Chapter 26

Studying Catabolism of Protein ADP-Ribosylation

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Abstract

Protein ADP-ribosylation is a conserved posttranslational modification that regulates many major cellular functions, such as DNA repair, transcription, translation, signal transduction, stress response, cell division, aging, and cell death. Protein ADP-ribosyl transferases catalyze the transfer of an ADP-ribose (ADPr) group from the β-nicotinamide adenine dinucleotide (β-NAD^+) cofactor onto a specific target protein with the subsequent release of nicotinamide. ADP-ribosylation leads to changes in protein structure, function, stability, and localization, thus defining the appropriate cellular response. Signaling processes that are mediated by modifications need to be finely tuned and eventually silenced and one of the ways to achieve this is through the action of enzymes that remove (reverse) protein ADP-ribosylation in a timely fashion such as PARG, TARG1, MACROD1, and MACROD2. Here, we describe several basic methods used to study the enzymatic activity of de-ADP-ribosylating enzymes.

Key words Poly(ADP-ribose) polymerase, ADP-ribosylation, PARG, Macrodomain, ADP-ribose, Biochemical assay, Hydrolases

1 Introduction

Protein ADP-ribosylation is a posttranslational modification that regulates many major cellular functions, such as DNA damage response and repair, transcription, translation, signal transduction, stress response, cell division, aging, and cell death [1, 2]. Protein ADP-ribosylation is synthesized by several families of enzymes that can act as protein ADP-ribosyl transferases (ART), namely poly ADP-ribosyl polymerases (PARPs), ARTCs (cholera toxin-like transferases), and sirtuins [1]. All these enzymes can utilize nicotinamide adenine dinucleotide (NAD^+) as a substrate to transfer ADP-ribose (ADPr) to target proteins. In response to an initial stimulus, the majority of ARTs can catalyze the addition of only one unit of ADPr (mADPr; MARylation) onto target proteins. However, several PARP family members such as PARP1, PARP2, and tankyrases can add long chains of the repeating ADPr units to their protein targets (polyADPr; pADPr; PARylation) (Fig. 1) [1]. Due to its highly negative charge and size, ADP-ribosylation can

Alexei V. Tulin (ed.), *Poly(ADP-Ribose) Polymerase: Methods and Protocols*, Methods in Molecular Biology, vol. 1608,
DOI 10.1007/978-1-4939-6993-7_26,

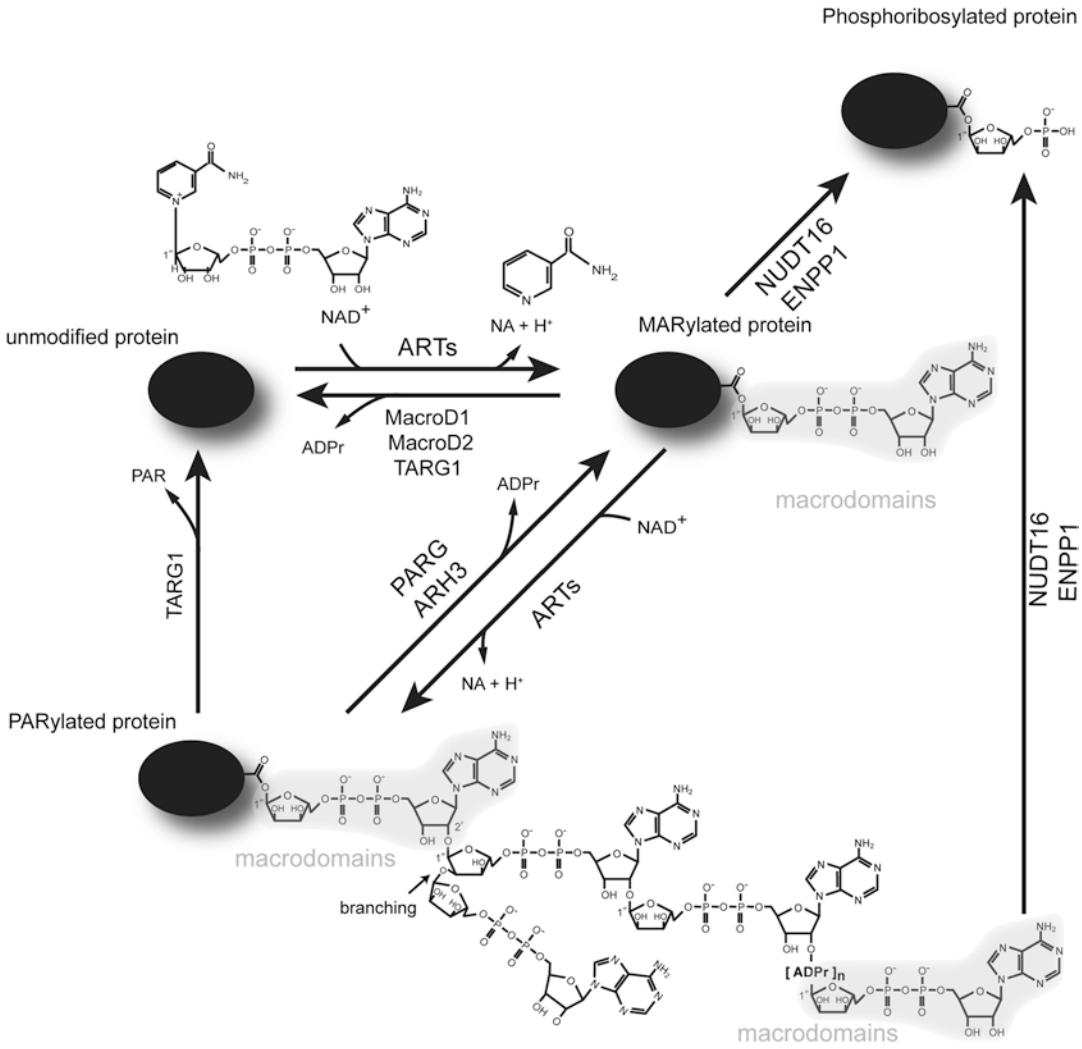

Fig. 1 Cycle of protein ADP ribosylation

strongly affect protein function, interactions, localization and stability [1, 2]. During the regulation of cellular processes, protein ADP-ribosylation signaling needs to be tightly controlled and one way of achieving this is through the timely action of the enzymes that remove (reverse) ADP-ribosylation [3, 4]. PAR glycohydrolase (PARG) is the most characterized enzyme in humans for PAR hydrolysis, which specifically cleaves the ribose–ribose bonds between the ADPr subunits of the PAR chains [5, 6]. Another enzyme able to reverse protein PARylation is ADP-ribosylhydrolase 3 (ARH3) (Fig. 1) [7]. However, these two enzymes are unable to process a single unit of ADPr attached to a protein [6, 7]. Indeed, terminal ADPr unit and MARylation directly linked to an acidic amino acid of the acceptor proteins can be removed by proteins,

such as terminal ADPr protein glycohydrolase (TARG1), MacroD1 and MacroD2 [1, 8–10] (Fig. 1). MARylation linked to arginine residues can be removed by ARH1 [11]. Recently, new classes of enzymes have been identified, capable of partially cleaving the ADP-riboylation producing the proteins carrying phosphoribosylated tags, such as NUDT16 and ENPP1 (Fig. 1) [12, 13].

In this chapter, we describe four commonly used assays to study biochemical activity of enzymes involved in degradation of protein ADP-ribosylation. These assays include: assessment of hydrolytic activity of candidate recombinant enzymes by SDS PAGE using autoADP-ribosylated PARPs as substrates, either using immunodetection (Fig. 2a) or autoradiography (Fig. 2b), and analysis of the reaction products of hydrolases by thin layer chromatography (TLC). We also describe a method to measure glycohydrolase activity of human PARG in cells using an immunofluorescence technique.

2 Materials

2.1 auto ADPribosylation of PARP1 and Western Blotting

1. PARP Enzyme.
2. 10× activated DNA.
3. 20 mM NAD^+ (*see* **Note 1**).
4. PARP Buffer 10×: 500 mM Tris–HCl pH 8.0, 40 mM $MgCl_2$, 500 mM NaCl, 2 mM DTT.
5. PD SpinTrap G-25 (GE Healthcare) (*see* **Note 2**).
6. 1 mM Olaparib (alternative names: AZD2281, KU0059436).
7. Recombinant PARG (*see* **Note 3**).
8. Recombinant NUDT16 (*see* **Note 4**).
9. Recombinant TARG1 (*see* **Note 5**).
10. NuPAGE LDS Sample Buffer (4×) (Life Technologies).
11. 2-Mercaptoethanol ≥99.0%.
12. LDS sample buffer 4× containing 2-mercaptoethanol (1.144 M).
13. NuPAGE 4-12% Bis-Tris Gel (Life Technologies).
14. NuPAGE MOPS SDS Running Buffer (20×) (Life Technologies).
15. Protein pre-stained standard.
16. Trans-Blot® Turbo™ Transfer System (Biorad).
17. Trans-Blot® Turbo™ Nitrocellulose Transfer Packs (Biorad).
18. Nonfat dry milk powder (Nonfat Marvel Dried Skimmed Milk, NFDM).
19. Rabbit Anti-PAR Polyclonal Antibody (Trevigen).
20. Rabbit Anti-PARP1 ab6079 (Abcam).

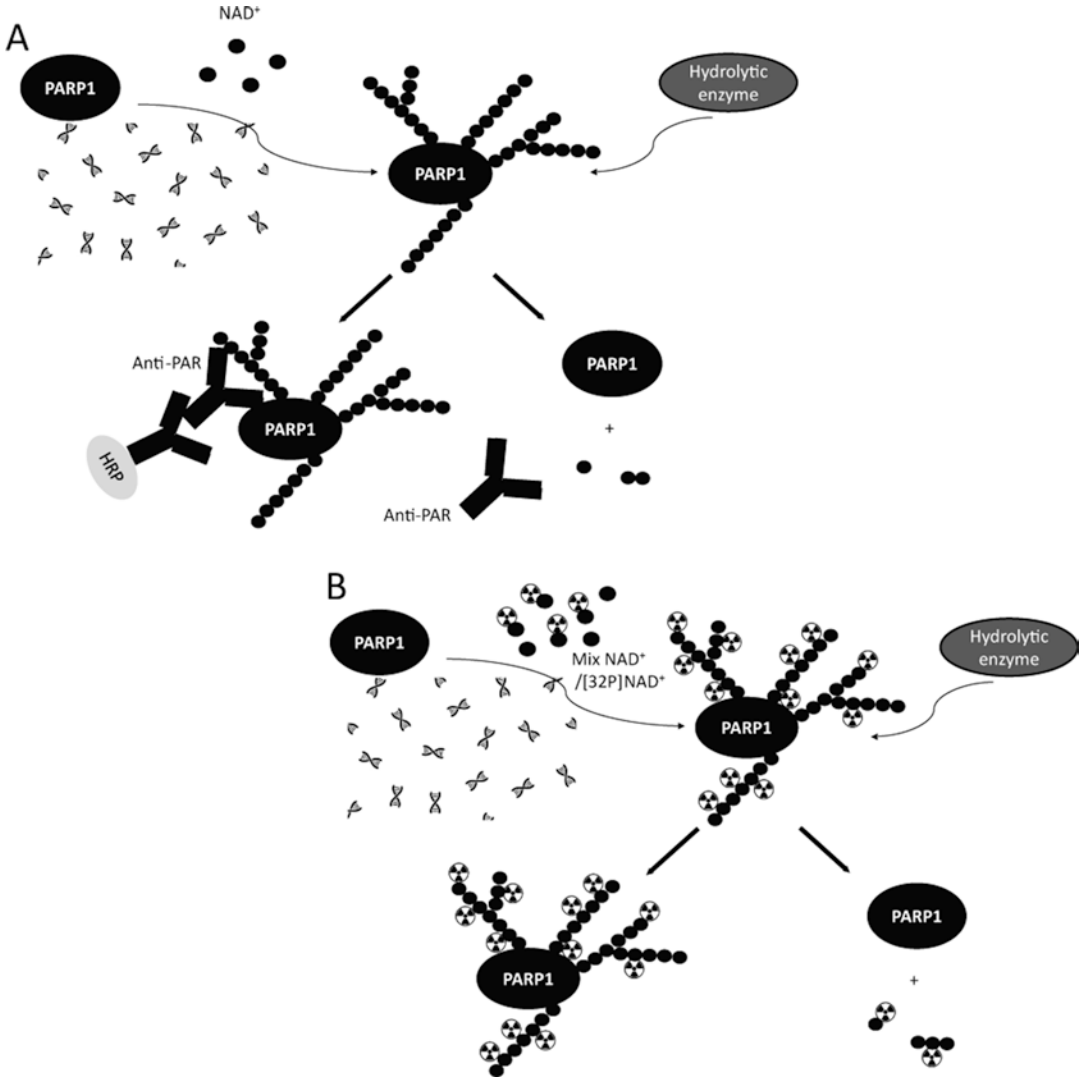

Fig. 2 Schematic illustration of some of the assays described in this chapter. (**a**) PARP1 recombinant protein is activated in vitro in presence of DNA and NAD^+. PARylated PARP1 is afterwards used as substrate to test hydrolytic activity of protein of interest. Read out is a western blot using anti-PAR antibody. If enzyme of interest is hydrolyzing protein PARylation, there will be no antibody recognition. (**b**) PARP1 recombinant protein is activated in vitro in presence of DNA and a mix of cold NAD^+ and radiolabeled NAD^+. In this case, read out is an autoradiography. TLC can be used to characterize chemical by-products of enzymatic reaction

21. Tween 20.
22. PBS 1×/Tween 20 0.1% (PBS-T).
23. PBS 1×/Tween 20 0.1% containing 5% NFDM (PBS-TM).
24. Polyclonal swine anti-rabbit immunoglobulins/HRP.
25. ECLWestern blotting substrate.
26. Amersham Hyperfilm ECL (GE Healthcare).

2.2 auto ADPribosylation of PARP1 in Presence of Radiolabeled NAD^+

1. NAD, [32P]- 800 Ci/mmol 5 mCi/ml (PerkinElmer).
2. Fixing solution: 10% (v/v) acetic acid, 10% (v/v) methanol.
3. Whatman™ 3MM Chr chromatography paper.
4. Gel dryer.
5. BIOMAX Maximum Sensitivity Film (Kodak).

2.3 Thin Layer Chromatography (TLC)

1. POLYGRAM CEL 300 PEI (MACHEREY-NAGEL).
2. TLC mobile phase: 0.15 M formic acid, 0.15 M lithium chloride (*see* **Note 6**).
3. TLC developing tank.

2.4 auto ADPribosylation of PARP1-E988Q Mutant

1. Recombinant PARP1-E988Q (*see* **Note 7**).

2.5 Measurement of Glycohydrolase Activity of Human PARG in Cells

2.5.1 Cell Culture and Plating

1. HeLa cells (CCL-2 from ATCC).
2. Cell culture media RPMI—1640.
3. Fetal bovine serum (FBS).
4. L-glutamine.
5. 75 cm^2 cell culture flasks.
6. BD Falcon 40 μM cell strainer.
7. ViaFlow Assist.
8. Black 384-well plates (Greiner).
9. Gloves.
10. Falcon 50 mL tubes.
11. Merck Millipore Muse cell counter or any other cell counter.
12. TrypLE Express.

2.5.2 Dosing Cells

1. Methyl methanesulfonate (MMS) (1.3 g/ml).
2. PARG inhibitor.
3. DMSO.
4. Labcyte Echo source plates.
5. Labcyte Echo 555.

2.5.3 Staining Cells

1. Phosphate buffered saline (PBS).
2. Methanol—HPLC grade.
3. Triton X-100.
4. Tween 20.
5. Anti-PAR mouse monoclonal antibody (10H) (Calbiochem AM80).

6. Alexa Fluor® 488 goat anti-mouse IgG (H + L).
7. Hoechst 33342.
8. Nunc aluminum seal tape for 96-well plates.

2.5.4 Capturing Images

1. Thermo Scientific™ CellInsight™ platform for high-content analysis.

3 Methods

3.1 auto ADPribosylation of PARP1 and Western Blotting

3.1.1 Activation of PARP1 and Automodification

1. Preparation of fresh PARP buffer 10×.
2. Calculate 10 μL of reaction for each experimental point (*see* **Note 8**).
3. Once determined the final volume of master mix, dilute PARP buffer 10× to 1× final, add NAD^+ to 200 μM final concentration, activated DNA 10× to 1× final, 1 μL of PARP1 (10 units/μL) every 50 μL of reaction, and add nuclease-free water to the final volume.
4. Incubate the reaction for 20 min at room temperature.
5. Add 1 μM olaparib (KU0059436) to inhibit PARP1

3.1.2 Desalting of Automodified PARP1 Mix (Optional)

1. Depending on the type of experiment, a desalting step may be required to remove excess of unreacted NAD^+. For this step, we use PD SpinTrap G-25 columns. They contain Sephadex G-25 Medium, which allows rapid group separation of high molecular weight substances from low molecular weight substances. Therefore, small molecules such as salts, unincorporated NAD^+, DNA fragments, and other impurities are efficiently separated from the high molecular weight substances of interest.
2. A number of three G-25 are suggested to enrich in highly modified PARP1. Thus, three G25 columns were equilibrated five times into the desired buffer, in according to manufacture protocol.
3. Pass the mix containing automodified PARP1 through three different equilibrated G-25 columns. The final flow through is then ready for downstream experiments.

*3.1.3 PARP1 as Substrate to Test Hydrolytic Activity of Candidate Enzymes (See **Note 9**)*

1. Aliquot 10 μL of PARP1 mix in clean tubes for each experimental point, including a negative control.
2. To test hydrolytic activity of candidate enzymes, consider a final volume of reaction of 15 μL (*see* **Note 10**).
3. Add desired concentrations of hydrolytic enzymes and buffer to 15 μL.
4. Incubate reaction at desired temperature and time (*see* **Note 11**).

5. Stop reaction by adding 4 μL of LDS sample buffer (4×) containing 2-mercaptoethanol and mix properly.
6. Boil sample 2 min at 90 °C.
7. Centrifuge samples at maximum speed for 30 s.
8. Load in duplicate 8 μL of each sample in two separate SDS-PAGE gels. One will be blotted against PAR and the second one against PARP1 (*see* **Note 12**) (Fig. 3a, b).
9. Run samples on SDS-PAGE gels at 180 V for ~1 h. Stop the gel when the migration dye front has arrived to the end of the gel length.
10. Transfer proteins from gels to nitrocellulose membranes. We use Trans-Blot® Turbo™ Transfer System, using Standard program (25 V, 1.0 A, 30 min) setting transfer.
11. Wash the nitrocellulose membranes in PBS-T and incubate with PBS-TM at room temperature for 1 h with gentle agitation.
12. Wash membranes with PBS-T twice for 5 min.
13. Incubate one nitrocellulose membrane with anti-PAR (1:2000), and the second one with anti-PARP1 (1:1000) diluted in PBS-TM at room temperature for 1 h.
14. Discard and regain the primary antibodies and wash membranes twice in PBS.
15. Wash membranes with PBS-T twice for 5 min.

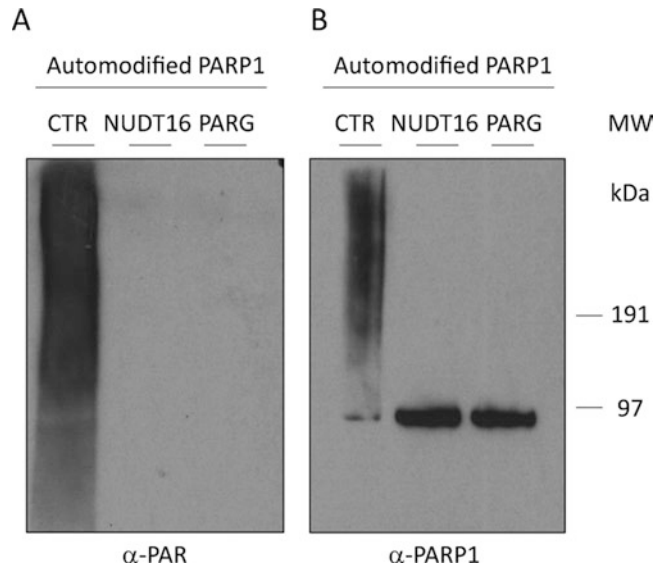

Fig. 3 AutoADP-ribosylation of PARP1 and analysis of pADPr hydrolytic activity by western blot

16. Incubate nitrocellulose membrane with polyclonal swine anti-rabbit immunoglobulins/HRP diluted 1:3000 in PBS-TM at room temperature for 1 h.
17. Wash membranes twice in PBS.
18. Wash membranes with PBS-T twice for 5 min.
19. Wash membranes twice in PBS.
20. Perform western blot chemiluminescent reactions.
21. Develop western blots.

3.2 auto ADPribosylation of PARP1 in Presence of Radiolabeled NAD+

3.2.1 Activation of PARP1 Wild Type and Automodification Using Radiolabeled NAD+

1. Preparation of fresh PARP buffer 10×
2. Calculate 10 μL of reaction for each experimental point (*see* **Note 8**).
3. Once determined the final volume of master mix, dilute PARP buffer 10× to 1× final, add cold NAD^+ to 2 μM final concentration, activated DNA 10× to 1× final, 0.5 μCi [32P]- NAD^+, and nuclease-free water to the final volume. Mix by pipetting.
4. Add 1 μL of PARP1 (10 units/μL) every 50 μL of reaction.
5. Incubate the reaction for 20 min at room temperature.
6. Add 1 μM olaparib (KU0059436) to inhibit PARP1.

3.2.2 Desalting of Automodified PARP1 Mix (Optional)

1. Perform desalting as described in Subheading 3.1.2. If desired to perform TLC as a downstream approach, this step is essential to get a clean chromatography pattern.

*3.2.3 Radiolabeled PARP1 as Substrate to Test Hydrolytic Activity of Candidate Enzymes (See **Note 9**)*

1. Aliquot 10 μL of PARP1 mix in clean tubes for each experimental point, including a negative control.
2. To test hydrolytic activity of candidate enzymes, consider a final volume of reaction of 15 μL (*see* **Note 10**).
3. Add desired concentrations of hydrolytic enzymes and buffer to 15 μL.
4. Incubate reaction at desired temperature and time.
5. If desired to perform TLC as downstream assay, save 2 μL of reaction in a clean tube.
6. Stop reaction by adding 4 μL of LDS sample buffer (4×) containing 2-mercaptoethanol and mix properly.
7. Boil sample 2 min at 90 °C.
8. Centrifuge samples at maximum speed for 30 s.
9. Load samples and run on SDS-PAGE gels at 180 V for ~1 h. Stop the gel when the migration dye front has arrived to the end of the gel length.
10. Wash gel twice in water for 5 min at room temperature with gentle agitation.

11. Add fixing solution to the gel and incubate it for at least 30 min.
12. Discard fixing solution and wash the gel in water with frequent water changes for at least 1 h.
13. Place gel on 3MM chromatography paper and dry it using gel dryer.
14. Expose the gel using normal ECL film or high sensitivity X-ray films (Fig. 4a).

3.3 Thin Layer Chromatography (TLC)

Separation of radiolabeled small compounds on TLC plate.

1. Prepare one POLYGRAM CEL 300 PEI TLC plate.
2. Use a ruler to define the input line. The input line has to be at 1.6 cm from the bottom of plate.
3. Using a thin pencil, mark on the input line the loading points. Every point has to be 1 cm spaced from the others.
4. Spot 1 μL of each sample on loading point.
5. Let the drop dry.
6. Decant the TLC mobile phase into the TLC developing tank and gently place TLC plate inner.
7. Cover the tank to avoid evaporation.

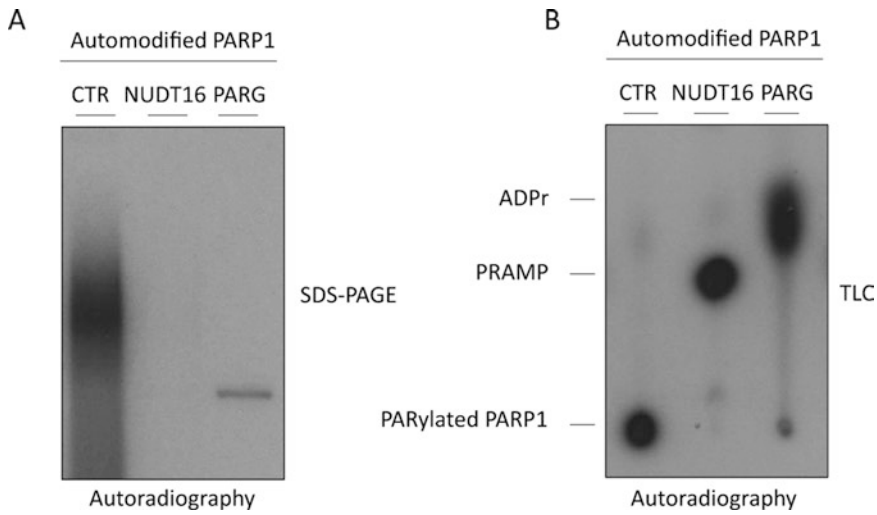

Fig. 4 AutoADP-ribosylation of PARP1 in presence of radiolabeled NAD^+ and analysis of pADPr hydrolytic activity by using autoradiography and TLC. (**a**) PARP1 was automodified as described in the text, and used as substrate for NUDT16 (5 μM) and PARG (1 μM). Samples were fractionated on SDS-PAGE as discussed in the relative paragraph and autoradiography shown in Fig. **b**. 1 μL of same samples shown in Fig. 4a were loaded on TLC plate. PARylated PARP1 was not able to move from the loading point. NUDT16 mainly released phosphoribose-AMP (PRAMP), PARG releases units of ADPr

8. When the migration arrives at 1 cm from the top of TLC plate, gently remove the plate from the tank and air-dry it (*see* **Note 13**).
9. Expose the gel using normal ECL film or high sensitivity X-Ray films (Fig. 4b).

3.4 auto ADPribosylation of PARP1-E988Q Mutant

3.4.1 Activation of PARP1-E988Q and Automodification

1. Preparation of fresh PARP buffer 10×
2. Calculate 10 μL of reaction for each experimental point (*see* **Note 8**).
3. Once determined the final volume of master mix, dilute PARP buffer 10× to 1× final, add cold NAD^+ to 2 μM final concentration, activated DNA 10× to 1× final, 0.5 μCi [32P]- NAD+, and nuclease-free water to the final volume. Mix by pipetting.
4. Add 1 μM of PARP1-E988Q (*see* **Note 7**).
5. Incubate the reaction for 20 min at room temperature.
6. Add 1 μM olaparib (KU0059436) to inhibit PARP1.

3.4.2 PARP1-E988Q as Substrate to Test Hydrolytic Activity of Candidate Enzymes (See **Note 9**)

1. Aliquot 10 μL of PARP1-E988Q mix in clean tubes for each experimental point, including a negative control.
2. To test hydrolytic activity of candidate enzymes, consider a final volume of reaction of 15 μL (*see* **Note 10**).
3. Add desired concentrations of hydrolytic enzymes and buffer to 15 μL. In Fig. 5, we probed NUDT16 (*see* **Note 4**) and TARG1 (*see* **Note 5**).
4. Incubate reaction at desired temperature and time.
5. Stop reaction by adding 4 μL of LDS sample buffer (4×) containing 2-mercaptoethanol and mix properly.
6. Boil sample for 2 min at 90 °C.
7. Centrifuge samples at maximum speed for 30 s.
8. Load samples and run on SDS-PAGE gels at 180 V for ~1 h. Stop the gel when the migration dye front has arrived to the end of the gel length.
9. Wash gel twice in water for 5 min at room temperature with gentle agitation.
10. Add fixing solution to the gel and incubate it for at least 30 min.
11. Discard fixing solution and wash the gel in water with frequent water changes for at least 1 h.
12. Place gel on 3MM chromatography paper and dry it using a gel dryer.
13. Expose the gel using normal ECL film or high sensitivity X-ray films (Fig. 5).

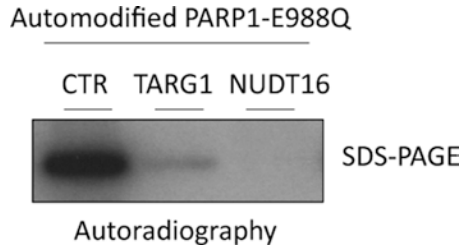

Fig. 5 AutoADP-ribosylation of PARP1-E988Q mutant and analysis of mADPr hydrolytic activity by autoradiography. PARP1-E988Q was used as substrate for hydrolytic activity of TARG1 (1 μM) and NUDT16 (5 μM). Samples were fractionated on SDS-PAGE as discussed in the relative paragraph and the autoradiography shown in figure

3.5 Measurement of Glycohydrolase Activity of Human PARG in Cells

3.5.1 Cell Preparation

1. Make complete media by adding 50 mL FBS and 5 mL glutamine to RPMI 1640.
2. Thaw Hela cells into media according to ATCC instructions
3. Maintain in culture in a T75 for 2 weeks and passage cells 2–3 times and do not let them become fully confluent (*see* **Note 14**).
4. When ready prepare cells for assay.
5. Aspirate media from flask and add 20 mL PBS to monolayer. Gently swirl PBS and then aspirate.
6. Add 2.5 mL TrypLE cell dissociation liquid and ensure that it covers all cells by tipping flask. Aspirate all but 0.5–1 mL TrypLE and return flask to incubator for 4 min.
7. Remove flask from incubator and check that cells have rounded up then tap gently to detach from the bottom of flask
8. Add 20 mL complete media and pipette up and down three times before passing through a 40 μM strainer in a 50 mL tube. Count cells (using two different samples from cell population—*see* **Note 15**).
9. Dilute cells to 4×10^4 cells/mL and plate 30 μL in each well of a 384-well plate and incubate cells overnight at 37 °C, 5% CO2 (*see* **Note 16**)
10. Visually check cells using an inverted microscope to ensure equal distribution.

3.5.2 Dosing Cells

The Echo 555 is an instrument that uses tipless, non-contact, acoustic fluid transfer for dispensing compounds. Using acoustic transfer eliminates the potential for samples or compounds to adhere to tips and helps to improve data quality. Compounds are typically pipetted into a low-dead volume (LDV) source plate and are fired using acoustic technology into an inverted plate (*see* **Note 17**). This should not be attempted unless the user has been thoroughly trained.

3.5.3 Dose Cells with Compound

1. Design dosing regimen using Echo Dose Response software (*see* **Note 18**) and add trial compounds, PARGinhibitor (PDD00017273) and DMSO to the source plate. Spin the source plate at 150 × *g* for 1 min.
2. Spin the destination plate (containing the cells) at 1000 rpm (164 × *g*) for 1 min (*see* **Note 19**) and use the Echo to dispense test compounds.
3. Incubate cells for 1 h at 37 °C, 5% CO2.

3.5.4 Dose Cells with DNA Damaging Agent

1. Prepare MMS: To 63 μL DMSO add 15 μL MMS from commercial stock. This gives a secondary stock of 250 mg/mL. Further dilute this using DMSO to 50 μg/mL. Add 50 μg/mL MMS or DMSO to the source plate as appropriate and spin the source plate at 150 × *g* for 1 min.
2. Spin destination plate (containing cells) again at 1000 rpm (164 × *g*) for 1 min
3. Use the Echo to dispense 30 nL MMS or DMSO into a 384-well plate (*see* **Note 18**).
4. Check Echo firing report for compounds and MMS
5. Incubate cells for exactly 1 h at 37 °C, 5% CO2 (*see* **Note 20**).

3.5.5 Staining Cells

1. After incubation with MMS, fix cells: Tip/flick off media into a large bowl/tray and immediately add 50 μL/well of ice-cold 95% methanol/PBS and incubate for 15 min at −20 °C.
2. Tip/flick off media/fixative and wash plates by adding 50 μL/well PBS. This can be done manually or by using an automated device (*see* **Notes 21**–**23**).
3. Tip/flick off PBS and permeabilize cells by adding 50 μL/well of 0.1% Triton X-100 in PBS and incubate for 20 min at RT.
4. Meanwhile prepare primary antibody stain by diluting anti-PARmouse monoclonal antibody (10H) at 1/4000 in 5% FBS in PBS + 0.05% Tween 20. Calculate approximately 8 mL per plate (including overage).
5. Tip/flick off PBS/Triton and wash plates once by adding 50 μL/well PBS.
6. Tip/flick off PBS and stain cells by adding 20 μL/well of primary antibody and incubate overnight at 4 °C
7. Prepare secondary antibody stain by diluting Alexa Fluor® 488 goat anti mouse IgG (H + L) at 1/1000 and Hoechst at 1:500 in 5% FBS in PBS + 0.05% Tween 20.
8. Using same technique as before wash plates three times with 50 μL/well PBS.
9. Add 20 μL/well of secondary antibody and incubate for 1 h at RT.

10. Wash plates three times with 50 μL/well PBS.
11. Add plate seal and read plates on Cell Insight or another similar high content screening system.

3.5.6 Data Analysis Using Cell Insight

1. Load Cellomics Scan software.
2. Click Load/Unload plate and insert plate into the machine. Ensure A1 is in the correct orientation.
3. Change the protocol to compartmental analysis and select the 10× objective.
4. Select two wells dosed at 30 μM with PARG inhibitor and MMS (positive control) and two wells dosed with PARG inhibitor alone. Select Channel 1 and click Autofocus. Click Channel 2 and then Acquire Image. Select 4 on the contrast setting. The image will be automatically saved.
5. Go to Group Configuration window to load the images. Work through the following:
6. Look at Primary Object Identification Ch1 and check that the nuclei have been correctly identified.
7. Look at Primary Object Validation Ch1 and check that the correct nuclei have been validated.
8. Look at Spot identification Ch2 and check that spots are observed in the nuclei of the max wells. Set the threshold low enough that some signal is detected in your PARG alone dosed cells.
9. Look at the statistics for the CircSpotTotalIntensity and check the values for positive control and PARG alone dosed cells. Save the protocol.
10. Select the area of the plate you want to scan and click to scan the plate.
11. After the scan has completed, load the Cellomics View software. Select the plate from the list and report the following: Valid object count, Mean object area, Mean circspot total intensity Ch2.
12. Export the data to Excel and analyze in an appropriate software to calculate IC_{50} values (*see* **Note 24**).

4 Notes

1. Store at −20 °C. Avoid freeze and thaw cycles.
2. PD SpinTrap G-25 desalting columns can be used to get rid of small compounds present into the PARP reaction of automodification, such as unreacted NAD^+ and DNA that can interfere with downstream enzymatic reactions.
3. Human recombinant PARG was expressed and purified as previously described in ref. [14].

4. NUDT16 was expressed and purified as previously described [12].
5. TARG1 was expressed and purified as previously described in ref. [8].
6. Always prepare fresh. First prepare 3 M Lithium Chloride (LiCl) solution and then prepare solution containing 10 mL of 3 M LiCl and 1.13 mL Formic Acid in water, 200 mL final volume.
7. pET28a PARP1-E988Q was expressed from and purified as previously described for wild-type PARP in ref. [15].
8. In order to avoid volume loss because of pipetting errors, always consider to prepare an activated PARP1 mix at least one sample (10 μL reaction for each sample) in excess to the effective number of experimental points.
9. Some hydrolytic enzymes require divalent cations. For example, NUDT16, ARH1 and ARH3 require $MgCl_2$. In this case, complementation in PARP1 master mix is necessary [12].
10. Final volume has to be decided by the experimenter depending on the starting concentration of recombinant hydrolytic enzymes to test. 15 μL final volume is an example in which 10 μL are represented by the substrate (PARP1 mix) and a maximum of 5 μL are available to treat ADP-ribosylated protein with desired concentrations of hydrolytic enzymes.
11. Incubation temperature and time depends on the efficiency of enzymes that have to be tested. PARG completely hydrolyses PARylate PARP1 at room temperature in 30 min. If nothing is known about the enzymes to be tested, incubate at 30 °C for at least 3 h.
12. Depending on the number of samples, we recommend to load the two set of samples into the same gel separated by a lane containing protein standard.
13. A fan heater can be used to quickly dry the TLC plate.
14. We have found that cells that have been in culture for less than 2 weeks or for more than 10 weeks tend to have greater variability (*see* James et al. [16], F1000Research)
15. Although there are many ways to count cells we find the most reliable for single cell populations is to use the Cell Viability stain on Merck Millipore Muse cell counter and count 5000 cells.
16. Although this can be achieved manually using an appropriate multichannel pipette we find that using and an automated method (Viaflow Assist) gives more consistent results.
17. The amount of media in the target or destination plate has to be demonstrated empirically. The plate is inverted in order to be dosed and then moves briskly whilst being dosed; too much media in each well will result in the media falling out of the well. Other fluid transfer instruments could be suitable but keep the percentage of DMSO added to the cells at less than 0.3%.

18. We use the Echo Dose Response software to dispense compounds into 304 wells of a 384-well plate—that is, all wells apart from the outer wells which are often more variable due to edge effects. The plate is dosed so that seven compounds are dosed with a ten point dose response (threefold dilutions from 30 μM to 0.001 μM) in duplicate in the top half of the plate and the same seven compounds are dosed in duplicate in the bottom half of the plate. This then allows the compounds to be dosed with single dose of MMS (or DMSO).
19. The wells in a 384-well plate are cuboid and we suspect that the vertices will draw media up over time and disrupt the surface tension.
20. It is critical that each plate of cells is incubated for exactly the same amount of time with compound and MMS.
21. We use the Biotek Elx 405 Select CW plate washer if more than three plates are being stained at any one time. This minimizes repetitive strain injury and ensures greater consistency of washing between plates.
22. After fixation and washing the plate containing the cells can be stored at 4 °C.
23. This assay should be run twice in the same week on cells with the same passage number. This can be achieved by splitting cells at different densities on the week previous to the assay. When carrying out this protocol twice, the first run can be fixed and the plates left with PBS at 4 °C after **step 2** (staining cells).
24. Note that the assay uses MMS at a final concentration of 50 μg/mL. If EC_{50}s shift, considering changing batch of MMS.

Acknowledgment

This work was supported by the Wellcome Trust (grant number 101794), the European Research Council (grant number 281739) and by Cancer Research UK (grant numbers C480/A1141, C5759/A17098, and C35050/A22284).

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