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Identifying Direct Protein Targets of Poly-ADP-ribose Polymerases (PARPs) Using Engineered PARP variants—orthogonal Nicotinamide Adenine Dinucleotide (NAD⁺) analogue pairs

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Abstract

Poly-ADP-ribose polymerases (PARPs) comprise a family of 17 distinct enzymes that catalyze the transfer of ADP-ribose from nicotinamide adenine dinucleotide (NAD⁺) to acceptor sites on protein targets. PARPs have been implicated in a number of essential signaling pathways regulating both normal cell function and pathophysiology. To understand the physiological role of each PARP family member in the cell we need to identify the direct targets for each unique PARP in a cellular context. PARP-family member specific target identification is challenging because of their shared catalytic mechanism and functional redundancy. To address this challenge, we have engineered a PARP variant that efficiently uses an orthogonal NAD⁺ analogue, an analogue that endogenous PARPs cannot use, as a substrate for ADP-ribosylation. The protocols in this article describe a general procedure for using engineered PARP variants—orthogonal NAD⁺ analogue pairs for labeling and identifying the direct targets of the poly-subfamily of PARPs (PARPs 1–3, 5, and 6).

Keywords

Poly-ADP-ribose polymerase; PARP; ADP-ribosylation; ADP-ribose; ADPr; click chemistry; proteins; post-translational modification

INTRODUCTION

This article describes a general method for the enrichment and identification of the direct protein targets of individual poly-ADP-ribose polymerase (PARP) family members. 17 individual PARPs have been identified in humans based on the presence of the conserved diphtheria toxin-like catalytic domain (Hottiger et al., 2010). The PARPs transfer ADP-ribose (ADPr) from nicotinamide adenine dinucleotide (NAD⁺) onto acceptor proteins (Burkle, 2005). A subset of the PARP family (PARPs 1–3, 5, and 6) can attach additional ADPr units onto the initially transferred ADPr, resulting in long chains of poly-ADP-ribose (PAR) (Vyas et al., 2014). ADP-ribosylation mediates many important cellular processes,

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including DNA repair (Kun et al., 2002; Okano et al., 2003), transcription, genome maintenance, stress response pathways (Hassa and Hottiger, 2008), and cell division (Krishnakumar and Kraus, 2010; Vyas et al., 2013). A complete understanding of ADP-ribosylation in physiology and pathophysiology requires the identification of the direct protein targets for each of the PARP family-members. The units in this report describe using engineered PARP variants—orthogonal NAD⁺ analogue pairs for the identification of the direct substrates of individual PARP family members (Figure 1A, see Commentary for further discussion) (Carter-O'Connell et al., 2014).

To generate engineered PARP variants—orthogonal NAD⁺ analogue pairs, we mutated a semi-conserved lysine residue (K903, human PARP1 numbering) in the nicotinamide-binding pocket to an alanine in an effort to create a unique pocket that can accommodate an ethyl substituent at the C-5 position of the nicotinamide ring of NAD⁺ (5-Et-6-a-NAD⁺, Figure 1B). The alkyne substituent at the N-6 position of the adenine ring of 5-Et-6-a-NAD⁺ serves as a latent tag: after incorporation into protein targets as 6-alkyne-ADPr (6-a-ADPr), the alkyne can be conjugated to an azide-bearing reporter (e.g. biotin-PEG₃-N₃) via a copper-catalyzed cycloaddition reaction (click chemistry); allowing direct protein targets to be labeled and identified. 6-a-NAD⁺ had previously been used to identify the global targets of ADP-ribosylation (Jiang et al., 2010), but it cannot be used to identify the direct protein targets of a given PARP family member because all PARPs use 6-a-NAD⁺ as a substrate with similar efficiency. By contrast, 5-Et-6-a-NAD⁺ is an efficient substrate for KA-PARPs, but not their wild-type counterparts. As such, 6-a-ADPr will only be transferred from 5-Et-6-a-NAD⁺ onto the direct protein targets for the specific KA-PARP variant. The labeling of direct protein targets using this technique has been confirmed for PARP1, 2, and 6; based on the presence of the K903 residue in both PARP3 and PARP5, it should be adaptable for each PARP member that catalyzes PAR formation. It is important to note that mutation of K903 to alanine in KA-PARPs converts them from a poly- to a mono-ADP-ribosyltransferase (Carter-O'Connell et al., 2014). This attribute reduces the complexity of the sample - facilitating target identification - and in our experience does not appear to affect targeting.

Labeling direct protein targets using this method presents a number of key advantages. The labeling reaction can be carried out in solution, is non-radioactive, and, most importantly, is specific for the KA-PARP variant. This makes the method well suited for visualizing the target profiles for individual PARP members. An important application of this method is the identification of direct protein targets using tandem mass spectrometry (LC-MS/MS). Following conjugation with biotin-azide via the click reaction, biotinylated proteins (i.e. direct targets of a given KA-PARP) can be enriched using NeutrAvidin agarose, proteolyzed, and the eluted peptides subject to LC-MS/MS. As target identification has proven essential for understanding PARP biology (Abd Elmageed et al., 2012), the ability to globally assess target profiles for an individual PARP family member represents a powerful tool for understanding this class of enzymes.

STRATEGIC PLANNING

Expression of KA-PARP and Synthesis of 5-Et-6-a-NAD⁺

The transfer of 6-a-ADPr to direct protein targets from 5-Et-6-a-NAD⁺ was demonstrated for the KA-PARP variants of PARP1, PARP2, and PARP6 (Carter-O'Connell et al., 2014). Based on: (i) the sequence similarity between the members of the PARP family capable of catalyzing PAR formation (PARP1–3, 5, and 6) (Smith, 2001); (ii) the high level of conservation in the catalytic domain between PARP5 and PARP6 (Gunaydin et al., 2012); and (iii) the presence of the conserved lysine residue at position 903 (PARP1 numbering), the following protocols should be applicable to all of the members of this sub-class of PARP enzymes (PARPs 1–3, 5, and 6). A source of pure, active recombinant KA-PARP is essential for successful generation of 6-a-ADPr labeled direct protein targets. Active PARPs can be expressed as various fusion proteins (e.g. His₆, GST, GFP, SBP) (Tan et al., 2012; Vyas et al., 2014; Wright et al., 2012), allowing for significant flexibility in the expression/purification system employed. Multiple protocols have been validated for the expression of recombinant PARP1, PARP2, and PARP3 in *E. coli* (Haikarainen et al., 2013; Lehtio et al., 2009; Tan et al., 2012). A distinct advantage for bacterial expression is the speed with which KA-PARP can be generated. After cloning the KA-PARP variant into the requisite *E. coli* expression vector, pure KA-PARP can be obtained within a week's time. Recombinant PARP1–3, 5, and 6 have also been purified using a baculovirus expression system in insect cells (Ame et al., 1999; Augustin et al., 2003; Giner et al., 1992; Smith et al., 1998). Recently, Chang and colleagues described the purification of each member of the PARP family using mammalian suspension cells (Vyas et al., 2014). While the expression of KA-PARP in either insect cells or mammalian suspension cells will involve a longer timeline prior to initiating the protocols below, they are currently the only methods for generating full-length, active PARP5 or PARP6.

Regardless of the method employed to generate the recombinant KA-PARP variant, the enzyme will need to be purified to ~95% purity to avoid background labeling of co-purified contaminants from the host system. Based on the recombinant tag selected for expression, it might be possible to achieve ~95% purity by first subjecting the tagged KA-PARP variant to affinity chromatography followed by cleanup with either size-exclusion chromatography or ion-exchange chromatography. Alternatively, the KA-PARP variant can be purified to a high degree of homogeneity using a previously described method (Giner et al., 1992; Tan et al., 2012) wherein activated sepharose resin is coupled to the pan-selective PARP inhibitor, 3-aminobenzamide (3AB). When used following the initial round of tag-specific affinity chromatography (i.e. Ni-NTA) the sepharose-3AB column routinely results in enzyme fractions with ~95% purity.

Identification of direct protein targets by LC-MS/MS using recombinant KA-PARP will require milligram quantities of the 5-Et-6-a-NAD⁺ probe. We recently reported a detailed synthesis of 5-Et-6-a-NAD⁺ (Carter-O'Connell et al., 2014). Sufficient quantities of 5-Et-6-a-NAD⁺ (5–10 mg) can be generated in ~1 month by an experienced chemist.

The protocols described in this article outline a general method for labeling and identifying the direct protein targets of an individual PARP enzyme. This process can be divided into

two basic procedures: (i) labeling of direct protein targets of an individual PARP enzyme using a KA-PARP orthogonal-NAD⁺ analogue pair (Basic Protocol 1); and (ii) enriching direct protein targets for identification using LC-MS/MS (Basic Protocol 2). The protocols can be generalized for PARPs 1–3, 5, and 6 in any cell lysate. As a specific example meant to illustrate the steps and expected results for the described protocols, KA-PARP1 ADP-ribosylation of direct protein targets using 5-Et-6-a-NAD⁺ is described (Carter-O'Connell et al., 2014). PARP1 plays an important role in the single-stranded DNA break repair pathway (Burkle, 2000; Gibson and Kraus, 2012). Due to this established role for PARP1, an intense effort was made to characterize the function of PARP1 and its direct protein targets. As such, the protein targets identified using the following protocols can be compared to previously identified targets (Dani et al., 2009; Gagne et al., 2012; Jiang et al., 2010; Jungmichel et al., 2013; Zhang et al., 2013), which confirms the identification of bona fide PARP1 targets using this strategy. The direct identification of PARP1 targets using the 'bump-hole' strategy also elucidated previously unknown PARP1 targets, highlighting the power of this method in revealing new roles for ADP-ribosylation in the cell.

BASIC PROTOCOL 1: *IN VITRO* LABELING OF THE DIRECT PROTEIN TARGETS OF KA-PARPS

Once the selected KA- and WT-PARP variants have been purified and the 5-Et-6-a-NAD⁺ probe has been synthesized (see Strategic Planning, above), the first step in assay development is to determine the conditions that will result in productive labeling of direct protein targets in the desired lysate. Sufficient biological material (i.e. cell tissue, nuclear lysate, etc.) should be prepared to facilitate a series of pilot experiments wherein the amount of mutant enzyme (usually 0.5 – 1.0 μ M), concentration of 5-Et-6-a-NAD⁺ (up to 250 μ M), and duration of the labeling reaction (usually 1 – 4 hours) are titrated to achieve optimum labeling efficiency for the given system. While the protocol below serves as a starting point for optimization, each individual labeling reaction will require an empirical determination to confirm productive protein target labeling. We find extensive protein labeling only when both the KA-PARP variant and 5-Et-6-a-NAD⁺ are present; we observe essentially no background labeling in the presence of 5-Et-6-a-NAD⁺ (up to 250 μ M) alone (Figure 2). The reaction should be carried out with the lowest concentration of mutant enzyme, modified NAD⁺, and shortest reaction time possible. This will decrease the likelihood of off-target and non-specific labeling, as well as conserving stocks of both enzyme and synthesized probe. The following protocol describes the generation of labeled direct protein targets in HEK 293T nuclear lysate using KA-PARP1 and 5-Et-6-a-NAD⁺, but it is generalizable for any member of the PAR-generating subclass of PARP enzymes.

Materials

HEK 293T cell pellet: A single 10 cm dish of 80–90% confluent cells is sufficient for multiple pilot experiments. Following cell harvesting, pellets can be flash frozen in liquid nitrogen and stored at –80°C for up to six months.

7× Protease inhibitor mix (see recipe)

Lysis buffer (see recipe)

Lysis buffer + NP-40 (see recipe)

Bradford reagent

BSA standards (1.0 mg/mL – 0.2 mg/mL in water)

10× R buffer (see recipe)

1 µg/µL activated DNA (Sigma, cat. no D4522)

Protein stock solution: 8.5 µM KA-PARP1 (Carter-O'Connell et al., 2014) in 1× R buffer

Analogue stock solution: 1.98 mM 5-Et-6-a-NAD⁺ (Carter-O'Connell et al., 2014) in 1× R buffer

3-[tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) (Sigma, cat. no 762342)

Copper (II) sulfate pentahydrate (Sigma, cat. no C8027)

Biotin-PEG₃-N₃ (Click Chemistry Tools, cat. no AZ104)

Sodium L-ascorbate (TCI America, cat. no A0539)

3× click buffer (see recipe)

4× sample buffer (see recipe)

Streptavidin-HRP buffer (see recipe)

10% Tris-HCl SDS-PAGE gel

1.7 mL microcentrifuge tubes

SDS-PAGE electrophoretic system

Nitrocellulose

Western blot transfer system

Prepare lysate for labeling

- 1 Resuspend the frozen HEK 293T pellet in 300 µL lysis buffer. Lyse cells gently by pipetting up and down until the cell pellet is solubilized fully in buffer. Transfer the homogenate to a sterile microcentrifuge tube and centrifuge for 15 minutes at 2,000 × g, 4°C.
- 2 Remove the supernatant and resuspend the pellet in 100 µL lysis buffer. Centrifuge for 5 minutes at 14,000 × g, 4°C. Transfer the supernatant to a sterile microcentrifuge tube.

The supernatant generated from step 1 can be saved and used to determine the extent of PARP labeling in the cytosol. As PARP1 is predominantly found in the nucleus, and this protocol focuses on the direct protein targets of PARP1, the cytoplasmic fraction is discarded. However, other PARPs (i.e. PARP5 and 6) are present in the

cytoplasmic fraction and their direct protein targets in both the nucleus and cytosol could be determined using this protocol.

- 3 Determine the total protein concentration in the nuclear lysate using a BSA standard curve and the Bradford assay.

The presence of protease inhibitors in the lysis buffer, as well as 1% NP-40, will produce a high level of background signal in the Bradford assay. Diluting the lysate 10-fold, as well as the lysis buffer for the blank measurement, will produce a reliable reading without interference from the buffer components.

Label direct protein targets with 5-Et-6-a-NAD⁺

- 4 For each labeling experiment, aliquot 60 µg of nuclear lysate to individual microcentrifuge tubes. Add 3 µL of 10× R buffer, 0.7 µL of activated DNA (1 µg/µL), and bring up the mixture to a total volume of 22.5 µL in deionized water.

While endogenous PARPs will not use 5-Et-6-a-NAD⁺ (up to 250 µM) at an appreciable rate, it is important to setup a control reaction for each optimization condition tested that includes 5-Et-6-a-NAD⁺ but lacks the KA-PARP variant. This control reaction provides a baseline for non-specific labeling due to endogenous activity or non-catalytic ADP-ribose transfer.

- 5 Add 3.8 µL of the protein stock solution to the reaction mixture. For the control reaction lacking mutant enzyme, add 3.8 µL of 1× R buffer. Following addition of the protein stock solution, add 3.8 µL of the analogue stock solution to each individual reaction mixture.
- 6 Incubate the reactions for 2 hours at 30°C with constant agitation. A standard orbital incubator set to 30°C, equipped with a microcentrifuge rack, and set to ~220 RPM is sufficient to provide constant agitation over the reaction incubation.
- 7 Add 15 µL of the 3× click buffer to each individual reaction mixture. Incubate the reactions for 1 hour at room temperature with constant agitation.
- 8 Immediately following the click conjugation, add 15 µL of 4× sample buffer to each individual reaction mixture. Boil the samples for 5 minutes at 95°C followed by centrifugation for 1 minute at 2,000 × g, 25°C.
- 9 Load the samples to a 10% Tris-HCl gel and fractionate via SDS-PAGE. Move the gel to a Western blot system and transfer the labeled direct protein targets to nitrocellulose. Perform immunoblot analysis with streptavidin-HRP to visualize biotinylated direct protein targets.
- 10 If necessary, repeat the protocol using alternative reaction conditions to determine the optimum labeling efficiency for the KA-PARP mutant in the desired experimental paradigm.

BASIC PROTOCOL 2: NEUTRAVIDIN ENRICHMENT OF LABELED DIRECT PROTEIN TARGETS FOR MASS SPECTROMETRY APPLICATIONS

Following optimization of the conditions required for direct protein labeling by a particular KA-PARP variant and the 5-Et-6-a-NAD⁺ probe, the reaction described in Basic Protocol 1 will first need to be scaled up to a sufficient volume to obtain enough labeled material for identification using LC-MS/MS. Given the broad range in LC-MS/MS sensitivity the amount of material required to achieve reliable and reproducible target identification will vary based on the instrumentation on hand. Therefore, while the protocol described below recommends starting with one mg of total protein from the HEK 293T nuclear lysate, the amount of material needed will require calibration based on the available LC-MS/MS. In this case, three 10 cm dishes of HEK 293T cells at 90–100% confluency are sufficient to generate one mg of total protein from the nuclear lysate for each condition tested. Given that each LC-MS/MS experiment requires a control sample containing 5-Et-6-a-NAD⁺ but lacking KA-PARP to determine the extent of background labeling, at least six 10 cm dishes should be prepared for each condition tested. We found that optimal click reactions occurred with smaller sample volumes, therefore cell pellets should be lysed in a scaled up volume of lysis buffer (i.e. 1.8 mL lysis buffer for six pellets) but the final reaction volume should not surpass 50 μ L. For the protocol described below, sixteen individual reactions - as detailed in Basic Protocol 1 - are performed in tandem and pooled following click conjugation for each condition assayed (i.e. sixteen reactions for the KA-PARP1 and 5-Et-6-a-NAD⁺ test and sixteen reactions for the control containing 5-Et-6-a-NAD⁺ alone). After generating the biotin-conjugated direct protein targets, NeutrAvidin agarose is used for enriching direct protein targets. Purified NeutrAvidin-bound protein targets are treated with trypsin, and the resultant peptides are eluted and subsequently subjected to tandem mass spectrometry for protein targeting identification. It is possible to substitute different proteases (e.g. chymotrypsin, endoproteinase lys-c, etc.) for trypsin to provide additional coverage in the mass spectrometry detection (Gupta et al., 2010). The following protocol details the generation of an enriched pool of peptides derived from direct KA-PARP1 protein targets from HEK 293T nuclear lysate for use with LC-MS/MS. Quality control samples taken throughout the protocol will aid in determining the extent of biotinylation, target enrichment, and protease digestion prior to downstream LC-MS/MS (Figure 3). The protocol can be adapted easily towards alternative downstream applications besides target identification by LC-MS/MS, such as immunoblot detection of direct targets using specific antibodies (stopping prior to trypsinization and eluting samples directly from the streptavidin beads) or ADP-ribose site-mapping (using a cleavable biotin-PEG₃-N₃ or by adding an additional purification step as discussed in Commentary below).

Materials

Sixteen identical HEK 293T nuclear lysate labeling reactions generated with KA-PARP1 and 5-Et-6-a-NAD⁺ from Basic Protocol 1, following click conjugation

Sixteen identical HEK 293T nuclear lysate labeling reactions generated with 5-Et-6-a-NAD⁺ (negative control) from Basic Protocol 1, following click conjugation

4 $\times$ sample buffer (see recipe)

Methanol (HPLC grade), kept at 4°C

Ni-NTA agarose (Qiagen, cat. no 30210)

Dilution buffer (see recipe)

Ni-NTA incubation buffer (see recipe)

2% sodium dodecyl sulfate (w/v) solution (SDS)

NeutrAvidin agarose (Thermo Scientific, cat. no 29200)

Wash buffer 1 (see recipe)

Urea wash buffer (see recipe)

Wash buffer 2 (see recipe)

1× phosphate buffered saline, pH 7.4 (PBS)

50 mM ammonium bicarbonate

6M urea

0.5M *tris*(2-carboxyethyl)phosphine

0.5M iodoacetamide

1.5× sample buffer + 1 mM biotin

10 mM CaCl₂

0.1 µg/µL sequencing grade trypsin in trypsin resuspension dilution buffer (Promega, cat. no V5111)

Formic acid

Streptavidin-HRP buffer (see recipe)

15 mL tubes

1.7 mL microcentrifuge tubes

End-over-end rotisserie

Spin columns (Pierce, cat. no 69705)

10% Tris-HCl SDS-PAGE gel

SDS-PAGE electrophoretic system

Nitrocellulose

Western blot transfer system

Prepare labeled direct protein targets for enrichment

- 1 Pool the sixteen individual labeling reactions from Basic Protocol 1, immediately following the click conjugation step, into a single 15 mL tube. Do the same for the negative control reactions in a separate 15 mL tube.

From now on, while the negative control will not be explicitly referenced, perform the same steps in this protocol on both the experimental sample (KA-PARP1 and 5-Et-6-a-NAD⁺) and the negative control sample (5-Et-6-a-NAD⁺).

- 2 Pipette 30 μ L of the reaction mixture into a fresh microcentrifuge tube. Add 10 μ L 4 $\times$ sample buffer. Label the sample as **Input** and store at -20°C until reaching step 38.
- 3 Add 8 mL of chilled methanol to the 15 mL tube. Precipitate the sample for at least 1 hour at -20°C .

The mixture at this point can be stored overnight at -20°C depending on time constraints. However, the reactions from Basic Protocol 1 should be carried through step 19 of this protocol on the same day to achieve optimal results.

- 4 While precipitating the labeled direct protein targets, pipette 200 μ L of Ni-NTA resin into a fresh microcentrifuge tube. Centrifuge the resin for 1 minute at $1,000 \times g$, at room temperature.

As the predominant target of PARP1 is itself, the addition of Ni-NTA resin prior to NeutrAvidin precipitation will help remove excess auto-labeled biotinylated His₆-KA-PARP1. This step may be omitted depending on the KA-PARP family member used in Basic Protocol 1 if the pilot experiments suggest that the KA-PARP variant added is not undergoing extensive auto-modification.

- 5 Remove the supernatant and resuspend the Ni-NTA beads in 500 μ L deionized water. Centrifuge the resin for 1 minute at $1,000 \times g$, at room temperature.
- 6 Repeat step 5 twice more; once with 500 μ L deionized water and once with 500 μ L dilution buffer. Centrifuge the resin for 1 minute at $1,000 \times g$, at room temperature and remove the remaining supernatant.
- 7 Resuspend the Ni-NTA beads in 300 μ L Ni-NTA incubation buffer. Incubate the beads at room temperature with end-over-end rotation for 1 hour.

The Ni-NTA beads are pre-incubated in excess HEK 293T nuclear lysate to prevent non-specific binding of the labeled direct protein targets from the experimental sample.

- 8 Centrifuge the Ni-NTA resin for 1 minute at $1,000 \times g$, at room temperature. Remove the supernatant and resuspend the beads in 500 μ L dilution buffer.
- 9 Repeat step 8 twice more. Store pre-incubated Ni-NTA beads in 500 μ L dilution buffer on ice until reaching step 14.

Affinity purify biotinylated direct protein targets

- 10 Centrifuge the labeled sample from step 3 for 30 minutes at $6,000 \times g$, at 4°C .

- 11 Carefully remove the methanol without disturbing the precipitate. Fully resuspend the precipitate in 1 mL chilled methanol by pipetting the entire sample up-and-down multiple times. Transfer the mixture to a fresh microcentrifuge tube. Centrifuge for 5 minutes at $12,000 \times g$, at 4°C .
- 12 Repeat step 11 twice more. Carefully remove the remaining methanol without disturbing the pellet. Lower levels of remaining methanol will decrease drying time. Air dry the precipitated protein pellet for 10–15 minutes.
- 13 Resuspend the precipitate in 250 μL 2% SDS. Gently pipette the pellet up-and-down in the SDS solution until the pellet is fully dissolved.
- During this step, the pellet may not readily dissolve in the SDS solution. If this happens, short (~ 1 – 2 minutes), repeated incubation of the pellet at 55°C should aid in pellet solubilization.
- 14 Transfer the mixture to a fresh 15 mL tube and add 4.5 mL dilution buffer. Add 200 μL of the Ni-NTA slurry from step 9 to the tube. Incubate the mixture for 1 hour, with end-over-end rotation, at room temperature.
- 15 While the reaction mixture is incubating, prepare the NeutrAvidin resin by first pipetting 200 μL of NeutrAvidin slurry to a fresh microcentrifuge tube. Centrifuge for 3 minutes at $2,000 \times g$, 4°C .
- 16 Remove the supernatant and bring the beads up in 1 mL wash buffer 1. Centrifuge for 3 minutes at $2,000 \times g$, 4°C .
- 17 Repeat step 16 twice more. Bring the beads up in 1 mL wash buffer 1 and store on ice until step 19.
- 18 Following Ni-NTA incubation, centrifuge the sample from step 14 for 5 minutes at $2,000 \times g$. Carefully transfer the supernatant to a fresh 15 mL tube. Pipette 30 μL of the supernatant into a fresh microcentrifuge tube. Add 10 μL $4\times$ sample buffer. Label the sample as **Load** and store at -20°C until reaching step 38.
- 19 Add the NeutrAvidin resin from step 17 - in wash buffer 1 - to the supernatant from step 18. Incubate overnight, with end-over-end rotation, at room temperature.

Wash the NeutrAvidin bound direct protein targets

- 20 Centrifuge the sample for 5 minutes at $2,000 \times g$, 4°C . Pipette 30 μL of the supernatant into a fresh microcentrifuge tube. Add 10 μL $4\times$ sample buffer. Label the sample as **Unbound** and store at -20°C until reaching step 38. Discard the remaining supernatant.
- 21 Resuspend the NeutrAvidin beads in 500 μL urea wash buffer. Transfer the mixture to a fresh microcentrifuge tube. Incubate the beads for 10 minutes, with end-over-end rotation, at room temperature. Centrifuge for 2 minutes at $2,000 \times g$, room temperature. Discard the supernatant.

Extensive washing of the beads to remove non-specifically bound proteins is possible because of the incredibly tight interaction between biotin-NeutrAvidin. The following wash protocol works well for removing background signal from HEK 293T nuclear lysate labeled with KA-PARP1 and 5-Et-6-a-NAD⁺. However, based on the needs of the particular application of this method, different wash conditions can be substituted. It is recommended that for altered wash protocols aliquots of NeutrAvidin slurry be taken at regular wash intervals for immunoblot analysis to confirm removal of background signal (by comparing the KA-PARP and 5-Et-6-a-NAD⁺ sample to the 5-Et-6-a-NAD⁺ sample alone) and retention of bona fide labeled direct protein targets. For suggested alterations to the wash protocol please see Commentary below.

- 22 Repeat step 21 once more.
- 23 Resuspend the NeutrAvidin beads in 500 μ L wash buffer 2. Incubate the beads for 10 minutes, with end-over-end rotation, at room temperature. Centrifuge for 2 minutes at $2,000 \times g$, room temperature. Discard the supernatant.
- 24 Repeat step 23 twice more.
- 25 Resuspend the NeutrAvidin beads in 500 μ L 50 mM ammonium carbonate. Incubate the beads for 10 minutes, with end-over-end rotation, at room temperature. Centrifuge for 2 minutes at $2,000 \times g$, room temperature. Discard the supernatant.
- 26 Repeat step 25 once more.
- 27 Resuspend the NeutrAvidin beads in 500 μ L 1 $\times$ PBS. Incubate the beads for 10 minutes, with end-over-end rotation, at room temperature. Centrifuge for 2 minutes at $2,000 \times g$, room temperature. Discard the supernatant.
- 28 Repeat step 27 once more.
- 29 Repeat step 25 twice more.

Trypsinize the labeled direct protein targets

- 30 Discard the supernatant from step 29. Resuspend the NeutrAvidin beads in 268 μ L 50 mM ammonium carbonate. Incubate for 10 minutes at 70°C, while agitating the tube.

The use of a heating block preheated to 70°C is recommended for this step. Gently flicking the tube every minute is sufficient to provide agitation.
- 31 Immediately add 132 μ L of 6M urea to the sample. Allow the suspension to cool to room temperature before proceeding to the next step.
- 32 Add 2.5 μ L of 0.5M TCEP to the sample. Mix briefly by pipetting up-and-down. Incubate for 30 minutes, with end-over-end rotation, at room temperature.

- 33** Add 9.0 μL of 0.5M iodoacetamide to the sample. Mix briefly by pipetting up-and-down. Incubate in the dark for 30 minutes, with end-over-end rotation, at room temperature.
- 34** Pipette 50 μL of the supernatant into a fresh microcentrifuge tube. Add 30 μL 1.5 $\times$ sample buffer + 1 mM biotin. Label the sample as **Bound** and store at -20°C until reaching step 38.
- 35** Add 3.7 μL of 10 mM CaCl_2 to the sample from step 33 followed by 20 μL 0.1 $\mu\text{g}/\mu\text{L}$ sequencing grade trypsin, mix by pipetting up-and-down. Incubate overnight, with end-over-end rotation, at room temperature.
- 36** Load the entire mixture to a new spin column. Centrifuge for 1 minute at $1,000 \times g$, room temperature into a separate collection tube. Add 20 μL formic acid to the eluate and store at -20°C until LC-MS/MS analysis.
- 37** Resuspend the leftover resin in the spin column in 370 μL 50 mM ammonium carbonate, transfer 50 μL to a fresh microcentrifuge tube. Add 30 μL 1.5 $\times$ sample buffer + 1 mM biotin. Label the sample as **Tryp** and store at -20°C until reaching step 38.
- 38** Boil the samples from steps 2 (**Input**), 18 (**Load**), 20 (**Unbound**), 34 (**Bound**), and 37 (**Tryp**) for 5 minutes at 95°C followed by centrifugation for 1 minute at $2,000 \times g$, 25°C . Load the samples to a 10% Tris-HCl gel and fractionate via SDS-PAGE. Move the gel to a Western blot system and transfer the labeled direct protein targets to nitrocellulose. Perform immunoblot analysis with streptavidin-HRP to visualize biotinylated direct protein targets.

REAGENTS AND SOLUTIONS

Use LC/MS grade water in all recipes and protocol steps.

7 $\times$ Protease inhibitor mix

Dissolve 1 mini-cOmplete, EDTA-free protease inhibitor tablet (Roche, cat. 11836170001) in 1.5 mL water. Aliquot and store at -20°C for 6 to 12 months.

Lysis buffer

25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) adjusted to pH 7.5 with HCl or NaOH

50 mM NaCl

10% glycerol (v/v)

1 $\times$ protease inhibitor

Once the protease inhibitor is added, keep on ice and use within 1 day

Lysis buffer + NP-40

25 mM HEPES adjusted to pH 7.5 with HCl or NaOH

50 mM NaCl

10% glycerol (v/v)

1% Nonidet P-40 (v/v)

1× protease inhibitor

Once the protease inhibitor is added, keep on ice and use within 1 day

10× R buffer

475 mM HEPES adjusted to pH 7.5 with HCl or NaOH

50 mM MgCl₂

5% Nonidet P-40 (v/v)

Solution can be stored at room temperature, but is made fresh prior to every reaction

3× Click buffer

Prepare fresh 3× click buffer immediately prior to its addition to the ADP-ribosylation reaction. Add the following components to the buffer in the exact order in which they are listed:

1.5 mM 3-[tris(3-hydroxypropyltriazolylmethyl)amine (THPTA)

750 μM CuSO₄

300 μM biotin-PEG₃-N₃

7.5 mM sodium ascorbate*

*Should be prepared fresh before every click reaction

Add 1× PBS, pH 7.4 to the final required volume and mix the solution by pipetting

4× Sample buffer

200 mM tris(hydroxymethyl)aminomethane-HCl (Tris-HCl) adjusted to pH 6.8 with HCl

8% SDS (w/v)

40% glycerol (v/v)

50 mM ethylenediaminetetraacetic acid (EDTA)

0.08% bromophenol blue (w/v)

The solution above can be stored indefinitely at room temperature

To make the working solution, add 50 μL of 14.7M β-mercaptoethanol to 950 μL of the 4× sample buffer

Once β-mercaptoethanol is added, store up to 6 weeks at −20°C

Streptavidin-HRP buffer

Dilute 2 μ L of 0.5 mg/mL streptavidin-HRP (Jackson ImmunResearch, cat. 016-030-084) in 5 mL Tris-Buffered Saline and Tween 20 (TBST). Solution can be stored at room temperature and should be used within 1 day of preparation.

Dilution buffer

1 $\times$ PBS, adjusted to pH 7.4

113 mM NaCl

1% NP-40 (v/v)

1 $\times$ protease inhibitor mix

Once the protease inhibitor is added, store on ice and use immediately

Ni-NTA incubation buffer

1 $\times$ PBS, adjusted to pH 7.4

113 mM NaCl

1% NP-40

Add HEK 293T nuclear lysate to a final concentration of 2 μ g/ μ L soluble protein

Once the lysate is added, store on ice and use immediately

Wash buffer 1

1 $\times$ PBS, adjusted to pH 7.4

1% NP-40 (v/v)

0.05% SDS (v/v)

Wash buffer can be stored at room temperature indefinitely

Urea buffer

4M urea

1 $\times$ PBS, adjusted to pH 7.4

Urea buffer can be stored at room temperature indefinitely

Wash buffer 2

1 $\times$ PBS, adjusted to pH 7.4

1% NP-40 (v/v)

Wash buffer 2 can be stored at room temperature indefinitely

COMMENTARY

Background Information

Exciting new findings suggest that ADP-ribosylation is more widespread in cell biology than previously appreciated (Feijs et al., 2013b; Vyas et al., 2013). A first step toward understanding the precise role for each of the 17 human PARP family members in the cell requires the identification of their direct protein targets. While non-radioactive NAD⁺ probes, such as 6-alkyne-NAD⁺ (Jiang et al., 2010) and biotin-NAD⁺ (Zhang, 1997), have proven useful in deciphering the global targets of PARPs, they can not provide a link between a specific PARP and its direct protein targets in a cellular context. This is because all PARPs use these NAD⁺ probes with similar catalytic efficiency. To address this challenge, the protocols above describe the adaptation of a 'bump-hole' methodology for linking an engineered PARP variant to its direct protein targets using an orthogonal NAD⁺ analogue.

The 'bump-hole' method was originally developed for the identification of the direct protein targets of protein kinases. Shokat and colleagues showed that mutation of I338 to an alanine or glycine in the active site of the protein kinase, v-Src, allowed access to a binding pocket not normally occupied by ATP (Liu et al., 1998; Shah et al., 1997). This 'hole' created by mutating I338 (now referred to as the "gatekeeper" position) to a smaller amino acid could accommodate substituents ('bump') at the N-6 position of ATP. One compound in particular, N-6 benzyl ATP, was an efficient substrate for I338G v-Src mutant and could be used to label (using radioactivity) the direct targets of v-Src. Importantly, this orthogonal ATP analogue was not used by wild type kinases. Subsequent work demonstrated that this strategy was generalizable across the protein kinase family (Tarrant and Cole, 2009). In recent years, the method has been adapted for the identification of direct targets of individual kinases using LC-MS/MS (Hertz et al., 2010). Following the work of Shokat and colleagues, the 'bump-hole' methodology spread to other post-translational modifications. Indeed, a 'bump-hole' technique has been used to study the targets of acetylation (Yang et al., 2013) and methylation (Islam et al., 2012). The protocols in this unit build on such previous advances to adapt the 'bump-hole' methodology for the PAR-generating subclass of PARP enzymes (PARP1–3, 5, and 6). This technique has already been successfully used to identify the direct protein targets of both PARP1 and PARP2 in HEK 293T cells (Carter-O'Connell et al., 2014).

One limitation of the current method is the inability to perform labeling studies in intact cells. This is because 5-Et-6-a-NAD⁺ is not membrane permeable. The requirement for prepared lysate should be considered when designing experiments using the KA-PARP and 5-Et-6-a-NAD⁺ pairs. In our view this requirement does not present a problem as previous studies using the 'bump-hole' methodology for protein kinases in cell lysates have identified bona fide protein targets. Another limitation of the current method is the inability to use the engineered PARP-modified NAD⁺ pair with the mono-PARPs. As the mono-PARPs lack the conserved lysine residue required to form the 'hole' they have thus far proven incompatible with the 'bump-hole' strategy. Current efforts are underway to identify

additional mutagenesis sites and NAD⁺ analogues that would allow the 'bump-hole' strategy to be adapted to the mono-PARPs.

Another strategy that has been describe for identifying the direct targets of a given PARP family member *in vitro* is the use of protein microarrays. Luscher and co-workers have developed a screening method that employs protein microarrays for the identification of mono-PARP targets (Feijs et al., 2013a). They purified recombinant PARP10 and PARP14 and applied the enzymes to the protein microarrays - along with biotin-PEG₃-NAD⁺ - detecting modified proteins using a fluorophore conjugated streptavidin. Targets identified in this manner are then be confirmed as bona fide *in vitro* and *in vivo* targets to avoid the identification of proteins that can be modified by the individual mono-PARP on the microarray but are not targeted in the cell. Nevertheless, this work led to the identification of GSK3 β as a PARP10 target and allowed the team to show that ADP-ribosylation of GSK3 β by PARP10 leads to decreased GSK3 β activity in the cell.

On a final note, many techniques have recently been described that aim to define the global ADP-ribosylated proteome in cells by enriching for endogenous ADP-ribosylated proteins and analyzing the enriched fraction using LC-MS/MS. The ADP-ribosylated targets are first enriched using either a poly-ADP-ribose antibody (a mono-ADP-ribose antibody does not exist) (Gagne et al., 2012), a resin-attached protein domain capable of binding the ADP-ribose moiety (e.g. WWE, PBZ, Macro, or PARG) (Dani et al., 2009; Gagne et al., 2012; Jungmichel et al., 2013), a boronic acid resin that can form a covalent complex with ADP-ribose (Zhang et al., 2013), or most recently, coupling Macrodome affinity purification with immobilized metal affinity chromatography of phosphodiesterase treated proteins (Daniels et al., 2014). Following elution of proteins and subsequent digestion, the samples are processed by LC-MS/MS and the ADP-ribosylated proteins are identified. Regardless of the method employed, the enriched fraction of ADP-ribosylated targets cannot be distinguished based on the identity of the PARP enzyme responsible for the post-translational modification. Nevertheless, this factor does not diminish the importance of describing the PARylome in cells using the techniques above. In fact, these strategies can be used to validate in cells the direct protein targets that are identified using engineered PARP variants—orthogonal NAD⁺ analogue pairs in cell lysates.

Critical Parameters

Choice of azide conjugated probe—The labeling of KA-PARP1 direct protein targets in HEK 293T nuclear lysate presented in the protocols above was all performed with biotin-PEG₃-N₃. The use of biotin-PEG₃-N₃ confers two distinct advantages: (i) the direct protein targets can be visualized via immunoblot detection with streptavidin-HRP; and (ii) the NeutrAvidin-biotin interaction can be subjected to stringent wash conditions during sample enrichment. However, there are multiple azide conjugates that have been synthesized that can be used with the protocols above depending on the required downstream applications. If visualization and screening of direct protein targets using Basic Protocol 1 is the primary goal, then the use of a fluorophore conjugated azide (e.g. TAMRA-N₃) might be preferable to biotin-PEG₃-N₃. While fluorophore based detection of direct protein targets might not be as sensitive as detection using biotin-streptavidin, the fluorophore can be detected in less

time than that required for immunoblot detection. Additionally, due to the nonlinear amplification caused by streptavidin-HRP the signals produced by immunoblot detection are not as readily quantifiable as those produced by fluorophore detection. Rhodamine-N₃ has been successfully used with 6-a-NAD⁺ to visualize the modified PARylome in MCF-7 cells (Jiang et al., 2010).

Recently, Hang and colleagues described a set of cleavable biotin-azobenzene-based affinity tags for use in site-mapping bioorthogonal chemical proteomes (Yang et al., 2010). As with other click chemistry methods, the cleavable biotin-azobenzene tag is conjugated to the alkyne handle on the labeled protein of interest. The tagged proteins are then enriched using streptavidin resin and treated exactly as in Basic Protocol 2 above. With non-cleavable azide tags (e.g. biotin-PEG₃-N₃) the peptide that has been modified with the biotin-azide probe remains trapped on the streptavidin resin and is not identified in the subsequent LC-MS/MS analysis. In contrast, incubating the biotin-azobenzene based tags with Na₂S₂O₄ reduces azobenzene and releases the modified peptide fragment from the streptavidin resin. The released peptide now carries a novel chemical signature that can be analyzed by LC-MS/MS and the precise site of modification can be determined. Diazo biotin-azide is commercially available (Click Chemistry Tools, cat. 1041-10) and can be substituted for biotin-azide in Basic Protocol 2 if the downstream applications will include ADP-ribosylation site-mapping.

Reagent purity—As with any method where the ultimate goal is accurate LC-MS/MS analysis, the reagents used to generate labeled direct KA-PARP targets should be of the highest available purity to avoid the presence of contamination in the peptide library. Purification of the modified NAD⁺ analogue should be performed by HPLC and the engineered KA-PARP variant should be purified to greater than 95% purity using standard chromatographic methods (see Strategic Planning above for further detail). All buffer solutions should be prepared with LC/MS grade water and should be filtered prior to use. Finally, care must be taken to avoid possible contamination from external sources (e.g. human keratin) by performing all wash/elution steps in a sterile environment. A laminar flow hood setup in an enclosed space is recommended, as is the vigilant cleaning of lab equipment (e.g. pipettes, gloves, tips, etc.) with LC/MS grade water prior to use.

Troubleshooting

An overall summation of potential problems and troubleshooting steps for the protocols described in this unit are collected in Table 1 below.

A lack of biotinylated direct protein target labeling—There may be a lack of detectable biotinylated direct protein targets in the immunoblot generated using Basic Protocol 1. A number of potential causes might lead to insufficient target labeling. The first step in troubleshooting low biotinylation signal is to confirm that the recombinant KA-PARP enzyme is active. PARPs 1–6 are all capable of catalyzing auto-ADP-ribosylation *in vitro* (Vyas et al., 2014). As such, the purified KA-PARP variant can be incubated in isolation with either 6-a-NAD⁺ or 5-Et-6-a-NAD⁺, conjugated to biotin-PEG₃-N₃, and auto-modification detected via immunoblot using streptavidin-HRP. If there is no detectable auto-

modification then the KA-PARP variant should be remade using the strategy outlined in Strategic Planning above. The synthesized 5-Et-6-a-NAD⁺ probe should be subjected to NMR and LC-MS analysis to rule out the potential inhibition of catalytic activity due to probe impurities. Finally, PARPs are activated by a number of different macromolecules (e.g. activated DNA for PARP1, ribosomal RNA for PARP2, 5'-phosphorylated DNA breaks for PARP3) (Krishnakumar and Kraus, 2010; Langelier et al., 2014; Leger et al., 2014), which are often required to achieve full catalytic activity. If there is a low level of auto-modification activity *in vitro*, then the appropriate stimulus might be required to achieve sufficient labeling with the 5-Et-6-a-NAD⁺ probe. Optimization should then be performed with these potential PARP activators using the *in vitro* auto-modification assay to titrate the level of activator required to achieve detectable auto-modification.

Another potential cause of low biotinylation signal might be a result of the buffer conditions used for both the initial labeling reaction and the click conjugation reaction. We have observed that the presence of certain detergents common in lysis buffers (i.e. SDS in RIPA buffer) cause a decrease in KA-PARP activity. If the KA-PARP variant is active in the *in vitro* auto-modification assay using the buffer system described in Basic Protocol 1, then the auto-modification experiment should be repeated in the desired lysis buffer to confirm that the lysis buffer is compatible with KA-PARP activity. Multiple reports have now expanded the repertoire of copper-catalyzed click ligands and reaction conditions that are compatible with the alkyne-azide conjugation described in this unit (Besanceney-Webler et al., 2011; Hong et al., 2009). Following confirmation of enzyme activity and probe purity, click conjugation conditions can be screened to determine the optimal click buffer that will produce the maximum amount of protein labeling in the desired experimental setup.

High background signal in the absence of the KA-PARP and 5-Et-6-a-NAD⁺ pair—Depending on the lysate conditions utilized in Basic Protocol 2, there may be an increase in background biotinylation signal in the absence of the KA-PARP variant—orthogonal NAD⁺ analogue pair. This background signal might be the result of an increased abundance of naturally biotinylated proteins or non-specific labeling by 5-Et-6-a-NAD⁺. If the background signal persists in the absence of the 5-Et-6-a-NAD⁺ probe, then it is most likely due to background levels of biotinylation. In this scenario, the concentration of KA-PARP and 5-Et-6-a-NAD⁺ can be increased in the labeling reaction in an effort to increase the signal to noise ratio for the LC-MS/MS analysis. Additionally a pre-clearing step using NeutrAvidin agarose can be performed prior to the incubation of the lysate with KA-PARP and 5-Et-6-a-NAD⁺. If the background labeling is occurring in the presence of 5-Et-6-a-NAD⁺ alone, then 5-Et-6-a-NAD⁺ is labeling proteins non-specifically (most likely due to non-enzymatic labeling). Either the concentration of the 5-Et-6-a-NAD⁺ probe can be lowered, or the incubation time can be decreased, or both, to address this problem. If the background signal is increased following NeutrAvidin enrichment, then it might be due to non-specific binding to the NeutrAvidin resin. This particular problem might not appear until the LC-MS/MS analysis. Should elevated levels of non-biotinylated, non-specific proteins appear in the final analysis, stricter wash steps can be added to Basic Protocol 2 to decrease non-specific NeutrAvidin binding. Specifically, three repeats of a single wash with

acetonitrile (up to 20% v/v in water) followed by a single wash with 1× PBS after step 28 in Basic Protocol 2 should help eliminate non-specific binding.

LC-MS/MS detection issues—When preparing samples for eventual analysis by LC-MS/MS it is imperative that work be completed in an environment that will minimize external contamination. As the levels of background contaminants (e.g. keratin) increase, the positively identified direct labeled protein targets will decrease. If background contaminants are interfering with the LC-MS/MS analysis, then sample preparation should be performed in a sterile environment with extensive washing of all the necessary tools with LC/MS grade water. If, instead, the overall levels of detectable peptides are low, then a number of troubleshooting steps might be required. The first option should be increasing either the amount of labeled lysate, the concentration of KA-PARP, the concentration of 5-Et-6-a-NAD⁺, or the amount of NeutrAvidin resin used for enrichment. It is possible that each of these variables will need to be optimized separately to achieve robust peptide detection. Secondly, care should be taken to monitor the enrichment process via immunoblot detection of biotinylated proteins at key points during Basic Protocol 2 (see Figure 3 for an example). Depending on the results of the immunoblot analysis it should be possible to identify whether the LC-MS/MS detection is suffering from a lack of initial labeling, issues with precipitation, resin binding, washing, or protease degradation and release. Alternatively, we have observed that excess KA-PARP variant can result in a detection bias towards the KA-PARP protein and away from other labeled endogenous proteins. As such, the KA-PARP protein serves as an important internal control for the LC-MS/MS analysis. Under optimal conditions, the KA-PARP variant should produce the highest peptide count due to auto-modification, but it should be within an order of magnitude of the other protein targets. If it appears that KA-PARP peptide count is too high, then it can be removed using excess affinity resin (e.g. Ni-NTA) prior to the NeutrAvidin enrichment step.

Anticipated Results

Basic Protocol 1 will produce sufficient quantities of labeled direct protein targets for visualization by either immunoblot or fluorophore detection (depending on the choice of azide probe). Screening the experimental variables that affect labeling efficiency using Basic Protocol 1 will determine the optimal conditions for ADP-ribose transfer. Comparing the labeled proteins generated by the engineered PARP-modified NAD⁺ analogue pair to the control using the modified NAD⁺ analogue alone will reveal the extent of background labeling in the chosen experimental setup. In Basic Protocol 2, these parameters will be used for labeling sufficient material for downstream LC-MS/MS analysis. Beginning with 1 mg of total soluble protein using this protocol has routinely resulted in the identification of 100 unique proteins for both KA-PARP1 and KA-PARP2 by LC-MS/MS.

Time Considerations

Depending on the expression system used to express the KA-PARP variant, sufficient recombinant enzyme can be obtained within 1–4 weeks time. The synthesis of 5-Et-6-a-NAD⁺ can be completed within a month by an experienced chemist. Once the reagents have been prepared, and the biological material that will be labeled has been collected, the steps in Basic Protocol 1 can be completed within a day of work. Enrichment of labeled targets,

and the generation of a trypsinized peptide library for LC-MS/MS analysis using Basic Protocol 2 will require at least 3 days of work. The preparation of the labeled protein pool on the first day will require a full day of hands-on work. The wash steps on the second day will only require a half-day at the bench, and the final elution of the trypsinized peptides will require less than an hour. Upon familiarization of the procedures in this unit, 5–10 individual samples can be prepared concurrently.

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Figure 1.

(A) Schematic for the modification of direct PARP protein targets using engineered KA-PARP and 5-Et-6-a-NAD⁺ analogues. Labeled direct protein targets are modified with a 6-a-ADPr adduct that is conjugated to an azide-linked probe using copper catalyzed azide-alkyne cycloaddition. Modified targets can then be visualized or enriched for downstream LC-MS/MS applications. P: phosphate. (B) 5-Et-6-a-NAD⁺ (**1**) used in this study. Adapted with permission from Carter-O'Connell, I., Jin, H., Morgan, R.K., David, L.L., and Cohen, M.S. 2014. Engineering the substrate specificity of ADP-ribosyltransferases for identifying direct protein targets. *Journal of the American Chemical Society* 136:5201–5204. Copyright 2014 American Chemical Society.

Figure 2.

Lysate labeling by PARPs and modified NAD⁺ analogues. The faint bands observed in the 5-Et-6-a-NAD⁺ only lane correspond to endogenous biotinylated proteins. The membrane stained with Ponceau S serves as a loading control. The asterisks mark the KA-PARP1 (upper) and KA-PARP2 (lower) bands. Adapted with permission from Carter-O'Connell, I., Jin, H., Morgan, R.K., David, L.L., and Cohen, M.S. 2014. Engineering the substrate specificity of ADP-ribosyltransferases for identifying direct protein targets. *Journal of the American Chemical Society* 136:5201–5204. Copyright 2014 American Chemical Society.

Figure 3.

Immunoblot analysis of the LC-MS/MS sample preparation. The indicated fractions from the NeutrAvidin enrichment protocol were fractionated by SDS-PAGE and imaged using streptavidin-HRP. The modified NAD⁺ analogue was spiked into HEK 293T nuclear extract (6-a-NAD⁺: 100 μ M; 5-Et-6-a-NAD⁺: 250 μ M) both without any additional source of exogenous PARP and with either KA-PARP1 or KA-PARP2, samples were labeled, conjugated to biotin, enriched, and submitted for LC-MS/MS analysis. Positions of the molecular-weight markers are indicated on the left of the blot. Reprinted with permission from Carter-O'Connell, I., Jin, H., Morgan, R.K., David, L.L., and Cohen, M.S. 2014. Engineering the substrate specificity of ADP-ribosyltransferases for identifying direct protein targets. *Journal of the American Chemical Society* 136:5201–5204. Copyright 2014 American Chemical Society.

Table 1

Troubleshooting Guide for Direct Protein Target Labeling and Analysis

Problem	Possible Cause	Solution
Low biotinylation signal	Low recombinant PARP-KA activity	Check <i>in vitro</i> auto-modification activity using the 6-a-NAD ⁺ probe
		Express fresh PARP-KA and purify following the suggestions in Strategic Planning
	5-Et-6-a-NAD ⁺ probe contamination	Confirm probe purity using NMR and LC/MS
	Reaction requires additional PARP activator	Repeat <i>in vitro</i> auto-modification assay using various PARP activators (e.g. activated DNA, rRNA, etc.)
	Incompatible lysate buffer	Perform <i>in vitro</i> auto-modification assay in the desired lysate buffer
	Incomplete click conjugation	Repeat <i>in vitro</i> auto-modification assay using alternative click ligands and conditions
Lack of labeling during lysate scale-up	Incomplete click conjugation in large volumes	Perform the labeling reaction in a series of identical small volume reactions and pool post click conjugation
Protein precipitate does not dissolve in SDS	Proteins remain aggregated	Heat pellet at 55°C until pellet dissolves
Low levels of biotinylation enrichment	NeutrAvidin resin is saturated	Increase amount of NeutrAvidin resin used
	Lack of direct targets in the lysate	Use different cell types that express high levels of suspected PARP targets
		Increase amount of lysate labeled
		Increase concentration of either PARP-KA and/or 5-Et-6-a-NAD ⁺ in the lysate
High background signal	High levels of endogenous biotinylation	Increase concentration of either PARP-KA and/or 5-Et-6-a-NAD ⁺ in the lysate
	Non-specific labeling	Decrease incubation time or concentration of 5-Et-6-a-NAD ⁺
	Non-specific enrichment	Insert an acetonitrile wash step following step 28 in Basic Protocol 2
Low target identification in the LC-MS/MS analysis	Background contamination	Perform all labeling steps in a sterile environment
	Saturation by PARP-KA	Decrease PARP-KA concentration
		Increase the amount of affinity resin used to remove excess PARP-KA in step 4 of Basic Protocol 2