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Identifying Genomic Sites of ADP-Ribosylation Mediated by Specific Nuclear PARP Enzymes Using Click-ChIP

Ryan A. Rogge, Bryan A. Gibson, and W. Lee Kraus

Laboratory of Signaling and Gene Regulation, Cecil H. and Ida Green Center for Reproductive Biology Sciences, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA.

Division of Basic Research, Department of Obstetrics and Gynecology, University of Texas, Southwestern Medical Center, Dallas, TX 75390, USA.

Abstract

Nuclear poly(ADP-ribose) polymerases (PARPs), including PARPs 1, 2, 3, and the Tankyrases, belong to a family of enzymes that can bind to chromatin, and covalently modify histones and chromatin-associated proteins with ADP-ribose derived from nuclear NAD⁺. The genomic loci where the nuclear PARPs bind and covalently modify chromatin is a fundamental question in PARP biology. Chromatin immunoprecipitation coupled with deep sequencing (ChIP-seq) has become an essential tool for determining specific sites of binding and modification genome-wide. Few methods are available, however, for localizing PARP-specific ADP-ribosylation events across the genome. Here we describe a variation of ChIP-seq, called Click-ChIP-seq, for identifying sites of ADP-ribosylation mediated by specific PARP family members. This method uses analog-sensitive PARP (asPARP) technology, including asPARP mutants and the alkyne-containing “clickable” NAD⁺ analog 8-Bu(3-yne)T-NAD⁺. In this assay, nuclei from cells expressing an asPARP protein of interest are incubated with 8-Bu(3-yne)T-NAD⁺, which is incorporated into ADP-ribose modifications mediated only by that specific asPARP protein. The nuclei are then subjected to crosslinking with formaldehyde, and the protein-linked analog ADP-ribose is clicked to biotin using copper-catalyzed alkyne-azide “click” chemistry. The chromatin is fragmented, and the fragments containing analog ADP-ribose are enriched using streptavidin-mediated precipitation. Finally, the enriched DNA is analyzed by qPCR or deep sequencing experiments to determine which genomic loci contain ADP-ribose modifications mediated by the specific PARP protein of interest. Click-ChIP-seq has proven to be a robust and reproducible method for identifying chromatin-associated, PARP-specific ADP-ribosylation events genome-wide.

Keywords

ADP-ribosylation; Analog-sensitivity; Automodification; Chromatin; Chromatin immunoprecipitation (ChIP); Click chemistry; Crosslink; Mono(ADP-ribosylation) (MARylation); Mutation; NAD⁺ analog; Nucleus; Nucleosome; Poly(ADP-ribose) polymerase (PARP); Poly(ADP-ribosylation) (PARylation); Post-translational modification (PTM)

Address correspondence to: W. Lee Kraus, Ph.D., Cecil H. and Ida Green Center for Reproductive Biology Sciences, The University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Boulevard, Dallas, TX 75390-8511, Phone: 214-648-2388, Fax: 214-648-0383, LEE.KRAUS@utsouthwestern.edu.

4. Notes

1. Introduction

Chromatin, the physiological template for nuclear processes, is bound by a host of nuclear proteins. Many of these proteins possess enzymatic activities that covalently modify nucleosomal histones, as well as other non-histone chromatin-associated proteins [2, 5]. These post-translational modifications can alter the structure and function of chromatin by altering nucleosomes locally, as well as higher-order chromatin structures globally [2, 4]. Nuclear poly(ADP-ribose) polymerases (PARPs), including PARPs 1, 2, 3, and the Tankyrases, belong to a family of enzymes that can bind to chromatin, and covalently modify histones and chromatin-associated proteins with ADP-ribose derived from nuclear NAD^+ [1, 12, 13]. PARP-mediated ADP-ribosylation can occur as mono or poly modifications, yielding mono(ADP-ribose) (MAR) or poly(ADP-ribose) (PAR), respectively [9, 24, 28]. The unique biologies of MAR and PAR are poorly understood.

Nuclear PARPs possess mono- or poly(ADP-ribosyl)transferase activity. For example, PARPs 1, 2, and the Tankyrases are polyenzymes [i.e., catalyzes poly(ADP-ribosyl)ation], while PARP-3 is a monoenzyme [i.e., catalyzes mono(ADP-ribosyl)ation] [1, 28]. PARP-1 in particular is a highly abundant protein, and the vast majority of PAR in the nucleus is associated with the automodification of PARP-1 [13, 24, 27]. Much less is known about the biology of other nuclear PARP enzymes. While first implicated as a facet of the cellular response to DNA damage [25], nuclear ADP-ribosylation (both MARylation and PARYlation) has now been shown to play key roles in transcription, as well as RNA processing and stability [11, 12, 13]. Identifying the location of ADP-ribosylation across the genome is important for understanding the role of PARPs and PAR in chromosome biology. In addition to core histones, other chromatin-associated proteins that are ADP-ribosylated include: the PARP enzymes (through automodification) [19]; linker histones [10, 16, 18]; and transcription-related factors such as NELF-E, C/EBP β , and CTCF [6, 10, 15]. Although identification of these and other substrates has been informative, identifying PARP-specific ADP-ribosylation events at specific genomic loci, however, has proven challenging.

The Kraus Lab recently developed a method for the isolation of chromatin fragments that have been modified by a specific PARP enzyme [10]. This method, which we call Click-ChIP, is a variation of chromatin immunoprecipitation (ChIP) [3, 7, 17]. It uses recently developed analog-sensitive PARP (asPARP) technology, including asPARP mutants (Fig. 1, A and B) and the alkyne-containing “clickable” NAD^+ analog 8-Bu(3-yne)T- NAD^+ [8, 10] (Fig. 1C). The use of 8-Bu(3-yne)T- NAD^+ for nuclear ADP-ribosylation reactions is made possible by an engineered analog-sensitive mutant of the PARP enzyme of interest [8, 10] (Fig. 1, A and B). Mutation of a “gatekeeper” residue in the adenine binding pocket of the PARP enzyme of interest to glycine or alanine (e.g., L877A in PARP-1) (Fig. 1B) creates a “hole” that is filled by the substituent added at the 8 position of the adenine ring in 8-Bu(3-yne)T- NAD^+ (Fig. 1C), allowing for its incorporation into ADP-ribosylated proteins [10].

In Click-ChIP, nuclei from cells expressing an asPARP mutant of interest are incubated with the NAD^+ analog, which is incorporated into ADP-ribose modifications mediated only by that specific asPARP protein [10] (Fig. 2). After crosslinking the nuclei with formaldehyde,

the protein-linked analog ADP-ribose is clicked to biotin using copper-catalyzed alkyne-azide “click” chemistry [20] (Fig. 2). The chromatin is fragmented, and the fragments containing analog ADP-ribose are enriched using streptavidin-mediated precipitation [10] (Fig. 2). The genomic DNA enriched by Click-ChIP can be analyzed by qPCR or deep sequencing to determine which genomic loci contain ADP-ribose modifications mediated by the specific PARP protein of interest [10]. The protocol described herein describes how to react isolated asPARP-containing nuclei with the NAD⁺ analog, click the analog to biotin, and enrich the fragmented chromatin. A brief description and references are provided for further analysis of the genomic DNA associated with enriched ADP-ribosylated chromatin by deep sequencing (Click-ChIP-seq) (Fig. 2).

2. Materials

All reagents should be the highest quality molecular biology and biochemistry grade.

2.1. Analog NAD⁺ labeling reaction

1. 18 MΩ double distilled H₂O (ddH₂O).
2. Protease inhibitor cocktail (e.g., Roche cOmplete™ EDTA-free; Tablets provided in glass vials, which can be dissolved to 1X) (see Note 1).
3. Nuclei Reaction Buffer: 30 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 5 mM CaCl₂, 10 mM KCl, 0.01% IGEPAL CA-630, 0.05 mM EDTA, 20% glycerol, 1 mM DTT, 1X protease inhibitor cocktail (see Note 1).
4. 8-Bu(3-yne)T-NAD⁺ solution: 10 mM 8-Bu(3-yne)T-NAD⁺ (analog NAD⁺; Biolog Life Science Institute) in ddH₂O.
5. β-NAD⁺ solution: 10 mM in ddH₂O. Store in small, single-use aliquots at −80°C. Dilute right before use.
6. Crosslinking Buffer: 10 mM HEPES (pH 8.0), 10 mM NaCl, 10 mM MgCl₂, 0.1% IGEPAL CA-630, 1 mM DTT, 1X protease inhibitor cocktail (see Note 1).
7. Formaldehyde, 16% (methanol-free).
8. Glycine Solution: 2.5 M in ddH₂O

2.2. Click reaction

1. Click Reaction Buffer: 10 mM HEPES (pH 8.0), 10 mM NaCl, 10 mM spermine, 0.1% IGEPAL CA-630, 1X protease inhibitor cocktail (see Note 1).
2. Azide-Biotin solution: 100 mM in ddH₂O
3. Copper sulfate (CuSO₄) solution: 100 mM dissolved in ddH₂O (see Note 1).
4. Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) solution: 500 mM in ddH₂O (see Note 2).

¹.Make fresh for each use.

².Make THPTA in ddH₂O and store in single use aliquots at −20°C.

5. Sodium ascorbate solution: 500 mM in ddH₂O (see Note 1).
6. 25X Click Catalyst Mix: 125 mM THPTA, 25 mM CuSO₄, 125 mM Sodium Ascorbate (see Section 3.2, Step 2 and Note 1).

2.3. Chromatin enrichment

1. ChIP Lysis Buffer: 50 mM Tris-HCl (pH 7.9), 1% SDS, 10 mM EDTA, 1X protease inhibitor cocktail (See Note 1).
2. Water bath sonicator (Diagenode).
3. 1X TBE: 89 mM Tris-HCl (pH 7.6), 89 mM boric acid, 2 mM EDTA.
4. 10X TBE Loading Solution: 400 mg/mL sucrose, 2 mg/mL Orange G.
5. ChIP Dilution Buffer: 20 mM Tris-HCl (pH 7.9), 2 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, 1 mM DTT, 1X protease inhibitor cocktail
6. Nitrocellulose membrane.
7. HRP-conjugated Streptavidin.
8. Enhanced chemiluminescent (ECL) reagent.
9. Streptavidin-coated beads (e.g., Dynabeads MyOne Streptavidin T1).
10. Magnetic isolation stand.
11. 2% Sodium dodecyl sulfate.
12. Low Salt Wash Buffer: 20 mM Tris-HCl (pH 7.9), 2 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, 1 mM DTT, 1X protease inhibitor cocktail (see Note 1).
13. High Salt Wash Buffer: 20 mM Tris-HCl (pH 7.9), 2 mM EDTA, 500 mM NaCl, 0.05% SDS, 1% Triton X-100, 1X protease inhibitor cocktail (see Note 1).
14. LiCl Wash Buffer: 10 mM Tris-HCl (pH 7.9), 1 mM EDTA, 250 mM LiCl, 1% IGEPAL, 1% sodium deoxycholate, 1X protease inhibitor cocktail (see Note 1).
15. TE buffer: 10 mM Tris-HCl (pH 7.9), 0.25 mM EDTA.

2.4. Decrosslinking the chromatin and purifying the genomic DNA

1. Decrosslinking Buffer: 100 mM Sodium bicarbonate, 100 mM NaCl, 1% SDS.
2. 500 µg/mL RNase, DNase-free.
3. 20 mg/ mL Proteinase K.
4. Phenol:chloroform:isoamyl alcohol mixture (25:24:1).
5. Chloroform.
6. Glycogen solution: 10 mg/ml in ddH₂O.
7. Sodium acetate (NaOAc) solution: 3M in ddH₂O.
8. Isopropanol.

9. Ethanol solution: 75% (v/v) in ddH₂O.
10. DNA purification resin (e.g., AMPure XP beads; Beckman-Coulter).

3.5. Genomic analyses

1. BOWTIE short read aligner (<http://bowtie-bio.sourceforge.net/index.shtml>) [14] or another appropriate short read aligner.
2. Custom scripts for analyzing Click-ChIP-seq data, as described in Gibson *et al* (2016) [10].

3. Methods

3.1. *In nuclei* labeling with analog NAD⁺ and formaldehyde crosslinking

The goal of this section of the protocol is to label PARP-specific sites of ADP-ribosylation across the genome using an analog-sensitive version of a nuclear PARP of interest and a “clickable” NAD⁺ analog. Since the cell membrane is impermeable to the NAD⁺ analog, the reactions are carried out in isolated nuclei from the cells of interest. The nuclei are incubated with the NAD⁺ analog, which is incorporated into post-translational modifications of chromatin proteins by the asPARP. The nuclei are then crosslinked with formaldehyde to preserve protein-DNA interactions in a standard chromatin immunoprecipitation (ChIP) protocol. After crosslinking, the nuclei contain proteins crosslinked to genomic DNA and other chromatin proteins, some of which are modified by clickable ADP-ribosylation from the activity of the asPARP.

1. For each experimental condition, resuspend approximately 5 million nuclei isolated from cells expressing an asPARP enzyme of interest in 500 μ L of room temperature Nuclei Reaction Buffer (see Note 3).
2. Set up both experimental and control reactions using analog NAD⁺ and natural β -NAD⁺. Add β -NAD⁺ or analog NAD⁺ to their respective reactions of 5 million nuclei to a final concentration of 250 μ M (i.e. add 12.5 μ L of 10 mM β -NAD⁺ or analog NAD⁺ to each 500 μ L reaction) (see Notes 4 and 5).
3. Allow the ADP-ribosylation reactions to proceed by incubating the nuclei at room temperature for 30 minutes with gentle mixing.
4. Collect the nuclei by centrifugation at 2,000 $\times$ *g* for 1 minute at room temperature in a microcentrifuge.

³. Isolate nuclei from cells expressing an asPARP of interest using a method appropriate for those cells in an amount appropriate for all downstream assays. The Click-ChIP protocol described herein has been used successfully with 1 to 10 million nuclei isolated from mouse embryonic fibroblasts (MEFs) and human MCF-7 cells.

⁴. Major factors that we have tested that affect the final enrichment and fold enrichment over control include (1) the number of nuclei, (2) the concentration of analog NAD⁺ with which the nuclei are reacted (0.001 to 250 μ M has been a useful range), and (3) the concentration of biotin-azide used in the click reaction (10 to 100 μ M has been a useful range). The conditions listed in the protocol were used in the published experiments from Gibson *et al.* (2016) [10]. Low concentrations of analog NAD⁺ for the labelling the isolated nuclei may result in low signal dot blots, but still yield good enrichment detectable by qPCR. Adjustment of the ratios noted above is a good place to start when optimizing for new biological systems or new experimental systems.

⁵. Additional controls include the same cells expressing GFP or wild-type version of the PARP enzyme. These controls will provide information about the background levels of analog NAD⁺ incorporation in the absence of a functional asPARP enzyme.

5. Wash the nuclei by gentle resuspension in 500 μ L of Nuclei Reaction Buffer without analog NAD^+ or natural $\beta\text{-NAD}^+$, with subsequent centrifugation at 2,000 x g for 1 minute at room temperature in a microcentrifuge.
6. Resuspend the nuclei in 750 μ L of Crosslinking Buffer.
7. To crosslink the chromatin within the nuclei, add 25 μ L of 16% formaldehyde to 750 μ L of Crosslinking Buffer. Incubate the samples at room temperature for 10 minutes with gentle mixing to prevent the nuclei from settling.
8. After the incubation, quench the formaldehyde crosslinking reaction by adding 100 μ L of 2.5 M glycine. Mix gently and incubate on ice for 10 minutes.
9. Collect the nuclei by centrifugation at 500 x g for 10 minutes at room temperature in a microcentrifuge. Aspirate the supernatant.
10. Wash the nuclei by resuspending in 100 μ L of Click Reaction Buffer.
11. Collect the nuclei by centrifugation at 500 x g for 5 minutes at room temperature in a microcentrifuge. Resuspend the nuclei in 100 μ L of Click Reaction Buffer.
12. Collect the nuclei by centrifugation at 500 x g for 5 minutes at room temperature in a microcentrifuge (see Note 6).

3.2. Clicking the ADP-ribosylated and crosslinked chromatin proteins to biotin

The goal of this section of the protocol is to click the ADP-ribosylated and crosslinked chromatin proteins to biotin using copper-catalyzed alkyne-azide click chemistry. The click reaction is carried out in intact, analog NAD^+ -labelled nuclei.

1. For the click reaction, resuspend the nuclei from Section 3.1, Step 12 in 100 μ L of Click Reaction Buffer containing 50 μ M of azide-biotin. (e.g. add 1 μ L of 100 mM azide to 2 mL of Click Reaction Buffer. Prepare a sufficient amount for two sequential click reactions).
2. Prepare 25X Click Catalyst Mix by adding the following stepwise to a tube: 1 part ddH₂O, 1 part 100 mM CuSO₄ (at this point the solution should be faint blue), 1 part 500 mM THPTA (at this point the solution should be intensely blue), and 1 part 500 mM sodium ascorbate (at this point the solution should be clear) (see Note 7).
3. To initiate the click reaction, add 4 μ L of 25X Click Catalyst Mix (from Step 2) and mix gently.

⁶After this step, the nuclei are labeled with analog NAD^+ , crosslinked with formaldehyde, and ready for the alkyne-azide click reaction.

⁷In order for the reaction to proceed at an appreciable rate, a copper catalyst is required, in particular Cu(I) ions. In this protocol, the Cu(I) ions are provided by CuSO₄ in the 25X click buffer. Sodium ascorbate is added to reduce Cu(II) to Cu(I) ions, and THPTA binds to and stabilizes Cu(I) ions. Prepare the 25X click buffer fresh for each use, especially the sodium ascorbate stocks. The particular order of addition used for the components in the 25X Click Catalyst Mix is to ensure binding of the copper ions to the copper ligand THPTA. If altering the mix, always pre-mix the CuSO₄ and the THPTA.

4. Let the click reaction proceed at room temperature with gentle mixing using a rotisserie-style mixer (or a nutator) at low speed (~10–15 RPM) for 1 hour (see Note 8)
5. Collect the nuclei by centrifugation at 500 x *g* for 5 minutes at room temperature in a microcentrifuge. Repeat the click reaction by resuspending the nuclei in fresh Click Reaction Buffer containing 50 µM biotin-azide with subsequent addition of 4 µL of 25X Click Reaction Buffer. Incubate at room temperature for 1 hour with gentle mixing using a rotisserie-style mixer.
6. Collect the nuclei by centrifugation at 500 x *g* for 5 minutes at room temperature in a microcentrifuge. Wash the nuclei by resuspending them in Click Reaction Buffer (without biotin-azide), then collect them by centrifugation at 500 x *g* for 5 minutes at room temperature in a microcentrifuge (see Note 9).

3.3. Enrichment of ADP-ribosylated chromatin fragments clicked to biotin

At this point in protocol, the chromatin within the nuclei contains ADP-ribosylated proteins that have been crosslinked to genomic DNA and clicked to biotin. This section of the protocol describes how to disrupt the nuclei, fragment the chromatin to a size that is appropriate for the generation of deep sequencing libraries, and isolate the fragmented chromatin using Streptavidin beads, which bind to the biotinylated, crosslinked chromatin proteins.

1. Resuspend the nuclei from Section 3.2, Step 4 in 300 µL of ChIP Lysis Buffer using a Biorupter bath sonicator (Diagenode). As a starting point, sonicate on high with 1 minute pulses on a 50% duty cycle (i.e., 30 seconds on, 30 seconds off), for a total time period of 3 minutes. Mix the tubes by inversion, and repeat the previous sonication program twice (i.e. run the 3 minute program 3 times). The goal of sonication is to generate DNA fragments with an average size of 200–300 bp (Fig. 3A) (see Note 10).
2. Perform a quality control check of the chromatin fragmentation. Deproteinize a small aliquot (10 µL) of each sample by adding 1 µL of 20 mg/mL proteinase K and incubating at 65° C for 2 hours. Add 1 µL of 10X TBE Loading Solution to each aliquot, mix, and run on a 1.5% agarose gel in 1X TBE. Stain the gel with ethidium bromide and visualize (Fig. 3A).

⁸.If altering the methods described herein, some important factors to keep in mind concerning the click reaction are as follows: (1) The click reaction is sensitive to dissolved oxygen, from which reactive oxygen species are produced. The addition of THPTA and sodium ascorbate helps to scavenge reactive oxygen species and (2) The oxidized form of the reducing agent, sodium ascorbate, can crosslink some amino acids. While unnecessary for this particular application, aminoguanidine hydrochloride can be added to prevent the crosslinking. (3)

⁹.After this step, the nuclei are labeled with analog NAD⁺, crosslinked with formaldehyde, and clicked to biotin. The chromatin is now ready to be fragmented by sonication. Alternatively, the nuclei can be collected by centrifugation and flash frozen. The specifics of the freezing process may affect the amount of sonication necessary for fragmentation of genomic DNA in the subsequent steps.

¹⁰.The sonication requires optimization for each cell type used. The parameters will vary based on the sonicator used. The goal of sonication is to generate DNA fragments with an average size of 200–300 bp. You may have to vary the intensity setting, duty cycle, duration of sonication, the number of cycles. When working out the optimal sonication conditions, collect analytical samples between cycles and analyze by gel electrophoresis (as in Section 3.3, Step 2; Fig. 3A).

3. Perform a quality control check of the biotin content of the chromatin. Adsorb 1 μ L of each sample onto dry nitrocellulose membrane by pipetting. Let the spots dry. Perform dot blotting using an HRP-streptavidin detection reagent (see Note 11). Biotin incorporation should only be observed in samples in which asPARP-containing nuclei were incubated with analog NAD⁺ (Fig. 3B) (see Note 4).
4. Add ChIP Dilution Buffer to the remaining sonicated chromatin samples from Step 1 above to a final volume of 1 mL and mix thoroughly, but gently.
5. Remove an aliquot from each sonicated chromatin sample as “input.” A 50 μ L aliquot will represent 5% of the total input. Do not add Streptavidin-coated beads to the input sample; this sample will not be enriched for biotin-containing chromatin.
6. The samples are now ready for enrichment using Streptavidin-coated magnetic beads. Resuspend the beads evenly, remove 50 μ L for each sample, and wash the total amount using 1 mL of ChIP Dilution Buffer in a microcentrifuge tube (see Note 12). Place the tube in magnetic stand and allow the magnet to collect the beads, aspirating the supernatant, and resuspend the beads in the original volume of ChIP Dilution Buffer. Add the 50 μ L of beads to each of your samples. Incubate overnight with gentle mixing at 4° C (see Note 13).
7. Place each sample tube in a magnetic stand and allow the magnet to collect the beads. Aspirate the supernatant.
8. Wash the beads by repeated magnetic isolation, aspiration of supernatant, and addition of 1 mL of wash buffer as described below, followed by a 5 minute incubation at 4°C with gentle mixing. The washes should be as follows:
 - a. Twice with 2% SDS.
 - b. Once with Low Salt Wash Buffer.
 - c. Three times with High Salt Wash Buffer.
 - d. Once with LiCl Wash Buffer.
 - e. Twice with TE.

3.4. Decrosslinking the chromatin and purifying the genomic DNA

In order to analyze the genomic DNA in the chromatin fragments, the chromatin must be decrosslinked, eluted from the Streptavidin beads, and purified. The steps below describe the protocol for doing so.

¹¹The choice of blocking agent for the dot blotting can affect the biotin detection results. We recommend BSA for blocking and for diluting the HRP-Streptavidin. Milk contains biotin, which can lead to an increased background if used as a blocking agent.

¹²When pipetting the Streptavidin-coated magnetic beads, use a wide bore plastic pipette tip, which can be prepared by severing a small portion at the end of the tip with a clean razor blade.

¹³If the background is too high after chromatin enrichment (i.e., a low signal to background ratio), consider pre-clearing samples using uncoated magnetic beads (or bead with a different affinity tag; e.g., Protein A Dynabeads), which can strip away non-specific interactions. For example, add 5 μ L of washed Protein A Dynabeads, incubate at 4°C with gentle mixing (e.g., on a nutator) for 1 hour, collect the beads using a magnetic stand, transfer the supernatant to a fresh tube, and continue as described in Section 3.3, Step 6.

1. Decrosslink the bead-bound chromatin from Section 3.3, Step 8 by resuspending the beads in 200 μ L of Decrosslinking Buffer. Incubate at 65° C overnight. Decrosslink the input samples from Section 3.3, Step 5 as well by bringing the volume to 200 μ L with Decrosslinking Buffer and incubating alongside the enriched samples at 65° C overnight.
2. Isolate the beads by placing the tubes in a magnetic stand. Collect and save the supernatants in fresh tubes.
3. Wash the beads with 300 μ L of TE and add the wash (containing additional decrosslinked genomic DNA released from the beads) to the cognate supernatants that were collected for each sample. Bring the input samples to a final volume of 500 μ L with TE as well (to match the enriched samples).
4. Digest the RNA in the samples by adding RNase to a final concentration of 3 μ g/mL (by adding 3 μ L of a 500 μ g/mL stock solution), mixing well, and incubating at 37° C for 30 minutes.
5. Digest the protein in the samples by adding proteinase K to a final concentration of 50 μ g/mL (by adding 1.25 μ L of 20 mg/mL stock solution), mixing well, and incubating at 55° C for 2 hours.
6. Extract the DNA samples with phenol:chloroform:isoamyl alcohol (25:24:1) as follows:
 - a. Add an equal volume of phenol:chloroform:isoamyl alcohol and vortex the samples for 20 seconds. The solution should turn cloudy.
 - b. Separate the phases by centrifuging the samples at full speed (21,000 x g) in a microcentrifuge for 5 minutes at room temperature.
 - c. Remove, transfer to a new tube, and save the aqueous phase, which should be the top layer containing the enriched genomic DNA.
7. Extract the samples in a similar manner using an equal volume of chloroform and collect the aqueous phase in a new tube.
8. Precipitate the DNA from the aqueous phase by the addition of 50 μ L of 3M NaOAc, 6 μ L of 10 mg/mL glycogen, and 500 μ L of isopropanol. Mix thoroughly and collect the precipitate by centrifuging the samples at full speed in a microcentrifuge for 25 minutes at 4°C.
9. Carefully remove the supernatant, making sure not to disturb the pellet, which will be translucent. Wash the pellet carefully with 500 μ L of 75% ethanol.
10. Pellet the DNA again by centrifuging the samples at full speed in a microcentrifuge for 10 minutes at 4°C. Carefully remove the supernatant, making sure not to disturb the pellet, which will be white at this point.
11. Dry the pellet for 5 minutes at room temperature to evaporate the alcohol by leaving the tubes open and inverted. Do not let the pellets dry completely, or they

will become difficult to dissolve. The enriched genomic DNA pellets can be stored at -20°C this point until further analysis.

12. Dissolve the DNA pellet in 100 μL of ddH₂O, TE, or your buffer of choice. At this point, the enriched genomic DNA can be stored at -20°C , analyzed by qPCR, or subjected to other analytical methods (such as deep sequencing; see Section 3.4 below).
13. Purify the DNA further using AMPure XP beads (or a similar bead product) according to the manufacturer's instructions.
14. Quantify the DNA from both the enriched and input fractions using a Qubit fluorimeter.
15. If desired, check the enrichment at specific genomic loci of interest using qPCR, as shown in Fig. 4.

3.5. Genomic analyses of genomic DNA enriched using Click-ChIP

The DNA isolated from the Click-ChIP protocol described above can be processed into genomic libraries for deep sequencing as described below, allowing a genome-wide view of PARP-specific ADP-ribosylation events.

1. Prepare Click-ChIP-Seq libraries from the enriched and input DNA samples using methods that we described previously [8] (see Note 14).
2. Sequence the libraries using an appropriate next generation sequencing technology (e.g., Illumina HiSeq or NextSeq) and collect the data (see Note 15).
3. Align the reads to a reference genome using the standard settings in BOWTIE [14].
4. Analyze the data and call peaks. Present the enrichment data in browser track representations, heatmaps, and boxplots (see for Fig. 5 for example) (see Note 16).

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¹⁴. Most standard ChIP-seq library preparation protocols will work [3, 7, 17].

¹⁵. Single-end 50 base reads should be sufficient.

¹⁶. For the browser tracks shown in Fig. 5, Gibson *et al.* [10] developed and used a custom script. The reference genome was divided into 2500 bp windows with a 250 bp step size. The 'intersectBed' tool in the bedTools package (<http://bedtools.readthedocs.io/en/latest/>) was used to count the number of input and treatment reads in each bin. The ratio of these read counts was calculated and then normalized by the total number of reads for each. The bins were then reduced to non-overlapping 250 bp segments. [10, 21, 22].

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Figure 1. Analog-sensitive PARP (asPARP) methodology used in Click-ChIP.

(A) Schematic representation of the asPARP methodology used in Click-ChIP, which allows detection of genomic loci ADP-ribosylated by a specific PARP family member. In the asPARP approach, an asPARP protein (*grey square*) uses an alkyne-containing NAD⁺ analog (e.g., 8-Bu(3-yne)T-NAD⁺ (*shown in purple*)) to catalyze the transfer of 8-Bu(3-yne)T-ADP-ribose (*also shown in purple*) onto a target protein (*black circle*).

(B) Multiple sequence alignment of a portion of the catalytic domains from PARPs 1, 2, and 3. The gatekeeper residues that confer NAD⁺ analog sensitivity are highlighted in red.

(C) Chemical structure of the clickable NAD⁺ analog 8-Bu(3-yne)T-NAD⁺. Adapted from Gibson and Kraus, 2017 [10].

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Figure 2. The Click-ChIP-seq workflow.

In Click-ChIP, nuclei from cells expressing an asPARP mutant of interest are incubated with the NAD⁺ analog, which is incorporated into ADP-ribose modifications mediated only by that specific asPARP protein. After crosslinking the nuclei with formaldehyde, the protein-linked analog ADP-ribose is clicked to biotin using copper-catalyzed alkyne-azide “click” chemistry. The chromatin is fragmented, and the fragments containing analog ADP-ribose are enriched using streptavidin-mediated precipitation. The genomic DNA enriched by Click-ChIP can be analyzed by qPCR or deep sequencing to determine which genomic loci contain ADP-ribose modifications mediated by the specific PARP protein of interest. Adapted from Gupte *et al*, 2017 [11].

Figure 3. Quality control assays of asPARP-labeled chromatin.

(A) Agarose gel analysis of sonicated chromatin from asPARP-1-labeled or control labeled nuclei. Nuclei from mouse embryo fibroblasts (MEFs) expressing asPARP-1, wtPARP-1, or GFP were incubated $\pm$ 8-Bu(3-yne)T-ADP-ribose. The nuclei were then crosslinked, and the protein-linked analog ADP-ribose was clicked to biotin using copper-catalyzed alkyne-azide “click” chemistry. The chromatin was then fragmented by sonication and analyzed on an ethidium bromide-stained 1.5% agarose gel run in 1X TBE.

(B) HRP-streptavidin dot blot on nitrocellulose for biotin detection of the same sonicated chromatin samples shown in (A).

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Figure 4. Gene-specific enrichment of PARP-1 ADP-ribosylated chromatin using Click-ChIP-qPCR in MEFs.

Bar graph showing Click-ChIP-qPCR results for a genomic locus (i.e., the promoter of the *Fkbp5* promoter) enriched in PARP-1 ADP-ribosylated chromatin in MEFs. The results are shown for nuclei expressing asPARP-1 with or without incubation with analog NAD⁺, as well as nuclei expressing wild-type PARP-1 or GFP incubated with analog NAD⁺. Each bar represents the mean $\pm$ SEM from three separate determinations.

Figure 5. Global enrichment of PARP-1 ADP-ribosylated chromatin using Click-ChIP-seq in MEFs.

Browser tracks showing Click-ChIP-seq results for two different genomic regions enriched in PARP-1 ADP-ribosylated chromatin in MEFs. Enrichment is displayed as the odds ratio (reads per bin treatment/reads per bin input, normalized by the ratio of their respective total read counts). The Click-ChIP-seq data are from Gibson et al, 2016 [10] and the browser track was generated using the Integrative Genomic Viewer (IGV) [23, 26]. Gene locations are displayed as horizontal blue block arrows. The region analyzed by qPCR in Figure 4 is marked by a black arrow.