFAM, and PPARGC1B transcript levels in wild-type and H2AX knockout MEFs by RT-PCR. Expression values are relative fold changes for gene transcripts normalized to GAPDH. Error bars represent SEM (n = 3). (F and G) Western blot analysis of OXPHOS subunits in wild-type and H2AX knockout MEFs. We performed the immunoblot detection using total OXPHOS Human WB Antibody Mixture, enabling concomitant analysis of main proteins of each complex in the electron transport chain, including complex I subunit NDUF88, complex II subunit 30 kDa, complex III subunit Core 2, complex IV subunit I, and ATP synthase subunit alpha; (F) representative image; (G) quantification. Data are means ± SEM (n = 3). (H and I) Western blot analysis of the OXPHOS subunits in the striatum of 4-mo-old wild-type and H2AX knockout mice; (H) representative image; (I) quantification. Data are means ± SEM (n = 3; *P < 0.05; **P < 0.001; ***P < 0.0001).
lesions (6, 8). These features are exemplified in AT, a form of progressive cerebellar degeneration associated with oxidative stress (12, 14). We have shown that diminution of ROS reduces phenotypic damage associated with AT (15). ATM kinase elicits DNA repair by phosphorylating the histone variant H2AX following double-stranded DNA breaks, which is consistent with the well-established role of H2AX in DNA damage repair (5). We observed neurobehavioral defects and impaired redox disposition in mice with genetic deletion of H2AX (18). In the present study, we report a role for H2AX in promoting mitochondrial homeostasis and mediating neuronal health.

Most mitochondrial proteins are encoded in the nucleus (22). Examples include basal transcription factors such as TFAM, TFB2M, TFB1M, and POLRMT, as well as the mitochondrial biogenesis gene PGC-1α (22, 23). PGC-1α is a transcription coactivator that regulates the expression of TFAM, TFB2M, TFB1M, and POLRMT, as well as the oxidative phosphorylation complex subunits (OXPHOS) through interaction with the transcription factor nuclear respiratory factor 1 (NRF1) (24, 25). It is well established that mitochondrial biogenesis and respiration are stimulated by PGC-1α through induction of NRF1 and NRF2 gene expression (26). Changes in chromatin configuration or alteration of chromatin-based DNA repair pathways are factors in regulating transcription factors (27, 28), but regulatory mechanisms are often unclear. As histone H2AX is a key component of chromatin and an important player in DNA repair pathways, we speculated that loss of H2AX can alter chromatin and DNA damage responses, thereby impacting mitochondrial biogenesis and eliciting oxidative stress. Deletion of H2AX led to pronounced diminution of PGC-1α protein in mouse embryonic fibroblasts (MEFs) as well as the brain (Fig. 1 A–D). Similar decreases occurred for the PGC-1α targets, TFAM, TFB2M, and POLRMT, as well as for PPARGC1B, another homolog of PGC-1α (Fig. 1E). Western blot analysis revealed substantial decreases of the key subunits of the five OXPHOS complexes in both H2AX-deficient MEFs and the brains of mutant mice (Fig. 1 F–I). The reduction in the OXPHOS complexes expression seems more pronounced in the striatum, since only a partial depletion of the OXPHOS complexes I and II was observed in the cortex (SI Appendix, Fig. S1A). Similar pattern of OXPHOS expression was detected in peripheral tissue such as the liver (SI Appendix, Fig. S1B), suggesting an increased vulnerability of the striatum to H2AX loss-induced mitochondrial damage. Taken together, H2AX deletion impairs expression of PGC-1α, the master regulator of mitochondrial biogenesis, leading to alteration of the principal components of mitochondrial function.

H2AX knockout cells displayed disorganized cristae and slightly enlarged mitochondria (Fig. 2 A and B). Cristae are functionally dynamic compartments, serving as sites for OXPHOS complexes. A substantial amount of the main...
subunits of the complex III and ATP synthase, and the cytochrome c, are stored in the cristae (29). Shapes of cristae and OXPHOS function are closely linked with direct impact on cellular metabolism (29). The loss of cristae in H2AX mutants fits with the diminished levels of OXPHOS subunits in mutant cells. We monitored OXPHOS function, employing the Seahorse technique. We observed diminished baseline oxygen consumption rate (OCR) in H2AX mutant cells, as well as reduced ATP production and impaired spare respiratory capacity (Fig. 2).

**Ectopic Expression of PGC-1α Restores OXPHOS Complexes and Reverses Mitochondria Damage-Induced Cytotoxicity.** Overexpressing PGC-1α in the H2AX knockout cells partially restored OXPHOS subunit levels (Fig. 3A) and diminished cytotoxicity elicited by the mitochondrial complex II inhibitor 3-nitropropionic acid (3-NP) (Fig. 3B). Enhanced mitochondrial functions, especially those involving the respiratory oxidative phosphorylation complexes, are associated with PGC-1α. PGC-1α is a master integrator of cellular signals that govern mitochondrial biogenesis, oxidative

---

**Fig. 3.** Ectopic expression of PGC-1α restores OXPHOS subunits and protects from the cytotoxicity induced by mitochondrial damage. (A) Immunoblot analysis of OXPHOS subunits in H2AX wild-type cells (H2AX WT), H2AX knockout cells (H2AX KO), and H2AX knockout cells in which PGC-1α expression was ectopically and transiently restored for 48 h. Actin was used as a loading control. Both wild-type and knockout cells were transfected with control vector. Most of the subunits were partially restored, except for complex IV and complex V. (Left) Representative image. (Right) Quantification. (B) Cells deficient in H2AX are vulnerable to 3-nitropropionic acid (3-NP), an inhibitor of OXPHOS complex II. This cytotoxicity was reversed by ectopic expression of PGC-1α in H2AX knockout cells. Viable cells were quantified using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were treated with increasing doses of 0, 0.1, 1, and 2 mM 3-NP for 48 h before MTT was added. Data are means ± SD; n = 3. Statistical significance was determined by a two-tailed, unpaired Student’s t test. (C–E) Mitochondrial complex inhibitors induce lower survival in H2AX mutant cells, and H2AX ectopic expression mitigates these cytotoxic effects. (C and D) Parental cells (H2AX WT), H2AX knockout cells (H2AX KO), and H2AX knockout cells in which H2AX expression was restored (REV) were treated with increasing concentrations of mitochondrial complex I inhibitors 1-methyl-4-phenylpyridinium (MPP+) and rotenone. Cell survival was estimated 48 h posttreatment using MTT assay. Cells were treated with increasing doses of 0, 10, 50, and 100 μM MPP+, or with 0, 0.1, 1, and 10 nM rotenone for 48 h before MTT was added. (E) Cell vulnerability to 3-nitropropionic acid (3-NP) was analyzed using MTT assay. Cells were treated with increasing doses of 0, 0.1, 1, and 2 mM 3-NP for 48 h before MTT was added. Data are means ± SD; n = 3. Statistical significance was determined by a two-tailed, unpaired Student’s t test; *P < 0.05; **P < 0.001; ns, nonsignificant.
H2AX KO
Materials and Methods
ples include proteins such as ATM, NBS1, RAD50, and 53BP1, matin remodeling following DNA double-strand breaks. Exam-
creased cytotoxicity which can be reversed by restoring markers
findings that H2AX knockout mice exhibit increased vulnerability
tivity in H2AX mutant mice and in mice treated with 3-NP
mice lacking H2AX following damage to mitochondria, further substantiating evidence that H2AX promotes neuronal health via control of mitochondrial homeostasis.
phosphorylation, adaptive thermoregulation, and fatty acid bio-
To further assess the link between the loss of H2AX and mi-
mitochondrial damage, we explored effects of additional inhibitors of mitochondrial complexes on the viability of cells deficient for H2AX. H2AX knockout cells were vulnerable to inhibitors of mitochondrialOXPHOS complexes I and II with toxicity reversed by overexpressing H2AX (Fig. 3 C–E). These observations show that the deficits observed in H2AX mutants are not the result of other unrelated differences between wild-type and mutant cells. Taken together, these findings establish that the mitochondrial defects induced by H2AX deletion lead to increased cytotoxicity which can be reversed by restoring markers of mitochondrial biogenesis. Histone H2AX is a key component of a cluster of proteins involved in genome stability and chro-
Among others. How changes in chromatin dynamic affect the metabolism of mitochondria via regulation of PGC-1α and its transcriptional targets is unclear.
H2AX Deficiency Increases Vulnerability to 3-Nitropropionic Acid That Targets Mitochondria in the Brain: Role in Neuroprotection. We challenged wild-type and H2AX mutant mice with 3-NP as illus-
our finding that H2AX knockout mice exhibit increased vulnerability to 3-NP in the striatum suggest elevated mitochondrial injury in mice lacking H2AX following damage to mitochondria, further substantiating evidence that H2AX promotes neuronal health via control of mitochondrial homeostasis.
In summary, the present study demonstrates that H2AX, a histone variant and classic DNA repair protein, promotes mitochondrial biogenesis and mediates responses to neurotoxic drugs targeting mitochondria. In addition, we show that cells lacking H2AX have impaired oxygen consumption, as well as disturbances in mitochondrial shape. Histone variant H2AX has been studied primarily as a mediator of DNA repair (5). The present study extends the role of this protein to the arena of mitochondrial function and neuronal health. Our findings reveal how alterations in the nuclear genome influence mitochondrial activity and cell metabolism. Other reports describe concomitant defects in DNA repair and mitochondrial activity in neurodegenerative diseases such as AT (7, 33). Several features of aging and neurodegeneration in AT are suppressed by complementation with antiaging compounds or selective activators of mitochondrial biogenesis (7). One of the hallmarks of neurodegenerative disease is the elevated risk of impaired mitochondrial function combined with the cell’s inability to properly handle DNA lesions. These phenomena occur in diverse diseases as well as during aging (21, 34). Our finding that deficiency of the DNA repair gene H2AX leads to impaired mitochondrial homeostasis and increased neuronal damage reflects a key role for histone H2AX and chromatin-based DNA repair in neurodegenerative diseases and aging. Accordingly, global genomic instability resulting from mutations or deficiencies in DNA repair genes may impact mitochondrial function leading to neurodegeneration.

Materials and Methods
Cell Culture. MEF from both wild-type and H2AX mutant mice were obtained from the Developmental Therapeutics Branch, Laboratory of Molecular Pharmacology, National Cancer Institute, National Institutes of Health. Cells were grown at 37 °C with 5% CO2 in DMEM (Invitrogen), supplemented with 10% FBS (Atlanta Biologicals). All media were supplemented with 2 mM glutamine, penicillin, and streptomycin (Invitrogen).

Fig. 4. H2AX deficiency leads to increased neuronal damage following mitochondrial damage. (A) Wild-type and H2AX knockout mice were injected with either 3-nitropropionic acid or PBS following instructions described in Materials and Methods. After 24 h, mice were killed, and brain tissues were either fixed in NBF or collected for biochemical analyses. (B) Coronal sections of the brain were taken through the striatum and analyzed for TH-positive cells by immunohistochemistry. Purple, TH; blue, DAPI. (Scale bars, 100 μm.) (C and D) Western blot analysis of TH in the striatum. (C) Representative image; (D) quantification. Data are means ± SD; n = 4 mice per group. Statistical significance was determined by a two-tailed, unpaired Student’s t test. (E) H2AX loss and 3-nitropropionic acid treatment cause reduction of body temperature. We measured the rectal temperature in wild-type and H2AX knockout mice. H2AX mutant mice display a significant reduction in body temperature, and this reduction progresses with 3-nitropropionic acid treatment. These changes are reflective of lower mitochondrial activity. Data are means ± SD; n = 4 mice per group. Statistical significance was determined by a two-tailed, unpaired Student’s t test; *P < 0.05; ns, nonsignificant.
Four to five-month-old animals were used to analyze effects of 3-Nitropropionic acid (3-NP). Injections were based on protocols previously reported for mice (32). In brief, 3-NP (Sigma) was dissolved at 10 mg/mL in sterile 0.1 ml PBS and adjusted to pH 7.4 with sodium hydroxide. For TH detection in the striatum, mice received i.p. injections of 60 mg/kg of 3-NP twice, with 2 h between injections, and tissues were collected 24 h postinjection.

Real-Time PCR. Total RNA was extracted from cells using RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. Quality of RNA preparation, based on the 28S/18S ribosomal RNAs ratio, was assessed by using the RNA 6000 Nano Lab-On-chip (Agilent Technologies). Reverse transcription and real-time PCR (RT-PCR) were performed as previously described (35). Oligonucleotides were predesigned and validated, and were considered to be proprietary information by Thermo Fisher Scientific. However, the assays IDs are available and are referenced as follow: PPARGC1B (Mm00504730_m1), TFAM (Mm00447485_m1), TFB2M (Mm01620397_s1), and POLRMT (Mm00553272_m1)

Mitochondrial Stress Assay Using Seahorse. Mitochondrial function was determined by measuring OCR of each cell line using XF Cell Mito Stress Test Kit (Agilent Technologies). Wild-type and HAZX knockout MEFs were seeded in an XFP6 cell culture microplate. Media were prepared by adding 1 mmol/L of pyruvate, 50 μmol/L of glutamine, and 10% of glucose and stored as per the manufacturer’s instructions. Seahorse assay was run in XF696 Extracellular Flux Analyzer (Agilent Technologies). Following three baseline OCR measurements, cells were exposed sequentially to oligomycin (0.5 μmol/L), carbonyl cyanide-4(trifluoromethoxy)phenylhydrazide (FCCP; 1 μmol/L), and rotenone/antimycin A (0.5 μmol/L). Oligomycin inhibits ATP synthase (complex V), and the following injection of oligomycin correlates with the mitochondrial respiration associated with cellular ATP production. FCCP is an uncoupling agent that collapses the proton gradient and disrupts the mitochondrial membrane potential, allowing cells to achieve maximal OCR. As a result, electron flow through the electron transfer chain is uninhibited, and oxygen is maximally consumed by complex IV. The FCCP-stimulated OCR can therefore be used to calculate spare respiratory capacity, defined as the difference between maximal respiration and basal respiration. Spare respiratory capacity is a measure of the ability of the cell to respond to increased energy demand. The third injection is a mix of rotenone, a complex I inhibitor, and antimycin A, a complex III inhibitor. This combination shuts down mitochondrial respiration and enables the calculation of nonmitochondrial respiration driven by processes outside the mitochondria. Data were normalized to total cell survival. The assay results were analyzed using Wave program 2.3.0 (Seahorse Bioscience), and data were exported in GraphPad Prism 7.

Brain Tissue Processing and Immunostaining. Mice were anesthetized by i.p. injection of sodium pentobarbital (80 mg/kg), then the heart was exposed, and transcardiac perfusion was performed using PBS for 5 min followed by ice-cold 10% neutral buffered formalin (NBF; vol/vol) for 30 min (using 3 mL/min rate of perfusion) and maintained in NBF for 24 h. Tissues were transferred to 30% sucrose and maintained at 4 °C for 24 h. Brains were sectioned on a freezing stage sliding microtome into a series of 35-μm sections. Sections were permeabilized with 0.5% Triton X-100 in Tris buffered saline, then blocked with 5% normal goat serum. Mouse anti-TH was used as a primary antibody at the dilution of 1:1,000. Anti-mouse IgG (H+L) Alexa Fluor 647 conjugated was used as a secondary antibody. ZEISS LSM 800 confocal microscopy was used to image immunostained sections.

Statistical Analysis. Statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software) and Microsoft Excel 2010. Parametric data were analyzed using a two-tailed t-test. A value of P < 0.05 was considered statistically significant. Data are presented as mean ± SD or as mean ± SEM.

Generation of cells expressing H2AX-wt (HA2X-rescued cells or revertant (REV)), Western blots, cell viability assay, Transmission Electron Microscopy (TEM), Mitochondrial extraction, and immunoblot can be found in SI Appendix, SI Materials and Methods.

ACKNOWLEDGMENTS. We thank Roxanne Barrow and Lauren Albacys of The Solomon H. Snyder Department of Neuroscience (Johns Hopkins University) for help with experiments. We thank Barbara Smith (TEM Specialist at Johns Hopkins School of Medicine) for her help in preparation and imaging of TEM samples. This work was supported by the National Institutes of Health and United States Public Health Service (USPHS) Grants GM188501 and DA000266.

11. Fukouri NB, et al. (September 20, 2018) Toward understanding genomic instability, mitochondrial dysfunction and aging. FEBS J 10:1119Feb:1463
16. Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM (1998) DNA double-stranded break induction in the striatum, mice received i.p. injections of 60 mg/kg of 3-NP twice, with 2 h between injections, and tissues were collected 24 h postinjection.