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Supplemental Information

Citron Kinase Deficiency Leads to Chromosomal Instability and TP53-Sensitive Microcephaly

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Supplemental data items

Figure S1.

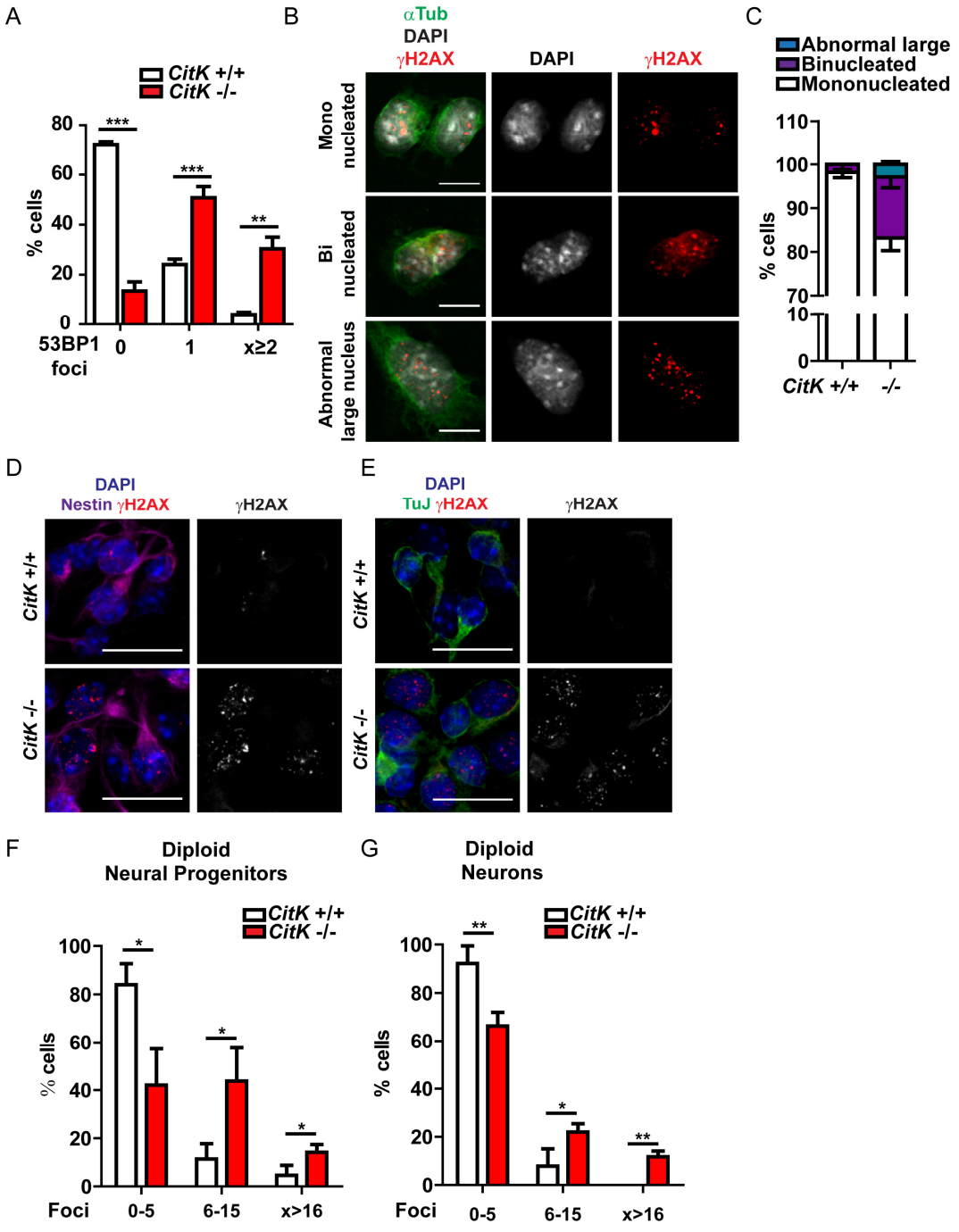

Figure S1. Additional data on DNA damage in *CitK* -/- NPC. Related to Figure 1.
A) Frequencies of cells negative (0 foci/cell), moderately positive (1 focus/cell) or strongly positive (>2 foci/cell) for 53BP1, in developing mouse cortex. **B)** Examples of mononucleated cells, binucleated cells and cells with an abnormally large nucleus from *CitK* -/- NPC cultures stained with alpha tubulin (Tub, green), gH2AX (red) and DAPI (gray). **C)** Relative frequency of the three types of cell populations described in B observed in control *CitK* +/+ and *CitK* -/- mice. **D-E)** NPCs from E12.5 embryo cortices stained for γH2AX (red), DNA (DAPI, blue) and either Nestin (magenta) or TUJ (green). Scale bars, 10 µm. **F-G)** Frequency of cells negative (0-5 foci/cell), moderately positive (6-15 foci/cell) or strongly positive (>15 foci/cell) for γH2AX in diploid NPCs positive for Nestin (Neural progenitors) or TUJ (post mitotic neurons). Two tails unpaired Student's t test were used for the statistical analysis of these experiments (n = 3–4 mice per group). Graphs show mean ± s.e.m. * p< 0.05; ** p < 0.01; *** p < 0.001.

Figure S2.

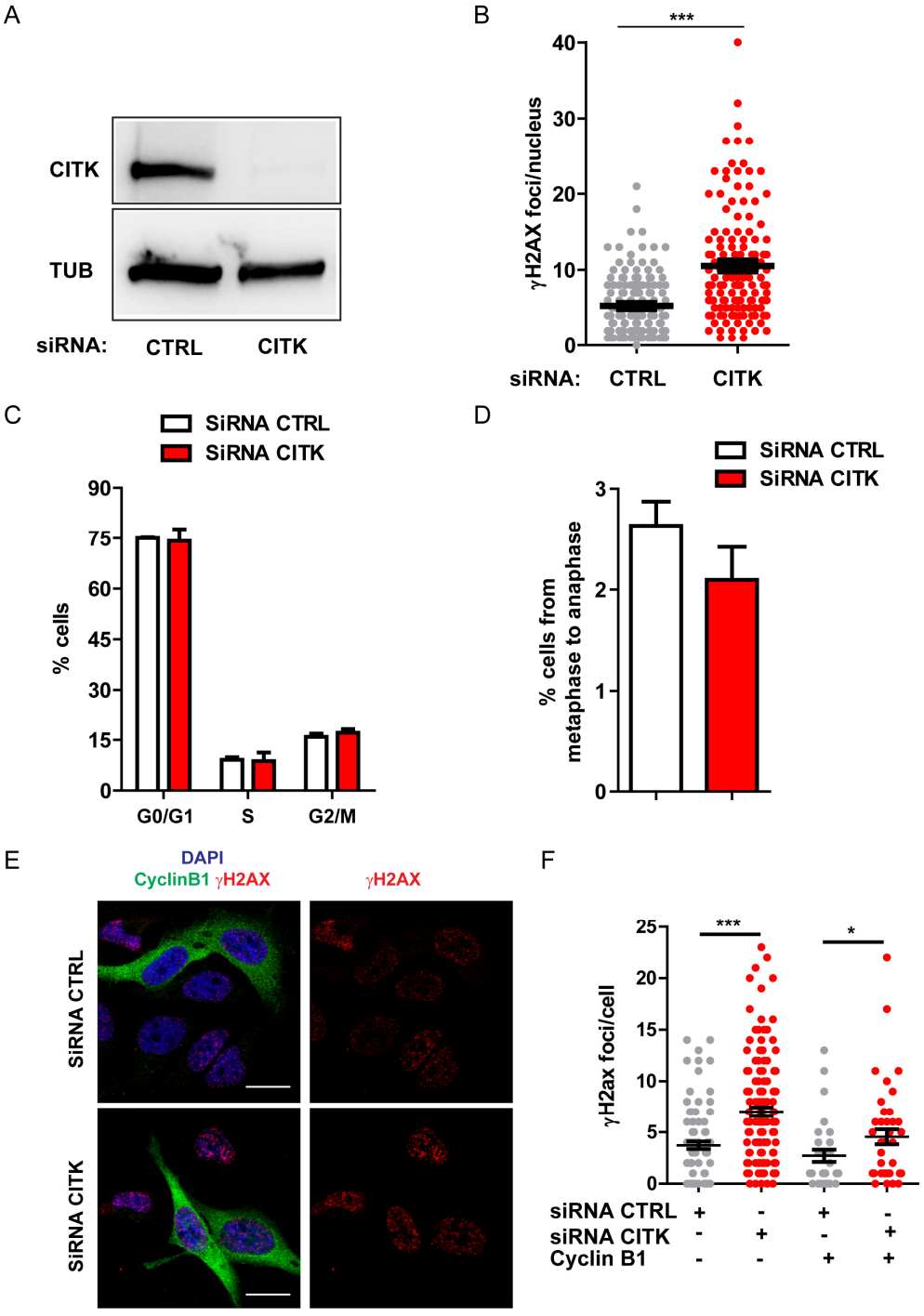

Figure S2. CITK leads to DSB accumulation or IR sensitivity independently of the cell cycle stage. Related to Figure 2 and Figure 3.

A) Western blot of lysates from ONS-76 cells, 48h after transfection with mock or CITK-specific siRNA. **B)** Quantification of γH2AX foci per cell in ONS-76 cells, 48h after transfection with the indicated siRNA. **C)** Flow cytometric analysis of cell cycle of HeLa cells 48 h after transfection with the indicated siRNA. **D)** Prometaphases, metaphases and anaphases were quantified by IF based on DAPI morphology in cultures obtained as in panel C. **E)** HeLa cells incubated 48 hour with the indicated siRNA and stained for γH2AX (red), cyclin B1 (green) and DNA (DAPI, blue) 1 hour after irradiation (4 Gy). Scale bars, 10 μm. **F)** Quantification of γH2AX foci in cells positive or negative for anti-Cyclin B1 staining obtained as in panel E. Two tails unpaired Student's t test was used for the statistical analysis of these experiments (n = 3 per group). Graphs show mean ± s.e.m. * $p< 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure S3

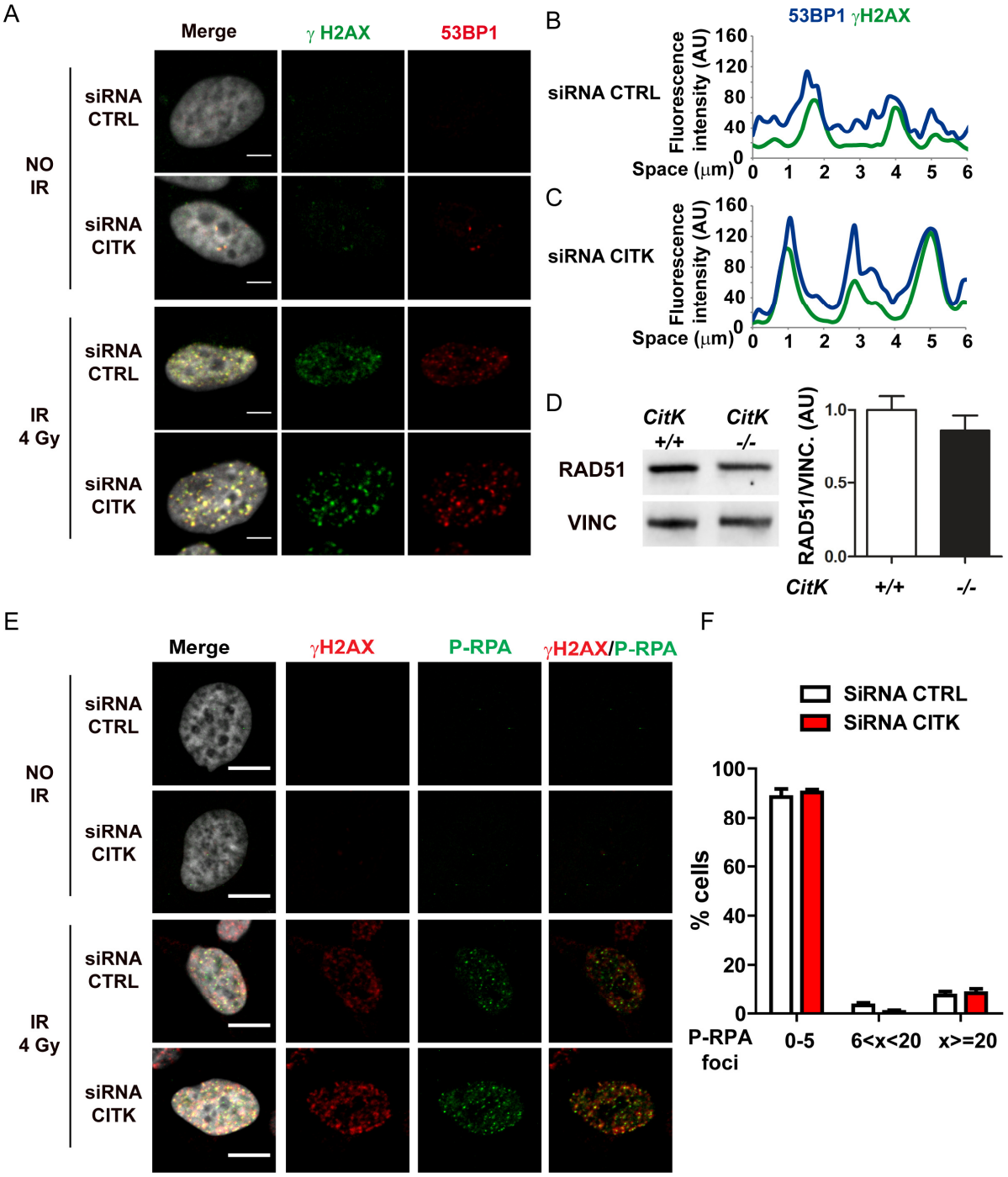

Figure S3. Localization of 53BP1 and P-RPA to γH2AX foci. Related to Figure 4.
A) HeLa cells incubated 48 hour with the indicated siRNA and stained for γH2AX (green), 53BP1 (red) and DNA (DAPI, gray) before (NO IR) or four hours after irradiation (IR 4 Gy)**.** Scale bars, 5 μm. **B-C)** Representative profiles of co-localization between γH2AX and 53BP1 signals in the above experiment. Fluorescence intensity was plotted for the two channels along a 6 μm-long line, randomly drawn in nuclei of exemplar cells. **D)** Total levels of RAD51 were analyzed by western blotting on P4 developing cerebella whole lysates. The histogram on the right shows quantification of the ratio between levels of RAD51 and Vinculin (VINC), which was used as internal loading control. **E)** HeLa cells incubated 48 hour with the indicated siRNA and stained for γH2AX (red), P-RPA (green) and DNA (DAPI, gray) before (NO IR) or 1 hour after irradiation (IR 4 Gy). Scale bars, 10 μm. **F)** Frequency of cells negative (0-5 foci/cell), moderately positive (6-19 foci/cell) or strongly positive (>=20 foci/cell) for P-RPA foci in cells obtained as in E. Two tails unpaired Student's t test was used for the statistical analysis of these experiments (n = 3 per group). Graphs show mean ± s.e.m.

Figure S4

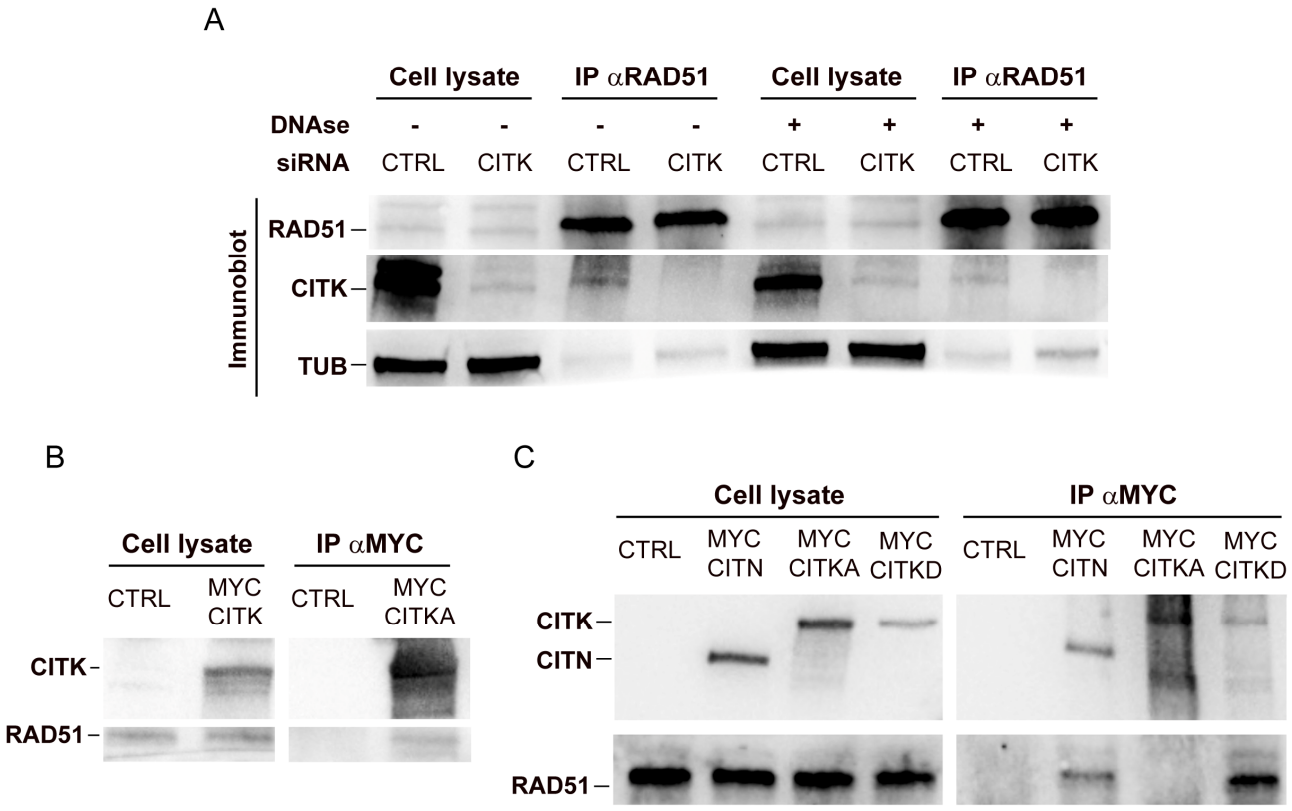

Figure S4. The association between CITK and RAD51 is DNA-independent and may be influenced by CITK catalytic activity. Related to Figure 4.
A) Western blot of total cell lysates or anti-RAD51immunoprecipitations obtained from ONS-76 medulloblasoma cells treated with control or CITK-specific siRNAs, in presence or absence of DNAse. Endogenous CITK, RAD51 and beta-tubulin (TUB) were revealed. **B)** Active MYC-tagged CITK (CTKA) or empty control vector were transfected in 293T cells. Total cell lysates were prepared from these cells 48 hours after transfection and then subjected to immunoprecipitation using anti-MYC antibodies. Lysates and immunoprecipitates were then analyzed by WB with anti CITK and RAD51 antibodies. **C)** The same experiment shown in panel B was performed on cells transfected with MYC-tagged CTKA, kinase dead mutant (CITKD) and CITN isoforms. Data are representative of 3 independent experiments.

Figure S5

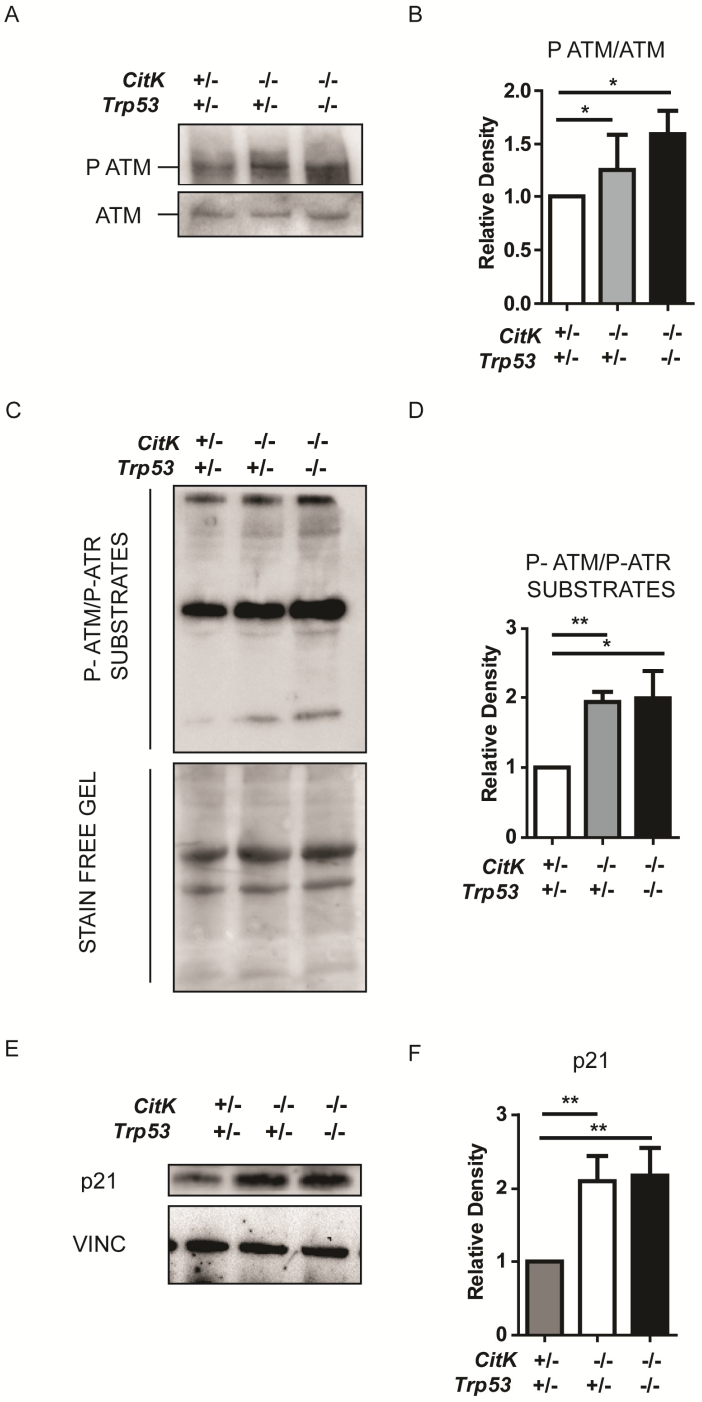

Figure S5. Activation of ATM and p21 in CitK knockout mice is independent of their p53 status. Related to Figure 5.

Western blots of total cell extracts from P4 cerebella of mice of the indicated genotypes, probed with antibodies against ATM and phosphorylated ATM (P ATM) (A), the ATM/ATR phosho-substrates (C), p21 and loading control Vinculin (E). **B)** The ratios between the phosphorylated (P ATM) and total ATM were quantified. **D)** The anti ATM/ATR phosho-substrate antibody (ATM/ATR P-substrates) recognizes proteins containing sites phosporylated by either of these kinases. We measured the global signal of these lanes (including all antibody-positive bands). The quantification measures the ratio between global signal and total amount of proteins in the lane (obtained using the BioRad fluoresence detection of proteins). **F)** The ratios between p21 and Vinculin were quantified**.** Two tails unpaired Student's t test were used for the statistical analysis of these experiments (n = 3–4 mice per group). Graphs show mean ± s.e.m. * $p< 0.05$; ** $p < 0.01$.

Figure S6.

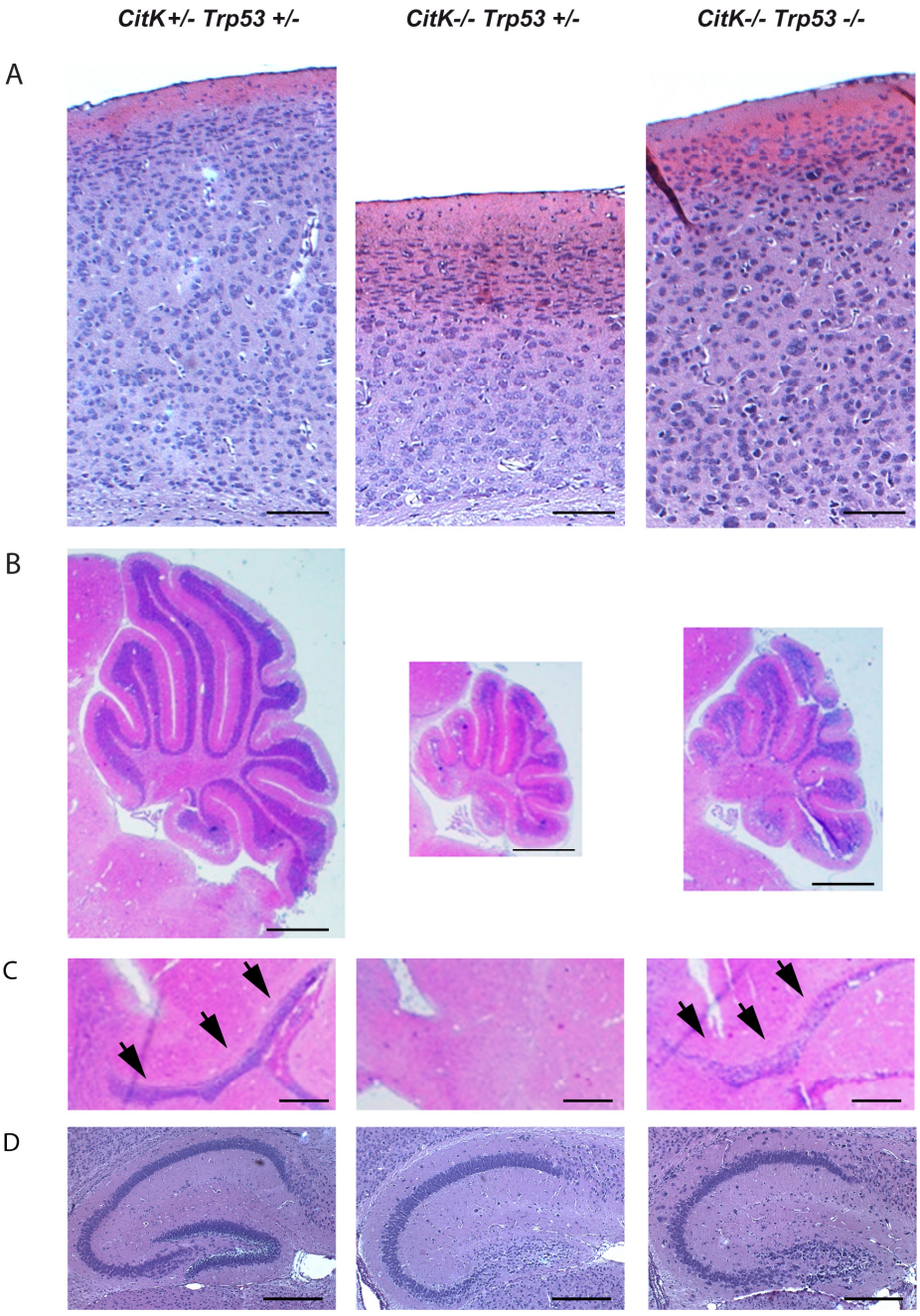

Figure S6. The inactivation of Trp53 in CitK -/- mice leads to partial rescue of the histological brain phenotype. Related to Figure 6.

A-E) Low magnification micrographs of cortices (A), cerebella (B), subventricular migratory stream (C, indicated by arrows) and hippocampal formation (D), obtained from brain sections of P21-old mice of the indicated genotypes, stained with hematoxylin/eosin (H&E). Scale bars correspond to 150 µm in A and 500 µm in the other panels.

Figure S7.

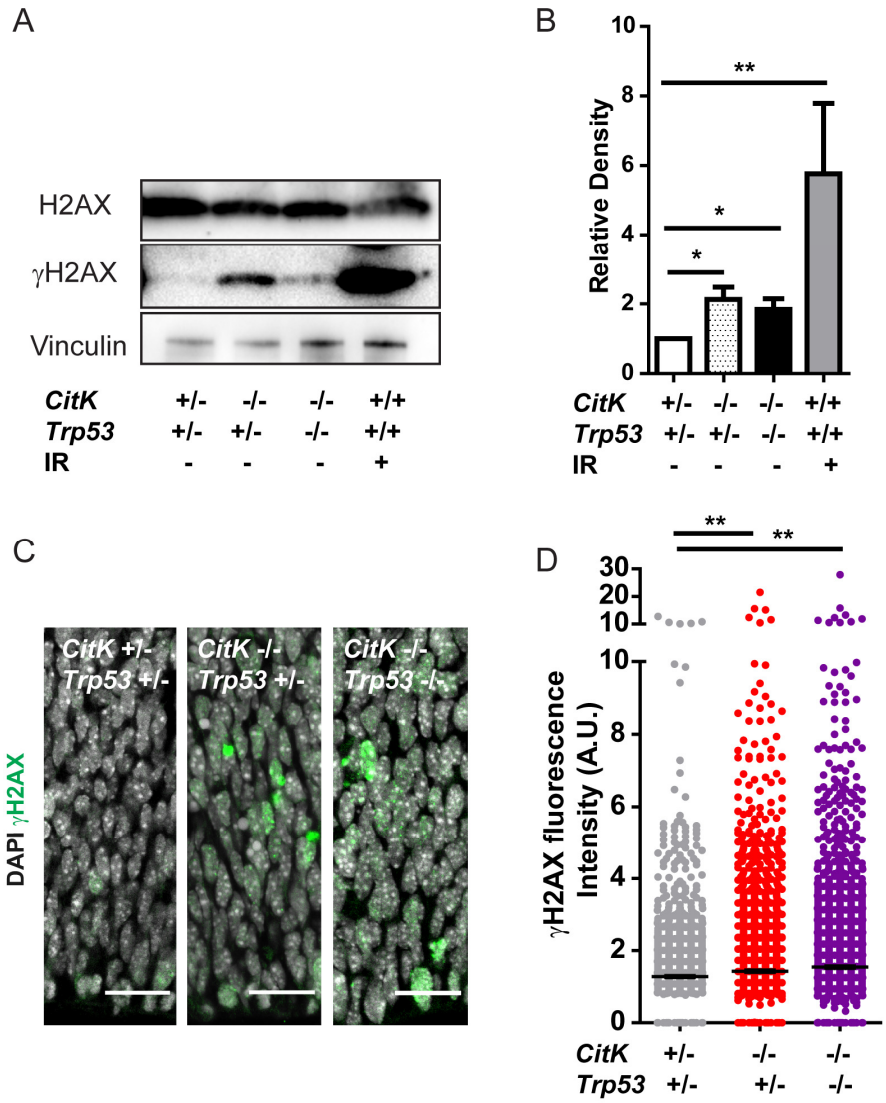

Figure S7. DNA damage in CitK mutant mice is p53-independent. Related to Figure 6.
A, B) Extended version of Figure1C, showing western blots of lysates obtained from P4 cerebella of the indicated genotypes, incubated with anti-γH2AX, anti-H2AX and anti-Vinculin antibodies. Cerebella obtained from IR-treated WT mice were used as a positive control. Quantification of the experiments is shown in B. **C-D)** Cerebral cortex sections from E14.5 mouse embryos of different genotypes were immunostained (C) for γH2AX (green) and DAPI (gray). Fluorescence intensities were quantified in (D). Scale bars = 50 µm. Two tails unpaired Student's t test was used for the statistical analysis of these experiments (n = 3–5 mice per group). Graphs show mean ± s.e.m. * $p < 0.05$; ** $p < 0.01$.

Movie S1. Spontaneous locomotor activity of P30 CitK +/- and Trp53 +/- mouse. Related to Figure 7.

Movie S2. Spontaneous locomotor activity of P30 CitK -/- and Trp53 +/- mouse. Related to Figure 7.

Movie S3. Spontaneous locomotor activity of P30 CitK -/- and Trp53 -/- mouse. Related to Figure 7.

Data S1. Differentially expressed genes, in pairwise comparisons of RNA-sequencing data obtained from P4 mouse cerebella of all the indicated genotypes. Related to Figure 6 and to Supplemental Table 1

Supplemental experimental procedures

Immunoprecipitations and western blotting.
For all immunoprecipitations, cells and tissue were extracted with lysis buffer containing 150 mM NaCl, 1 mM MgCl2 , 50 mM Tris pH 7, 1% NP40, 5% Glycerol protease inhibitors (Roche, Basel; Switzerland) and 1mM phenylmethylsulfonyl fluoride (PMSF). Antibodies and Dynabeads protein G (GE Healthcare Life Science, Little Chalfont, UK) were added to cleared lysates and incubated for 2 hours at 4°C. Pellets were washed four times with lysis buffer and analyzed by SDS-PAGE. When specified we added RQ1 DNAse at the concentration of 10U/ml (Promega, Madison, WI, USA). For immunoblots, immunoprecipitates or equal amounts of proteins from total cell lysates were resolved by reducing SDS-PAGE and transferred to nitrocellulose or PVDF filters that were then incubated with the indicated antibodies (see Supplemental Table S5) and developed using the the Luminata Forte HRP (Merck Millipore, Billerica, MA, USA).

Staining of mouse embryonic cortex and adult mouse brain.
Embryonic brains were dissected at E14.5 and fixed for 12-16 hours (h) at 4°C in. Postnatal mice were trans-cardially perfused with 4% PFA. Brains were post-fixed overnight at 4°C equilibrated in 30% sucrose in PBS for 12-24h at 4°C, embedded with Tissue-TEK (O.C.T, Sakura Finetek, Alphen aan den Rijn, The Netherlands), frozen in liquid nitrogen and stored at -20°C. Sectioning was then performed with a cryostat (20µm). For staining, cryo-sections were subjected to antigen retrieval using 10mM Na Citrate pH 6.5, 1% Glycerol in PBS. Sections were then permeabilized with 0.3% Triton X-100 in PBS for 30 minutes (min) and quenched with 0.1 M glycine for 30 min Sections were then incubated with primary antibodies overnight at 4°C, and then with secondary antibodies for 1 h at room temperature (RT) in a solution of 0.2% gelatin, 300 mMNaCl, and 0.3% Triton X-100 in PBS. DNA was stained in the last wash using 4´,6-diamidino-2-phenylindole (DAPI) 30 min at RT and sections were mounted using ProLong anti-fade reagent (Thermo Fisher Scientific, Waltham, MA, USA). Fluorescent Nissl staining was performed using the NeuroTrace™Fluorescent Nissl Stains (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's protocol.. Apoptosis was measured by the TUNEL assay using "In Situ cell death detection kit, TMD red" (Roche, Basel; Switzerland) according to manufacturer's protocol. Following TUNEL staining, sections were counterstained with DAPI and mounted using ProLong anti-fade reagent (Thermo Fisher Scientific, Waltham, MA, USA).

Cell culture.
HeLa and ONS-76 cells were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. 293T cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin cells. Embryonic brains were isolated from E12 timed pregnant mice. The lateral portion of the dorsal telencephalon was dissected, dissociated using a Papain based kit (Neural Tissue Dissociation Kit (P), Miltenyi Biotec, Bergisch Gladbach, Germany) and plated at the concentration of 5 x 10^4 cells/cm^2 on Poly-L-lysine/ Laminin-treated glass coverslip. Cells were analyzed 18h after plating. Culture medium comprised DMEM F12, supplemented with 2% B27 w/o Retinoic Acid,10ng/ml EGF and 40 ng/ml bFGF (all from Thermo Fisher Scientific, Waltham, MA). Cells were grown at 37° C in a humidified incubator with 5% CO2.

Plasmids, recombinant proteins, siRNAs and cell transfection.
The CITK -Cherry wild-type, MYC-CITK wild-type, MYC-CITN wild-type and MYC-CITKD constructs have been previously described(Gai et al., 2011). 293T cells were transfected with Trans-IT-LT1 transfection reagent (Mirus Bio, Madison, WI, USA Bio) according to manufacturers' instruction.
In this study, we used a previously validated siRNA sequence (CK1 =AUGGAAGGCACUAUUUCUCAA) (Gai et al., 2011). siRNAs were obtained from GE-Healthcare (Dharmacon, Lafayette, CO, USA). The ON-TARGET-plus non-targeting siRNA #1 was used as a negative control for potential off-target effects. HeLa or ONS-76 cells plated on a six-well plate were transfected using 1 µg of the required plasmid DNA and either 3 µl of Trans-IT-LT1 transfection reagent (Mirus Bio, Madison, WI, USA Bio), or 6.25 µl of the required siRNA (20 µM) and 2.5 µl Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA), according to manufacturers' specifications. For HeLa cells efficient knockdown was obtained after 48 h (Gai et al., 2011).

Antibodies.
We used the following antibodies: 53BP1 (Abcam), ATM (Cell Signaling Technologies, Danvers, MA), ATM / ATR substrate (Cell Signaling Technologies), Phospho ATM (Cell Signaling Technologies), ATR (Cell Signaling Technologies), Phospho ATR (Cell Signaling Technologies), Calbindin (Swant, Marlyn, Switzerland), Calretinin (Swant), Citron (Mouse, BD – Transduction laboratories), Citron (Rabbit, Abcam), Cyclin B1 (Santa Cruz Biotechnology), H2AX (Cell Signaling Technologies), PH3 (Rat, Abcam), HSP90 (Santa Cruz Biotechnology, Dallas, TE), Myc (Home-made from clone 9E10), Nestin (Abcam), p21 (Santa Cruz Biotechnology), p53 (Mouse clone 1C12, Cell Signaling Technologies), Phospho p53 (ser15, Cell Signaling Technologies), RAD51 (GeneTex), RPA32/RPA2 (Phospho S4+S8, Abcam), TUJ (Covance, Princeton, NJ), TUJ 488 Alexa labeled (Covace), Vinculin (Home-made),

αTubulin (Sigma-Aldrich), γH2AX (Rabbit S139, Cell Signaling Technologies), γH2AX (Rabbit 20E3, S139, Cell Signaling Technologies), γH2AX (Mouse clone 9F3).

***In vivo* and *in vitro* X-ray treatment.**
X-ray treatment was performed using a RADGILL irradiator Stationary anode X-ray tube, 200kV (Gilardoni, Mandello del Lario Lecco, Italy). Newborn mice were irradiated with 12 Gy, and sacrificed after 30' or 5 h; cerebella were extracted and processed for Western blotting. HeLa cells were irradiated at indicated doses and then processed for immunofluorescence at differentpost irradiation times.

Clonogenic assay.
24 h after transfection of CITK-specific and control siRNA, HeLa cells were irradiated at the indicated doses and seeded in six-well plates 30 min later, at the concentration of 500 cells per well. Cells were cultured for 10 to 14 days to allow for colony formation. Colonies were washed twice in PBS, stained for 15 min with Nissl staining (0.1% Cresyl Violet Acetate, 0.6% glacial acetic acid) and rinsed in water. The surviving fraction was determined by counting colonies of more than 50 cells.

Metaphase Spread Analysis.
Two days after transfection with appropriate siRNA, ONS-76 cells were treated with KaryoMAX Colcemid Solution (0.1 μg/ml, GIBCO), for 90 min. Cells were hypotonically swollen with 0.075 M KCL (15 min, 37° C), fixed with cold methanol/acetic acid (3:1) for 10 minutes at RT, dropped onto microscope slide and flame burned for two second. The slides were dried at 60°C for 1h and then stained with DAPI and mounted with Prolong (Thermo Fisher Scientific, Waltham, MAnologies, Carlsbad, CA, USA). Metaphases were imaged using a HCX PL APO 63x/1.4 NA OIL immersion objective on a Microscope Nikon ViCo. Our karyotypic analyses revealed that most metaphases of control and CITK-depleted cells contain between 74 to 80 chromosomes.

Cell staining.
For RAD51, γH2AX and 53BP1 immunostaining cells were fixed 5 min at RT using PFA 2% then treated 10 min at RT using CSK buffer [100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 10 mM PIPES (pH 6.8)] 0.7% Triton, and finally fixed again 5 min at RT using PFA 2% . Cells were then permeabilized in 0,1% Triton X 100 in PBS for 10 min, saturated in 5% BSA in PBS for 30 min and incubated with the desired primary antibody (see Table1) for 1h at RT. Primary antibodies were detected with anti–rabbit Alexa Fluor 488 or 568 (Molecular Probes, Thermo Fisher Scientific, Waltham, MA), anti–mouse Alexa Fluor 488 or 568 (Molecular Probes, Thermo Fisher Scientific, Waltham, MA) used at 1:500 dilution for 30 min Cells were counterstained with 0.5 μg/ml DAPI for 10 min and washed with PBS.

***Drosophila* strains.**
The dck^1 and dck^2 mutant alleles, were described in (Gatti and Baker, 1989; Naim et al., 2004). Both alleles were kept in stocks over the balancer chromosome TM6 C carrying the dominant markers *Stubble (St)* and *Tubby (Tb).* The tsr^1 (Gunsalus et al., 1995) and sqh^1 (Karess et al., 1991) mutant alleles were kept balanced over the *FM7-TbA, (*carrying the dominant markers *Bar* and *Tb*; (Lattao et al., 2011)) and *TSTL* (carrying the dominant markers *Curly (Cy)* and *Tb)* chromosomes, respectively (for details, see http://flybase.bio.indiana.edu/). Homozygous mutant larvae were always recognized for their non-*Tubby* phenotype. All stocks were maintained on standard *Drosophila* medium at 25ºC.

***Drosophila* chromosome cytology and γH2Av immunostaining.**
To analyze metaphase chromosomes, brains from third instar larvae were dissected in saline (NaCl 0.7) and incubated for 1h with colchicine (10^{-5} M in saline). Brains were treated for 8 min with hypotonic solution (0.5% Na Citrate), squashed in 45% acetic acid under a 20 x 20 mm coverslip and then frozen in liquid nitrogen. After removal of the coverslip, preparations were mounted in Vectashield H-1200 (Vector Laboratories), containing the DNA dye DAPI. To test X-ray sensitivity, wild type and homozygous *dck* mutant larvae were irradiated with 2.5 Gy and analyzed as above 3 h after irradiation. For immunostaining, brains from third instar larvae were dissected and fixed as previously described in (Bonaccorsi et al., 2000). Brain preparations were then rinsed in PBS containing 0.1% Triton-X (PBST), incubated overnight at 4°C with rabbit anti-Histone H2AvD pS137 (1:100 in PBST; Rockland code #600-401-914), rinsed in the same buffer and then incubated for 1h at RT with Alexa-Fluor-555-conjugated anti-rabbit secondary antibody (1:300 in PBST; Molecular Probes). After two rinses in PBST preparations were mounted in DAPI-containing Vectashield H-1200 (Vector Laboratories). To quantify the γH2Av-positive foci at least 800 cells were analyzed from at least 3 brains. All cytological preparations were examined with a Zeiss Axioplan fluorescence microscope, equipped with an HBO100W mercury lamp and a cooled charged-coupled device (CCD camera; Photometrics CoolSnap HQ). Images were converted to Photoshop (Adobe System), pseudo-colored and merged.

RNA-sequencing and bioinformatic analysis.
RNA-seq was performed as previously described (Neri et al., 2015) with some modifications; RNA quality was checked using the Bioanalyzer instrument. Libraries were prepared from total RNA using TruSeq RNA Sample Preparation v2

according to the Illumina's protocol and sequenced on Illumina HiScanSQ platform. Sequencing reads were trimmed out of the low-quality bases with Fastx Toolkit. Filtered sequences were mapped on mm9 genome assembly by using TopHat v2.0.6 (Trapnell et al., 2009) and mRNAs quantification were performed using Cuffdiff v2.0.2 (Kim et al., 2013). For downstream analysis, genes with RPKM < 1 in all the samples were filtered out. Primary data have been deposited in the Gene Expression Omnibus database with accession number GSE83465. Gene Ontology was analysed using DAVID web software (Huang et al., 2009).

Mice behavior testing.
An activity cage (Ugo Basile Biological Research Apparatus, Varese, Itlay) was used to track the movement in horizontal or vertical direction using an infrared sensor. After a two h acclimation period, animals were individually tracked for 24 h.

Mice EEG.
We recorded quantitative Electro-Encephalogram (qEEG) at three different ages: 12 days, 4 weeks and 12 weeks. For each recording, a 3 min period of artefact-free background EEG was selected. Subsequent power spectral analyses were performed by means of LabChart™ 6.2 software (ADInstruments™). Each period was subjected to Fast Fourier Transform (FFT), epochs of 4s, tapered with Hanning window; the frequencies spectrum of the EEGs used for the FFT ranged from 0,5 to 20Hz. Absolute power values were normalized to the total absolute power to obtain relative values, comparable among the different recordings. Mean dominant frequency (MDF), defined as the "center of mass" of a frequency band, was extracted from power spectra within delta (0.5-4 Hz), theta (4-8 Hz),alpha (8-12 Hz) and beta (12-20 Hz) bands using the formula:
$MDF = \sum (P_i \cdot f_i) / \sum P_i$
where P_i is the power at frequency f_i and i is the index sweeping the whole frequency band samples.

Microscopy and image analysis in mouse and human cells
Imaging was performed using a Leica TCS SP5-AOBS 5-channel confocal system (Leica Microsystems GmbH, Germany) equipped with a 405nm diode, an argon ion, a561nm DPSS and a HeNe laser. Fixed cells were imaged using a PL APO 40x/1.2 NA oil immersion objective. All the images were analyzed by using Fiji Software. Foci were quantified using the command "Find Maxima" after the appropriate setting of the threshold and noise. Colocalization analysis was performed using the Fiji's plugin "Coloc 2", and calculating the Mander's Overlap Coefficient (MOC) of the RAD51 signal over γH2AX signal. MOC, similarly to Pearson Correlation Coefficient is used to quantify the degree of colocalization between fluorophores. MOC value varies between 0 and 99, with 0 corresponding to mutually exclusive signals and 99 to complete overlap. For ploidy measurements, nuclei were manually identified as regions of interest (ROI) in maximum projections of confocal stacks and "integrated density" was measured for each ROI. Diploid and tetraploid nuclear fluorescence intensities were defined by analyzing the distribution in control cultures.

Supplemental references

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