Chapter 4

Compartment-Specific Poly-ADP-Ribose Formation as a Biosensor for Subcellular NAD Pools

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Abstract

Nicotinamide adenine dinucleotide (NAD) is vital to many cellular processes and is distributed between distinct subcellular pools in the compartmentalized eukaryotic cell. The detection and relative quantification of these individual pools is difficult because of the methods usually applied, which require cell disruption and fractionation.

Here, we describe an immunochemical method to visualize and relatively quantify subcellular NAD^+ pools, which relies on the NAD^+-consuming activity of poly-ADP-ribose polymerase 1 (PARP1). We demonstrate that this system can be readily applied to detect changes in the mitochondrial, Golgi, endoplasmic reticulum, and peroxisomal NAD^+ pools.

Key words Compartmentalization, Mitochondria, PARP1, Poly-ADP-ribose, NAD, Biosensor, Immunocytochemistry, Immunoblot analysis

1 Introduction

NAD plays important roles both in redox reactions and in many signaling events. The distribution of these NAD-dependent processes throughout the different cellular compartments reflects the presence of NAD in different organelles [1]. Studying NAD-dependent reactions requires insights into NAD homeostasis in the compartmentalized cell. The NAD-dependent reactions themselves have been widely studied in the context of the relevant enzymes (e.g., by altering gene expression) [2]. However, investigation of the impact of the distribution of NAD itself on these reactions is difficult due to the lack of appropriate methods allowing for selective detection, quantification, and modulation of the different NAD pools.

The method described here allows for relative quantification and modulation of subcellular NAD^+ pools. It relies on the NAD^+-consuming nature of poly-ADP-ribose polymerase 1

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(PARP1) and its preference for automodification [3–5]. In this assay, the constitutively active catalytic domain of PARP1 (PARP1cd) is targeted to a compartment of interest where it is automodified in an NAD^+ concentration-dependent manner (Fig. 1a, b). The resulting polymers can be immunodetected, and the extent of PAR formation reflects NAD^+ availability in any given organelle.

To assess the mitochondrial NAD^+ pool, PARP1cd can be targeted to these organelles by fusion to an appropriate targeting sequence. Expression of PARP1cd in the mitochondrial matrix leads to in situ polymer formation (Figs. 2 and 3a, c). Furthermore, the system is responsive to changes in NAD^+ content, as shown by both incubation of bacterially expressed, purified PARP1cd with increasing NAD^+ concentrations and the effect of an inhibitor of NAD biosynthesis on cells expressing PARP1cd, thereby making it a suitable approach for relative quantification of mitochondrial NAD^+ contents (Fig. 3b, c). In addition, this tool can also be used to constitutively lower mitochondrial NAD^+ levels by stable expression of PARP1cd in these organelles [6–8].

Using appropriate targeting sequences, PARP1cd can also be expressed in the Golgi apparatus, endoplasmic reticulum, peroxisomes, nucleus, and cytosol (Fig. 2) [9]. Similarly to mitochondria, PAR formation can be detected in the Golgi apparatus, endoplasmic reticulum, and peroxisomes. However, expression of PARP1cd in the nucleus or the cytosol does not result in detect-

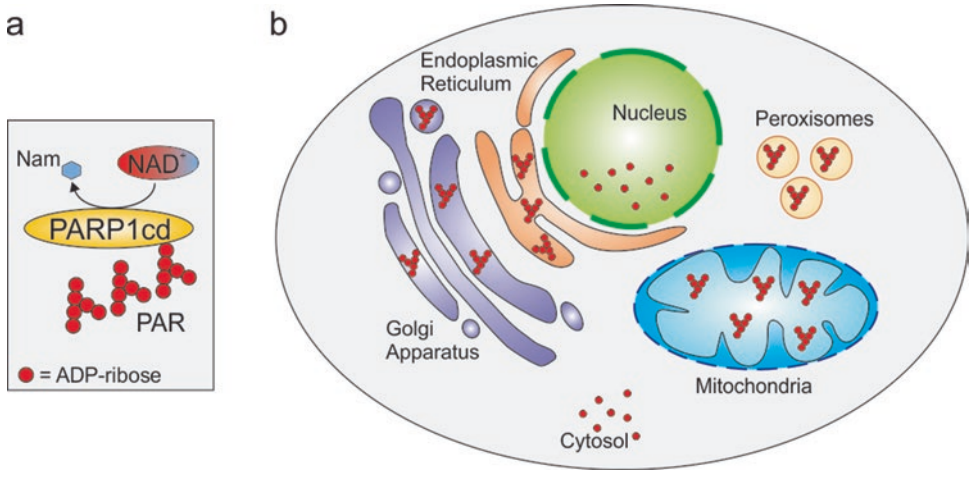

Fig. 1 Principle of PAR-assisted NAD^+ detection. (**a**) The catalytic domain of PARP1 (PARP1cd) is constitutively active and cleaves NAD^+ into nicotinamide (Nam) and ADP-ribose (spheres) to generate poly-ADP-ribose (PAR). The extent of PAR formation is dependent on NAD^+ concentration and availability. (**b**) Fusion proteins consisting of a targeting signal and PARP1cd generate PAR in membrane-bound organelles such as the mitochondria (in the matrix), peroxisomes, endoplasmic reticulum, and the Golgi apparatus. PAR formation is not detectable upon PARP1cd expression in the cytosol and the nucleus most likely due to high PAR-degrading activities carried out primarily by poly-ADP-ribose glycohydrolase isoforms in these compartments

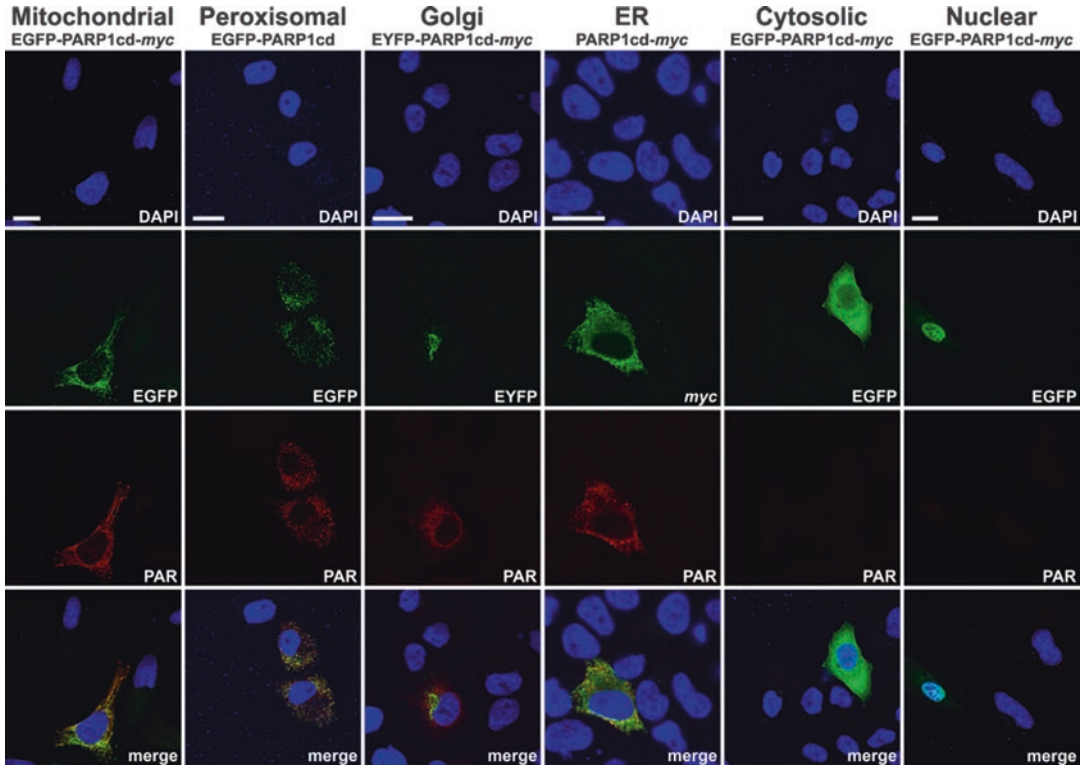

Fig. 2 Compartment-specific expression of PARP1cd leads to immunodetectable PAR formation in membrane-bounded organelles but not in the cytosol and the nucleus of human cells. HeLa S3 cells transiently expressing PARP1cd in the cytosol, nucleus, mitochondria, peroxisomes, Golgi apparatus, and endoplasmic reticulum (ER) were subjected to PAR immunocytochemistry. The cytosolic, nuclear, mitochondrial, peroxisomal, and Golgi-localized proteins were visualized using the intrinsic fluorescence resulting from the fusion of PARP1cd to EGFP or EYFP. The PARP1cd-construct targeted to the ER was co-immunostained using the myc epitope. Images show confocal laser scanning micrographs. Scale bars: 20 μm

able PAR formation (Fig. 2). This is likely due to PAR degradation primarily carried out by poly-ADP-ribose glycohydrolase isoforms present in the cytosol and nucleus [10, 11].

In conclusion, organelle-targeted expression of the catalytic domain of PARP1 enables visualization, relative quantification, and modulation of subcellular NAD^+ pools in a rather straightforward way.

2 Materials

All solutions are to be prepared using ultrapure water (18 μΩ-cm at 25 °C). Unless otherwise stated, solutions can be stored at room temperature.

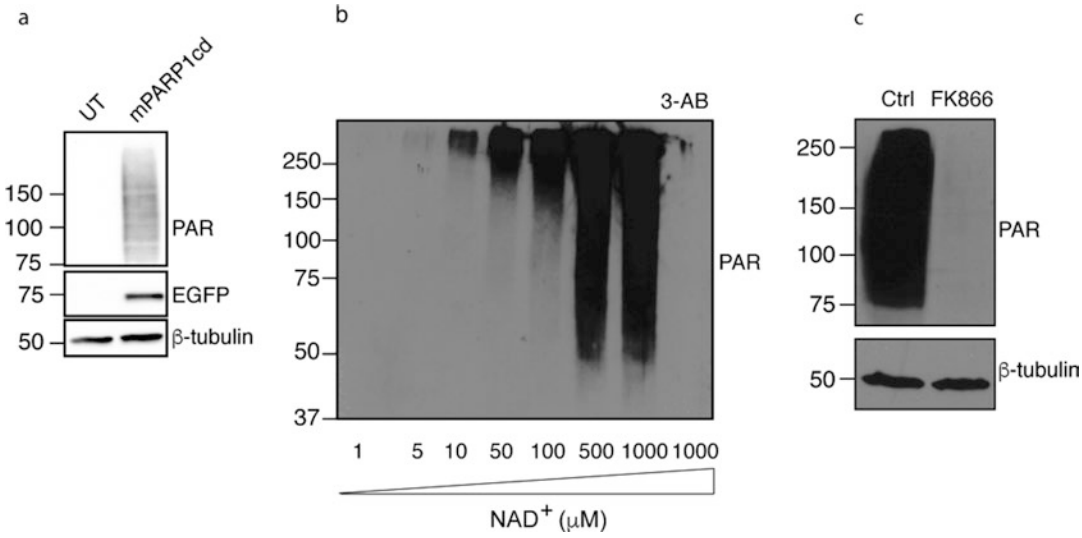

Fig. 3 Expression of the catalytic domain of PARP1 is a biosensor for NAD^+ availability. Heterogenic length and degree of branching of PAR causes PAR immunoreactivity to appear as smeared signal over a wide range of molecular masses. (**a**) PAR formation is detectable in 293 cells expressing mitochondrial PARP1cd (mPARP1cd) but not in untransfected (UT) control cells. PAR immunoreactivity is also detectable upon PARP1cd expression in the endoplasmic reticulum, peroxisomes, and the Golgi apparatus (not shown, [9]). (**b**) Extent of PAR formation is dependent on NAD^+ concentration. The PAR immunoblot shows automodification of purified, recombinant PARP1cd (overexpressed in E. coli) after incubation with the indicated concentrations of NAD^+. PAR formation is precluded by the PARP inhibitor 3-aminobenzamide (3-AB). (**c**) Decrease of mitochondrial NAD^+ content is monitored by PAR formation. Relative NAD^+ contents in mitochondria were detected in lysates of 293 cells expressing mitochondrial PARP1cd in absence or presence of the NAD biosynthesis inhibitor FK866. Panels **b** and **c** are reproduced with permission from [7]

2.1 Plasmid Generation

Detailed maps and sequences of the mammalian expression vectors used in this method are provided on request (Marc.Niere@uib.no).

2.2 Cell Culture

1. Complete Ham's F12 growth medium: Ham's F12 medium supplemented with 10% (v/v) fetal bovine serum, 100 U/ml potassium penicillin, and 100 μg/ml streptomycin sulfate. Store at 4 °C.
2. Complete DMEM medium: DMEM medium supplemented with 10% (v/v) fetal bovine serum, 100 U/ml potassium penicillin, and 100 μg/ml streptomycin sulfate. Store at 4 °C.
3. Neubauer counting chamber.
4. Transfection reagent.
5. 12- and 24-well cell culture plates.

2.3 Immunocytochemistry

1. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4 (*see* **Note 1**).
2. Fixing solution: 4% (v/v) ice-cold formaldehyde in PBS (*see* **Note 2**).

3. PBST/0.5: 0.5% Triton X-100 (v/v) in PBS (*see* **Note 3**).
4. PBST/0.1: 0.1% Triton X-100 (v/v) in PBS (*see* **Note 4**).
5. DAPI solution: 0.5 μg/ml DAPI in PBS (*see* **Note 5**).
6. 24-well cell culture plate.
7. Cover slip no. 1.5, Ø 12 mm.
8. Mounting solution (*see* **Note 6**).

2.4 Immunoblot Analysis

1. SDS lysis buffer: 20 mM Tris–HCl pH 7.4, 150 mM NaCl, 2% (w/v) sodium dodecyl sulfate (SDS), 1 mM ethylenediaminetetraacetate (EDTA), 1 mM 3-aminobenzamide (3-AB) (*see* **Notes 7** and **8**).
2. 5× SDS loading buffer: 300 mM Tris–HCl pH 6.8, 10% (w/v) SDS, 50% (v/v) glycerol, 0.5 M DTT, 0.025% (w/v) bromophenol blue (*see* **Note 9**).
3. 7% SDS-polyacrylamide resolving gel: 7% (w/v) acrylamide/bisacrylamide (37.5:1), 390 mM Tris–HCl pH 8.8, 0.1% (w/v) SDS (*see* **Note 10**).
4. 3% SDS-polyacrylamide stacking gel: 3% (w/v) acrylamide/bisacrylamide (37.5:1), 172 mM Tris–HCl pH 6.8, 0.1% (w/v) SDS (*see* **Note 11**).
5. SDS-PAGE running buffer: 25 mM Tris–HCl pH 8.3, 192 mM glycine, 0.1% (w/v) SDS (*see* **Note 12**).
6. Mini-PROTEAN® Tetra Cell electrophoresis system or similar equipment.
7. Mini-PROTEAN® Spacer plates with 1.0 mm integrated spacers or similar equipment.
8. Mini-PROTEAN® Short Plates or similar equipment.
9. Mini-PROTEAN® Tetra Cell casting stand with clamp kit or similar equipment.
10. Blotting buffer: 25 mM Tris, 192 mM glycine, 20% (v/v) methanol (*see* **Note 13**).
11. Nitrocellulose blotting membrane.
12. Whatman paper.
13. Sponges.
14. Ice unit.
15. Ponceau S solution: 0.1% (w/v) Ponceau S in 5% (v/v) acetic acid (*see* **Note 14**).
16. TBS: 50 mM Tris–HCl pH 7.4, 150 mM NaCl (*see* **Note 15**).
17. TBST/0.1: 0.1% Triton X-100 (v/v) in TBS (*see* **Note 16**).
18. Blocking solution: 5% (w/v) nonfat dry milk in TBST/0.1.

2.5 Antibodies

1. Mouse (IgG3) monoclonal PAR antibody (10H).
2. Chicken (IgY) polyclonal c-Myc tag antibody.
3. Rabbit polyclonal c-*Myc* tag antibody.
4. β-tubulin monoclonal antibody (2–28–33).
5. Living Colors® A.v. monoclonal antibody (JL-8).
6. Alexa Fluor® 594-conjugated goat anti-mouse (IgG H + L) secondary antibody.
7. Alexa Fluor® 488-conjugated goat anti-chicken IgY (IgG H + L) secondary antibody.
8. Alexa Fluor® 488-conjugated goat anti-rabbit (IgG H + L) secondary antibody.
9. HRP-conjugated goat anti-mouse (IgG H + L) secondary antibody.

3 Methods

3.1 PAR Immunocytochemistry

1. Grow HeLa S3 cells in complete Ham's F12 growth medium (*see* **Note 17**).
2. Place a cover slip (no. 1.5, Ø 12 mm) into the cavity of a 24-well flat-bottom tissue culture test plate.
3. One day before transfection, seed 75,000 HeLa S3 cells per well of a 24-well plate in 1 ml complete Ham's F12.
4. One day after cell seeding, replace the medium with fresh one, and transfect the cells with the respective PARP1cd plasmid using, for instance, Effectene® transfection reagent according to the manufacturer's recommendations. Transfect control cells with the corresponding control plasmid, and use untransfected cells to control for nonspecific binding of secondary antibodies.
5. After 24 h, wash the cells with 1 ml PBS.
6. Remove the PBS and incubate the cells with 500 µl fixing solution for 20 min at 4 °C.
7. Remove the fixing solution and wash the cells with 1 ml PBS (*see* **Note 18**).
8. Permeabilize the cells with 500 µl PBST/0.5 for 10 min at room temperature.
9. Remove the PBST/0.5 and wash the cells once with 1 ml PBS.
10. Incubate the cells with 1 ml complete growth medium for 1 h at room temperature in order to avoid nonspecific antibody binding.
11. Dilute primary mouse monoclonal (10H) PAR antibody 1:1000 in complete growth medium, and add 250 µl antibody

solution to each well. Incubate overnight at 4 °C (*see* **Note 19**). Protect the cells from light. For PARP1cd constructs that do not display intrinsic fluorescence, perform co-immunocytochemistry with antibodies specific for the C-terminal *myc* epitope.

12. Remove the antibody solution and wash the cells once for 3 min with 1 ml PBS.
13. Remove the PBS and wash the cells once for 3 min with PBST/0.1.
14. Remove the PBST/0.1 and wash the cells once with 1 ml PBS.
15. Dilute Alexa Fluor® 594-conjugated goat anti-mouse secondary antibody 1:1000 in complete growth medium, remove the PBS from the cells, and add 250 µl antibody solution to each well. Incubate for at least 1 h at room temperature. Protect the cells from light. For co-immunocytochemistry using the *myc* epitope, add appropriate Alexa Fluor® 488-conjugated secondary antibodies.
16. Remove the antibody solution, and stain the chromatin by incubation with 500 µl DAPI solution for 10 min at room temperature (*see* **Note 20**).
17. Remove the DAPI solution and wash cells once for 3 min with 1 ml PBS.
18. Remove the PBS and wash the cells once for 3 min with PBST/0.1.
19. Remove the PBST/0.1 and wash cells once with 1 ml PBS.
20. Add one drop (ca. 5 µl) of mounting solution per cover slip to a microscope slide. Mount and press the cover slip firmly and upside down onto the microscope slide.
21. Clean the microscope slides with water after the mounting solution has dried, and analyze the cells using an inverted epi-fluorescence microscope or by confocal laser scanning microscopy.

3.2 PAR Immunoblot Analysis

1. Cultivate 293 cells in complete DMEM medium.
2. One day before transfection, seed 250,000,293 cells per well of a 12-well plate in 1 ml complete DMEM medium (*see* **Note 21**).
3. One day after seeding, replace the medium with fresh one, and transfect the cells with the mammalian expression vector encoding compartment-specific PARP1cd (e.g., using Effectene® (Qiagen) transfection reagent according to the manufacturer's recommendations). Transfect control cells with the corresponding control plasmid, and use untransfected cells to control for nonspecific binding of secondary antibodies.

4. After 24 h, carefully aspirate the cell culture medium (*see* **Notes 22** and **23**).
5. Carefully wash the cells once with 1 ml PBS.
6. Remove the PBS and add 120–150 µl SDS lysis buffer to the cells (*see* **Note 24**).
7. Collect the lysate with a cell scraper, and transfer the sample into a 1.5 ml reaction tube using a 1 ml pipette (*see* **Note 25**).
8. Pass the sample 10× through a 23-gauge needle attached to a 1 ml plastic syringe until a homogeneous lysate is achieved. Let the lysate rest for some time until the foam produced from the detergent in the SDS lysis buffer has settled (*see* **Note 26**).
9. If many samples are to be processed, keep the samples at room temperature until further use (*see* **Note 27**).
10. For determination of the protein concentration, transfer 10 µl of each sample into a new tube, dilute this further 1:5 in SDS lysis buffer, and use this diluted sample (*see* **Note 28**).
11. Add the adequate volume of 5× SDS loading buffer to the remaining original sample (e.g., for 100 µl sample, add 25 µl 5× SDS loading buffer). Mix briefly by vortexing (*see* **Note 29**).
12. Incubate samples at 95 °C for 5 min.
13. Cool samples briefly (avoid precipitation!), and spin down samples briefly to remove condensate from the lid of the 1.5 ml tube (*see* **Note 30**).
14. Load a volume corresponding to 30 µg total protein on a discontinuous 7% SDS-polyacrylamide gel covered with a 3% SDS-polyacrylamide stacking gel (*see* **Notes 31** and **32**).
15. Run the SDS-polyacrylamide gel electrophoresis until the blue running front has just exited the gel.
16. Transfer the proteins onto a nitrocellulose membrane using a standard Western blot protocol.
17. Confirm homogenous protein transfer by reversible protein staining, for example, using Ponceau S stain (*see* **Note 33**).
18. Incubate the membrane in blocking solution for 1 h at room temperature.
19. Dilute mouse anti-PAR (10H) antibody 1:5000 in 10 ml blocking solution, and incubate the membrane with the antibody overnight at 4 °C.
20. Remove the antibody solution.
21. Wash the membrane 4× with 10 ml TBST for 5 min.
22. Incubate the membrane with secondary anti-mouse HRP conjugate in blocking solution for at least 1 h at room temperature.

23. Remove the antibody solution.
24. Wash the membrane with 10 ml TBST 3× for 5 min.
25. Rinse the membrane with 10 ml TBS.
26. Remove TBS completely.
27. Develop the blot using enhanced chemiluminescence with increased sensitivity using, for example, SuperSignal West Pico Chemiluminescent Substrate according to the manufacturer's recommendations.

4 Notes

1. Prepare 10× PBS by dissolving 80 g NaCl, 2 g KCl, 26.8 g Na_2HPO_4-$7H_2O$, and 2.4 g KH_2PO_4 in 800 ml of water. Adjust the pH to 7.4 with HCl, and add water to a final volume of 1 l. Use autoclaved 1:10 dilutions in cell culture.
2. Prepare freshly by adding 925 µl 10× PBS and 1 ml 37% (v/v) formaldehyde to 7.325 ml ice-cold water.
3. Add 500 µl Triton X-100 to 99.5 ml 1× PBS.
4. Add 100 µl Triton X-100 to 99.9 ml 1× PBS.
5. Prepare freshly from a 5 mg/ml stock solution dissolved in N,N-dimethylformamide, stored at 4 °C in the dark.
6. Add 2.4 g Mowiol® 4-88 (G488) to 4.8 ml 100% glycerol and then add 6 ml of water and stir at room temperature for several hours. Add 12 ml 0.2 M Tris–HCl pH 8.5, heat at 50 °C, and stir until Mowiol is dissolved. Add 0.45 g 1,4-diazabicyclo[2.2.2] octane (DABCO), and stir at 4 °C until DABCO is dissolved. Store aliquots at −20 °C.
7. Prepare this buffer from the following stock solutions: 1 M Tris–HCl pH 7.4 solution, 20% (w/v) SDS, 0.5 M EDTA, and 4 M NaCl. To prepare 1 M Tris–HCl, dissolve 121.14 g of Tris(hydroxymethyl)-aminomethane (Tris) in 800 ml of water, adjust to the desired pH with HCl, and add water to a final volume of 1 l. For the 20% (w/v) SDS stock solution, dissolve 100 g of SDS in 300 ml of water, and then add water to a final volume of 500 ml. For 0.5 M EDTA, dissolve 93.06 g of disodium ethylenediaminetetraacetate-$2H_2O$ (EDTA) in 400 ml of water, adjust to pH 8.0 with NaOH, and add water to a final volume of 500 ml. For 4 M NaCl, dissolve 233.76 g of sodium chloride in 800 ml of water, and add water to a final volume of 1 l.
8. To prepare 50 ml of lysis buffer, mix 30 ml of water with 1 ml of 1 M Tris–HCl pH 7.4, 1.875 ml of 4 M NaCl, 5 ml of 20% (w/v) SDS solution, and 100 µl of EDTA solution, and add water to a final volume of 50 ml. To exclude the possible activation of endogenous PARP enzymes upon lysis, we recommend

adding the PARP1 inhibitor 3-aminobenzamide to the lysis buffer to a final concentration of 1 mM.

9. To prepare 100 ml of 5× SDS loading buffer, mix 30 ml of 1 M Tris–HCl pH 6.8, 10 g of SDS, 50 ml of glycerol, and 25 mg of bromophenol blue. Add water to a final volume of 80 ml, and prepare aliquots of 800 µl (store at −20 °C). Prior to use, add 200 µl of a 2.5 M DTT stock solution.
10. For a 11.25 ml gel solution (sufficient for two 1-mm-thick mini gels), mix 5.568.75 ml of water with 2.925 ml of 1.5 M Tris–HCl pH 8.8, 2.625 ml of a 30% (w/v) acrylamide/bisacrylamide (37.5:1) stock solution, 56.25 µl of 20% (w/v) SDS solution, 60 µl of a 10% (w/v) ammonium persulfate (APS) stock solution, and 15 µl of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED). Immediately pour the solution between the glass plates of the Mini-PROTEAN® system, leaving about 1 cm for the stacking gel. To assure an even surface between the stacking and resolving gel, overlay with 1 ml isopropanol during polymerization. Let the gel polymerize for at least 15 min.
11. For a 5 ml gel solution (sufficient for two mini gels), mix 3.565 ml of water with 860 µl of 1 M Tris–HCl pH 6.8, 500 µl of 30% (w/v) acrylamide/bisacrylamide (37.5:1) stock solution, 25 µl of 20% (w/v) SDS solution, 40 µl of a 10% (w/v) ammonium persulfate (APS) stock solution, and 10 µl of *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED). Remove the isopropanol from the resolving gel, pour the stacking gel on top of the resolving gel, and insert the comb. Let the gel polymerize for at least 15 min.
12. Prepare 10× stock solution common to SDS-PAGE running buffer and blotting buffer. Dissolve 30.3 g Tris and 144 g of glycine in a final volume of 1 l water. Do not adjust the pH. For the preparation of 1 l SDS-PAGE running buffer, dilute 100 ml of this 10× buffer with 895 ml water, and add 5 ml of 20% (w/v) SDS solution.
13. Dilute 100 ml of the 10× buffer described in **Note 6** with 800 ml water, and add 200 ml of methanol. Keep at 4 °C. This solution can be reused up to five times.
14. Dissolve 0.5 g of Ponceau S in 500 ml 5% (v/v) acetic acid. This solution can be reused.
15. To prepare a 10× TBS stock solution, dissolve 60.57 g of Tris and 87.66 g of NaCl in 700 ml of water. Adjust pH to 7.4 with HCl, and add water to a final volume of 1 l. Prior to use, dilute 100 ml of the 10× TBS stock solution with 900 ml of water.
16. For 1 l, dilute 100 ml of the 10× TBS stock solution described in **Note 9** with 899 ml of water, and add 1 ml of Triton X-100.

17. This protocol is established for HeLa S3 cells. For other cell types, growth conditions, cell counts, transfections, etc., have to be adapted individually.
18. The procedure can be interrupted at this step and continued on the next day.
19. Alternatively, incubation with the primary antibodies can be performed at room temperature for at least 2 h.
20. Other fluorescent nucleic acid stains may be used.
21. Seeding, transfection, and incubation conditions (e.g., number of cells, cell type, time of incubation) depend on the experimental setting and have to be established for the specific experiment. The protocol described here is valid for 293 cells.
22. Increasing incubation time (e.g., 48 h) may increase the PAR signal.
23. When many samples using 293 cells are processed, we suggest to work "well by well" from **steps 5–8**.
24. If protein concentration is limiting, use smaller volume (down to 80 µl).
25. The cell lysate may be very viscous. Make sure to transfer the whole sample.
26. This step, in combination with the incubation step at 95 °C, shears the genomic DNA and avoids the need to spin down cell debris.
27. Storage of the samples at room temperature is safe due to the high SDS concentration in the SDS lysis buffer. Storage of the samples on ice is possible but leads to precipitation of SDS in the sample. This does not affect the outcome of the experiment; however, before proceeding with any subsequent steps (e.g., protein concentration determination or loading of samples onto an SDS gel), ensure that all precipitate has been dissolved by incubation at room temperature or heating the sample to 37 °C.
28. Use a method that is compatible with the composition of the SDS lysis buffer, e.g., the bicinchoninic (BCA) assay. If experiencing problems with protein concentration determination, dilute your sample with H_2O.
29. Adjusting all samples to the same protein concentration using additional 1× SDS loading buffer allows for equal loading volume and may improve SDS-polyacrylamide gel electrophoresis and Western blotting.
30. If samples are not processed immediately, they can be stored at −20 °C until further use. When thawing samples, ensure complete resuspension of SDS precipitates that may occur upon freezing, maybe include a brief heating step at 60 °C.

31. If detection of PAR is weak, increase the total protein amount.
32. If detection of other proteins (e.g., the PARP1cd construct and control proteins) is necessary and not possible because of the low acrylamide concentration in the SDS gel, run the same amount of sample in parallel on another SDS gel with a higher acrylamide concentration or use gradient gels.
33. The membrane can be cut horizontally at this point to allow for detection of an EGFP-PARP1cd fusion construct using the JL-8 antibody or of a loading control such as β-tubulin. PAR formation is mostly an automodification of the recombinant protein.

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