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Phenotypic Characterization of Parp-1 and Parp-2 Deficient Mice and Cells 2 3

Christian Boehler, Laurent Gauthier, Jose Yelamos, Aurélie Noll, Valérie Schreiber, and Françoise Dantzer 4
5

Abstract 6

Poly(ADP-ribosyl)ation is a post-translational modification of proteins mediated by Poly(ADP-ribose) polymerases (Parps), a family of 17 members. Among them, Poly(ADP-ribose) polymerase-1 (Parp-1) and Parp-2 are so far the sole enzymes whose catalytic activity has been shown to be induced by DNA strand breaks. The generation and characterization of Parp-1 and Parp-2 deficient cellular and animal models have largely contributed to describe both proteins as active players of the base excision repair/single-strand break repair (BER/SSBR) process with both redundant and more specific functions. Double Parp-1^{-/-}Parp-2^{-/-} embryos die at gastrulation demonstrating the crucial role of poly(ADP-ribosyl)ation during embryonic development, whereas a specific female lethality related to X chromosome instability is associated with the Parp-1^{+/-}Parp-2^{-/-} genotype. Finally, recent research discovered emerging unique functions of Parp-2 in physiological processes including spermatogenesis, T-cell maturation, and adipogenesis although with distinct mechanisms. In this chapter, we describe standard operating procedures used to genotype and phenotype both mouse lines and the derived mouse embryonic fibroblasts. 7
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Key words: Poly(ADP-ribose) polymerases, Genome integrity, DNA damage, Knockout mice, Mouse embryonic fibroblasts 19
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1. Introduction 21

Generation and characterization of Parp-1 and Parp-2 deficient mouse models and derived cell lines provided the Parp field with remarkable research models to experimentally elucidate the in vivo functions of both proteins. More particularly, they revealed both cooperative and emerging unique functions of Parp-1 and Parp-2 in various cellular pathways in which both proteins are involved including cellular response to DNA damage, cell proliferation, cell differentiation, cell death, energy metabolism, and tumorigenesis (for review, see refs. 1, 2). 22
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31 Both mutant models display sensitivity to ionizing radiation
32 and alkylating agents associated with defects in DNA-damage pro-
33 cessing and cell cycle progression but at different extent. Whereas
34 Parp-1^{-/-} mice and cells are more sensitive to high-dose ionizing
35 radiation and alkylating agents, Parp-2^{-/-} cells display hyper-
36 radiosensitivity to low-dose irradiation (0–2 Gy) (3). These results
37 support key shared protective roles of both Parps with regard to
38 cell survival and maintenance of genomic integrity but also reflect
39 possible specific functions of both enzymes in these pathways. This
40 is further strengthened by the observation that Parp-1 rather acts
41 at early steps of the repair process in the detection and signalling of
42 single-strand breaks and recruitment of XRCC1, whereas Parp-2
43 functions in later steps of the repair process.

44 Parp-1^{-/-}/Parp-2^{-/-} double mutant mice are not viable and die
45 at the onset of gastrulation thus demonstrating the crucial role of
46 poly(ADP-ribosyl)ation during embryonic development but also
47 suggesting partial redundant functions of both proteins with regard
48 to cell survival (4). Accordingly, a particular role of both proteins
49 in the maintenance of constitutive and facultative heterochromatin
50 integrity is suggested by previous research. Indeed, Parp-1 and
51 Parp-2 localize to and associate with specific partners at telomeres
52 (5, 6), centromeres (7, 8), and in the nucleolus (9). In addition,
53 the Parp-1^{+/-}Parp-2^{-/-} background displays specific female embry-
54 onic lethality associated with X chromosome instability (4).

55 More recently, further characterization of the mutant mice
56 revealed particular roles of Parp-2 both in the maintenance of
57 heterochromatin integrity and in various differentiation processes
58 including spermatogenesis (10), adipogenesis (11), and immune
59 cell development (12, 13).

60 Altogether, the in-depth characterization of Parp-1^{-/-} and
61 Parp-2^{-/-} mice clearly complemented biochemical and cellular stud-
62 ies to confirm the essential role of both proteins in cellular response
63 to DNA damage but also revealed unknown unique functions of
64 both enzymes and particularly Parp-2 in various physiological
65 processes. In this chapter, we detail some of the most common
66 methodologies used to genotype and phenotype both mouse models.

67 2. Materials

68 **2.1. Parp-1 and Parp-2** 69 **Deficient Mouse** 70 **Models**

71 The generation of Parp-1 and Parp-2-deficient mouse strains has
72 been described previously (4, 14). 8- to 10-week-old mice are
73 routinely used in the described experiments. Animals are main-
74 tained in accordance with the National Institutes of Health
Guide for the Care and Use of Laboratory Animals, and the exper-
iments need to be approved by the local Institutional Animal Care
Committee.

2.2. Genotyping of Mouse Tail by PCR

1. Microcentrifuge tubes (1.5 mL) for extraction. 75
2. Dissecting instruments: small dissecting scissors and forceps. 76
3. Ethanol 70%, see Subheading 2.4.3. 77
4. REDExtract-N-Amp™ Tissue PCR kit (Sigma, Saint Louis, MO, USA) containing extraction solution, tissue preparation solution, neutralization solution B and PCR mix. Store at 4°C. 78-80
5. 0.2-mL Thermo-Strips. 81
6. Thermal cycler (BioMetra Professional Basic, Labgene, Archamps, France). 82-83
7. dH₂O. 84
8. TE buffer: 10 mM Tris and 1 mM ethylenediamine tetraacetic acid (EDTA), pH 8.0. 85-86
9. Primer sequences (Table 1): 1 µg/µL in TE buffer. Store at -20°C. 87-88
10. Ethidium bromide stock solution: 5 µg/µL in dH₂O. Store at 4°C. 89-90
11. 1× TAE buffer: dilute 20 mL of 50× TAE (5Prime GmbH, Hamburg, Germany) in dH₂O to 1 L. 91-92
12. DNA loading marker solution: 200 µL 1 kb DNA ladder (Invitrogen, Carlsbad, USA), 1,500 µL TE buffer, and 300 µL of Bromophenol blue (Merck, Darmstadt, Germany). 93-95
13. Ultra Pure™ Agarose (Invitrogen, Carlsbad, USA). 96
14. UV lamp (Electronic Dual Light™ TransIlluminator, Bioprobe systems, Montreuil-sous-Bois, France). 97-98

[AU1]

Table 1 t1.1
Primer sequences t1.2

Gene	Primers name	Primer sequence	Amplicon size (bp)	
<i>Parp-1</i> t1.4				
Wild-type allele	A	TT GAT GGC CGG GAG CTG CTT CTT	200	t1.5
	B	GGC CAG ATG CGC CTG TCC AAG AAG		t1.6
Knockout allele	A	TT GAT GGC CGG GAG CTG CTT CTT	700	t1.7
	C	GGC GAG GAT CTC GTC GTG ACC CAT G		t1.8
<i>Parp-2</i> t1.9				
Wild-type allele	A	GGG ACT CTG GTT TGG AGC CT	330	t1.10
	B	TGC TGC CGT CCC TTA TTC TAA GCT		t1.11
Knockout allele	A	GGG ACT CTG GTT TGG AGC CT	250	t1.12
	C	TTA GGG CCA GCT CAT TCC TCC CA		t1.13

[AU2]

99 **2.3. Exposure of Mice**
 100 **to Genotoxic Stress**
 101 **and Survival Curves**

- 102
1. 8- to 10-week-old Parp-1^{-/-} mice and age-matched control Parp-1^{+/+} mice. 8- to 10-week-old Parp-2^{-/-} mice and age-matched control Parp-2^{+/+} mice (see Note 1).
 2. DNA damaging agents:
 - 103 (a) X-irradiation: we used a Pantax Seifert X-ray system
 104 (Ahrensburg, Germany) and a UNIDOS E Universal
 105 Dosemeter (PTW, La Ville du Bois, France) (see Note 2).
 - 106 (b) γ -irradiation: we used a Cobalt ⁶⁰Co source irradiator.
 - 107 (c) *N*-Methyl-*N*-nitrosourea (MNU): dissolve at 2 M in
 108 DMSO and store at -20°C. *MNU is carcinogenic, manip-*
 109 *ulate with precaution following the NIH Guidelines for the*
 110 *Laboratory Use of Chemical Carcinogens.*

111 **2.4. Necropsy and**
 112 **Histological Analysis**

113 2.4.1. Tissue Fixation
 with Bouin's Solution

1. Bouin's solution (Picroformol Bouin Aqueous, Carlo Erba, Val de Reuil, France).
2. Ethanol 70%, see Subheading 2.4.3.

114 2.4.2. Tissue Fixation
 115 with 10% Neutral Buffered
 116 Formalin

1. Dulbecco's phosphate-buffered saline (PBS): for a 10× stock solution, dissolve the content of one bottle of powdered salts (96 g, #D5652, Sigma-Aldrich) into 1 L of dH₂O. For 1× PBS, add 100 mL of 10× PBS to 900 mL of dH₂O. Autoclave and store at 4°C.
2. Formaldehyde stock solution (36.5% w/v) (i.e., concentrated formalin) (Sigma-Aldrich, St. Louis, USA). For a 10% buffered formalin solution: dilute 10 mL concentrated formalin in 90 mL 1× PBS. *Formaldehyde is carcinogenic, manipulate with precaution following the NIH Guidelines for the Laboratory Use of Chemical Carcinogens.*

125 2.4.3. Paraffin Embedding
 126 and Sectioning

1. Ethanol 100%.
2. Ethanol 95%(v/v): add 950 mL ethanol 100% to 50 mL dH₂O.
3. Ethanol 70%(v/v): add 700 mL ethanol 100% to 300 mL dH₂O.
4. Paraffin solvent (Histosol clearing agent, Roti®-Histol, Carl Roth GmbH, Karlsruhe, Germany).
5. Paraffin wax with a 55°C melting point.
6. Embedding moulds of different sizes.
7. Microtome.
8. Gelatine coated glass slides.

135 2.4.4. Haematoxylin
 136 and Eosin Staining
 137 of Histological Sections

1. Coplin jars.
2. Harris haematoxylin solution: dissolve and heat 5 g haematoxylin Certistain® (C.I. 75290) in 50 mL ethanol 100% using a stirrer hotplate. Dissolve and heat 100 g potassium aluminium

138

- sulphate in 950 mL dH₂O using a stirrer hotplate. Add slowly the haematoxylin to the potassium aluminium sulphate under stirring and heat until near boiling. Add 370 mg sodium iodate, cool down, and add 4 mL acetic acid (glacial, 100%). Filter before use.
3. Eosin Y solution 0.1%: dissolve 1 g Eosin Y Certistain® (C.I. 45380) in 1 L dH₂O while stirring. Add 1.6 mL acetic acid (glacial, 100%). Filter before use.
 4. Hydrochloric acid HCl 37%.
 5. Ethanol 100%.
 6. Ethanol 95%, see Subheading 2.4.3.
 7. dH₂O.
 8. Histosol clearing agent, see Subheading 2.4.3.
 9. Glass coverslips (24×60 mm) (Menzel-Glaser).
 10. Acid alcohol (v/v): add 1 mL of 37% HCl to 100 mL of ethanol 70%.
 11. Mounting solution containing antifading reagent (Mowiol/Dabco): add 2.4 g of MOWIOL 4–88 (Calbiochem; VWR, Fontenay sous Bois, France) to 6 g of glycerol, stir to mix. Add 6 mL of dH₂O and stir at room temperature for several hours. Add 12 mL of 0.2 M Tris-Cl, pH 8.5, and heat to 50°C for 10 min with occasional mixing. Clarify by centrifugation at 5,000×g for 15 min. Add 1,4-diazobicyclo-(2.2.2)-octane (DABCO, Sigma-Aldrich) to 2.5% (w/v). Aliquot into airtight containers and store at –20°C.
- 2.4.5. Microscopic In Situ Detection of Cell Death Using the TUNEL Assay*
1. ApoAlert™ DNA Fragmentation Assay kit (BD biosciences Clontech, Palo Alto, US) containing equilibration buffer, Nucleotide Mix, TdT terminal transferase, 20× SSC, and proteinase K (10 mg/mL).
 2. Coplin jars.
 3. Xylene.
 4. Ethanol 100%.
 5. Ethanol 95% (v/v): add 950 mL ethanol 100% to 50 mL dH₂O.
 6. Ethanol 85% (v/v): add 850 mL ethanol 100% to 150 mL dH₂O.
 7. Ethanol 70% (v/v): add 700 mL ethanol 100% to 300 mL dH₂O.
 8. Ethanol 50% (v/v): add 500 mL ethanol 100% to 500 mL dH₂O.
 9. NaCl 0.85% (w/v): add 0.85 g NaCl to 100 mL dH₂O. Mix, autoclave, and store at 4°C.

- 181 10. 1× PBS, see Subheading 2.4.2.
- 182 11. 10% Buffered formalin solution, see Subheading 2.4.2
- 183 *Formaldehyde is carcinogenic, manipulate with precaution*
- 184 *following the NIH Guidelines for the Laboratory Use of Chemical*
- 185 *Carcinogens.*
- 186 12. Proteinase K working solution (20 µg/mL): dilute 2 mL of
- 187 10 mg/mL proteinase K in 998 mL of 100 mM Tris-HCl, pH
- 188 8.0, 50 mM EDTA. Aliquot and store at -20°C.
- 189 13. SSC 2×: add 10 mL of 20× SSC to 90 mL dH₂O.
- 190 14. Propidium iodide-RNase solution: prepare a 10 mg/mL propi-
- 191 pidium iodide stock solution: dissolve 10 mg of propidium
- 192 iodide in 10 mL of PBS. Protect from light and store in the
- 193 dark at 4°C. Propidium iodide-RNase working solution:
- 194 0.5 µg/mL propidium iodide, 0.5 µg/mL DNase-free RNase.
- 195 *Propidium iodide is carcinogenic, manipulate with precaution*
- 196 *following the NIH Guidelines for the Laboratory Use of Chemical*
- 197 *Carcinogens.*
- 198 15. 4',6-Diamidino-2-phenylindole (DAPI, Sigma-Aldrich) stock
- 199 solution (5 mg/mL): dissolve 10 mg in 2 mL dH₂O. For long-
- 200 term storage, the stock solution can be aliquoted and stored
- 201 at -20°C. For short-term storage, the solution can be kept at
- 202 4°C, protected from light. *DAPI is a known mutagen and*
- 203 *should be handled with care.*
- 204 16. Mounting solution, see Subheading 2.4.4.
- 205 17. Fluorescence microscope with oil-immersion objectives and a
- 206 digital CDD camera (e.g., ORCA ER; Hamamatsu Photonics,
- 207 Massy, France).

2.5. Isolation of Mouse Embryonic Fibroblasts

- 208 1. Parp-1^{+/-} or Parp-2^{+/-} pregnant female 13.5 days post-coitum.
- 209 2. Mouse embryonic fibroblasts (MEFs) medium: Dulbecco's
- 210 modified Eagle's medium (DMEM) (Gibco/BRL, Bethesda,
- 211 MD, USA) supplemented with 10% (v/v) foetal bovine serum
- 212 (FBS, AdGenix, Voisins le Bretonneux, France) and 1% (w/v)
- 213 gentamicin (Gibco/BRL).
- 214 3. Trypsin (0.05% w/v) and EDTA (0.53 mM) solution (Gibco/
- 215 BRL).
- 216 4. Ethanol 100%.
- 217 5. 1× PBS, see Subheading 2.4.2.
- 218 6. Dissecting instruments: fine scissors, tweezers, sterile razor
- 219 blades.

2.6. Clonogenic Survival Assays on MEFs After DNA Damage

- 220 1. 100-mm Diameter plastic cell culture dishes.
- 221 2. MEFs medium, see Subheading 2.5.
- 222 3. Trypsin (0.05% w/v) and EDTA (0.53 mM) solution (Gibco/
- 223 BRL).

	4. 1× PBS, see Subheading 2.4.2.	224
	5. DNA damaging agents:	225
	(a) X-irradiation, see Subheading 2.3	226
	(b) γ -irradiation, see Subheading 2.3	227
	(c) MNU, see Subheading 2.3	228
	(d) H ₂ O ₂ : prepare a 100× working solution by diluting 10 μ L of 8.8 M stock H ₂ O ₂ (Sigma–Aldrich; stored at 4°C) with 870 μ L dH ₂ O.	229 230 231
	6. Mitomycin C (×20): add 10 mL DMEM to 2 mg/ampoule of Mitomycin C (Sigma) to obtain a final stock solution of 200 μ g/mL (w/v). Filter sterilize and store at 4°C. <i>Mitomycin C is carcinogenic, manipulate with precaution following the NIH Guidelines for the Laboratory Use of Chemical Carcinogens.</i>	232 233 234 235 236
	7. 10% Buffered formalin solution, see Subheading 2.4.2.	237
	8. Crystal violet: for a 0.1% solution, dilute 10 mg of crystal violet powder (Crystal Violet, Sigma–Aldrich) in 10 mL of dH ₂ O.	238 239
	9. dH ₂ O.	240
2.7. Genome Integrity on MEFs by Fluorescent In Situ Hybridization		
<i>2.7.1. Metaphase Spreads of Adherent Cells</i>		
	1. T75 flasks.	241
	2. Colchicin stock solution (# C9754, Sigma): add 10 mL dH ₂ O to obtain a 10 μ g/ μ L stock solution. <i>Colchicin is carcinogenic, manipulate with precaution following the NIH Guidelines for the Laboratory Use of Chemical Carcinogens.</i>	242 243 244 245
	3. Trypsin (0.05% w/v) and EDTA (0.53 mM) solution (Gibco/BRL).	246 247
	4. 13-mL Conical tubes.	248
	5. Hypotonic solution pre-warmed at 37°C: mix 1 volume of 75 mM KCl with 1 volume of diluted human serum (1/6 ^c dilution in dH ₂ O; PAA, #C11-061).	249 250 251
	6. Fixative solution: add 3 volumes of ethanol to 1 volume of acetic acid.	252 253
	7. Superfrost microscope slides (Menzel-Glaser).	254
	8. Phase-contrast microscope.	255
<i>2.7.2. Telomeric and Centromeric Fluorescent In Situ Hybridization</i>		
	1. Coplin jars.	256
	2. 1× PBS, see Subheading 2.4.2.	257
	3. dH ₂ O.	258
	4. 10% Buffered formalin solution, see Subheading 2.4.2.	259
	5. 1 M HCl solution adjusted to pH 2: add 8.4 mL HCl fuming 37% to 91.6 mL of dH ₂ O and adjust pH with NaOH.	260 261
	6. 0.1 M HCl solution, pH 2: add 100 μ L 1 M HCl, pH 2 to 900 μ L of dH ₂ O.	262 263

- 264 7. Pepsin 0.5 mg/mL: add 25 μ L of pepsin (20 mg/mL, Sigma
265 6887) in 975 μ L of 0.1 M HCl, pH 2.
- 266 8. Glass coverslips 24 \times 60 mm (Menzel-Glaser).
- 267 9. Laboratory hot air oven at 37°C.
- 268 10. Ethanol 100%.
- 269 11. Ethanol 80% (v/v): mix 800 mL of 100% ethanol with 200 mL
270 of dH₂O.
- 271 12. Ethanol 70%, see Subheading 2.4.5.
- 272 13. Hybridization buffer: 70% deionized formamide, 10 mM Tris,
273 pH 7.2 with 1% BSA (Sigma, A7906). *Formamide is carcino-*
274 *genic, manipulate with precaution following the NIH Guidelines*
275 *for the Laboratory Use of Chemical Carcinogens.*
- 276 14. Wet dark chamber: a wet paper towel is placed in a plastic petri
277 dish covered with aluminium foil.
- 278 15. Telomeric PNA: Cy-3-labeled (CCCTAA)₃ PNA probe
279 (Applied Biosystems).
- 280 16. Conc. Human Pan Centromeric paint FITC (Cambio, starfish,
281 #1695-F-02).
- 282 17. Hot plate (Stuart Scientific, SH1D1).
- 283 18. Formamide 50%: SSC ~~2~~¹ solution: mix 100 mL formamide,
284 20 mL 20 \times SSC, and 80 mL dH₂O. Pre-heat in a water bath
285 at 45°C.
- 286 19. 0.1 \times SSC: mix 1 mL of 20 \times SCC and 199 mL dH₂O. Pre-heat
287 in a water bath at 45°C.
- 288 20. 2 \times SSC, 0.05% Tween 20 solution: mix 5 mL of 20 \times SCC with
289 50 μ L of Tween 20 and 95 mL of dH₂O.
- 290 21. 4',6-Diamidino-2-phenylindole (DAPI): stock solution at
291 1 mg/mL DAPI (Sigma) in PBS and working solution
292 at 0.5 μ g/mL.
- 293 22. Fluoromount-G (SouthernBiotech).
- 294 23. Fluorescent microscope with oil-immersion objectives, appro-
295 priate filters, and a digital CCD camera (e.g., ORCA ER;
296 Hamamatsu Photonics, Massy, France).
- 297 **2.8. Detection of DNA**
- 298 **Damage on MEFs**
- 299 **by COMET Assay**
- 300 1. 35-mm Diameter plastic cell culture dishes, coverslips (0.17-mm
301 thickness), microscope slides, cut 101–1,000 μ L tips.
- 302 2. MEFs medium, see Subheading 2.5.
- 303 3. Trypsin (0.05% w/v) and EDTA (0.53 mM) solution (Gibco/
304 BRL).
- 305 4. DNA damaging agents: see Subheading 2.3.
- 306 5. Ethanol 100%.
- 307 6. 1 \times PBS, see Subheading 2.4.2.

7. 1% Agarose: mix 100 mg of agarose in 10 mL of PBS. 305
8. 1% Low melting point agarose: mix 100 mg of LMP Agarose (Sigma–Aldrich) in 10 mL of MEFs medium. 306
307
9. Denaturing lysis buffer: 10 mM Tris–OH, 2.5 M NaCl, and 0.1 M EDTA in dH₂O. Add 150 mM of NaOH and dissolve overnight. Add 34 mM of sodium lauroyl sarcosinat and adjust pH to 10. Immediately before use add 1% (v/v) Triton X-100 and 10% (v/v) dimethylsulphoxide (DMSO). 308
309
310
311
312
10. Non-denaturing lysis buffer: 87 mM sodium dodecyl sulphate (SDS), 34 mM sodium lauroyl sarcosinat, 25 mM EDTA, pH 9.5 in dH₂O. Adjust pH to 9.5. 313
314
315
11. Migration buffer: 30 mM NaOH and 10 mM EDTA, pH 8.0 in 2 L of dH₂O. 316
317
12. Unwinding buffer: 500 mM NaCl in 500 mL of migration buffer. 318
319
13. Neutralization buffer: 400 mM Tris–HCl, pH 7.5 in dH₂O. 320
14. 1× TBE buffer: dilute 10.8 g of Tris–OH and 5.5 g of boric acid in 1 L of dH₂O. Add 4 mL of 0.5 M EDTA solution (pH 8.0). Adjust pH to 8.4. 321
322
323
15. Ethidium bromide (2 µg/mL): dilute 4 µL of 5 mg/mL ethidium bromide solution in 10 mL of dH₂O. 324
325
16. Fluorescence microscope equipped with a digital CDD camera (e.g., ORCA ER; Hamamatsu Photonics, Massy, France). 326
327

3. Methods

328

3.1. Genotyping of Parp-1 and Parp-2 Deficient Mouse Models

Pups are tailed (for DNA extraction) and toed (for identification) between 8 and 14 days of age. 329
330

3.1.1. Mouse Tailing

1. Cut off a tail piece of approximately 2–4 mm length using dissecting scissors and place it in 1.5-mL microcentrifuge tubes for digestion. 331
332
333
2. Scissors are washed liberally with ethanol 70% between cuts to prevent cross contamination. 334
335
3. Tails can be frozen or processed immediately for DNA extraction. 336
337

3.1.2. Tail Lysis, DNA Extraction, and PCR (see Note 3)

DNA Extraction

1. Add 100 µL of extraction solution and 25 µL of tissue preparation Solution to the tube and mix thoroughly by vortexing. 338
339
2. Incubate sample at room temperature for 10 min. 340
3. Incubate sample at 95°C for 3 min. 341
4. Add 100 µL of neutralization solution B to sample and mix by vortexing. 342
343

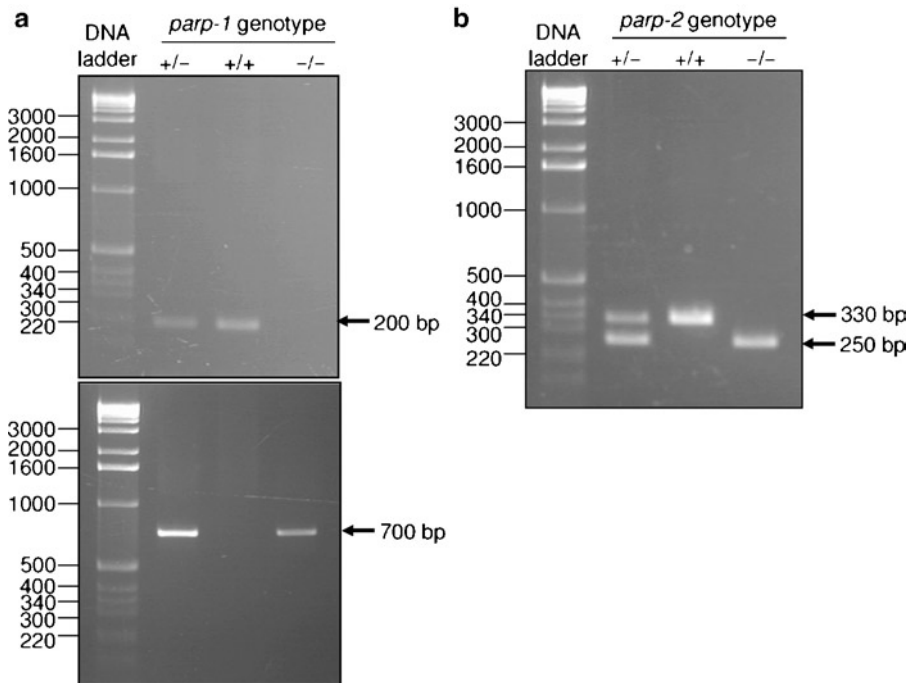
344 5. The neutralized DNA tissue extract can be stored at 4°C or
345 processed immediately in PCR.

346 Polymerase Chain Reaction

- 347 1. Add the following reagents to the PCR microcentrifuge plate.
348 (1) For Parp-1: DNA extract (3 µL); dH₂O (4.26 µL); Primer
349 (A+B) wild-type allele (0.12 µL each) OR Primer (A+C)
350 knockout allele (0.12 µL each); REExtract-N-Amp PCR Mix
351 (7.5 µL). Total volume should be 15 µL. *Note: Identification*
352 *of wild-type or knockout alleles have to be processed separately.*
353 (2) For Parp-2: DNA extract (2 µL); dH₂O (5.06 µL); Primer
354 A (0.20 µL); Primer B (0.12 µL); Primer C (0.12 µL);
355 REExtract-N-Amp PCR Mix (7.5 µL). Total volume should
356 be 15 µL. *Note: Identification of wild-type and knockout alleles*
357 *are processed in a single reaction.*
- 358 2. Mix gently and perform thermal cycling as following: (1) For
359 Parp-1: Step 1 (94°C, 2 min); Step 2 (94°C, 40 s; 61°C, 45 s;
360 73°C, 1 min; 30 cycles); Step 3 (72°C, 4 min); Step 4
361 (4°C, hold). (2) For Parp-2: Step 1 (94°C, 3 min); Step 2
362 (94°C, 50 s; 64°C, 50 s; 72°C, 1 min; 30 cycles); Step 3
363 (72°C, 4 min); Step 4 (4°C, hold).
- 364 3. Prepare a 1.5% Agarose gel by adding 1.5 g Agarose to 100 mL
365 1× TAE. Microwave or heat until near boiling to dissolve the
366 agarose. Cool down for 5 min and add 5 µL of ethidium
367 bromide stock solution.
- 368 4. After polymerization, carefully remove the comb, place the gel
369 in a running chamber and add 1× TAE buffer containing
370 250 µg/L ethidium bromide to cover the gel.
- 371 5. Load the PCR samples into sample wells.
- 372 6. Load 5 µL of DNA loading marker solution into one well.
- 373 7. Connect the power supply. The gel is run at 12 V/cm until the
374 dye front reaches the bottom of the gel.
- 375 8. Analyse the results under UV-light. An example of results is
shown in Fig. 1.

376 **3.2. Exposure of Mice**
377 **to Genotoxic Stress**
378 **and Survival Curves**

- 379 1. Mice treatment:
- 380 (a) Using ionizing radiation: place mice on a horizontally
381 rotating tray to produce a homogeneous irradiation under
382 the beam of X or γ source. Adjust the setting of the source
383 according to the protocol used and stop at the desired
384 dose measured by a dosimeter (see Note 2).
- 385 (b) With a monofunctional alkylating agent: use a 1-mL syringe
386 equipped with a 26-gauge needle and inject intraperitoneally
387 100 µL/mice of the appropriate dose of MNU diluted
in saline solution. *Dispose of used alkylating agent in an*
appropriate waste container according to the NIH Guidelines
for the Laboratory Use of Chemical Carcinogens.



[AU3] Fig. 1. Genotyping of Parp-1^{-/-} and Parp-2^{-/-} mice by PCR. *Left panel*, PCR products of mouse tail DNA of respectively Parp-1^{+/-}, Parp-1^{+/+} and Parp-1^{-/-} mice. *Top*, wild-type allele (200 bp); *bottom*, knockout allele (700 bp). *Right panel*, PCR products of mouse tail DNA of, respectively, Parp-2^{+/-}, Parp-2^{+/+}, and Parp-2^{-/-} mice (wild-type allele: 330 bp; knockout allele: 250 bp).

2. House mice in static cages in a quiet environment, without excessive noise or vibration. 388 389
3. Check mice for signs of weakness daily, starting on day 1 after the irradiation. As soon as the first signs of weakness appear, provide mice with wet food pellets on the floor of the cage. 390 391 392
4. The percentage of deceased mice from each genotype is evaluated according to Kaplan–Meier analysis. Statistical analysis is done using ordinary ANOVA tests and StatView software. The comparative sensitivity of Parp-1^{-/-} and Parp-2^{-/-} male and female mice to cumulative doses of whole body X-irradiation (1 Gy/day for 2 weeks) is shown in Fig. 2. 393 394 395 396 397 398
5. Euthanize moribund mice for gross examination, necropsy, and histological analysis (see Subheading 3.3). 399 400

3.3. Necropsy and Histological Analysis

3.3.1. Necropsy

1. Kill the moribund mouse by cervical dislocation. 401
2. Swab the mouse with ethanol to reduce aerosols of hair or potential allergens. 402 403
3. Make a ventral incision through the skin from the lower abdomen to the apex of the lower jaw and extend down the limbs. 404 405
4. Remove the ventral rib cage at the level of the costochondral junction and cut through the clavicles to expose the trachea. 406 407

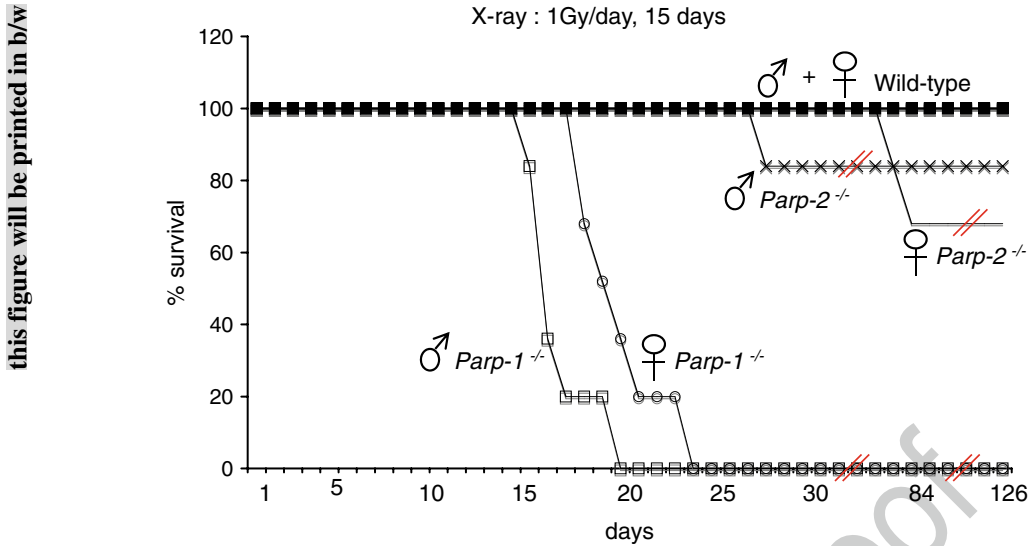


Fig. 2. Survival of wild-type males and females ($n=12$), Parp-1^{-/-} males ($n=6$), Parp-1^{-/-} females ($n=6$), Parp-2^{-/-} males ($n=6$), and Parp-2^{-/-} females ($n=6$) after X-irradiation with 1 Gy/day for 2 weeks. The percentage of alive mice is plotted against time.

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5. Identify gross pathology of the mouse and produce a written and photographic record of macroscopic characteristics including organ weights.
6. Collect selected fresh tissues for histology.

412 3.3.2. Tissue Fixation with
413 Bouin's Solution
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Bouin's solution fixative is used for histopathological analysis of mouse embryos, foetuses, and adult testis. It is not recommended for immunohistochemistry or in situ hybridization studies.

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1. Following dissection, immediately place the tissue into Bouin's fixative to prevent autolytic alterations. Ensure that the volume of the fixative is 10–20 times the volume of the tissue samples.
2. Fix at room temperature for a minimum of 24 h under mild agitation on a rocking platform. *Dispose of used fixatives in an appropriate waste container according to the NIH Guidelines for the Laboratory Use of Chemical Carcinogens.*
3. Wash in ethanol 70% for 1 h followed by overnight incubation, at room temperature, under mild agitation on the rocking platform. Store at 4°C before embedding into paraffin.

425 3.3.3. Tissue Fixation with
426 10% Buffered Formalin

10% Buffered formalin fixative is used for histopathological analyses of adult tissues, immunohistochemistry, and in situ detection of cell death (see Note 5). Tissues may be stored in the fixative indefinitely without any morphological damage, but should not be fixed for longer than 24 h if immunohistochemistry will be subsequently performed.

[AU4]

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1. Immediately immerse the tissue into 10% neutral buffered formalin fixative. Ensure that the volume of the fixative is 10–20 times the volume of the tissue samples. 431–433
 2. Fix at room temperature for 24 h under mild agitation on the rocking platform. *Dispose of used fixatives in an appropriate waste container according to the NIH Guidelines for the Laboratory Use of Chemical Carcinogens.* 434–437
 3. Wash in 1× PBS for 1 h at room temperature under mild agitation on the rocking platform and transfer in ethanol 70%. Store at 4°C before embedding into paraffin. 438–440
- 3.3.4. Paraffin Embedding and Sectioning
- Dehydration: Dehydrate in a graded series of ethanol as following: 441–442
1. Wash in ethanol 70% for 1 h. 443
 2. Wash in ethanol 95% for 2 h (change the ethanol solution after 1 h). 444–445
 3. Wash in ethanol 100% for 2 h (change the ethanol solution after 1 h). 446–447
- Clearing: 448
4. Clear with HistoSol for 30 min, 1 h and then a further 1 h (change the HistoSol between each time period). 449–450
- Infiltration: 451
5. Fill glass containers (one container/sample) with the embedding agent: paraffin and melt the paraffin in an oven at 60°C. Transfer the cleared samples into the containers once the paraffin is melted. 452–455
 6. Infiltrate with paraffin for 1–2 h, overnight, and then a further 5 h (change the paraffin between each time period). 456–457
- Paraffin Embedding: 458
7. Transfer the pre-heated mould onto a hot plate and fill the mould with fresh molten paraffin. 459–460
 8. Transfer the paraffin-infiltrated tissues into the appropriate sized embedding mould. Orientate the tissue into the desired position in relation to the cutting axis. Centre the tissue in the mould ensuring that paraffin entirely surrounds the edge of the tissue. The tissue must not come into contact with the edge of the mould (see Note 4). 461–466
 9. Carefully transfer the mould onto a cooling plate. Cool down for 15 min until the paraffin is set. 467–468
 10. Snap off the mould to present the paraffin embedded tissue underneath the cassette (called a paraffin block). 469–470

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Sectioning:

11. Prepare tissue sections of 5 μm thickness using a microtome. Transfer tissue ribbon onto glass slide coated with a drop of gelatine-water and pre-heated at 42°C.
12. Dry the slides horizontally on a warm plate for 10 min and then vertically in a laboratory oven overnight at 37°C. Store in dry boxes at room temperature before use.

478 *3.3.5. Haematoxylin and*
479 *Eosin Staining of Tissue*
480 *Sections (see Note 6)*

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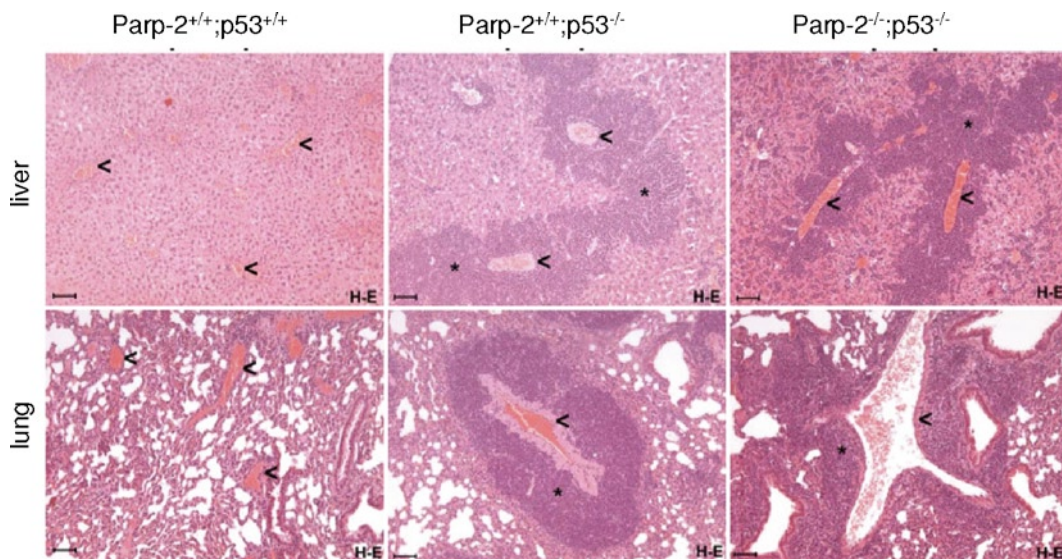
Deparaffinise and rehydrate tissue sections as following:

1. Wash in Histosol twice for 3 min each.
2. Rehydrate twice for 3 min each with ethanol 100%.
3. Rehydrate twice for 3 min each with ethanol 95%.
4. Wash twice with dH₂O for 3 min each.
5. Stain for 3 min in Harris haematoxylin.
6. Wash in running tap water for 3 min.
7. Decolourise briefly in acid alcohol for 2 s.
8. Wash and blue the sections in running tap water for 3 min.
9. Stain for 30 s in Eosin Y solution 0.1%.
10. Rinse in tap water for 30 s.
11. Dehydrate 2 \times for 2 min each in ethanol 95%.
12. Dehydrate 2 \times for 2 min each in ethanol 100%.
13. Clear sections in Histosol 2 \times for 2 min each.
14. Immediately apply 2–3 drops of the mounting medium and place a coverslip on the slide carefully avoiding the formation of bubbles. Dry the slides overnight at room temperature on a flat surface. An illustration showing Haematoxylin–Eosin staining of tumours collected from Parp-2^{+/+} p53^{-/-} and Parp-2^{-/-} p53^{-/-} mice is shown in Fig. 3.

498 *3.3.6. In Situ Detection*
499 *of Cell Death by TUNEL*
500 *Assays (see Note 7)*

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1. Immerse slides in xylene at room temperature for 5 min to remove paraffin.
2. Repeat using fresh xylene.
3. Immerse slides in 100% ethanol at room temperature for 5 min.
4. Rehydrate the slides by sequential 3 min-incubations at room temperature in ethanol 100%, ethanol 95%, ethanol 85%, ethanol 70% and ethanol 50%.
5. Immerse the slides in 0.85% NaCl at room temperature for 5 min.
6. Wash the slides in 1 \times PBS at room temperature for 5 min.
7. Fix the slides in 10% buffered formalin at room temperature for 15 min.



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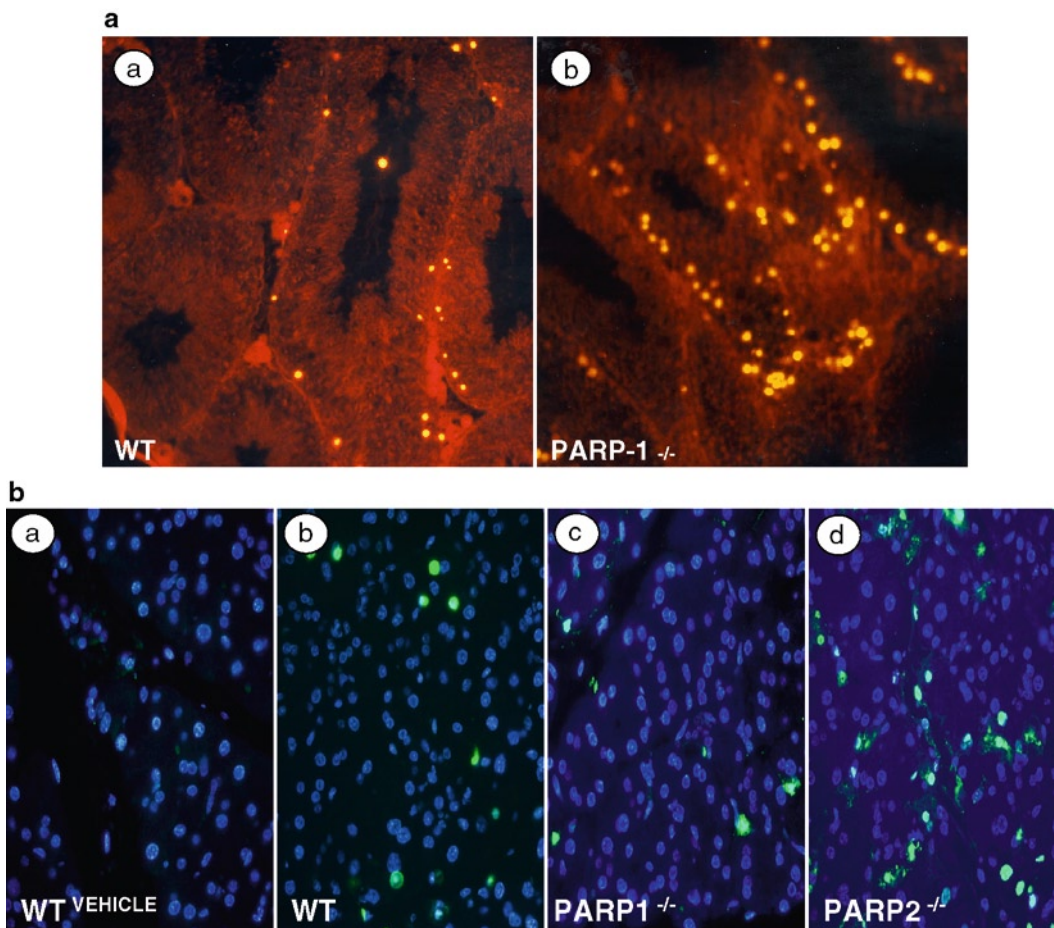
Fig. 3. Lymphoma dissemination in Parp-2^{+/+}p53^{-/-} and Parp-2^{-/-}p53^{-/-} mice. Representative light micrographs of infiltration and growth of lymphoma (*) in the liver and the lung (severe perivascular (<) infiltration). *H-E* Haematoxylin-Eosin.

8. Wash the slides in 1× PBS at room temperature for 5 min. 510
9. Repeat using fresh 1× PBS. 511
10. Allow the liquid to drain thoroughly and place slides on a flat surface. 512
11. Cover tissue sections on the slides with 100 μL of proteinase K working solution. Incubate at room temperature for 5 min. 514
12. Wash the slides in 1× PBS at room temperature for 5 min. 516
13. Incubate the slides in 10% buffered formalin at room temperature for 5 min. 517
14. Wash the slides in 1× PBS at room temperature for 5 min. 519
15. Remove slides from 1× PBS, allow the liquid to drain thoroughly and place slides on a flat surface. 520
16. Cover the tissue samples with 100 μL of equilibration buffer. Gently place a piece of plastic coverslip on top of the section to evenly spread the liquid. Equilibrate at room temperature for 10 min. 522
17. Prepare TdT incubation buffer containing for one reaction 45 μL equilibration buffer, 5 μL Nucleotide Mix, 1 μL TdT enzyme. Wrap with aluminium foil to protect from light. 526
18. Carefully remove coverslip and excess of liquid. 529
19. Gently cover the tissue section with 50 μL of the TdT incubation buffer. Place a plastic coverslip on top of the sections to spread the liquid. Place the slides in a dark, humidified 37°C incubator for 1 h. 530

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20. Gently remove the plastic coverslip and immerse slides in 2× SSC at room temperature for 15 min. Wrap with aluminium foil to protect from light.
 21. Wash the slides in 1× PBS at room temperature for 5 min. Repeat this step twice. Allow the liquid to drain thoroughly and place slides on a flat surface.
 22. DNA staining:
 - (a) With propidium-iodide-RNase: cover the tissue samples with 100 μL propidium-iodide-RNase working solution and stain for 10 min at room temperature in the dark.
 - (b) With DAPI: cover the tissue samples with 100 μL of DAPI working solution (diluted 1: 10,000 in PBS) and incubate for 10 min at room temperature in the dark.
 23. Wash the slides in dH₂O at room temperature for 5 min. Repeat this step twice.
 24. Add a drop of Mowiol mounting solution (35 μL) and cover the treated tissues with a glass coverslip. Store in the dark at room temperature for several hours to dry, then at -20°C to minimize background fluorescence.
 25. Observe the slides under a fluorescence microscope. Two examples of microscopic detection of cell death in X-irradiated testis or pancreas upon acute pancreatitis induction are shown in Fig. 4.

3.4. Isolation of MEFs

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1. Sterilize dissecting instruments by dipping in ethanol 100% and let stand under the hood until dry.
 2. Sacrifice the pregnant female at day 13.5 post-coitum by cervical dislocation. Dissect out the uterine horns and place into a Petri dish containing sterile 1× PBS.
 3. Separate each embryo from its placenta and surrounding membranes.
 4. Process each embryo separately. In a new Petri dish containing a minimal amount of 1× PBS, cut away brain and red organs. Wash the embryo, remove as much blood as possible. Cut the tail and keep it for genotyping, see Subheading 3.1.
 5. Place the embryo in a 15-cm Petri dish containing 2 mL Trypsin. Finely mince the embryo using the razor blades until it becomes “pipettable.” Incubate the cells/tissue suspension at 37°C, 5% CO₂ for 15 min.
 6. Pipette the resulting viscous suspension up and down several times to disintegrate any larger pieces of tissue left. Add 20 mL of warmed MEFs medium, pipette up and down again several times and incubate at 37°C, 5% CO₂. This corresponds to MEFs at passage ~~NO~~_X. Each further trypsination will increment the passage number.



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[AU5] Fig. 4. In situ detection of cell death by TUNEL assay. (a) Increased TUNEL staining (green nuclear signal) in Parp-1^{-/-} testis compared to WT testis after whole body X-irradiation at 2 Gy. DNA is stained with propidium iodide (red). (b) In situ detection of cell death (green nuclear signal) in pancreas sections from WT, Parp-1^{-/-}, and Parp-2^{-/-} mice administered 12-h intraperitoneal injections of 50 µg/kg cerulein to induce acute pancreatitis. Cell nuclei are stained with DAPI (blue). Magnifications ×40.

7. Change medium on the following day and any second day. The fibroblasts will be the only cells that attach to the dishes. Plates will be confluent within one to a few days. 578
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3.5. Cell Survival Assay After Genotoxic Stress by Clonogenicity

3.5.1. Mitomycin C Treatment of MEFs

1. Add 1:20 volume (v/v) Mitomycin C stock solution to sub-confluent wild-type MEFs and incubate at 37°C for 2 h. 581
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2. Wash monolayer twice with 1× PBS at room temperature. 583
3. Trypsinize and seed feeder cells at appropriate concentration or aliquot to freeze. 584
585

3.5.2. Cell Survival After DNA Damage

1. Seed 300,000 cells in 100-mm diameter plastic cell culture dishes 2 days before DNA damaging. 586
587
2. Damaging DNA: 588

- 589 (a) With a monofunctional alkylating agent: replace the
 590 medium with medium containing 1.5 mM of MNU (see
 591 Subheading 2.3) and incubate for 30 min at 37°C.
- 592 (b) With H₂O₂: wash the cells 2× with 5 mL of 1× PBS at
 593 room temperature. Add to the cells 10 mL of 1× PBS con-
 594 taining 2.5 μL of freshly diluted 100× H₂O₂ (25 μM) and
 595 incubate for 30 min at 37°C.
- 596 (c) Using ionizing radiation (X- or γ-rays). Place Petri dishes
 597 horizontally on a rotating tray to produce a homogeneous
 598 irradiation under the beam of X or γ source. Adjust the set-
 599 ting of the source (see Note 2) and stop at the desired dose
 600 measured by a dosimeter according to the protocol used.
- 601 3. Remove medium, rinse cells twice with 5 mL of 1× PBS and
 602 trypsinize.
- 603 4. Seed 1,500 treated cells in triplicate into 100-mm diameter
 604 plastic cell culture dish together with 500,000 Mitomycin-
 605 treated feeder cells. Complete to 10 mL of medium.
- 606 5. After 8–12 days of incubation, rinse the plates with 1× PBS.
- 607 6. Fix the cells with 5 mL of 10% formalin buffer per dish for
 608 30 min.
- 609 7. Remove formalin solution. *Dispose of used fixatives in an appro-*
 610 *priate waste container according to the NIH Guidelines for the*
 611 *Laboratory Use of Chemical Carcinogens.*
- 612 8. Stain the cells with 5 mL of 0.1% crystal violet solution per dish
 613 for 30 min.
- 614 9. Remove crystal violet solution.
- 615 10. Rinse dishes two times with dH₂O.
- 616 11. Air-dry overnight.
- 617 12. Count stained clones using a light box.

618 3.6. Genome Integrity

619 3.6.1. Metaphase Spreads 620 of Adherent Cells

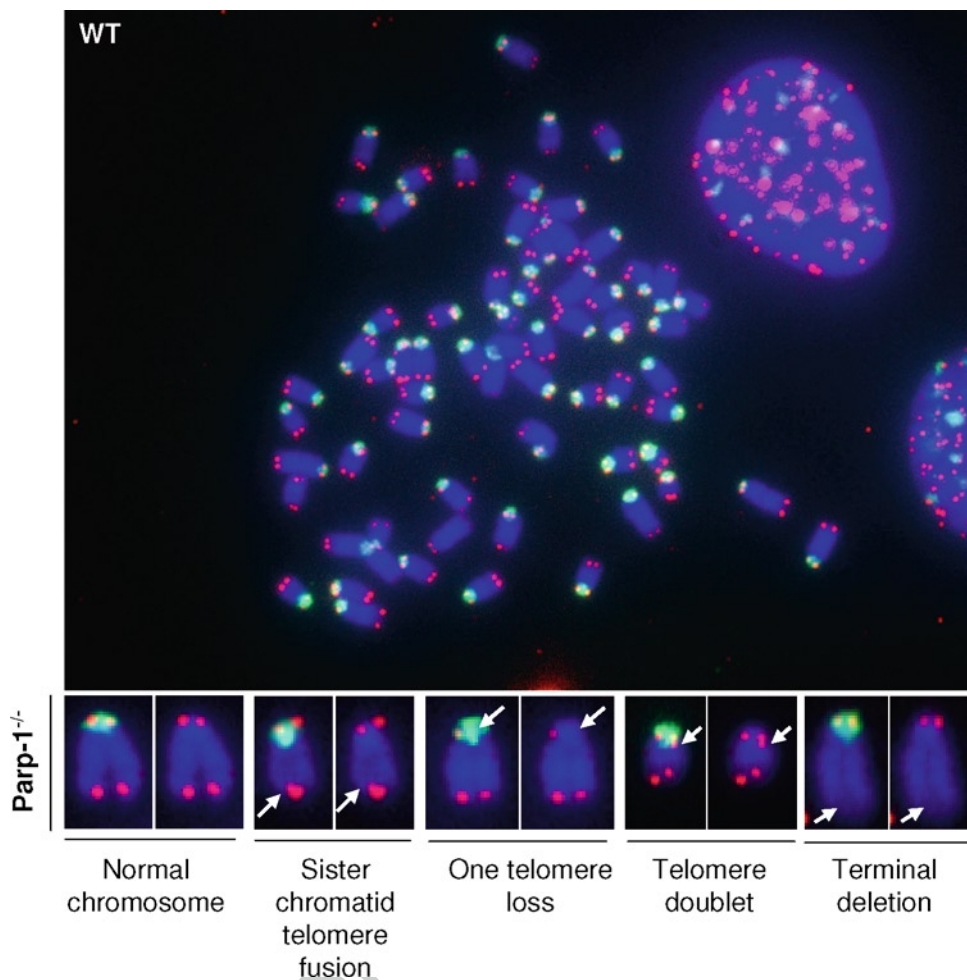
- 621 1. Split cells into T75 flask (10 mL of volume culture) in order to
 622 obtain 60–80% of cell confluence after 24 h of culture (depending
 623 on cell type).
- 624 2. Add Colchicin to the cultures at a final concentration of
 625 0.1 μg/mL.
- 626 3. Incubate at 37°C for 2–4 h (depending on cell type).
- 627 4. Transfer the medium into 13-mL conical tubes (sample tubes).
- 628 5. Rinse flasks with 2 mL Trypsin–EDTA and add to the sample
 629 tubes.
6. Centrifuge the sample tubes for 7 min at ~~1,400 rpm~~. [AU6]
7. During centrifugation, add 2 mL of fresh Trypsin–EDTA to
 the flasks and incubate at 37°C for 2–5 min.

8. When cells begin to detach, add 10 mL of hypotonic solution (pre-warmed at 37°C) to the flasks. 630
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 9. At the end of centrifugation (step 6), remove supernatants, leaving 0.5 mL of medium and gently resuspend the pellet by flicking tubes with fingers. 632
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 10. Transfer the cell suspension (12 mL, step 8) to the sample tubes while gently tapping the tube to mix. 635
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 11. Incubate at 37°C for 20 min. 637
 12. During incubation, prepare fresh fixative solution (10 mL per sample tube). 638
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 13. Add 15 drops of fresh fixative solution to stop the reaction while vortexing (~~1,400 rpm~~). 640
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 14. Centrifuge the sample tubes for 7 min at ~~1,400 rpm~~. 642
 15. Remove as much of the supernatant as possible. 643
 16. Resuspend the pellet in 8 mL of fresh fixative solution (vortex ~~1,400 rpm~~). 644
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 17. Repeat steps 14–16. 646
 18. Store sample tubes overnight at 4°C. 647
 19. Rinse superfrost slides with dH₂O and store slides overnight in dH₂O at 4°C. 648
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 20. The next day, centrifuge the sample tubes for 7 min at ~~1,400 rpm~~. 650
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 21. Remove supernatants and add 8 mL of fresh fixative solution, vortex (1,400 rpm). 652
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 22. Centrifuge the sample tubes for 7 min at ~~1,400 rpm~~. 654
 23. Discard the supernatant and resuspend in 200 µL of fresh fixative solution. 655
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 24. In a chemical hood, apply 20 µL of cells to the top of inclined pre-cleaned cooled superfrost slides. 657
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 25. Air-dry the slides in a chemical hood. 659
 26. Check for metaphase spreads with a phase-contrast microscope. If necessary, add fresh fixative solution in the sample tubes to adjust cell concentration to obtain an appropriate cell density. 660
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 27. Prepare 5–10 slides per sample as described in steps 5 and 6. 664
 28. Store slides at least 24 h at room temperature before fluorescent in situ hybridization (FISH). Slides with metaphase spreads can be stored for at least 1 year at –20°C. 665
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1. Immerse the slides in 1× PBS for 5 min at room temperature. 668
 2. Fix with 4% formaldehyde for 2 min. 669
 3. Wash three times in 1× PBS for 5 min each. 670

- 671 4. Pre-treated slides with 50 μ L of pepsin per slide (0.5 mg/mL
672 in HCl 0.1 M, pH 2), cover slides with coverslips and incubate
673 at 37°C in hot air oven for 5–10 min (depending on cell type).
674 5. Slide off coverslips.
675 6. Rinse gently in 1 \times PBS during 30 s.
676 7. Fix with 4% formaldehyde for 2 min (in a 100 mL coplin jar).
677 8. Wash three times in 1 \times PBS for 5 min each.
678 9. Dehydrate the slide preparations in ice-cold 70%, 80%, 100%
679 ethanol for 5 min each.
680 10. Air-dry the slides for 15 min.
681 11. Add 15 μ L of hybridization mixture containing 0.3 μ g/mL of
682 telomere PNA-Cy3 probe to each slide. Cover slides with
683 coverslips.
684 12. Denature on a hot plate for 3 min at 80°C.
685 13. Leave slides in wet dark chamber for 2 h at room temperature.
686 14. Denature centromeric probe for 10 min at 85°C.
687 15. Add 12 μ L of denatured centromeric probe under the coverslips.
688 16. Leave slides overnight in a wet dark chamber at 37°C.
689 17. Wash the slides twice with 50% formamid, 2 \times SCC, pH 7 at
690 45°C.
691 18. Wash the slides twice with 0.1 \times SSC, pH 7 at 45°C.
692 19. Wash once with 2 \times SSC, 0.05% Tween 20, pH 7 at room
693 temperature.
694 20. Counterstain with DAPI (1 μ g/mL, final concentration) for
695 3 min.
696 21. Wash the slides for 5 min in 1 \times PBS.
697 22. Add a drop of Fluoromount-G solution on metaphase
698 spreads.
699 23. Cover with a glass coverslip.
700 24. Observe the slides under a fluorescent microscope. Spontaneous
701 telomeric abnormalities detected in Parp-1^{-/-} MEFs are illus-
702 trated in Fig. 5.

703 **3.7. Detection of DNA**
704 **Damage by COMET**
705 **Assay**

- 706 1. Prepare 1% Agarose (100 mg/10 mL) in PBS. Microwave or
707 heat until near boiling and the agarose dissolves. While agarose
708 is hot, dip conventional microscope slides completely and
709 gently remove. Wipe underside of slides to remove agarose and
710 lay the slide on a flat surface to dry. The slides are air dried.
Store the slides at room temperature until needed; avoid high
humidity conditions. Slides are prepared the day before use.
Slides should be labelled prior to dipping.



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Fig. 5. Detection of telomere abnormalities in Parp-1^{-/-} MEFs. Metaphase spreads prepared from either wild-type (WT) or Parp-1 deficient (Parp-1^{-/-}) MEFs were hybridized successively with a telomeric PNA probe (in red) and a centromeric DNA probe (in green) and counterstained with DAPI (blue). The different types of telomere aberrations observed in Parp1^{-/-} cells are illustrated in the bottom line (white arrows). Chromosomes are shown with (left panels) or without (right panels) centromeric signal.

2. Prepare 1% LMP Agarose (100 mg/10 mL) in MEFs medium. 711
 Microwave or heat without boiling and until the agarose 712
 dissolves. Aliquot 5 mL samples into suitable containers and 713
 refrigerate until use. 714
 When needed, briefly melt agarose in microwave or by 715
 another appropriate method. Place LMP Agarose vial in a 37°C 716
 water bath to cool and stabilize the temperature. 717
3. Seed 100,000 cells in 35-mm diameter plastic cell culture dish 718
 the day before experiment. 719
 Handlings are done in darkness. Pipetting is done with cut 720
 tips. Tubes containing cells are covered with aluminium foil. 721

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4. Treat cells with DNA damaging agents
 - (a) With a monofunctional alkylating agent: replace the medium with medium containing 1 mM of MNU (see Subheading 2.3) and incubate for 30 min at 37°C.
 - (b) Using ionizing radiation (X-rays). Place Petri dishes horizontally on a rotating tray to produce a homogeneous irradiation under the beam of X or γ source. Adjust the settings of the source (see Note 2) and stop at the desired dose measured by a dosimeter according to the protocol used.
 5. Apply 500 to 1,000 μ L of diluted Trypsin in 1 \times PBS (10% v/v) on cells for 2 min at 37°C in order to unstick them. Add 500 μ L to 1.5 mL (depending on the cellular density) of fresh medium to stop reaction.
 6. Mix 500 μ L of the cell suspension with 500 μ L of melting LMP Agarose. Lay four drops of mix per coated slide. Place a cover slip and lay the slide on a slide tray resting on the ice until the agarose layer hardens (3–5 min).

739 *3.7.1. Detection of DNA*
740 *Single-Strand Breaks*

This protocol allows detecting mainly DNA single-strand breaks but few DNA double-strand breaks will be detected too.

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1. After solidification, remove cover slips.
 2. Put slides in denaturing lysis buffer for 1 h at 4°C. Cells can be kept in this buffer during extended time.
 3. Put slides in unwinding buffer for 40 min at room temperature.
 4. Place slides in a horizontal gel electrophoresis unit filled with migration buffer. Gently cover slides with buffer at 0.5-cm high.
 5. Electrophorese the slides for 15 min at 0.56 V/cm and adjust the current to 50 mA by raising or lowering the buffer level.
 6. Rinse slides two times with neutralization buffer for 5 min at room temperature.
 7. Dehydrate slides for 10 min in cold 100% ethanol at –20°C.
 8. Dry by exposing to the air.
 9. Stain with 100 μ L ethidium bromide (2 μ g/mL) per slide, drain off, and put a cover slip.
 10. Observe the slides under a fluorescence microscope with 550 nm wavelength. An example is shown in Fig. 6.

757 *3.7.2. Detection of DNA*
758 *Double-Strand Breaks*

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1. After solidification, remove cover slips.
 2. Put slides in non-denaturing lysis buffer for 15 min at room temperature.
 3. Rinse slides with dH₂O for 5 min at room temperature.
 4. Place slides in a horizontal gel electrophoresis unit filled with 1 \times TBE buffer (pH 8.4). Gently cover slides with buffer at 0.5-cm high.

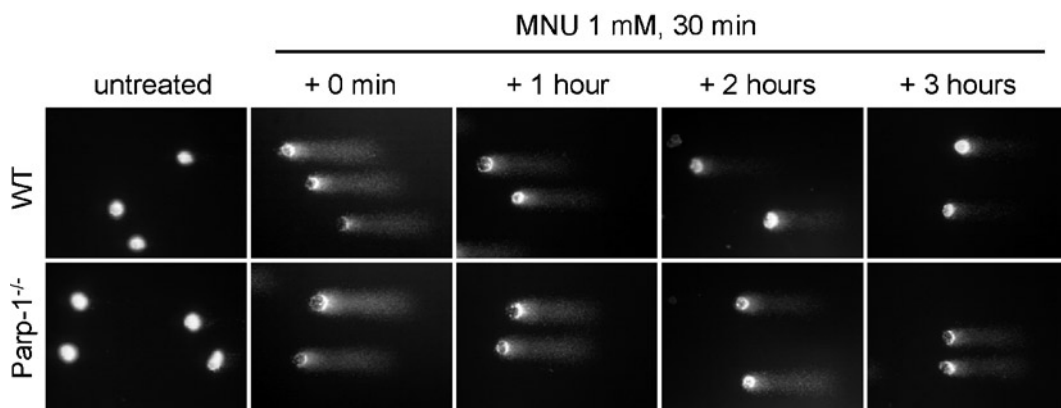


Fig. 6. Delayed repair of MNU-induced DNA damage in Parp-1^{-/-} MEFs compared to WT MEFs as measured by COMET assay.

5. Electrophorese the slides for 4 min at 2.5 V/cm and adjust the current to 16 mA by raising or lowering the buffer level. 764
6. Dehydrate slides for 10 min in cold 100% ethanol at -20°C. 765
7. Dry by exposing to the air. 766
8. Stain with 100 μ L ethidium bromide (2 μ g/mL) per slide, drain off, and put a cover slip. 767
9. Observe the slides under a fluorescence microscope with 550-nm wavelength. 768

4. Notes 772

1. Control wild-type groups should share the same genetic background and breeding conditions of the mutant mice under investigation. Ideally, control and mutant mice should be littermates. 773
2. For a Pantak Seifert X-ray system, settings for irradiating mice are: 200 kV and 4.5 mA with a metallic shutter in place and settings for cells are: 100 kV and 4.5 mA with the metallic shutter removed. 774
3. We have found this kit to be excellent for genotyping. Numerous competitive kits are available from other commercial sources. 775
4. If the tissue is poorly orientated in the final block, the block can be melted and the tissue re-embedded. 776
5. 10% Buffered formalin is not recommended for PAR detection by immunohistochemistry, as it can damage DNA and induce PAR synthesis independently of physiological breaks or damaging agents. 777

- 790 6. This protocol assumes to use manual staining. Alternatively an
791 automatic staining device might be used.
- 792 7. We have found this kit to be excellent for microscopic detec-
793 tion of spontaneous as well as DNA damage-induced cell death
794 in tissue sections. Numerous competitive kits are available from
795 other commercial sources.

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Author Queries

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Queries	Details Required	Author's Response
AU1 ^λ	Please check whether Tris can be changed to Tris-HCl, here and in subsequent occurrences.	
AU2 ^λ	Please clarify whether "pb" represents "bp (base pair)" in Table 1.	
AU3 ^λ	In Fig. 1, Please provide part labels (a) & (b) in caption.	
AU4 ^λ	As per publisher style specification, cross references of Notes should be in sequential order. Please check and renumber if necessary.	
AU5 ^λ	As per publisher style specification, color figures are not allowed. Therefore, all figures have been changed to black and white. Please reword the text accordingly.	
AU6 ^λ	RPM values are not allowed for centrifugation. Please change the value in units of g-force throughout the chapter.	
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