SUPPLEMENTARY METHODS AND FIGURES

Kinetics of endogenous mouse FEN1 in base excision repair.

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Supplementary methods

(Material and methods for Figure S1A-F are also found in the main article).

Mice, organs, RNA and protein isolation

Tissues, snap frozen in liquid nitrogen or on dry ice, were homogenized using Lysing Matrix D in either Trizol or modified RIPA buffer (20 mM Tris HCl, 150 mM NaCl, 0,5 mM EDTA, 0.5 % NP-40, 0.1 % SDS, + phenylmethanesulfonylfluoride (PMSF) and protease inhibitor cocktail (PIC) added fresh), for total RNA and whole protein cell extract (WCE), respectively, in a FastPrep FP120 homogenizer (Thermo, Electron Corporation). For RNA isolation the TRIzol Plus RNA Purification/PureLink Micro-to-Midi Total RNA Purification kit was used (Invitrogen, Catalog # 12183-555). Concentration of isolated RNA was measured on Nanodrop spectrophotometer (Saveen Werner), aliquoted and stored at -80°C. For WCE the lysed cells were incubated in RIPA buffer on ice for 30 minutes, followed by centrifugation at 13200 rpm, at 4°C, for 15 minutes. The protein concentration was measured with the BioRad protein assay, followed by storage of protein aliquots at -80°C. Imaging of FEN1-YFP was performed on organotypic slices of brain and skin. Organotypic slices of brain and liver were produced by cutting 300 µm of the organ with a TissueChopper (McIllwain). The brain slices were kept in Neurobasal-A medium. Skin from the back of an adult mouse was prepared by pulling out hair and cutting out small squares for microscopy, hairside facing the glass in a small amount of cell culture medium (CnT07, Bioconnect) to avoid drying. Intestine was dissected out, flushed, a 2-3 cm long piece was cut open and placed with the muscle facing up and villi down on the glass for imaging within 1 hour. Both brain and skin were imaged within 2 hours following preparation. Testis, spleen and kidney were cut in half (testis) or sliced with a scalpel before imaging within 2-4 hours, while the whole thymus was imaged within 2-4 hours. All organs were kept in Neurobasal-A medium on ice before imaging.

Western blot analysis

Western analysis was carried out on WCE from spleen, thymus, testis and lung as previously described (1). Primary antibodies used for the analysis were: a polyclonal rabbit anti-FEN1 (Cat. # ab17993, Abcam), diluted 1:10 000, and a monoclonal rat

anti-HA (Clone 3F10, Cat. # 11 867 423 001 Roche), diluted 1:500, with over night incubation at 4°C. The secondary antibodies goat anti-rabbit IgG AP (Sigma), diluted 1:30 000, and goat anti-rat (Southern Biotech), diluted 1:2000, were incubated for 1 hour at room temperature. After antibody incubations, the PVDF membranes were washed 2 times in 0.1 % PBS-T, and once with 0.9 % NaCl / 0.1 M Tris buffer, both for 10 minutes at room temperature. ECF substrate was added and KODAK Image Station 4000MM Pro used for fluorescence detection. Image processing was performed with the Image Quant program.

Quantitative real-time PCR

The cDNA template was produced from 1.7 μg DNase treated (Turbo DNA free kit from Ambion) total RNA in a 20 μl cDNA reaction volume, using the High capacity cDNA reverse transcription kit (Applied Biosystems). For SYBR green quantitative real-time (qRT) PCR, 2 μl 1:10 diluted cDNA for each sample was used per reaction. The real-time PCR program was run on a StepOnePlus (Applied Biosystems) machine; using Power SYBR green PCR 2x master mix (Applied Biosystems). Standard run conditions were used, including a one step holding stage at 95°C for 10 minutes, a two step cycling stage with 40 cycles of one step at 95°C for 15 seconds followed by a step at 60°C for 1 minute, and ending with a three step melt curve stage, 95°C for 15 seconds, 60°C for 1 minute and 95°C for 15 seconds. Primers used for qRT-PCR on target gene *Fen1* and reference gene *Gapdh* are given in Supplementary Table 1. At least four samples each of wt *Fen1* and knock-in $Fen1^{y/y}$ spleen samples were analyzed, and qRT-PCR was performed in triplicate and was repeated in three separate experiments. P-values were calculated using the two-tailed unpaired Student's T-test with equal variances.

FEN1 endonuclease activity on a synthetic DNA flap substrate

Nuclear extracts used for the flap assays were prepared as described previously (2). The flap assay was performed with the oligonucleotide sequences provided in the supplementary Table I, by the method previously described (3).

Immunofluorescence

For Ki-67-staining of MEFs, cultured *Fen1*$^{y/y}$ primary MEFs were washed with PBS, fixed, permeabilized and blocked in PBS+ as described in the main manuscript (Immunofluorescence). Primary antibody was anti Ki-67 (Abcam, rabbit polyclonal) at 1:500 dilution, and secondary antibody was Alexa Fluor 633 goat anti-rabbit (Invitrogen, Molecular Probes) at 1:400 dilution in PBS+. Ki-67 and DAPI stained *Fen1*$^{y/y}$ primary MEFs were imaged using the Zeiss LSM 710 multiphoton laser scanning microscope. For immunofluorescent staining of paraffin sections, see Materials and methods in main manuscript.

Immunohistochemistry

Immunohistochemistry was performed on cerebellum from postnatal day 7.5 (p7.5) mice. The tissue was fixed with 10 % buffered formalin (21154, Chemi-Teknik) and embedded in paraffin. 4 µm sections were deparaffinised in Clear Rite 3 (6901, Richard Allan Scientific) and rehydrated. The sections were treated with antigen retrieval buffer pH 9.0 (Tris-EDTA) at 95ºC for 25 minutes in microwave oven and permeabilized in 0.1 % Triton X-100 (T8787, Sigma) for 15 minutes at room temperature. To detect FEN1, rabbit polyclonal anti-FEN1 (ab17993, Abcam) was used at 4 µg/ml at 4ºC over night. For visualization EnVision + HRP (DAB) (K4010, Dako) was used. Monoclonal mouse anti-rat Ki-67 antigen, clone MIB5 (M7248, Dako) at 4.5 µg/ml at 4ºC over night was used to detect proliferation after blocking of endogenous avidin/biotin activity with Endogenous avidin/biotin blocking kit (00-4303, Zymed Histochemical Reagents). Dako ARK (Animal Research Kit) Peroxidase for Mouse Primary Antibodies (K3954, DAKO) was used for visualization. The sections were stained with hematoxylin, dehydrated in alcohol and cleared in Clear Rite 3 (6901, Richard Allan Scientific). Mounting medium (4111, Richard Allan Scientific) and coverslips were applied. The sections were examined with light microscope (Axioplan 2 Zeiss mounted with an AxioCam HRc Zeiss). Positive signal stained brown.

Cell line stably expressing GFP-Ku80

Chinese hamster lung fibroblast cell line XR-V15B, deficient in non-homologous end-joining protein Ku80 and stably expressing GFP-Ku80 (4), was cultured (as described in (4) and reference therein) and used for our "high" laser power local damage experiments (Supplementary data Figure S1N).

Tables

Supplementary Table I Sequences of primers and oligonucleotides used in this study

Methods	Sequence (5'-3')	Details (paired with number)
PCR verification of homologous recombination	1. CACTCTGTGACAGATGGTGCCATTGC	*Fen1* forward intron 1-2, 5'-3' targeting, PCR (5.) (Data not shown)
PCR genotyping and verification of homologous recombination	2. GCGAGCCAAATGAAGAAGAG	*Fen1* forward exon 2 wt/knock-in allele genotyping (8.) and 5'-3' targeting, PCR (6.)
PCR verification of homologous recombination	3. AAAGACTGGGGGAGCGGGGAAGTTCCGAAG	*Fen1* forward exon 2 wt/knock-in allele, 5'-3' targeting, PCR (5.)
PCR verification of homologous recombination	4. CACAACGTCTATATCATGGCCGACA	*YFP* forward knock-in allele, 5'-3' targeting, PCR (7.)
PCR verification of homologous recombination	5. CCCCCACCGTATAGTGGAAT	*Fen1* reverse wt/knock-in allele, 5'-3' targeting, PCR (1. and 3.)
PCR verification of homologous recombination	6. AAGGCATGGCAGGTAAAAGA	*Fen1* reverse wt/knock-in allele, 5'-3' targeting, PCR (2.) (Data not shown)
PCR verification of homologous recombination	7. GGGGGGGGACAACTCAAATTTAATCTGGTA	*Fen1* reverse wt/knock-in allele, 5'-3' targeting, PCR (4.)
PCR genotyping	8. GAACTTCAGGGTCAGCTTGC	*YFP* reverse knock-in allele (2.)
PCR genotyping	9. GGCATTAAGTGTGCCACTGACCT	*Fen1* forward wt allele (10.)
PCR genotyping	10. GCTGCTGTGGCCACCTTACAT	*Fen1* reverse wt allele (9.)
PCR genotyping	11. TCAAGACCGACCTGTCCGGTG	*Neo* reverse knock-in allele (9.) (Data not shown)

Quantitative real-time PCR	12. TCAGCAATTAGTTTGGCAAGGCCG	SYBR green *Fen1*
Quantitative real-time PCR	13. ATTCGCTCTGCTCCGAACATTCCT	SYBR green *Fen1*
Quantitative real-time PCR	14. TCGTCCCGTAGACAAAATGGT	SYBR green *Gapdh*
Quantitative real-time PCR	15. CGCCCAATACGGCCAAA	SYBR green *Gapdh*
DNA flap substrate, 16-mer	16. CAGCAACGCAAGCTTG	The three oligonucleotides 16.-18. align to form the flap substrate. The 19+14 nt oligonucleotide is 5'^{32}P labeled to reveal flap endonuclease activity.
DNA flap substrate, 19+14-mer	17. ATGTGGAAAATCTCTAGCAGGCTGCAGGTCGAC	See 16.
DNA flap substrate, 30 nt	18. GTCGACCTGCAGCCCAAGCTTGCGTTGCTG	See 16.
Probe for Southern, 3' verification of homologous recombination	19. GTAGAGGGAGGGCAGGTCT	*Fen1* 3'-targeting, Southern (20.)
Probe for Southern, 3' verification of homologous recombination	20. GGTGACAGATGGGTCTGCGG	*Fen1* 3'-targeting, Southern (19.)

Legends to supplementary figure

Figure S1

A. Verification of 3' homologous recombination in ES cells. Southern blot hybridization of wild-type and *Fen1*$^{yNeo/WT}$ ES cells. Expected DNA fragment sizes for wild-type and knock-in ES after restriction enzyme digestion are indicated, together with location of the 3' probe. See B. for location of BamHI restriction sites in the ES cell DNA.

B. 5'-3' targeting PCR to verify correct targeting of the knock-in *Fen1-YFP* construct and Cre-excision of *Neo*. Primers P3 (*Fen1* forward exon 2) and P5 (reverse in 3' region downstream *Fen1*, and downstream *YFP* and *Neo*) amplified one (homozygous) or two (heterozygous) DNA fragments, with or without *Neo*, in the 5'-3' targeting PCR.

C. Location of PCR primers within the mouse *Fen1* locus and knock-in construct.

D. PCR verifying germline transmission of the targeted *Fen-YFP* allele without *Neo* after Cre excision. Primers P4 (*YFP* forward) and P7 (*Fen1* reverse) amplified a DNA fragment 3' of *YFP* with or without *Neo*.

E. PCR genotyping of a typical litter after heterozygous mating ($Fen1^{WT/y}$ x $Fen1^{WT/y}$). Primers P2 (*Fen1* forward exon 2) and P8 (*YFP* reverse) amplified a DNA fragment from the knock-in allele, while P9 (*Fen1* reverse) and P10 (*Fen1* forward) amplified a fragment from the wild-type allele.

F. Expression of the FEN1-$YFP^{His6\text{-}HA}$ fusion protein in heterozygous and homozygous knock-in mice (with or without *Neo* removed, as indicated). Representative western blot analysis with 60 µg of whole cell extracts (WCE) from spleen, testis and lung of mice of the indicated genotypes. (Wild-type (spleen only), $Fen1^{yNeo/WT}$ (spleen, testis, lung), $Fen1^{WT/y}$ (spleen, testis, lung) and $Fen1^{yNeo/yNeo}$ (spleen only).) The anti-HA antibody detects the 70kDa FEN1-$YFP^{His6\text{-}HA}$ fusion protein. Anti-HA also binds to a smaller, unspecific band. Anti-GAPDH detects the 36kDa GAPDH protein, and is used as a loading control.

G. Quantitative real-time PCR (qRT-PCR) confirmed no significant difference in expression of *Fen1* and *Fen1-YFP* mRNA. We compared the relative *Fen1*/*Gapdh* expression in total RNA from spleen from at least four wild-type mice and four $Fen1^{y/y}$ mice, using two-tailed unpaired Student's T-test with equal variances, $P>0.05$. The qRT-PCR samples were run in triplicates and repeated in three different experiments.

H. Flap assay with nuclear extracts from wild-type and $Fen1^{y/y}$ testis and MEFs. No significant difference in flap cleavage activity could be observed between FEN1 and FEN1-YFP proteins.

I. Endogenous expression and immunofluorescence anti-Ki-67 demonstrating FEN1-$YFP^{His6\text{-}HA}$ fusion protein expression in proliferating primary mouse embryonic fibroblast (MEF) cells. $Fen1^{y/y}$ primary MEFs were stained with an antibody anti-Ki-67 (proliferation marker, red) and DAPI (staining nuclear DNA, turquoise). In $Fen1^{y/y}$ primary MEFs both endogenous eYFP (green) signal from the fusion protein and Ki-67 immunostaining were detected in the nucleus. The cells were analyzed using a Zeiss LSM 710 microscope with a 40x/1.3 objective.

J. FEN1 and Ki-67 expression in proliferating cells of the external granule layer (EGL) in sections of mouse cerebellum (P7.5). No significant difference in FEN1 or Ki-67 (both brown, see indicated antibody next to panels) expression could be observed between wild-type mice and $Fen1^{y/y}$ mice. Stained cryo-sections from one representative $Fen1^{y/y}$ mouse cerebellum and one representative wild-type mouse cerebellum are shown, along with negative controls as indicated. There is no EGL in the mature cerebellum of the adult mouse. The scale bars for 20x and 63x zoom are 50 µm and 20 µm, respectively.

K. Immunofluorescent staining of FEN1, PAR, NeuN, GFAP, PARP1 and Ki-67 expression in wild-type and $Fen1^{y/y}$ mouse cerebellum (p7.5) shown at 40x and 100x (not PARP1) magnification. The scale bars for 40x and 100x zoom are 50 µm and 20 µm, respectively.

L. FEN1-YFP expression in organs from a 6 weeks old $Fen1^{y/y}$ mouse. Kidney, spleen and testis were imaged using a multiphoton LSM 710 microscope. Transmitted light (DIC in grey)

and YFP is shown merged in the left panels, and YFP expression (green) alone in the middle and right panels, the latter is a higher magnification of a part of the organ (indicated by the dashed white boxes/lines). The scale bar is 200 μm.

M. FEN1-YFP nuclear expression in *Fen1*$^{y/y}$ primary MEFs with and without genotoxic treatment. Transmitted light (DIC in grey) visualized the cellular shape, including cytoplasm, and YFP (green) visualized FEN1-YFP, which was found in the nucleus. Representative images of *Fen1*$^{y/y}$ primary MEFs are shown, including untreated cells and cells treated with either MMS, H_2O_2 or $KBrO_3$ The scale bar is 10 μm.

N. FEN1-YFP showed a strong accumulation to "low" power laser-induced DNA damage, whereas the double strand break (DSB) repair protein Ku80 barely accumulated with "low" power settings. "High" power laser settings give accumulation of both FEN1 and Ku80 to the site of local damage. "Low" laser power corresponds to the settings used to induce local damage and see accumulation of FEN1 in *Fen1*$^{y/y}$ MEFs (this study, Figure 2). "High" laser power corresponds to the settings used to induce local damage with accumulation of Ku80 to DSBs in a previous study (4).

Reference List

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2. Klungland,A., Rosewell,I., Hollenbach,S., Larsen,E., Daly,G., Epe,B., Seeberg,E., Lindahl,T. and Barnes,D.E. (1999) Accumulation of premutagenic DNA lesions in mice defective in removal of oxidative base damage. *Proc. Natl. Acad. Sci. U. S. A*, **96**, 13300-13305.

3. Spiro,C. and McMurray,C.T. (2003) Nuclease-deficient FEN-1 blocks Rad51/BRCA1-mediated repair and causes trinucleotide repeat instability. *Mol. Cell Biol.*, **23**, 6063-6074.

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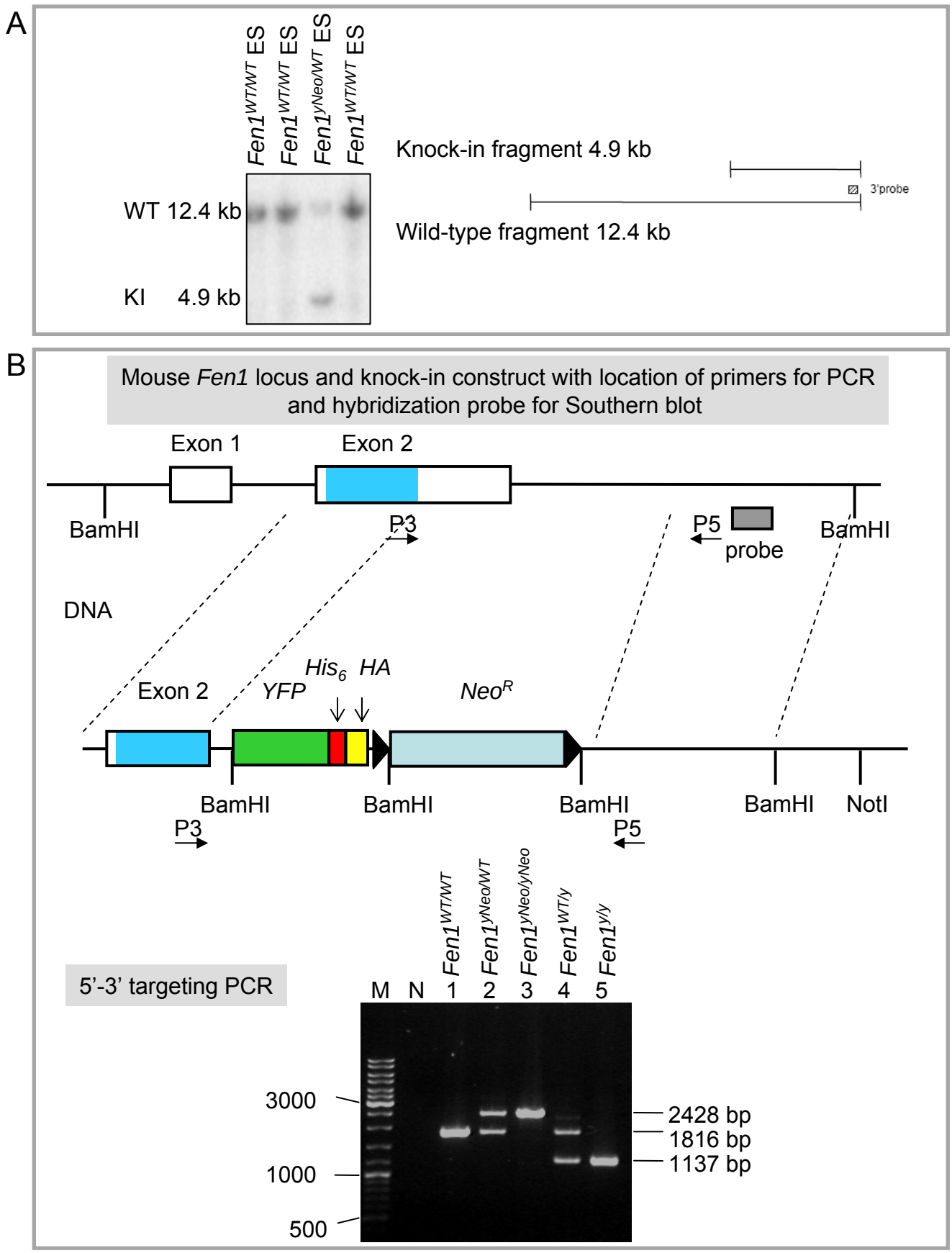
Supplementary info Figure S1

Southern blot analysis for 3' homologous recombination in ES cells



Supplementary info Figure S1

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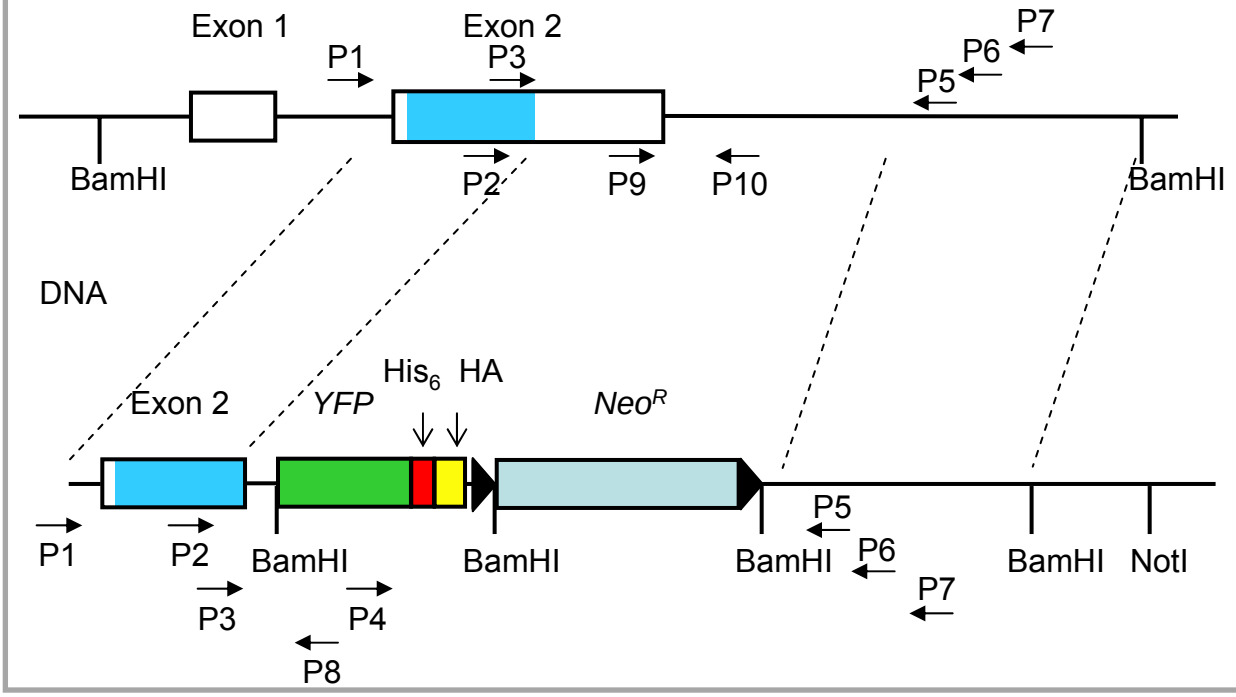

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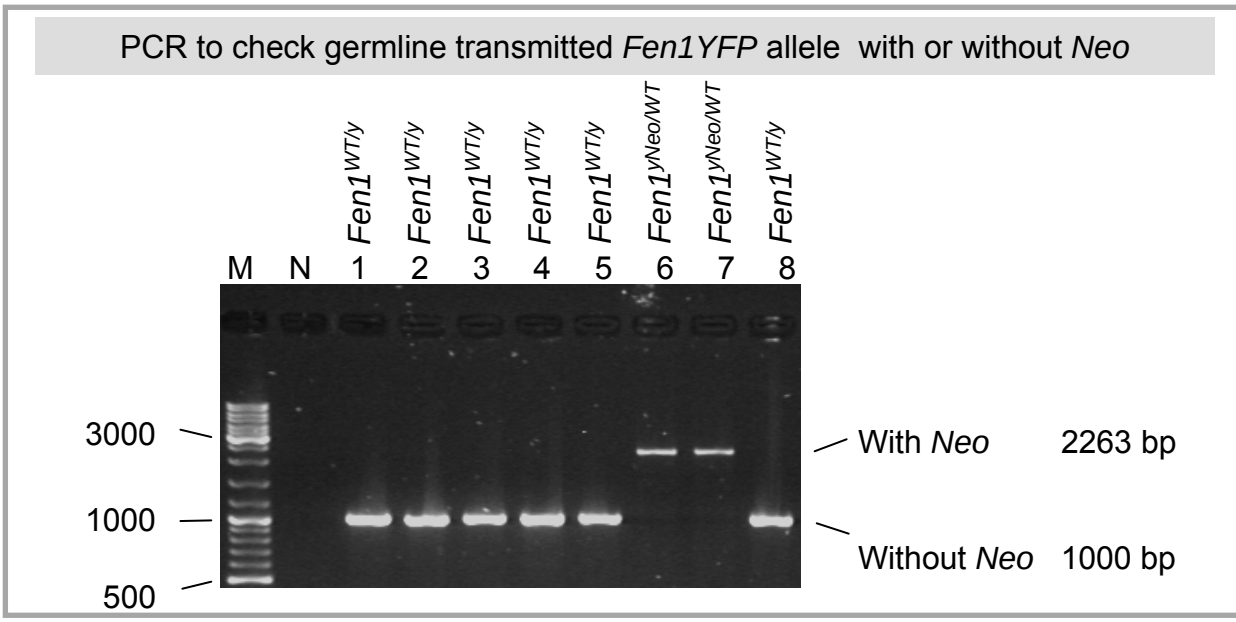

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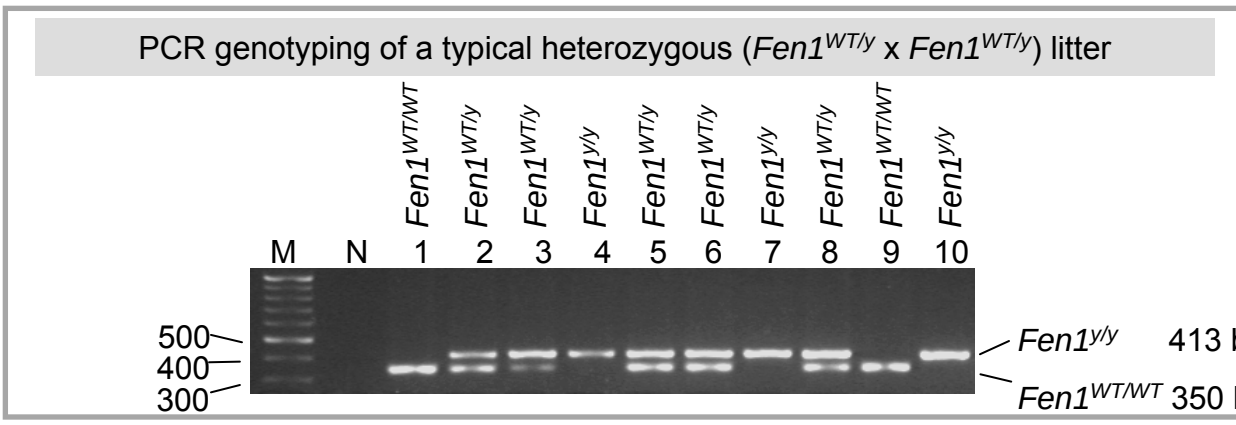

Supplementary info Figure S1

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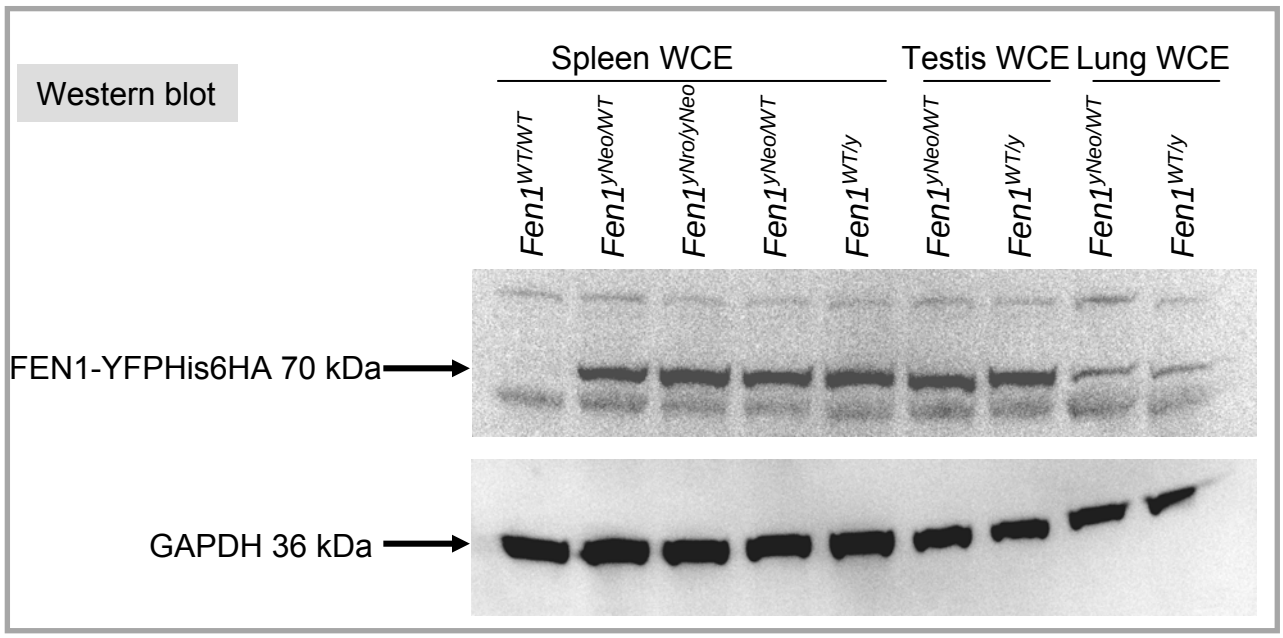

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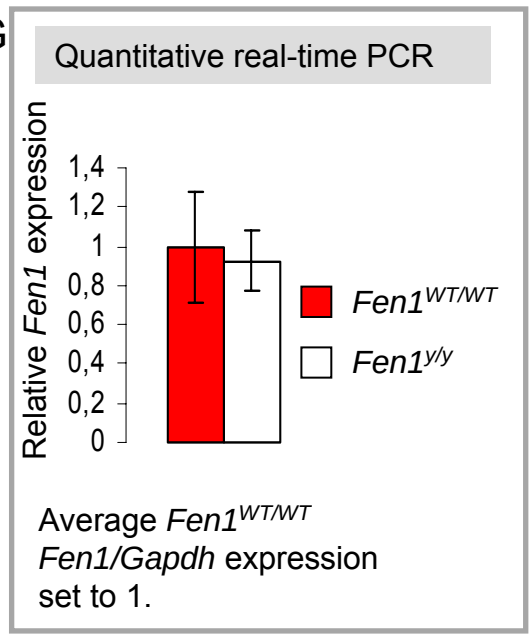

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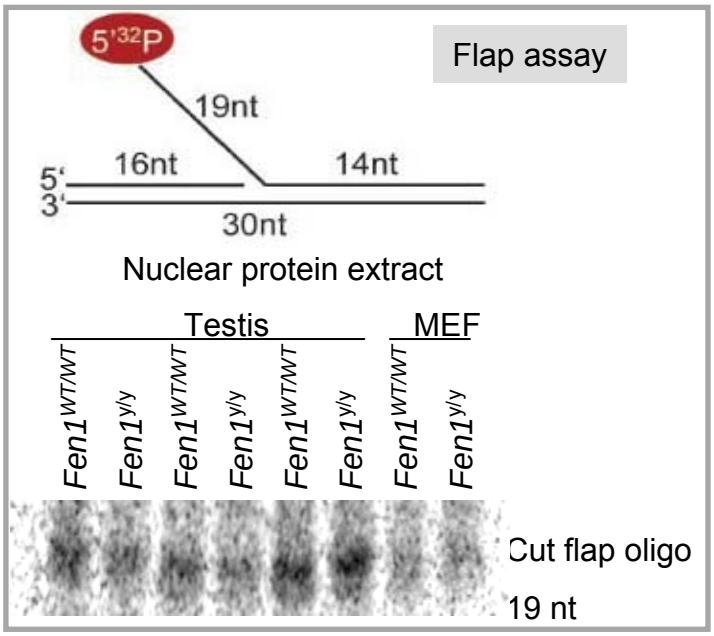

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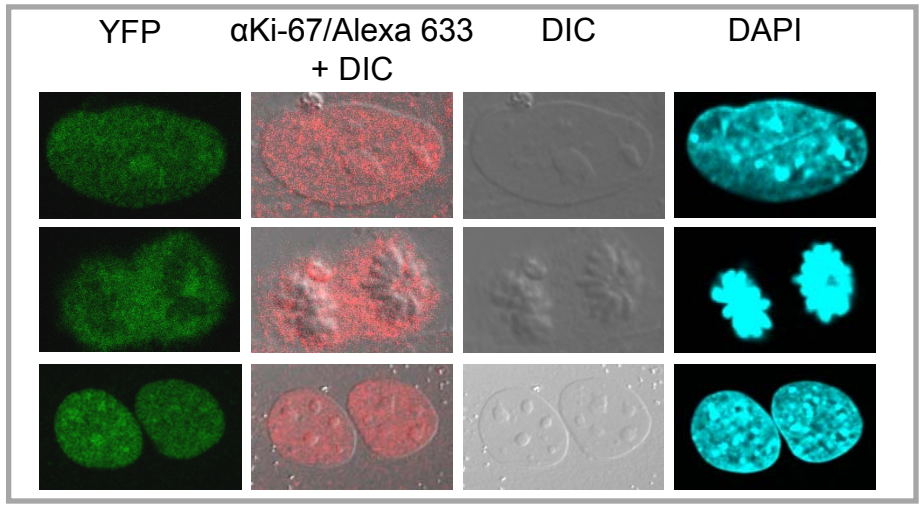
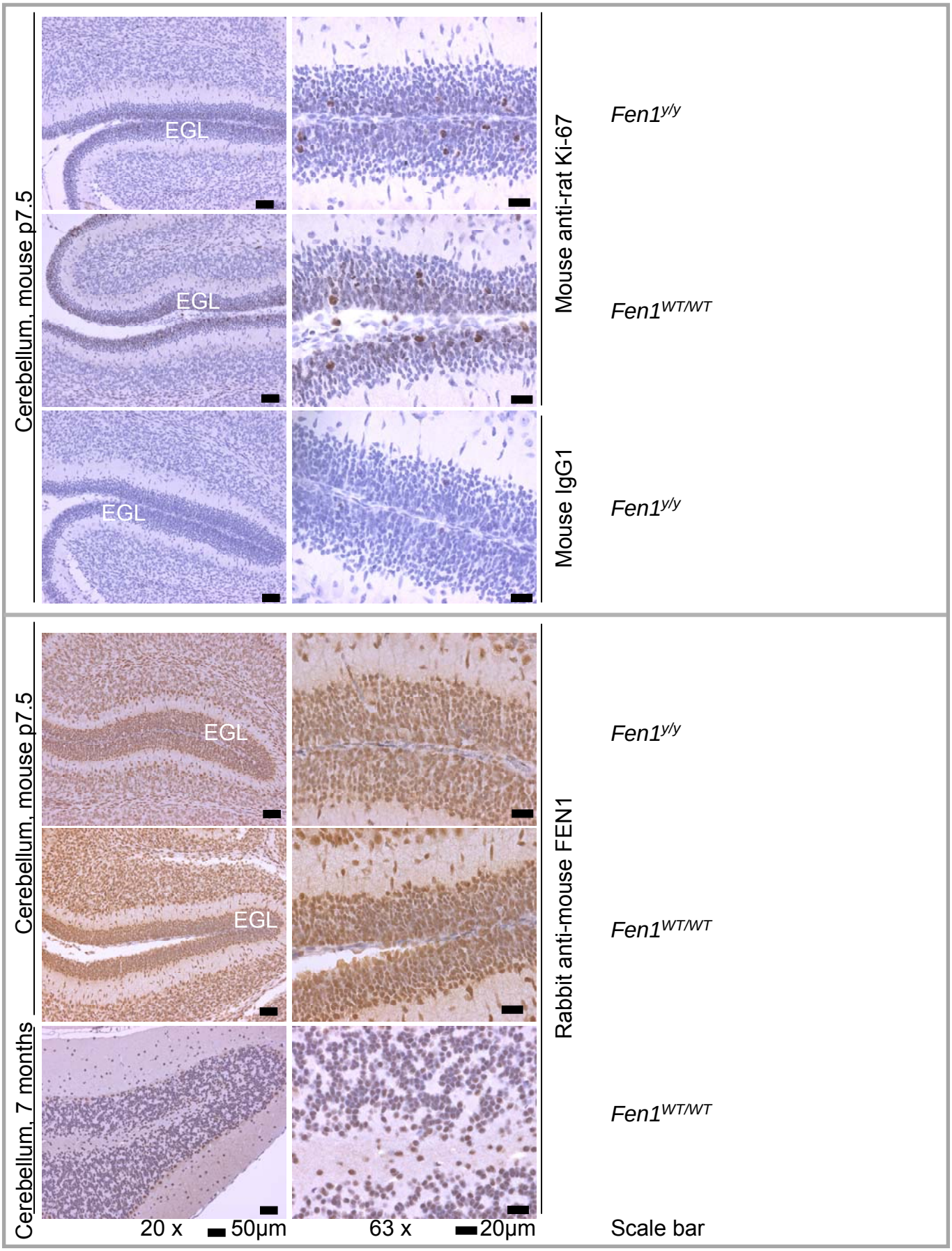


Supplementary info Figure S1

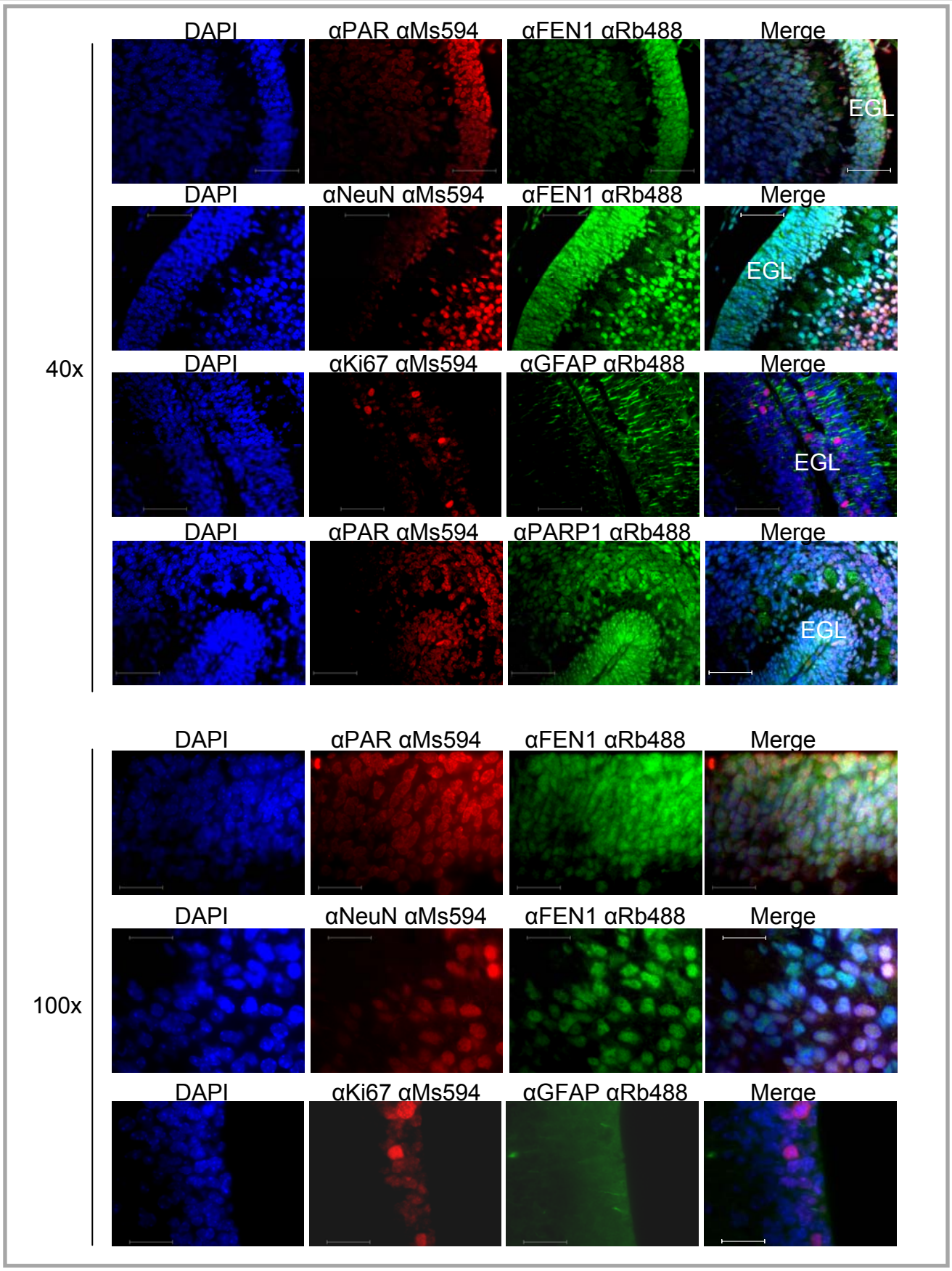
FEN1 and Ki-67 expression in proliferating cells of wild-type and *Fen1$^{y/y}$* mouse cerebellum (p7.5)

J



Supplementary info Figure S1

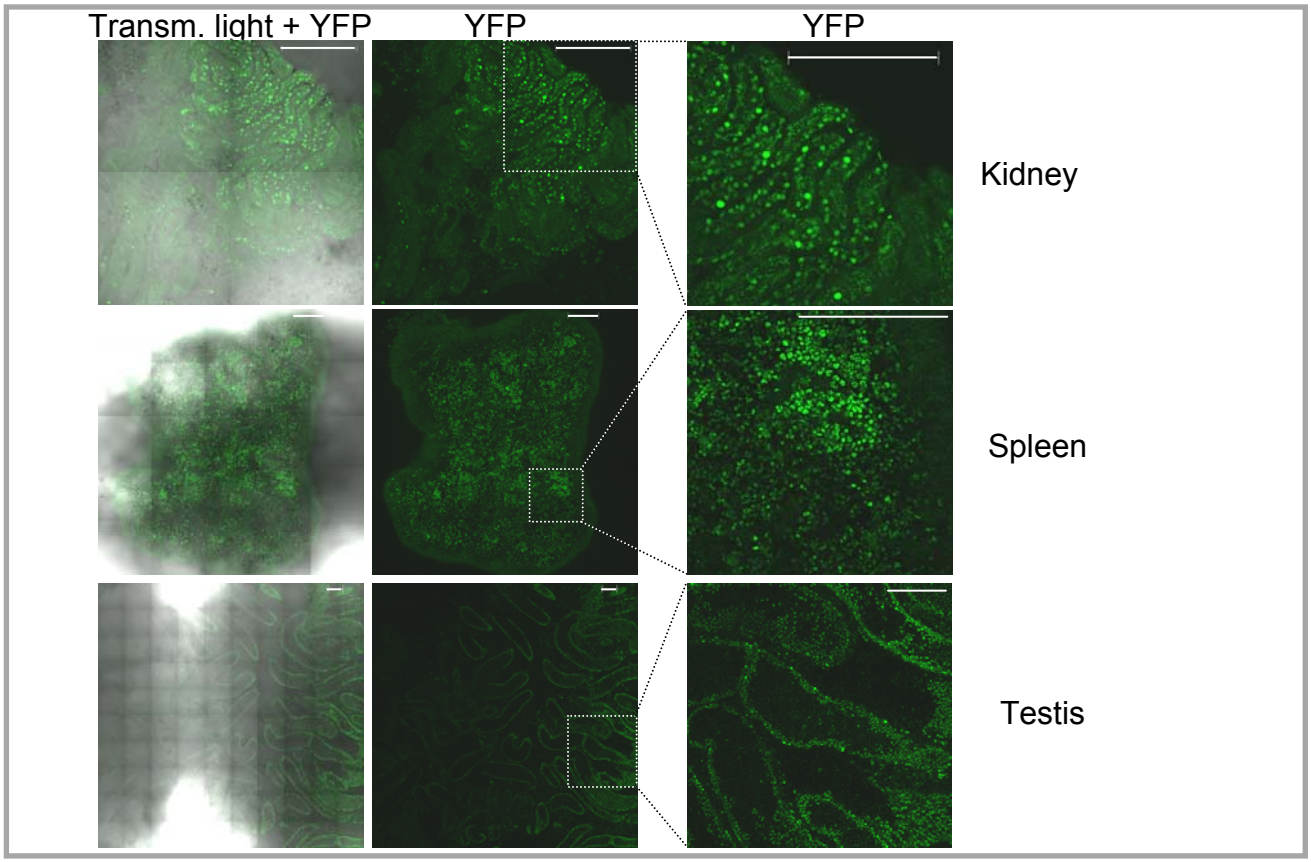
FEN1, PAR, NeuN, GFAP, PARP1 and Ki-67 expression in wild-type and *Fen1*$^{y/y}$ mouse cerebellum (p7.5)



Supplementary info Figure S1

FEN1 expression in *Fen1*$^{y/y}$ mouse organs

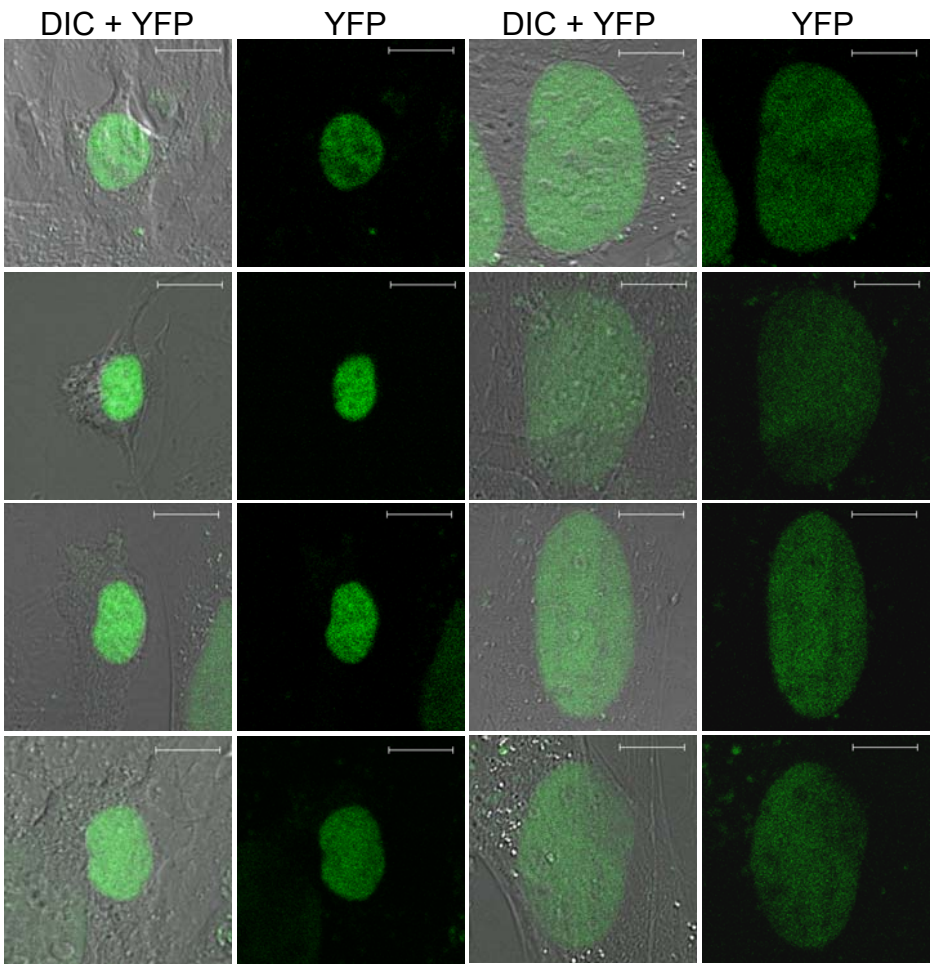
L



Supplementary info Figure S1

FEN1-YFP nuclear expression in *Fen1$^{y/y}$* primary MEFs with and without genotoxic treatment

M



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FEN1-YFP and GFP-Ku80 accumulation to ”low” vs ”high” power laser-induced DNA damage