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PARPs and ADP-ribosylation: Deciphering the Complexity with Molecular Tools

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SUMMARY

PARPs catalyze ADP-ribosylation—a post-translational modification that plays crucial roles in biological processes, including DNA repair, transcription, immune regulation, and condensate formation. ADP-ribosylation can be added to a wide range of amino acids with varying lengths and chemical structures, making it a complex and diverse modification. Despite this complexity, significant progress has been made in developing chemical biology methods to analyze ADP-ribosylated molecules and their binding proteins on a proteome-wide scale. Additionally, high-throughput assays have been developed to measure the activity of enzymes that add or remove ADP-ribosylation, leading to the development of inhibitors and new avenues for therapy. Real-time monitoring of ADP-ribosylation dynamics can be achieved using genetically encoded reporters, and next-generation detection reagents have improved the precision of immunoassays for specific forms of ADP-ribosylation. Further development and refinement of these tools will continue to advance our understanding of the functions and mechanisms of ADP-ribosylation in health and disease.

eTOC blurb

Dasovich and Leung's review highlights the latest technical advances in characterizing ADP-ribosylation, such as identifying ADP-ribosylated proteins and sites, utilizing NAD⁺ analogs to determine PARP substrate specificity, using chemical probes to identify the ADP-ribose binding proteome, and monitoring ADP-ribosylation dynamics both in vitro and in vivo.

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DECLARATION OF INTERESTS

Anthony K. L. Leung holds a patent related to the ELTA technology used for labeling ADP-ribosylated molecules.

BACKGROUND

Post-translational modifications are ideally suited for rapid responses to the environment by using the chemistry of ubiquitous metabolites, such as ATP, Acetyl-CoA, and NAD⁺, to alter protein function. Without the need to synthesize new molecules, protein modifications can be initiated immediately. An example is ADP-ribosylation, an NAD⁺-dependent protein modification best known for its role in the DNA damage response. DNA damage-dependent ADP-ribosylation is catalyzed by PARP1, an abundant nuclear protein associated with chromatin.¹ Once a DNA strand break occurs, PARP1 rapidly (~1 s) binds the break, activating the enzyme by opening its NAD⁺-binding pocket.² PARP1 then uses NAD⁺ to ADP-ribosylate proteins in the vicinity of the DNA break, which initiates a signaling cascade that repairs DNA. While ADP-ribosylation research has historically focused on PARP1 and DNA repair, humans express 16 additional PARPs of emerging biological importance and therapeutic relevance. ADP-ribosylation also plays critical roles in functions unrelated to DNA repair, such as RNA metabolism, protein turnover, and the immune response. PARP inhibitors have been FDA-approved for treating multiple cancers, and pre-clinical data support their use in immune-related diseases, viral infection, and neurodegeneration. Due to intense medical interest over the last two decades, an expanded toolkit is now available for studying ADP-ribosylation with chemical precision. Here, we review the chemical and regulatory complexity of ADP-ribosylation, then delve into the molecular tools developed to uncover its substrates, binding partners, and dynamics.

The Chemical Complexity of ADP-ribosylation

The genetic fingerprints of ADP-ribosylation are found across all kingdoms of life and viruses, with enzymes ADP-ribosylating proteins, small molecules, DNA, and RNA.³ This review focuses on protein ADP-ribosylation catalyzed by human PARPs, though there are other “writer” enzymes that add this modification, including sirtuins, bacterial toxins, and ecto-enzymes.⁴ PARPs contain the eponymous catalytic domain responsible for ADP-ribose transfer (Figure 1A). PARP domains are found in all six eukaryotic supergroups and knockout of PARPs 1/2 or PARPs 5a/b causes embryonic lethality in mice, hinting that PARPs have conserved and essential functions in eukaryotes.⁵⁻⁷ In humans, PARPs represent the largest group of NAD⁺-consuming enzymes and can use almost any nucleophilic amino acid (CDEHKRSTY) as acceptor sites for ADP-ribosylation,⁸ encompassing diverse functional groups from sulfides and ethers to ketoamines and esters (Figure 1B). Mass spectrometry analyses suggest that PARPs display promiscuity. For example, PARP7 modifies C, D, and E in cells and PARP11 ADP-ribosylates C, D, E, and K in an *in vitro* reaction (Figure 1B).⁹⁻¹¹ However, sites identified from *in vitro* reactions should be interpreted with caution,¹¹ as it is now apparent that PARPs do not always act alone, and instead form complexes with accessory proteins that regulate PARP activity and specificity in cells (*e.g.*, HPF1, discussed below).

The seventeen human PARPs distinguish themselves with several accessory domains that regulate their functions.¹² Notably, most PARPs (except for 5a/b, 6, 8, and 16) contain one or more DNA-, RNA-, and/or ADPr-binding domains, suggesting a direct link between nucleic acid metabolism and PARP-mediated ADP-ribosylation (Figure 2). Many PARPs

are transcriptionally regulated by interferon,¹³ and PARP activity can be altered by NAD⁺ availability, nucleic acid binding, and accessory factors. For example, regulation of PARP activity can be achieved through NAD⁺ synthesis by nicotinamide adenyl transferases (NMNATs 1–3), which are localized to different subcellular locations and control the NAD⁺ concentration in specific compartments.¹⁴ PARP activity can also be tuned by their nucleic acid or protein partners. PARP1 can be activated by nucleic acids (*e.g.*, DNA strand breaks or small nucleolar RNAs), and it can perform catalysis on its own or in complex with either NMNAT1 or histone PARylation factor (HPF1).^{15–18} Each one of these ternary nucleic acid–PARP1–accessory factor complexes targets different proteins, and in the case of PARP1/HPF1, switching the specificity to Ser.¹⁹

The Structural Complexity of ADP-ribosylation

Adding to this complexity, PARPs 1, 2, 5a, and 5b can synthesize ADP-ribose–ADP-ribose bonds to create poly(ADP-ribose) (PAR). Polymers as large as ~200 units have been reported in cells treated with high concentrations of DNA-damaging agents,²⁰ while a sizable population of PAR oligomers between 2 and 20 units is detected in more biologically relevant conditions.^{21–23} PAR can also have branch points every 20–30 ADPr units.²⁴ The potential for ADP-ribose modifications on nine different amino acids, combined with the possibility of mono-, oligo-, or poly(ADP-ribose), results in hundreds of possible structures. Therefore, ADP-ribosylation is grouped with other complex post-translational modifications, such as glycosylation and ubiquitination, that have been historically difficult to characterize.^{25,26}

The length of the ADP-ribosylation is tightly regulated by opposing classes of enzymes and cofactors.^{15,27} PAR is primarily degraded by PAR glycohydrolase (PARG).²⁸ While PARP activity is activated by stress, PARG is constitutively active, keeping PAR concentration low under most circumstances. PARG knockout increases DNA breaks and causes embryonic lethality in mice.^{29,30} Therefore, the proper dynamics of PARylation—both synthesis and degradation—are critical for DNA repair and cell survival. Interestingly, human PARG does not cleave the ADPr-protein bond. The final mono(ADP-ribose) (MAR) is instead left for several eraser enzymes, each with a preference for specific ADPr-protein linkages (Figure 1B). The eraser specificity reveals a possible regulatory mechanism where the half-life of the modification depends on the conjugated amino acid. For example, MAR on Asp and Glu have a half-life of <1 hour in cells, whereas MAR on Cys persists for several hours.¹⁰

The structural diversity of ADP-ribosylation is broadly categorized as MAR or PAR (Figure 1A). It was previously thought that PAR was the most abundant form in cells, but recent studies have suggested that MAR is more common, even after DNA damage.^{31,32} However, PAR is observed upon PARG knockdown, suggesting some proteins are initially modified with PAR, then PARG rapidly trims the polymer to MAR.³¹ In situations unrelated to DNA damage (*e.g.*, proteotoxic stress), MARYlation may be the most abundant form.³³ Consistent with this premise, most PARPs appear to exclusively synthesize MARYlation when incubated with NAD⁺ *in vitro*.¹¹ Mirroring the stepwise process of PAR removal, a MARYlating PARP could transfer the first ADP-ribose, then a second PARylating PARP could add additional ADPr. In this model, a MAR–PAR equilibrium could exist at a single ADP-ribosylation site,

regulated by the relative concentrations and rates of PARPs, PARG, MAR-erasing enzymes, and NAD⁺ (Figure 1A). Accessory factors can also influence the structure of ADPr modifications; PARP1 alone synthesizes PAR, whereas the PARP1/HPF1 complex reduces the ADPr length to primarily MAR.^{34–38} Notably, unrestrained Ser MARYlation induced by ARH3 knockout is viable, yet unrestrained PARYlation through additional PARG inhibition is acutely toxic.³⁹ Taken together, these data suggest that MAR and PAR are discrete signaling molecules with profoundly different consequences for cellular function. Detection reagents that differentiate between MAR and PAR have been invented recently.^{31,40,41} As detection technologies improve, we may uncover the biological significance and mechanism behind the balance between MAR and PAR.

This review will summarize current techniques for teasing apart the molecular details of PARP-related processes, including proteomic methods for identifying ADP-ribosylated proteins, sites, and non-covalent ADPr-protein interactions, as well as the development of detection reagents, small molecule inhibitors, and genetically encoded reporters for monitoring ADP-ribosylation dynamics in living cells.

TECHNIQUES FOR IDENTIFYING ADP-RIBOSYLATED PROTEINS AND SITES

Large-scale proteomic studies of phosphorylation and acetylation have been essential for establishing the function of specific sites and delineating their roles in diseases.^{42,43} Analyzing ADP-ribosylation to a similar depth has proved challenging due to its chemical heterogeneity and low abundance. However, several methods have recently been developed that overcame these challenges and revealed thousands of ADP-ribosylated proteins and sites.

Key techniques to identify ADP-ribosylation sites

Enrichment—The classic ADP-ribose enrichment method uses boronate agarose to covalently bind *cis*diols on ribose at alkaline *pH* (Figure 3). This method successfully identified thousands of ADP-ribosylated proteins in breast cancer cell lines.^{44,45} Boronate enrichment has the potential to be highly effective in identifying peptides modified with long PAR, thanks to the increased avidity from the higher number of *cis*-diols. To further enrich PARYlated sites, PARG may be knocked down to boost the amount and length of PAR modifications for analyses.

Another enrichment method relies on the archaeal Af1521 macrodomain (Figure 3),⁴⁶ a protein that binds to MAR and the terminal ADPr of PAR.³⁷ This enrichment is robust, with multiple labs reporting hundreds to thousands of ADP-ribosylated proteins and sites.^{8,47–50} Af1521 enrichment was recently bolstered by protein engineering to increase the macrodomain–ADPr affinity to low nM.⁵⁰ However, the Af1521 macrodomain is also an eraser capable of removing MAR at Asp and Glu.⁵¹ Therefore, it is important to mitigate the hydrolysis by performing enrichment at 4°C.⁴⁹ Despite the inherent differences between boronate and macrodomain enrichment, a direct comparison revealed that the majority of the ADP-ribosylated proteins are identified by both techniques.³²

The final method discussed here is mediated through enzymatic labeling of terminal ADP-ribose (ELTA; Figure 3). This method relies on oligoadenylate synthase 1, a human protein that polymerizes ATP into 2'–5' oligoadenylate as part of the immune response.⁵² OAS1 can efficiently add a variety of ATP analogs to ADP-ribose,⁵³ and using 2'-dATP prevents oligoadenylation to allow attachment of a single analog to ADP-ribosylated molecules.³⁴ To identify ADP-ribosylation sites, ELTA labels ADP-ribosylated peptides with N⁶-azido-dATP, followed by enrichment on dibenzocyclooctyne-agarose via copper-free click chemistry. With this method, as little as 1 fmol of modified peptide can be detected in cell lysates, and hundreds of ADP-ribosylated proteins were identified. The diversity of these sites and the surrounding sequences can be enhanced by varying the ion-pairing reagents used during solid-phase extraction of tryptic peptides.⁵⁴

Derivatization—Once ADP-ribosylated peptides are enriched, the next step is to simplify the chemical complexity of ADP-ribosylation for site identification. Variable lengths of PAR on ADP-ribosylated peptides complicate mass spectra interpretation, and their negative charges make them difficult to analyze with the positive ion mode routinely used for proteomics. To circumvent these issues, a chemical or enzymatic reaction is used to derivatize the ensemble of ADPr modifications into a single, defined mass (Figure 3). Hydroxylamine reacts with the Asp/Glu-ADPr ester bond, replacing MAR and PAR modifications with hydroxamic acid.^{44,45} Hydroxamic acid is a stable, neutrally charged modification that can be sequenced with fragmentation methods such as higherenergy collisional dissociation (HCD). However, hydroxylamine can only identify Asp/Glu-ADPr and may react with unmodified Asp, Glu, Asn, and Gln residues at high concentrations.³² Therefore, sites from hydroxylamine-based proteomics should be supplemented with mutagenesis experiments to ensure that the identified Asp/Glu are *bona fide* ADP-ribosylation sites.^{16,17,33} A more general method of derivatizing ADPr is treatment with hydrofluoric acid, which cleaves the phosphate-oxygen bonds, leaving a single ribose at the modified site. While hydrofluoric acid-based derivatization could theoretically identify ADPr at any amino acid, it has only been used to analyze *in vitro* PARylated PARP1.⁵⁵

An alternative approach to simplifying ADPr involves enzymatic derivatization. MAR and PAR can be digested with phosphodiesterase to produce phosphoribose.⁵⁶ While it requires purification before use, a highly potent phosphodiesterase from snake venom is commercially available. On the other hand, human enzymes such as NudT16 and ENPP1, which exhibit lower enzymatic activity, can be produced in significant amounts in laboratories.^{57–59} Phosphoribose derivatization allows for standard phospho-enrichment, streamlining co-analysis of ADP-ribosylation and phosphorylation sites.⁶⁰ Many Ser and Tyr sites are co-targeted by these modifications, and their cross-talk on histone proteins has important consequences for gene expression.^{8,17} However, because phosphorylation is more abundant than ADP-ribosylation, such co-enrichment may reduce sensitivity.

Another enzyme commonly used is PARG, which converts PAR to MAR, resulting in a +541 Da mass tag at all modified sites. Mono(ADP-ribose) modifications are labile during HCD fragmentation, making it difficult to determine the modification site from the mass spectrum.⁶¹ Instead, electron transfer dissociation (ETD), which specifically fragments the peptide backbone, provides higher localization probabilities for ADP-ribosylated peptides,

albeit with lower sensitivity than HCD. However, hybrid methods such as EThcD and AI-ETD have been developed to improve the sensitivity of ETD-based methods and have confidently localized hundreds of ADP-ribosylation sites in a single experiment.⁴⁷

Diverse ADP-ribosylation sites revealed by various techniques

Together, these proteomics techniques have extensively characterized ADP-ribosylated proteins in cultured human cells treated with DNA-damaging agents. Under these conditions, the majority of ADP-ribosylation occurs on PARP1 itself, histone proteins, and to a lesser extent on proteins involved in DNA repair, cell cycle control, and RNA metabolism.⁸ Precisely which sites are modified is, however, less clear. Initial *in vitro* experiments found Asp and Glu are the acceptor sites on histones and PARP1,^{62,63} which inspired the development of the boronate/hydroxylamine method.^{44,45} However, when the PARG/macrodomain method is used and intact ADP-ribose is searched for on all nine amino acids, most ADPr is found on Ser (~90% of sites, 97% of intensity),⁸ primarily through the activity of the PARP1/HPF1 complex,^{19,32} while Asp and Glu only constitutes a minor fraction of total ADPr (~20 sites, <0.1% intensity).⁸

The discrepancy may be explained due to the technical details of the chosen methods. As hydroxylamine-based methods can only detect ADPr on Asp and Glu, they may provide excellent depth and sensitivity for a minor fraction of the total ADP-ribosylome. These studies also often used PARG knockdown cells, which may reveal modifications that would not stably exist in cells with normal PAR turnover.^{44,45} On the other hand, Asp/Glu sites have an ester, which is more sensitive to hydrolysis than other sites, particularly at basic *pH* (Figure 1B). ADP-ribosylomics experiments typically involve multiple overnight steps above *pH* 7 and have employed off-line high-*pH* fractionation to increase the analysis depth.⁸ The extent of Asp/Glu-ADPr signal loss due to hydrolysis during these lengthy sample preparation steps has not been assessed. Recently, the synthesis of defined MARylated peptides has been described for Ser, Thr, Tyr, Cys, and Arg.^{31,64–66} If available for all sites, these chemically defined peptides would be valuable standards for evaluating the stability of different ADPr-peptide linkages during proteomics procedures.

Orthogonal western blot analyses found that Ser ADP-ribosylation is the most common after DNA damage, confirming the proteomics results. However, the same western blots also detected a minor fraction of DNA damage-dependent ADP-ribosylation on Asp/Glu.⁶⁷ Asp/Glu ADP-ribosylation also has functions unrelated to DNA damage, such as promoting transcription.⁶⁸ Western blot analyses found most ADP-ribosylation occurred at Asp/Glu in pre-adipocytes, where Asp/Glu-ADPr on histones controls gene expression during differentiation.¹⁷ The relative abundance of ADP-ribosylation on other amino acids, such as Lys, Arg, His, and Tyr, can vary depending on the tissue, cell type, cellular compartment, or stress condition (*e.g.*, interferon response).^{47–49} Bolstered by these proteomics efforts, recent studies have explored the biochemical effects and biological outcomes of site-specific ADP-ribosylation on proteins, highlighting how PARPs regulate essential cellular components, like histones and ribosomes (Table 1).^{9,16,18,19,32,33,37,68–77} While the biological functions of most ADP-ribosylation sites are still unknown, proteomics studies have revealed a chemical diversity of ADPr-protein linkages, suggesting that ADP-ribosylation plays a role

in multiple functional niches, with each linkage type regulated by a unique set of writers and erasers.

Harnessing NAD⁺ analogs to identify PARP substrate specificity

Which PARP enzymes are responsible for these diverse site modifications? PARPs have diverse accessory domains, unique site preferences, and appear to target specific subsets of proteins (Figure 2). While proteome arrays can be used to identify *in vitro* substrates of individual PARPs,^{78,79} several labs have used NAD⁺ analogs to study PARP substrate specificity in cellular contexts (Figure 4).^{80,81} Early work found that PARPs tolerate modifications at the nicotinamide and adenine rings of NAD⁺.^{82,83} Jiang and colleagues extended this work by demonstrating that PARPs can accept N⁶-alkyne-NAD⁺, which is then linked through click chemistry to various moieties, including fluorescent dyes and enrichment handles to detect and purify ADP-ribosylated proteins.^{84,85} However, N⁶-alkyne-NAD⁺ alone does not differentiate which targets are modified by specific PARPs because multiple PARPs can use this analog.

Inspired by “analog-sensitive” approaches used to identify targets of other protein-modifying enzymes,⁸⁶ Carter-O’Connell and colleagues applied similar technology to investigate modifications catalyzed by specific PARPs.⁸⁷ By mutating a lysine within the active site, PARP1 and PARP2 can accept NAD⁺ analogs with an ethyl group at the C⁵ position of the nicotinamide ring (Figure 4), while the wild-type enzymes cannot modify proteins with this analog. By combining this approach with the N⁶-alkyne modification, they obtained the first proteomics data set of PARP1 and PARP2 targets from human cell lysates.⁸⁷ The Cohen lab went on to characterize the targets of PARPs 7, 10, 11, and 14.^{10,88,89} This revealed that these six PARPs have distinct target preferences and occupy specific cellular niches. Notably, they created a chimera of the PARP11 accessory domains fused to the PARP10 catalytic domain. Their PARP10–PARP11 chimera modified a subset of PARP10 and PARP11 targets, indicating both the accessory and catalytic domains regulate target preferences.⁸⁸

N⁶-alkyne-NAD⁺, however, inhibits PAR elongation,^{85,87} which is a significant issue for PARylating PARPs 1, 2, 5a, and 5b.¹¹ Gibson and colleagues solved this problem by exploring the C⁸ position of the adenine ring. Interestingly, amine substitutions at C⁸ result in poor reactivity, but sulfide substitutions are well tolerated by PARP1 once an active-site leucine is mutated to alanine (Figure 4).^{68,85} They applied their C⁸ analog to intact nuclei to identify PARP1, PARP2, and PARP3 targets and modification site preferences with proteomics, as well as which regions of the genome are ADP-ribosylated by PARP1 by coupling enrichment of ADP-ribosylated histones with DNA sequencing. This method detected strong correlations between PARP1 ADP-ribosylation and actively transcribed promoters, where PARP1 releases paused RNA polymerase II through ADP-ribosylation of negative elongation factor E.⁶⁸

Rational analog design is possible, now that the structure of the wild-type PARP1 catalytic domain bound to a non-hydrolyzable NAD⁺ has been solved.⁹⁰ The structure agrees well with previous findings: the C⁵ nicotinamide and C⁸ adenine positions are buried deep within the domain, and therefore mutation of residues near these positions produces analog-

sensitive PARPs. On the other hand, the C², N³, and C⁴ positions on adenine and the ribose hydroxyls are exposed to solvent, suggesting that modifications at these positions will be tolerated by wild-type PARP1. For example, C²-functionalized NAD⁺ analogs are broadly compatible, as they have shown activity with all PARPs tested thus far (Figure 4).^{10,91–94} Zhang and colleagues replaced the 3'-hydroxyl with an azide to create NAD⁺ analogs with high PARylation activity.^{95,96} The 3'-azide-NAD⁺ specifically showed activity with PARP1 and little activity with PARP2 or PARP5a.⁹⁶ Yet, 3'-azide-NAD⁺ modified proteins in lysates when PARP1 was knocked out,⁹⁶ suggesting this analog can also be used by other ADP-ribosylation enzymes in cells.

NAD⁺ analogs have traditionally been difficult to use in living cells due to their inability to cross the cell membrane. However, the Marx and Zhang labs have developed methods for delivering NAD⁺ analogs into living cells, including partially permeabilizing the membrane with detergent or using the cationic lipid DOTAP as a transfection reagent.^{91,94,95} Through combination with analog-sensitive technologies, NAD⁺ analog delivery is an exciting development for studying the substrates and functions of individual PARPs in living cells, especially in contexts such as the immune response, where ADP-ribosylation levels are low and multiple PARPs are involved.

SYNTHETIC ADP-RIBOSYLATED PROBES TO IDENTIFY THE ADP-RIBOSE BINDING PROTEOME

Besides altering the activities of covalently modified substrates, ADP-ribosylation can also affect the function of unmodified proteins through non-covalent interactions. PAR serves as a scaffold for macromolecular complexes and biomolecular condensates.⁹⁷ For example, PARP1 ADP-ribosylates proteins surrounding DNA breaks, which can then recruit DNA repair proteins through their PAR-binding domains.⁹⁸ Some E3 ubiquitin ligases, such as RNF146, activate their catalytic activity upon binding to PARylated proteins, resulting in PAR-dependent ubiquitination.^{99,100} Emerging data indicate ADPr-binding domains govern the specificity and timing of PARP signaling events (Figure 5). For instance, macrodomains bind ADPr, but significant sequence divergence within the family leads to unique binding preferences and functions (*e.g.*, the PARG macrodomain degrades PAR chains, but has no affinity for MAR, while the PARP14 macrodomains have no catalytic activity and only bind to MAR).^{101–104} The fact that the PARP14 macrodomains are specific for MAR suggests the sequence context surrounding the MARylation site may also be important for macrodomain recognition.^{40,41,102} The WWE domain in RNF146 binds to the internal repeating unit of PAR,¹⁰⁵ while the tandem PARP13 WWE domain prefers to “cap” the PAR chain by binding to the end.¹⁰⁶ The PBZ domains within APLF specifically bind to PAR, with a preference for branch points.^{107,108} Altogether, these examples suggest that the length and branching of PAR modifications control which proteins are recruited at which times, introducing the possibility of a “PAR code” where the structure of the ADPr modification impacts downstream signaling.⁹⁷ Therefore, tools are much needed to evaluate the extent and specificity of ADPr–protein interactions on a proteome-wide scale.

Chemical probes reveal the MAR- and PAR-binding proteomes

Pioneering efforts identified PAR-binding proteins by overlaying radioactive PAR on protein membranes transferred from gels of cell extracts and used peptide arrays to define clusters of basic and hydrophobic amino acids as a PAR-binding motif.^{110,111} Later proteomics studies identified proteins within PARylated complexes from cells with anti-PAR antibodies.^{112,113} However, these pulled-down proteins may not necessarily be direct binders or “readers”, as they could be covalently PARylated, PAR-binding, or pulled down through indirect protein-protein interactions. Recently, a range of synthetic MAR and PAR probes have been developed and used to directly identify ADPr readers with proteomics (Figure 6).¹¹⁴

One challenge in developing these probes has been the difficulty of synthesizing ADP-ribosylated molecules. Commercial PAR is costly and contains a mixture of lengths (~10–300+), making it impractical for use in large-scale proteomics. However, recent advances have made it possible to synthesize milligrams of single-length PAR oligomers using chemical or enzymatic methods. The core building block for chemical synthesis contains a protected phosphoramidite and an $\alpha(2\rightarrow1)$ adenosine-ribose bond, which can be linked through solid-phase P(III)-P(V) couplings and terminated with an adenosine phosphoramidite.^{115,116} Using this synthetic strategy, Kliza and colleagues used biotinylated mono-, di-, and tri-ADP-ribose to identify their respective binders from human cell lysates with mass spectrometry (Figure 6A).¹⁰⁰ The tri-ADP-ribose probe identified many proteins involved in DNA strand break repair, while the mono-ADP-ribose probe identified proteins involved in metabolism and transcription initiation. These data suggest unique functions for MAR and PAR, highlighting how structurally defined probes improve our understanding of ADPr signaling.

The low yield of pyrophosphate coupling (~70%, compared to >99% for DNA/RNA synthesis) has, however, limited the longest chemically synthesized PAR to a pentamer.¹¹⁶ To obtain longer lengths of PAR, several labs have used *in vitro* reactions with NAD^+ and purified PARP1 or PARP5a.^{21,34,117} Enzymatically synthesized PAR can then be functionalized with enrichment handles and photoaffinity labels through organic synthesis. Lam and colleagues synthesized a bifunctional NAD^+ analog with an azide for click chemistry-based enrichment and a diazirine for efficient photo-cross-linking (Figure 6B).⁹³ PARP1 PARylated with this analog is then added to human cell lysates and irradiated to covalently capture PAR readers for identification. One advantage of this method is that the synthesized PAR remains protein-conjugated, which is more biologically relevant than purified PAR that has been detached from the protein. However, a disadvantage is that the synthesized PAR has varying lengths and branch points, which provides no insight into the structural specificity of interactions identified with this approach.

Alternatively, PAR can be synthesized as a mixed chain polymer using PARPs and NAD^+ and detached from proteins with hydroxylamine or hydroxide. The PAR mixture is fractionated into defined lengths using anion exchange HPLC.^{34,117} While this method is inherently inefficient for synthesizing a single length of PAR, the range of polymer lengths produced in the enzymatic reaction can be adjusted by adding HPF1, histones, or PARP1 fragments.^{34,117} This enzymatic approach remains the only method for obtaining PAR longer than pentamer, with lengths up to 63-mer reported.¹¹⁸ Our group has used

the enzymatic method to synthesize 8- and ~40-mer PAR for identifying length-specific PAR binding proteomes (Figure 6C).¹¹⁹ These polymers were converted into probes for protein capture by ELTA with biotin-dATP and chemical acylation with a photo-cross-linker. The probes were incubated with human cell lysate, cross-linked to capture PAR readers, enriched, and analyzed with mass spectrometry. Many proteins were identified with both lengths of the probe, but some readers were only captured by the 8-mer oligomer or the 40-mer polymer. The 8-mer readers were involved in central metabolism (*e.g.*, glycolysis), while the 40-mer readers were often associated with RNA metabolism and DNA repair. These data, along with those from Kliza and colleagues,¹⁰⁰ support a hypothesis where the length of PAR regulates the timing and specificity of PARP signaling pathways.

Other proteome-based screens complement existing approaches

While cell lysates are a useful source of endogenous human proteins, they provide poor sampling of proteins that have low solubility or abundance in the cell types typically used for lysate preparation. To address this issue, Kang and colleagues used a microarray-based method that individually expresses and purifies ~17,000 human proteins from yeast and spots them onto chips for analysis.¹²⁰ The chip is then overlaid with biotin-PAR and detected with fluorescent streptavidin (Figure 6D). This method has the advantage of directly observing each interaction, and it offers a better sampling of proteins with low protein copy numbers that may be difficult to detect with mass spectrometry-based proteomics. Indeed, this array-based method uncovered several PAR-protein interaction motifs not found in other screens, including the cysteine-rich motifs CNXC and CPXC.

Although these probes have made considerable progress by identifying thousands of ADPr-binding candidates, the probes in use mostly lack the native peptide context. Even for the study by Lam and colleagues, the use of *in vitro* PARylated PARP1 may not recapitulate biologically relevant PARP1 modification states. To address these issues, future research may tap into new reagents such as peptides MARYlated at specific residues^{31,64–66} or proteins with site-specific MAR and PAR modifications.^{37,71,72} If used in pull-downs in cell extracts, these recombinant ADP-ribosylated substrates may reveal new readers that require both ADPr and the surrounding protein for recognition.

METHODS FOR MONITORING ADP-RIBOSYLATION DYNAMICS *IN VITRO* AND *IN VIVO*

Covalent substrates and their non-covalent interactions with unmodified proteins form an ADPr-centric network that regulates various cellular processes. This network can be fine-tuned by the form of ADP-ribosylation (MAR or PAR). To better understand the function of this dynamic network, researchers are developing tools including antibodies and genetically encoded reporters to detect specific forms of ADPr. Additionally, high-throughput assays have led to the discovery of small molecule inhibitors that can be used to study enzymes that control ADPr dynamics and assess their potential as therapeutic targets.

Reagents detect specific forms of ADP-ribosylation

Historically, ADP-ribosylation was studied using bulk detection methods, such as radioactively labeled precursors (*e.g.*, ATP, NMN, NAD⁺), which were used to discover PARylation in isolated nuclei.¹²¹ The sensitivity of PAR detection was further improved by boronate enrichment and chloroacetaldehyde-mediated conversion to fluorescent poly(ethenoADP-ribose), which established a link between PAR synthesis and DNA repair.¹²² Monoclonal and polyclonal antibodies against PAR, such as 10H, were developed for immunoassays (western blotting, immunofluorescence, ELISA, etc.).¹²³ However, these methods tend to bias towards PARylation. The radioactive and etheno-ADP-ribose-based methods have a linear relationship between signal and PAR length, and the 10H antibody prefers PAR. As a result, the first five decades of PARP research largely excluded mono- and oligo(ADP-ribose) from their analyses.

Engineering more specific ADPr detection reagents became possible following the discovery of protein domains that bind defined ADPr structures (Figure 5). Detection reagents based on fusing WWE or macrodomains to antibody moieties are now available for use in immunoassays.⁴⁰ Anti-ADPr antibodies were also generated with phage display of MARylated peptides.³¹ This approach created antibodies that are MAR-specific, bind both MAR and PAR, and for the first time, antibodies that recognize MARylation at specific sites. The increased specificity of these detection reagents improves the precision of experiments and, more crucially, their commercial availability makes ADP-ribosylation research accessible to the broader scientific community.

High-throughput assays monitor the activity of ADPr writer and erasers

While antibody-based methods are useful for basic biological research, they may not be suitable for drug discovery, which requires quantitative, high-throughput detection of ADPr activity. High-throughput enzymatic assays typically convert the enzymatic activity of interest into an optical signal (*e.g.*, fluorescence and luminescence) that can be easily adapted to automated drug screening platforms in 384-well formats.

Strategies for converting PARP activity to light take advantage of NAD⁺ analogs (Figure 4). In general, recombinant PARPs are expressed and immobilized on plate. Biotin-NAD⁺ is added to initiate the PARP reaction, which is then converted to a fluorescent signal using detection reagents like europium-labeled streptavidin (Figure 7A).^{124,125} However, the low auto-modification activity of some PARP family members can be an issue, which is addressed through forced proximity or an active-site displacement assay.^{126,127} Potent and selective inhibitors for PARP1/2, 7, 10, 11, and 14 are now available, which are important tools for understanding the functions of individual PARPs.¹²⁸ These inhibitors are also the foundation for new therapeutics, with PARP1/2 inhibitors achieving FDA approval and a PARP7 inhibitor currently in phase I clinical trials for cancer.^{129,130}

While PARP inhibition has been studied for over 40 years,¹²⁹ the inhibition of ADP-ribosylhydrolases, enzymes that remove ADPr, is less studied.¹³¹ There is now significant interest in discovering small-molecule inhibitors of ADPr eraser enzymes as their roles in the progression of cancer and viral infections have recently been recognized. Multiple

high-throughput assays for ADP-ribosylhydrolase activity are now available. The conversion of ADPr removal into light can be achieved through multiple routes: the enzymatic cleavage of ADP-ribose into phosphoribose and AMP, which then converts AMP to luminescence with the luciferase-based AMP-Glo (Figure 7B), chemical conversion of α -NAD⁺ into a fluorescent compound, or the use of a bespoke ADP-ribose analog that releases a fluorophore upon hydrolysis.^{64,132–136}

The most well-developed inhibitor program for ADP-ribosylhydrolases targets human PARG, and a cell-permeable compound with nanomolar potency is now commercially available.¹³⁸ This inhibitor has been used to study the function of PARG in living cells and effectively kills breast and prostate cancer cells.^{139,140} Additionally, viral macrodomains, which are conserved in some RNA virus families (e.g., *Coronaviridae*) and play a critical role in viral replication and pathogenesis, are another druggable ADPr eraser of therapeutic interest.^{141–143} Viruses expressing catalytically-deficient macrodomains are less lethal in mouse models, suggesting inhibitors of viral macrodomains could reduce the severity of infection.^{141,142,144} Several inhibitors of the SARS-CoV-2 macrodomain have recently been reported and are being evaluated as a new strategy for antiviral therapy.^{134,145,146}

Visualization of ADPr dynamics in cells

ADPr is a reversible, dynamic modification. For example, PARP1 is recruited to DNA breaks within seconds, and PAR synthesis begins immediately, reaches a maximum level within minutes before degrading within 1 hour.¹⁴⁷ The temporal changes of PAR length can be followed by isolating the polymers from other nucleic acids and separating them through electrophoresis or chromatography. In the past, the length of intact PAR chains was visualized by feeding cells radiolabeled adenine,^{20,148} but this approach may induce unintended radio-damage and trigger PARYlation. Instead, isolated PAR can be stained with nucleic acid dyes or labeled with fluorescent or radioactive moieties with ELTA.³⁴ However, ADP-ribosylation can be difficult with traditional assays, as they often involve cell lysis or fixation, and may not reveal the full repertoire of ADPr structures. To directly visualize ADPr modifications in cells, fluorescent NAD⁺ analogs can be delivered through mild permeabilization or transfection (Figure 4 and 7C).^{92,94,95} However, this method may also detect other NAD⁺-dependent processes unrelated to PARP activity.

Genetically encoded reporters, such as fluorescent proteins fused to ADPr reader domains (Figure 5 and 7C), enable the real-time monitoring ADP-ribosylation dynamics in live cells.^{149–151} PAR-specific biosensors have been developed using split-protein systems, where fluorescence only occurs when the two halves of the split fluorophore complement each other to form a fully folded structure on the same PAR molecule. Split PAR sensors were validated in a proof-of-principle experiment using cell lysate,¹⁵² and later confirmed the rapid metabolism of PARYlation following DNA damage in live cells.¹⁵³ Notably, a PAR sensor can also be used to detect *specific* PARYlated proteins by fusing one half of the sensor to the gene of interest and the other half to a PAR-binding domain. The fluorescence produced by this technique acts as a “memory” of the PARYlation event, even after the PAR has been degraded, due to the longer half-life of split fluorescent protein dimerization compared to the PARYlation event (~10 minutes).¹⁵³

Challa, Ryu, and colleagues recently improved the design of split-GFP PAR sensors by systematically analyzing different combinations of ADPr-binding domains.¹⁵⁴ They also developed a split nanoluciferase (NanoLuc) reagent with improved signal-to-noise ratio for use in tissues and animals. These sensors were used to study the PARylation dynamics in cellular differentiation and in live animals following gamma-irradiation. While the dynamic range of these sensors is relatively modest (~3–5-fold), their direct detection of PAR *in vivo* simplifies experimental design and reduces the risk of artifacts.¹⁵⁴ Together, the high spatial and temporal resolution of these sensors complements the proteomic techniques used to identify ADP-ribosylation substrates and binders, providing a more comprehensive, temporal view of PAR signaling networks in living systems.

REMAINING CHALLENGES AND FUTURE DIRECTIONS

Over the past several years, techniques for studying ADP-ribosylation have advanced rapidly and delved into the targets, binding partners, and metabolism of a once enigmatic protein modification (Table 2). Proteomics advances have illuminated a surprisingly complex view of ADP-ribosylation with thousands of protein targets and nine possible modification sites that vary depending on cell type, cellular compartment, and environmental cues. However, current techniques for identifying ADP-ribosylation sites derivatize the heterogeneous mixture of ADPr modifications into defined mass tags (Figure 3). As a result, details of the interplay between MAR and PAR and their regulation in cells remain largely uncharacterized. While techniques have been developed to preserve both the length and identity of repeating units in other complex post-translational modifications, traditional mass spectrometry is not set up for analyzing the negative charge of PAR chains due to the use of positive ion mode, which is optimized for basic tryptic peptides. One possible solution is to explore the negative ion mode, which has been successful in sequencing negatively charged glycans and glycopeptides.¹⁵⁵

With the identification of nearly 2,000 ADPr reader candidates, we are now in a position to make significant progress in understanding the non-covalent interactions between proteins and ADP-ribosylation. By determining the binding sites of these readers and studying the effects of binding-deficient mutant proteins in live cells and tissues,^{16,156} we will gain valuable insights into the biological roles of ADPr-protein interactions. Additionally, transfecting a photo-cross-linking NAD⁺ analog into a cell line expressing an analog-sensitive PARP can provide further information about the coordination of both covalent and non-covalent ADP-ribosylation events by specific PARPs. Mapping ADPr-binding sites to pathological mutations may uncover the physiological significance of these binding interfaces and potential drug-targeting opportunities.

Another revelation is the importance of protein MARYlation. MAR-specific detection reagents are now available,^{40,41} including antibodies that recognize specific MARYlation sites within histone and PARP1.³¹ Further development of MAR-specific reagents and their use in imaging will complement proteomics data by measuring the cellular dynamics of MARYlation. Emergent data also revealed that the chain length of PAR is linked to pathological states (*e.g.*, slower PAR degradation in cancerous tissue),^{147,157} highlighting the need to develop tools for monitoring changes in both the site and length of ADP-

ribosylation as clinical biomarkers. These tools could have a significant impact on patient selection and treatment, particularly in light of ongoing efforts to pursue drugs targeting the synthesis and degradation of ADP-ribosylation.

One outstanding question remaining in the field is how PAR—a homopolymer—achieves specificity with its binding partners.^{97,158} The length of PAR seems to be a crucial regulator during the initiation and elongation steps of building an ADPr-centric network. However, the three-dimensional structure of this homopolymer has remained elusive, with previous efforts only able to resolve dimeric ADP-ribose.¹⁰¹ Recent developments in synthesizing length-defined PAR and labeling both termini have enabled the use of biophysical techniques that precisely measure PAR-protein interactions with single-molecule precision.^{34,159–161} Fully synthetic PAR synthesis allows for complete control over its structure, including the ability to add defined branch points and alter chemical moieties.¹⁶² Creating chemical probes to identify branch readers and developing them into biosensors will enable us to explore the significance of this unique PAR structure. By synthesizing PAR analogs, we can also determine the importance of specific components, such as the phosphate group, base, and sugar, in binding interactions.^{83,93,95}

Lastly, exciting recent work has revealed that DNA and RNA are ADP-ribosylated in mammalian cells.^{163,164} MARYlated proteins can also be modified with ubiquitin through a noncanonical O-linkage between the C-terminal glycine of ubiquitin and the 3'-hydroxyl of ADPr.¹⁶⁵ These examples illustrate the exceptional versatility and high-energy nature of NAD⁺, which facilitates chemical reactions with diverse biomolecules that challenge our conventional definitions of post-translational modifications. The biological significance of non-canonical ADP-ribosylation in human cells is just beginning to emerge, but this new direction supports further development of detection reagents that are specific for DNA-ADPr, RNA-ADPr, or ubiquitin-ADPr. Coupling these reagents with proteomics, sequencing, and live-cell imaging will drive cutting-edge basic science efforts in the field and may reveal novel applications for ADPr in biotechnology and medicine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Krishnakumar R, & Kraus WL (2010). The PARP Side of the Nucleus: Molecular Actions, Physiological Outcomes, and Clinical Targets. *Mol. Cell*, 39, 8–24. 10.1016/j.molcel.2010.06.017 [PubMed: 20603072]
2. Pascal JM (2018). The comings and goings of PARP-1 in response to DNA damage. *DNA Repair (Amst.)* 71, 177–182. 10.1016/j.dnarep.2018.08.022 [PubMed: 30177435]

3. Schuller M & Ahel I (2022). Beyond protein modification: the rise of non-canonical ADP-ribosylation. *Biochem. J* 479, 463–477. 10.1042/BCJ20210280 [PubMed: 35175282]
4. Lüscher B, Bütepage M, Ecke L, Krieg S, Verheugd P, & Shilton BH (2018). ADP-Ribosylation, a Multifaceted Posttranslational Modification Involved in the Control of Cell Physiology in Health and Disease. *Chem. Rev* 118, 1092–1136. 10.1021/acs.chemrev.7b00122 [PubMed: 29172462]
5. Perina D, Miko A, Ahel J, etkovi H, Žaja R, & Ahel I (2014). Distribution of protein poly(ADP-ribosylation) systems across all domains of life. *DNA Repair (Amst.)*, 23, 4–16. 10.1016/j.dnarep.2014.05.003 [PubMed: 24865146]
6. Ménissier de Murcia J, Ricoul M, Tartier L, Niedergang C, Huber A, Dantzer F, Schreiber V, Amé JC, Dierich A, LeMeur M, Sabatier L, Chambon P, & de Murcia G (2003). Functional interaction between PARP-1 and PARP-2 in chromosome stability and embryonic development in mouse. *EMBO J*. 22, 2255–2263. 10.1093/emboj/cdg206 [PubMed: 12727891]
7. Chiang YJ, Hsiao SJ, Yver D, Cushman SW, Tessarollo L, Smith S, & Hodes RJ (2008). Tankyrase 1 and Tankyrase 2 Are Essential but Redundant for Mouse Embryonic Development. *PLoS One*, 3, e2639. 10.1371/journal.pone.0002639 [PubMed: 18612384]
8. Hendriks IA, Larsen SC, & Nielsen ML (2019). An Advanced Strategy for Comprehensive Profiling of ADP-ribosylation Sites Using Mass Spectrometry-based Proteomics. *Mol. Cell. Proteomics*, 18, 1010–1026. 10.1074/mcp.TIR119.001315 [PubMed: 30798302]
9. Palavalli Parsons LH, Challa S, Gibson BA, Nandu T, Stokes MS, Huang D, Lea JS, & Lee Kraus W (2021). Identification of PARP-7 substrates reveals a role for MARYlation in microtubule control in Ovarian cancer cells. *eLife*, 10, 60481. 10.7554/eLife.60481
10. Rodriguez KM, Buch-Larsen SC, Kirby IT, Siordia IR, Hutin D, Rasmussen M, Grant DM, David LL, Matthews J, Nielsen ML, & Cohen MS (2021). Chemical genetics and proteome-wide site mapping reveal cysteine MARYlation by PARP-7 on immune-relevant protein targets. *eLife*, 10, e60480. 10.7554/eLife.60480 [PubMed: 33475084]
11. Vyas S, Matic I, Uchima L, Rood J, Zaja R, Hay RT, Ahel I, & Chang P (2014). Family-wide analysis of poly(ADP-ribose) polymerase activity. *Nature Communications* 5, 4426. 10.1038/ncomms5426
12. Lüscher B, Ahel I, Altmeyer M, Ashworth A, Bai P, Chang P, Cohen M, Corda D, Dantzer F, Daugherty MD, et al. (2021). ADP-ribosyltransferases, an update on function and nomenclature. *FEBS J*. 289, 7399–7410. 10.1111/febs.16142 [PubMed: 34323016]
13. Sanderson DJ, & Cohen MS (2020). Mechanisms governing PARP expression, localization, and activity in cells. *Crit. Rev. Biochem. Mol. Biol* 55, 541–554. 10.1080/10409238.2020.1818686 [PubMed: 32962438]
14. Cambronne XA, & Kraus WL (2020). Location, Location, Location: Compartmentalization of NAD⁺ Synthesis and Functions in Mammalian Cells. *Trends Biochem. Sci* 45, 858–873. 10.1016/j.tibs.2020.05.010 [PubMed: 32595066]
15. Gibbs-Seymour I, Fontana P, Rack JGM, & Ahel I (2016). HPF1/C4orf27 Is a PARP-1-Interacting Protein that Regulates PARP-1 ADP-Ribosylation Activity. *Mol. Cell*, 62, 432–442. 10.1016/j.molcel.2016.03.008 [PubMed: 27067600]
16. Kim DS, Camacho C. v., Nagari A, Malladi VS, Challa S, & Kraus WL (2019). Activation of PARP-1 by snoRNAs Controls Ribosome Biogenesis and Cell Growth via the RNA Helicase DDX21. *Mol. Cell*, 75, 1270–1285.e14. 10.1016/j.molcel.2019.06.020 [PubMed: 31351877]
17. Huang D, Camacho C. v., Setlem R, Ryu KW, Parameswaran B, Gupta RK, & Kraus WL (2020). Functional Interplay between Histone H2B ADP-Ribosylation and Phosphorylation Controls Adipogenesis. *Mol. Cell*, 79, 934–949.e14. 10.1016/j.molcel.2020.08.002 [PubMed: 32822587]
18. Huang D, Kim DS, & Kraus WL (2020). Specific Binding of snoRNAs to PARP-1 Promotes NAD⁺-Dependent Catalytic Activation. *Biochemistry*, 59, 1559–1564. 10.1021/acs.biochem.0c00100 [PubMed: 32293172]
19. Bonfiglio JJ, Fontana P, Zhang Q, Colby T, Gibbs-Seymour I, Atanassov I, Bartlett E, Zaja R, Ahel I, & Matic I (2017). Serine ADP-Ribosylation Depends on HPF1. *Mol. Cell*, 65, 932–940.e6. 10.1016/j.molcel.2017.01.003 [PubMed: 28190768]

20. Alvarez-Gonzalez R & Jacobson MK (1987). Characterization of Polymers of Adenosine Diphosphate Ribose Generated in Vitro and in Vivo. *Biochemistry*, 26, 3218–3224. 10.1021/bi00385a042 [PubMed: 3038179]
21. Kiehlbauch CC, Aboul-Ela N, Jacobson EL, Ringer DP, & Jacobson MK (1993). High Resolution Fractionation and Characterization of ADP-Ribose Polymers. *Anal. Biochem* 208, 26–34. 10.1006/abio.1993.1004 [PubMed: 8434792]
22. Kleczkowska HE, Malanga M, Szumiel I, & Althaus FR (2002). Poly ADP-ribosylation in two L5178Y murine lymphoma sublines differentially sensitive to DNA-damaging agents. *Int. J. Radiat. Biol* 78, 527–534. 10.1080/095530002317577349 [PubMed: 12065056]
23. Malanga M, & Althaus FR (1994). Poly(ADP-ribose) molecules formed during DNA repair in vivo. *J. Biol. Chem* 269, 17691–17696. 10.1016/S0021-9258(17)32496-1 [PubMed: 8021281]
24. Miwa M, Saikawa N, Yamaizumi Z, Nishimura S, & Sugimura T (1979). Structure of poly(adenosine diphosphate ribose): identification of 2'-[1''-ribosyl-2''-(or 3''-)(1'''-ribosyl)]adenosine-5',5'',5'''-tris(phosphate) as a branch linkage. *Proc. Natl. Acad. Sci. USA* 76, 595–599. 10.1073/pnas.76.2.59 [PubMed: 218210]
25. Aebersold R, Agar JN, Amster IJ, Baker MS, Bertozzi CR, Boja ES, Costello CE, Cravatt BF, Fenselau C, Garcia BA, et al. (2018). How many human proteoforms are there? *Nat. Chem. Biol* 14, 206–214. 10.1038/nchembio.2576 [PubMed: 29443976]
26. Seydel C (2022). Diving deeper into the proteome. *Nat. Methods* 19, 1036–1040. 10.1038/s41592-022-01599-9 [PubMed: 36008631]
27. Kozłowski M, Corujo D, Hothorn M, Guberovic I, Mandemaker IK, Blessing C, Sporn J, Gutierrez-Triana A, Smith R, Portmann T, et al. (2018). MacroH2A histone variants limit chromatin plasticity through two distinct mechanisms. *EMBO Rep.* 19, e44445. 10.15252/embr.201744445 [PubMed: 30177554]
28. O'Sullivan J, Tedim Ferreira M, Gagné JP, Sharma AK, Hendzel MJ, Masson JY, & Poirier GG (2019). Emerging roles of eraser enzymes in the dynamic control of protein ADP-ribosylation. *Nat. Commun* 10, 1182. 10.1038/s41467-019-08859-x [PubMed: 30862789]
29. Koh DW, Lawler AM, Poitras MF, Sasaki M, Wattler S, Nehls MC, Stöger T, Poirier GG, Dawson VL, & Dawson TM (2004). Failure to degrade poly(ADP-ribose) causes increased sensitivity to cytotoxicity and early embryonic lethality. *Proc. Natl. Acad. Sci. USA* 101, 17699–17704. 10.1073/pnas.040618210 [PubMed: 15591342]
30. Shirai H, Poetsch AR, Gunji A, Maeda D, Fujimori H, Fujihara H, Yoshida T, Ogino H, & Masutani M (2013). PARG dysfunction enhances DNA double strand break formation in S-phase after alkylation DNA damage and augments different cell death pathways. *Cell Death Dis.* 4, e656. 10.1038/cddis.2013.133 [PubMed: 23744356]
31. Bonfiglio JJ, Leidecker O, Dauben H, Longarini EJ, Colby T, San Segundo-Acosta P, Perez KA, & Matic I (2020). An HPF1/PARP1-Based Chemical Biology Strategy for Exploring ADP-Ribosylation. *Cell*, 183, 1086–1102.e23. 10.1016/j.cell.2020.09.055 [PubMed: 33186521]
32. Hendriks IA, Buch-Larsen SC, Prokhorova E, Elsborg JD, Rebak AKLFS, Zhu K, Ahel D, Lukas C, Ahel I, & Nielsen ML (2021). The regulatory landscape of the human HPF1- and ARH3-dependent ADP-ribosylome. *Nat. Commun* 12, 5893. 10.1038/s41467-021-26172-4 [PubMed: 34625544]
33. Challa S, Khulpateea BR, Nandu T, Camacho C. v., Ryu KW, Chen H, Peng Y, Lea JS, & Kraus WL (2021). Ribosome ADP-ribosylation inhibits translation and maintains proteostasis in cancers. *Cell*, 184, 4531–4546.e26. 10.1016/j.cell.2021.07.005 [PubMed: 34314702]
34. Ando Y, Elkayam E, McPherson RL, Dasovich M, Cheng S-J, Voorneveld J, Filippov D. v, Ong S-E, Joshua-Tor L, & Leung AKL (2019). ELTA: Enzymatic Labeling of Terminal ADP-Ribose. *Mol. Cell*, 73, 845–856.e5. 10.1016/j.molcel.2018.12.022 [PubMed: 30712989]
35. Langelier M-F, Billur R, Sverzhinsky A, Black BE, & Pascal JM (2021). HPF1 dynamically controls the PARP1/2 balance between initiating and elongating ADP-ribose modifications. *Nat. Commun* 12, 6675. 10.1038/s41467-021-27043-8 [PubMed: 34795260]
36. Rudolph J, Roberts G, Muthurajan UM, & Luger K (2021). HPF1 and nucleosomes mediate a dramatic switch in activity of PARP1 from polymerase to hydrolase. *eLife*, 10, e65773. 10.7554/eLife.65773 [PubMed: 33683197]

37. Mohapatra J, Tashiro K, Beckner RL, Sierra J, Kilgore JA, Williams NS, & Liszczak G (2021). Serine ADP-ribosylation marks nucleosomes for ALC1-dependent chromatin remodeling. *eLife*, 10, e71502. 10.7554/eLife.71502 [PubMed: 34874266]
38. Suskiewicz MJ, Zobel F, Ogden TEH, Fontana P, Ariza A, Yang JC, Zhu K, Bracken L, Hawthorne WJ, Ahel D, et al. (2020). HPF1 completes the PARP active site for DNA damage-induced ADP-ribosylation. *Nature* 579, 598–602. 10.1038/s41586-020-2013-6 [PubMed: 32028527]
39. Prokhorova E, Agnew T, Wondisford AR, Tellier M, Kaminski N, Beijer D, Holder J, Gros Lambert J, Suskiewicz MJ, Zhu K, et al. (2021). Unrestrained poly-ADP-ribosylation provides insights into chromatin regulation and human disease. *Mol. Cell*, 81, 2640–2655.e8. 10.1016/j.molcel.2021.04.028 [PubMed: 34019811]
40. Gibson BA, Conrad LB, Huang D, & Kraus WL (2017). Generation and Characterization of Recombinant Antibody-like ADP-Ribose Binding Proteins. *Biochemistry*, 56, 6305–6316. 10.1021/acs.biochem.7b00670 [PubMed: 29053245]
41. Weixler L, Ikenga NJ, Voorneveld J, Aydin G, Mhr Bolte T, Momoh J, Bütepage M, Golzmann A, Lüscher B, Filippov D. v, et al. (2023). Protein and RNA ADP-ribosylation detection is influenced by sample preparation and reagents used. *Life Sci. Alliance*, 6, e202201455. 10.26508/lsa.202201455 [PubMed: 36368907]
42. Chunaram C, Chanchal K, Florian G, Nielsen LM, Michael R, Walther CT, Jesper VO, & Matthias M (2009). Lysine Acetylation Targets Protein Complexes and Co-Regulates Major Cellular Functions. *Science*, 325, 834–840. 10.1126/science.1175371 [PubMed: 19608861]
43. Ochoa D, Jarnuczak AF, Viéitez C, Gehre M, Soucheray M, Mateus A, Kleefeldt AA, Hill A, Garcia-Alonso L, Stein F, et al. (2020). The functional landscape of the human phosphoproteome. *Nat. Biotechnol* 38, 365–373. 10.1038/s41587-019-0344-3 [PubMed: 31819260]
44. Zhang Y, Wang J, Ding M, & Yu Y (2013). Site-specific characterization of the Asp- and Glu-ADP-ribosylated proteome. *Nat. Methods*, 10, 981–984. 10.1038/nmeth.2603 [PubMed: 23955771]
45. Zhen Y, Zhang Y, & Yu Y (2017). A Cell-Line-Specific Atlas of PARP-Mediated Protein Asp/Glu-ADP-Ribosylation in Breast Cancer. *Cell Rep*. 21, 2326–2337. 10.1016/j.celrep.2017.10.106 [PubMed: 29166620]
46. Karras GI, Kustatscher G, Buhecha HR, Allen MD, Pugieux C, Sait F, Bycroft M, & Ladurner AG (2005). The macro domain is an ADP-ribose binding module. *EMBO J*. 24, 1911–1920. 10.1038/sj.emboj.7600664 [PubMed: 15902274]
47. Buch-Larsen SC, Hendriks IA, Lodge JM, Rykær M, Furtwängler B, Shishkova E, Westphall MS, Coon JJ, & Nielsen ML (2020). Mapping Physiological ADP-Ribosylation Using Activated Ion Electron Transfer Dissociation. *Cell Rep*. 32, 108176. 10.1016/j.celrep.2020.108176 [PubMed: 32966781]
48. Higashi H, Maejima T, Lee LH, Yamazaki Y, Hottiger MO, Singh SA, & Aikawa M (2019). A Study into the ADP-Ribosylome of IFN-I-Stimulated THP-1 Human Macrophage-like Cells Identifies ARTD8/PARP14 and ARTD9/PARP9 ADP-Ribosylation. *J. Proteome Res* 18, 1607–1622. 10.1021/acs.jproteome.8b00895 [PubMed: 30848916]
49. Martello R, Leutert M, Jungmichel S, Bilan V, Larsen SC, Young C, Hottiger MO, & Nielsen ML (2016). Proteome-wide identification of the endogenous ADP-ribosylome of mammalian cells and tissue. *Nat. Commun* 7, 12917. 10.1038/ncomms12917 [PubMed: 27686526]
50. Nowak K, Rosenthal F, Karlberg T, Bütepage M, Thorsell AG, Dreier B, Grossmann J, Sobek J, Imhof R, Lüscher B, et al. (2020). Engineering Af1521 improves ADP-ribose binding and identification of ADP-ribosylated proteins. *Nat. Commun* 11, 5199. 10.1038/s41467-020-18981-w [PubMed: 33060572]
51. Jankevicius G, Hassler M, Golia B, Rybin V, Zacharias M, Timinszky G, & Ladurner AG (2013). A family of macrodomain proteins reverses cellular mono-ADP-ribosylation. *Nat. Struct. Mol. Biol* 20, 508–514. 10.1038/nsmb.2523 [PubMed: 23474712]
52. Donovan J, Dufner M, & Korennykh A (2013). Structural basis for cytosolic double-stranded RNA surveillance by human oligoadenylate synthetase I. *Proc. Natl. Acad. Sci. USA*, 110, 1652–1657. 10.1073/pnas.1218528110 [PubMed: 23319625]

53. Cayley PJ, & Kerr IM (1982). Synthesis, Characterisation and Biological Significance of (2'-5')Oligoadenylate Derivatives of NAD⁺, ADP-Ribose and Adenosine(5')Tetraphospho(5')Adenosine. *Eur. J. Biochem* 122, 601–608. 10.1111/j.1432-1033.1982.tb06481.x [PubMed: 6277637]
54. McPherson RL, Ong S-E, & Leung AKL, (2019). Ion-Pairing with Triethylammonium Acetate Improves Solid-Phase Extraction of ADP-Ribosylated Peptides. *J. Proteome Res* 19, 984–990. 10.1021/acs.jproteome.9b00696
55. Gagné JP, Langelier MF, Pascal JM, & Poirier GG (2018). Hydrofluoric Acid-Based Derivatization Strategy to Profile PARP-1 ADP-Ribosylation by LC-MS/MS. *J. Proteome Res* 17, 2542–2551. 10.1021/acs.jproteome.8b00146 [PubMed: 29812941]
56. Daniels CM, Ong SE, & Leung AKL (2014). Phosphoproteomic approach to characterize protein mono- and poly(ADP-ribosylation) sites from cells. *J. Proteome Res* 13, 3510–3522. 10.1021/pr401032q [PubMed: 24920161]
57. Daniels CM, Thirawatananond P, Ong SE, Gabelli SB, & Leung AKL (2015). Nudix hydrolases degrade protein-conjugated ADP-ribose. *Sci. Rep* 5, 18271. 10.1038/srep18271 [PubMed: 26669448]
58. Palazzo L, Thomas B, Jemth AS, Colby T, Leidecker O, Feijs KLH, Zaja R, Loseva O, Vert JC, Matic I, Helleday T, & Ahel I (2015). Processing of protein ADP-ribosylation by Nudix hydrolases. *Biochem. J* 468, 293–301. 10.1042/BJ20141554 [PubMed: 25789582]
59. Palazzo L, Daniels CM, Nettleship JE, Rahman N, McPherson RL, Ong SE, Kato K, Nureki O, Leung AKL, & Ahel I (2016). ENPP1 processes protein ADP-ribosylation *in vitro*. *FEBS J.* 283, 3371–3388. 10.1111/febs.13811 [PubMed: 27406238]
60. Daniels CM, Kaplan PR, Bishof I, Bradfield C, Tucholski T, Nuccio AG, Manes NP, Katz S, Fraser IDC, & Nita-Lazar A (2020). Dynamic ADP-Ribosylome, Phosphoproteome, and Interactome in LPS-Activated Macrophages. *J. Proteome Res* 19, 3716–3731. 10.1021/acs.jproteome.0c00261 [PubMed: 32529831]
61. Leidecker O, Bonfiglio JJ, Colby T, Zhang Q, Atanassov I, Zaja R, Palazzo L, Stockum A, Ahel I, & Matic I (2016). Serine is a new target residue for endogenous ADP-ribosylation on histones. *Nat. Chem. Biol* 12, 998–1000. 10.1038/nchembio.2180 [PubMed: 27723750]
62. Ogata N, Ueda K, Kagamiyama H, & Hayaishi O (1980). ADP-ribosylation of histone H1. Identification of glutamic acid residues 2, 14, and the COOH-terminal lysine residue as modification sites. *J. Biol. Chem* 255, 7616–7620. 10.1016/S0021-9258(19)43873-8 [PubMed: 6772638]
63. Tao Z, Gao P, & Liu HW (2009). Identification of the ADP-ribosylation sites in the PARP-1 automodification domain: Analysis and implications. *J. Am. Chem. Soc* 131, 14258–14260. 10.1021/ja906135d [PubMed: 19764761]
64. Voorneveld J, Rack JGM, Ahel I, Overkleeft HS, van der Marel GA, & Filippov DV (2018). Synthetic α - And β -Ser-ADP-ribosylated Peptides Reveal α -Ser-ADPr as the Native Epimer. *Org. Lett* 20, 4140–4143. 10.1021/acs.orglett.8b01742 [PubMed: 29947522]
65. Voorneveld J, Rack JGM, van Gijlswijk L, Meeuwenoord NJ, Liu Q, Overkleeft HS, van der Marel GA, Ahel I, & Filippov DV (2021). Molecular Tools for the Study of ADP-Ribosylation: A Unified and Versatile Method to Synthesise Native MonoADP-Ribosylated Peptides. *Chemistry*, 27, 10621–10627. 10.1002/chem.202100337 [PubMed: 33769608]
66. Voorneveld J, Kloet MS, Wijngaarden S, Kim RQ, Moutsopoulos A, Verdegaal M, Misra M, iki I, van der Marel GA, Overkleeft HS, et al. (2022). Arginine ADP-Ribosylation: Chemical Synthesis of Post-Translationally Modified Ubiquitin Proteins. *J. Am. Chem. Soc* 144, 20582–20589. 10.1021/jacs.2c06249 [PubMed: 36318515]
67. Palazzo L, Leidecker O, Prokhorova E, Dauben H, Matic I, & Ahel I (2018). Serine is the major residue for ADP-ribosylation upon DNA damage. *eLife*, 7.e34334. 10.7554/eLife.34334 [PubMed: 29480802]
68. Gibson BA, Zhang Y, Jiang H, Hussey KM, Shrimp JH, Lin H, Schwede F, Yu Y, & Kraus WL (2016). Chemical genetic discovery of PARP targets reveals a role for PARP-1 in transcription elongation. *Science*, 353, 45–50. 10.1126/science.aaf7865 [PubMed: 27256882]

69. Gupte R, Nandu T, & Kraus WL (2021). Nuclear ADP-ribosylation drives IFN γ -dependent STAT1 α enhancer formation in macrophages. *Nat. Commun* 12, 3931. 10.1038/s41467-021-24225-2 [PubMed: 34168143]
70. Jones A, & Kraus WL (2022). Multiomics analysis of the NAD⁺-PARP1 axis reveals a role for site-specific ADP-ribosylation in splicing in embryonic stem cells. *Genes Dev.* 36, 601–617. 10.1101/gad.349335.121 [PubMed: 35654456]
71. Tashiro K, Mohapatra J, Brautigam CA, & Liszczak G (2022). A Protein Semisynthesis-Based Strategy to Investigate the Functional Impact of Linker Histone Serine ADP-Ribosylation. *ACS Chem. Biol* 17, 810–815. 10.1021/acscchembio.2c00091 [PubMed: 35312285]
72. Hananya N, Daley SK, Bagert JD, & Muir TW (2021). Synthesis of ADP-Ribosylated Histones Reveals Site-Specific Impacts on Chromatin Structure and Function. *Journal of the American Chemical Society*, 143, 10847–10852. 10.1021/jacs.1c05429 [PubMed: 34264659]
73. Bartlett E, Bonfiglio JJ, Prokhorova E, Colby T, Zobel F, Ahel I, & Matic I (2018). Interplay of Histone Marks with Serine ADP-Ribosylation. *Cell Rep.* 24, 3488–3502.e5. 10.1016/j.celrep.2018.08.092 [PubMed: 30257210]
74. Liszczak G, Diehl KL, Dann GP, & Muir TW (2018). Acetylation blocks DNA damage-induced chromatin ADP-ribosylation. *Nat. Chem. Biol* 14, 837–840. 10.1038/s41589-018-0097-1 [PubMed: 30013063]
75. Prokhorova E, Zobel F, Smith R, Zentout S, Gibbs-Seymour I, Schützenhofer K, Peters A, Gros Lambert J, Zorzini V, Agnew T, et al. (2021). Serine-linked PARP1 auto-modification controls PARP inhibitor response. *Nat. Commun* 12, 4055. 10.1038/s41467-021-24361-9 [PubMed: 34210965]
76. Yang CS, Jividen K, Kamata T, Dworak N, Oostdyk L, Remlein B, Pourfarjam Y, Kim IK, Du KP, Abbas T, Sherman NE, Wotton D, & Paschal BM (2021). Androgen signaling uses a writer and a reader of ADP-ribosylation to regulate protein complex assembly. *Nat. Commun* 12, 2705. 10.1038/s41467-021-23055-6 [PubMed: 33976187]
77. Leutert M, Menzel S, Braren R, Rissiek B, Hopp AK, Nowak K, Bisceglie L, Gehrig P, Li H, Zolkiewska A, et al. (2018). Proteomic Characterization of the Heart and Skeletal Muscle Reveals Widespread Arginine ADP-Ribosylation by the ARTC1 Ecto-enzyme. *Cell Rep.* 24, 1916–1929.e5. 10.1016/j.celrep.2018.07.048 [PubMed: 30110646]
78. Feijs KL, Kleine H, Braczynski A, Forst AH, Herzog N, Verheugd P, Linzen U, Kremmer E, & Lüscher B (2013). ARTD10 substrate identification on protein microarrays: Regulation of GSK3 β by mono-ADP-ribosylation. *Cell Commun. Signal* 11, 5. 10.1186/1478-811x-11-5 [PubMed: 23332125]
79. Troiani S, Lupi R, Perego R, Re Depaolini S, Thieffine S, Bosotti R, & Rusconi L (2011). Identification of candidate substrates for poly(ADP-ribose) polymerase-2 (PARP2) in the absence of DNA damage using high-density protein microarrays. *FEBS J.* 278, 3676–3687. 10.1111/j.1742-4658.2011.08286.x [PubMed: 21812934]
80. Depaix A, & Kowalska J (2019). NAD Analogs in Aid of Chemical Biology and Medicinal Chemistry. *Molecules* 24, 4187. 10.3390/molecules24224187 [PubMed: 31752261]
81. Rodriguez KM, & Cohen MS (2021). Chemical genetic methodologies for identifying protein substrates of PARPs. *Trends Biochem Sci.* 47, 390–402. 10.1016/j.tibs.2021.07.002 [PubMed: 34366182]
82. Bauer PI, Hakam A, & Kun E (1986). Mechanisms of poly(ADP-ribose) polymerase catalysis; mono-ADP-ribosylation of poly(ADP-ribose) polymerase at nanomolar concentrations of NAD. *FEBS Lett.* 195, 331–338. 10.1016/0014-5793(86)80188-0 [PubMed: 2935422]
83. Oei SL, Griesenbeck J, Buchlow G, Jorcke D, Mayer-Kuckuk P, Wons T, & Ziegler M (1996). NAD⁺ analogs substituted in the purine base as substrates for poly(ADP-ribosyl) transferase. *FEBS Lett.* 397, 17–21. 10.1016/s0014-5793(96)01137-4 [PubMed: 8941705]
84. Meldal M, & Tomøe CW (2008). Cu-catalyzed azide-alkyne cycloaddition. *Chemical Reviews*, 108, 2952–3015. 10.1021/cr0783479 [PubMed: 18698735]
85. Jiang H, Kim JH, Frizzell KM, Kraus WL, & Lin H (2010). Clickable NAD analogues for labeling substrate proteins of poly(ADP-ribose) polymerases. *J. Am. Chem. Soc* 132, 9363–9372. 10.1021/ja101588r [PubMed: 20560583]

86. Islam K (2018). The Bump-and-Hole Tactic: Expanding the Scope of Chemical Genetics. *Cell Chemical Biology*, 25, 1171–1184. 10.1016/j.chembiol.2018.07.001 [PubMed: 30078633]
87. Carter-O’Connell I, Jin H, Morgan RK, David LL, & Cohen MS, (2014). Engineering the Substrate Specificity of ADP-Ribosyltransferases for Identifying Direct Protein Targets. *J. Am. Chem. Soc* 136, 5201–5204. 10.1021/ja412897a [PubMed: 24641686]
88. Carter-O’Connell I, Jin H, Morgan RK, Zaja R, David LL, Ahel I, & Cohen MS (2016). Identifying Family-Member-Specific Targets of Mono-ARTDs by Using a Chemical Genetics Approach. *Cell Rep.* 14, 621–631. 10.1016/j.celrep.2015.12.045 [PubMed: 26774478]
89. Carter-O’Connell I, Vermehren-Schmaedick A, Jin H, Morgan RK, David LL, & Cohen MS (2018). Combining Chemical Genetics with Proximity-Dependent Labeling Reveals Cellular Targets of Poly(ADP-ribose) Polymerase 14 (PARP14). *ACS Chem. Biol* 13, 2841–2848. 10.1021/acscchembio.8b00567 [PubMed: 30247868]
90. Langelier MF, Zandarashvili L, Aguiar PM, Black BE, & Pascal JM (2018). NAD⁺ analog reveals PARP-1 substrate-blocking mechanism and allosteric communication from catalytic center to DNA-binding domains. *Nat. Commun* 9, 844. 10.1038/s41467-018-03234-8 [PubMed: 29487285]
91. Wallrodt S, Buntz A, Wang Y, Zumbusch A, & Marx A (2016). Bioorthogonally Functionalized NAD⁺ Analogues for In-Cell Visualization of Poly(ADP-Ribose) Formation. *Angew. Chem. Int. Ed. Engl* 55, 7660–7664. 10.1002/anie.201600464 [PubMed: 27080423]
92. Wallrodt S, Simpson EL, & Marx A (2017). Investigation of the action of poly(ADP-ribose)-synthesising enzymes on NAD⁺ analogues. *Beilstein J. Org. Chem* 13, 495–501. 10.3762/bjoc.13.49 [PubMed: 28382184]
93. Lam AT, Zhang X-N, Courouble VV, Strutzenberg TS, Pei H, Stiles BL, Louie SG, Griffin PR, & Zhang Y (2021). A Bifunctional NAD⁺ for Profiling Poly-ADP-Ribosylation-Dependent Interacting Proteins. *ACS Chem. Biol* 16, 389–396. 10.1021/acscchembio.0c00937 [PubMed: 33524253]
94. Lehner M, Rieth S, Höllmüller E, Spliesgar D, Mertes B, Stengel F, & Marx A (2022). Profiling of the ADP-Ribosylome in Living Cells. *Angew. Chem. Int. Ed. Engl* 61, e202200977. 10.1002/anie.202200977 [PubMed: 35188710]
95. Zhang X-N, Cheng Q, Chen J, Lam AT, Lu Y, Dai Z, Pei H, Evdokimov NM, Louie SG, & Zhang Y (2019). A ribose-functionalized NAD⁺ with unexpected high activity and selectivity for protein poly-ADP-ribosylation. *Nat. Commun* 10, 4196. 10.1038/s41467-019-12215-4 [PubMed: 31519936]
96. Zhang XN, Lam AT, Cheng Q, Courouble V. v., Strutzenberg TS, Li J, Wang Y, Pei H, Stiles BL, Louie SG, Griffin PR, & Zhang Y (2022). Discovery of an NAD⁺ analogue with enhanced specificity for PARP1. *Chem. Sci* 13, 1982–1991. 10.1039/d1sc06256e [PubMed: 35308855]
97. Leung AKL (2020). Poly(ADP-ribose): A Dynamic Trigger for Biomolecular Condensate Formation. *Trends Cell Biol.* 30, 370–383. 10.1016/j.tcb.2020.02.002 [PubMed: 32302549]
98. Chaudhuri AR, & Nussenzweig A (2017). The multifaceted roles of PARP1 in DNA repair and chromatin remodelling. *Nat. Rev. Mol. Cell Biol* 18, 610–621. 10.1038/nrm.2017.53 [PubMed: 28676700]
99. DaRosa PA, Wang Z, Jiang X, Pruneda JN, Cong F, Klevit RE, & Xu W (2014). Allosteric activation of the RNF146 ubiquitin ligase by a poly(ADP-ribosylation) signal. *Nature*, 517, 223–226. 10.1038/nature13826 [PubMed: 25327252]
100. Kliza KW, Liu Q, Roosenboom LWM, Jansen PWTC, Filippov DV, & Vermeulen M (2021). Reading ADP-ribosylation signaling using chemical biology and interaction proteomics. *Mol. Cell*, 81, 4552–4567.e8. 10.1016/j.molcel.2021.08.037 [PubMed: 34551281]
101. Barkauskaite E, Brassington A, Tan ES, Warwicker J, Dunstan MS, Banos B, Lafite P, Ahel M, Mitchison TJ, Ahel I et al. (2013). Visualization of poly(ADP-ribose) bound to PARG reveals inherent balance between exo- and endo-glycohydrolase activities. *Nat. Commun* 4, 2164. 10.1038/ncomms3164 [PubMed: 23917065]
102. Forst AH, Karlberg T, Herzog N, Thorsell AG, Gross A, Feijs KLH, Verheugd P, Kursula P, Nijmeijer B, Kremmer E, et al. (2013). Recognition of Mono-ADP-Ribosylated ARTD10 Substrates by ARTD8 Macrodomains. *Structure*, 21, 462–475. 10.1016/j.str.2012.12.019 [PubMed: 23473667]

103. Rosenthal F, Feijs KLH, Frugier E, Bonalli M, Forst AH, Imhof R, Winkler HC, Fischer D, Cafilisch A, Hassa PO, et al. (2013). Macrod domain-containing proteins are new mono-ADP-ribosylhydrolases. *Nat. Struct. Mol. Biol* 20, 502–507. 10.1038/nsmb.2521 [PubMed: 23474714]
104. Rack JGM, Perina D, & Ahel I (2016). Macrod domains: Structure, Function, Evolution, and Catalytic Activities. *Annu. Rev. Biochem* 85, 431–454. 10.1146/annurev-biochem-060815-014935 [PubMed: 26844395]
105. Wang Z, Michaud GA, Cheng Z, Zhang Y, Hinds TR, Fan E, Cong F, & Xu W (2012). Recognition of the iso-ADP-ribose moiety in poly(ADP-ribose) by WWE domains suggests a general mechanism for poly(ADP-ribosyl)ation-dependent ubiquitination. *Genes Dev.* 26, 235–240. 10.1101/gad.182618.111 [PubMed: 22267412]
106. Kuttiyatveetil JRA, Soufari H, Dasovich M, Uribe IR, Mirhasan M, Cheng SJ, Leung AKL, & Pascal JM (2022). Crystal structures and functional analysis of the ZnF5-WWE1-WWE2 region of PARP13/ZAP define a distinctive mode of engaging poly(ADP-ribose). *Cell Rep.* 41, 111529. 10.1016/j.celrep.2022.111529 [PubMed: 36288691]
107. Eustermann S, Brockmann C, Mehrotra PV, Yang JC, Loakes D, West SC, Ahel I, & Neuhaus D (2010). Solution structures of the two PBZ domains from human APLF and their interaction with poly(ADP-ribose). *Nat. Struct. Mol. Biol* 17, 241–243. 10.1038/nsmb.1747 [PubMed: 20098424]
108. Chen Q, Kassab MA, Dantzer F, & Yu X (2018). PARP2 mediates branched poly ADP-ribosylation in response to DNA damage. *Nat. Commun* 9, 3233. 10.1038/s41467-018-05588-5 [PubMed: 30104678]
109. Singh HR, Nardoza AP, Möller IR, Knobloch G, Kistemaker HAV, Hassler M, Harrer N, Blessing C, Eustermann S, Kotthoff C, et al. (2017). A Poly-ADP-Ribose Trigger Releases the Auto-Inhibition of a Chromatin Remodeling Oncogene. *Mol. Cell*, 68, 860–871.e7. 10.1016/j.molcel.2017.11.019 [PubMed: 29220653]
110. Pleschke JM, Kleczkowska HE, Strohm M, & Althaus FR (2000). Poly(ADP-ribose) Binds to specific domains in DNA damage checkpoint proteins. *J. Biol. Chem* 275, 40974–40980. 10.1074/jbc.m006520200 [PubMed: 11016934]
111. Gagné JP, Hunter JM, Labrecque B, Chabot B, & Poirier GG (2003). A proteomic approach to the identification of heterogeneous nuclear ribonucleoproteins as a new family of poly(ADP-ribose)-binding proteins. *Biochem. J* 371, 331–340. 10.1042/bj20021675 [PubMed: 12517304]
112. Gagné JP, Isabelle M, Lo KS, Bourassa S, Hendzel MJ, Dawson VL, Dawson TM, & Poirier GG (2008). Proteome-wide identification of poly(ADP-ribose) binding proteins and poly(ADP-ribose)-associated protein complexes. *Nucleic Acids Res.* 36, 6959–6976. 10.1093/nar/gkn771 [PubMed: 18981049]
113. Gagné JP, Pic É, Isabelle M, Krietsch J, Éthier C, Paquet É, Kelly I, Boutin M, Moon KM, Foster LJ, et al. (2012). Quantitative proteomics profiling of the poly(ADP-ribose)-related response to genotoxic stress. *Nucleic Acids Res.* 40, 7788–7805. 10.1093/nar/gks486 [PubMed: 22669911]
114. Cohen MS (2021). Catching mono- and poly-ADP-ribose readers with synthetic ADP-ribose baits. *Mol. Cell*, 81, 4351–4353. 10.1016/j.molcel.2021.10.016 [PubMed: 34739826]
115. Kistemaker H. A. v., Lameijer LN, Meeuwenoord NJ, Overkleeft HS, van der Marel GA, & Filippov DV (2015). Synthesis of Well-Defined Adenosine Diphosphate Ribose Oligomers. *Angew. Chem. Int. Ed. Engl* 127, 4997–5000. 10.1002/anie.201412283
116. Liu Q, Knobloch G, Voorneveld J, Meeuwenoord NJ, Overkleeft HS, van der Marel GA, Ladurner AG, & Filippov DV (2021). Chemical synthesis of linear ADP-ribose oligomers up to pentamer and their binding to the oncogenic helicase ALC1. *Chem. Sci* 12, 12468–12475. 10.1039/d1sc02340c [PubMed: 34603678]
117. Tan ES, Krukenberg KA, & Mitchison TJ (2012). Large-scale preparation and characterization of poly(ADP-ribose) and defined length polymers. *Anal. Biochem* 428, 126–136. 10.1016/j.ab.2012.06.015 [PubMed: 22743307]
118. Fahrer J, Kranaster R, Altmeyer M, Marx A, & Bürkle A (2007). Quantitative analysis of the binding affinity of poly(ADP-ribose) to specific binding proteins as a function of chain length. *Nucleic Acids Res.* 35, e143. 10.1093/nar/gkm944 [PubMed: 17991682]

119. Dasovich M, Beckett MQ, Bailey S, Ong S-E, Greenberg MM, & Leung AKL, (2021a). Identifying Poly(ADP-ribose)-Binding Proteins with Photoaffinity-Based Proteomics. *J. Am. Chem. Soc* 143, 3037–3042. 10.1021/jacs.0c12246 [PubMed: 33596067]
120. Kang BG, Kang S-U, Kim JJ, Kwon J-S, Gagné J-P, Lee SY, Kim S, Sangwon KL, Ha S, Jeong JS, et al. (2022). Proteome-wide microarray-based screening of PAR-binding proteins. *BioRxiv*, 2022.06.06.494829. 10.1101/2022.06.06.494829
121. Chambon P, Weill JD, Doly J, Strosser MT, & Mandel P (1966). On the formation of a novel adenylic compound by enzymatic extracts of liver nuclei. *Biochem. Biophys. Res. Commun* 25, 638–643. 10.1016/0006-291X(66)90502-X
122. Juarez-Salinas H, Sims JL, & Jacobson MK (1979). Poly(ADP-ribose) levels in carcinogen-treated cells. *Nature*, 282, 740–741. 10.1038/282740a0 [PubMed: 229416]
123. Kawamitsu H, Hoshino HO, Okada H, Miwa M, Sugimura T, & Momoi H (1984). Monoclonal Antibodies to Poly(adenosine diphosphate ribose) Recognize Different Structures. *Biochemistry*, 23, 3771–3777. 10.1021/bi00311a032 [PubMed: 6206890]
124. Kirby IT, Sanderson DJ, & Cohen MS (2021). PASTA: PARP activity screening and inhibitor testing assay. *STAR Protoc.* 2, 100344. 10.1016/j.xpro.2021.100344 [PubMed: 33665624]
125. Wigle TJ, Church WD, Majer CR, Swinger KK, Aybar D, Schenkel LB, Vasbinder MM, Brendes A, Beck C, Prahm M, et al. (2020). Forced Self-Modification Assays as a Strategy to Screen MonoPARP Enzymes. *SLAS Discov.* 25, 241–252. 10.1177/2472555219883623 [PubMed: 31855104]
126. Wigle TJ, Blackwell DJ, Schenkel LB, Ren Y, Church WD, Desai HJ, Swinger KK, Santospage AG, Majer CR, Lu AZ, Niepel M, Perl NR, Vasbinder MM, Keilhack H, & Kuntz KW (2020). In Vitro and Cellular Probes to Study PARP Enzyme Target Engagement. *Cell Chem. Biol* 27, 877–887.e14. 10.1016/j.chembiol.2020.06.009 [PubMed: 32679093]
127. Galera-Prat A, Alaviuhkola J, Alanen HI, & Lehtiö L (2022). Protein engineering approach to enhance activity assays of mono-ADP-ribosyltransferases through proximity. *Protein Eng. Des. Sel* 35, gzac006. 10.1093/protein/gzac006
128. Nizi MG, Maksimainen MM, Lehtiö L, & Tabarrini O (2022). Medicinal Chemistry Perspective on Targeting Mono-ADP-Ribosylating PARPs with Small Molecules. *J. Med. Chem* 65, 7532–7560. 10.1021/acs.jmedchem.2c00281 [PubMed: 35608571]
129. Curtin NJ, & Szabo C (2020). Poly(ADP-ribose) polymerase inhibition: past, present and future. *Nat. Rev. Drug Discov* 19, 711–736. 10.1038/s41573-020-0076-6 [PubMed: 32884152]
130. Gozgit JM, Vasbinder MM, Abo RP, Kunii K, Kuplast-Barr KG, Gui B, Lu AZ, Molina JR, Minissale E, Swinger KK, et al. (2021). PARP7 negatively regulates the type I interferon response in cancer cells and its inhibition triggers antitumor immunity. *Cancer Cell*, 39, 1214–1226.e10. 10.1016/j.ccell.2021.06.018 [PubMed: 34375612]
131. Glumoff T, Sowa ST, & Lehtiö L (2022). Assay technologies facilitating drug discovery for ADP-ribosyl writers, readers and erasers. *BioEssays*, 44, 2100240. 10.1002/bies.202100240
132. Drown BS, Shirai T, Rack JGM, Ahel I, & Hergenrother PJ (2018). Monitoring Poly(ADP-ribosyl)glycohydrolase Activity with a Continuous Fluorescent Substrate. *Cell Chem. Biol* 25, 1562–1570.e19. 10.1016/j.chembiol.2018.09.008 [PubMed: 30318463]
133. Kasson S, Dharmapriya N, & Kim IK (2021). Selective monitoring of the protein-free ADP-ribose released by ADP-ribosylation reversal enzymes. *PLoS One*, 16, e0254022. 10.1371/journal.pone.0254022 [PubMed: 34191856]
134. Dasovich M, Zhuo J, Goodman JA, Thomas A, McPherson RL, Jayabalan AK, Busa VF, Cheng SJ, Murphy BA, Redinger KR, et al. (2022). High-Throughput Activity Assay for Screening Inhibitors of the SARS-CoV-2 Mac1 Macromodain. *ACS Chem. Biol* 17, 17–23. 10.1021/acscchembio.1c00721 [PubMed: 34904435]
135. Wazir S, Maksimainen MM, Alanen HI, Galera-Prat A, & Lehtiö L (2021). Activity-Based Screening Assay for Mono-ADP-Ribosylhydrolases. *SLAS Discov.* 26, 67–76. 10.1177/2472555220928911 [PubMed: 32527186]
136. Rack JGM, & Ahel I (2023). A Simple Method to Study ADP-Ribosylation Reversal: From Function to Drug Discovery. *Methods Mol. Biol* 2609, 111–132. 10.1007/978-1-0716-2891-1_8 [PubMed: 36515833]

137. Lin C-Y, Romei MG, Oltrogge LM, Mathews II, & Boxer SG, (2019). Unified Model for Photophysical and Electro-Optical Properties of Green Fluorescent Proteins. *J. Am. Chem. Soc* 141, 15250–15265. 10.1021/jacs.9b07152 [PubMed: 31450887]
138. James DI, Smith KM, Jordan AM, Fairweather EE, Griffiths LA, Hamilton NS, Hitchin JR, Hutton CP, Jones S, Kelly P, et al. (2016). First-in-class chemical probes against poly(ADP-ribose) glycohydrolase (PARG) inhibit DNA repair with differential pharmacology to olaparib. *ACS Chem. Biol* 11, 3179–3190. 10.1021/acscchembio.6b00609 [PubMed: 27689388]
139. Houll JH, Ye Z, Brosey CA, Balapiti-Modarage LPF, Namjoshi S, Bacolla A, Laverty D, Walker BL, Pourfarjam Y, Warden LS, et al. (2019). Selective small molecule PARG inhibitor causes replication fork stalling and cancer cell death. *Nat. Commun* 10, 5654. 10.1038/s41467-019-13508-4 [PubMed: 31827085]
140. Waszkowycz B, Smith KM, McGonagle AE, Jordan AM, Acton B, Fairweather EE, Griffiths LA, Hamilton NM, Hamilton NS, Hitchin JR, et al. (2018). Cell-Active Small Molecule Inhibitors of the DNA-Damage Repair Enzyme Poly(ADP-ribose) Glycohydrolase (PARG): Discovery and Optimization of Orally Bioavailable Quinazolinone Sulfonamides. *J. Med. Chem* 61, 10767–10792. 10.1021/acs.jmedchem.8b01407 [PubMed: 30403352]
141. Fehr AR, Channappanavar R, Jankevicius G, Fett C, Zhao J, Athmer J, Meyerholz DK, Ahel I, & Perlman S (2016). The conserved coronavirus macrodomain promotes virulence and suppresses the innate immune response during severe acute respiratory syndrome coronavirus infection. *mBio*, 7, e01721–16. 10.1128/mbio.01721-16 [PubMed: 27965448]
142. McPherson RL, Abraham R, Sreekumar E, Ong SE, Cheng SJ, Baxter VK, Kistemaker HAV, Filippov DV, Griffin DE, & Leung AKL (2017). ADP-ribosylhydrolase activity of Chikungunya virus macrodomain is critical for virus replication and virulence. *Proc. Natl. Acad. Sci. USA*, 114, 1666–1671. 10.1073/pnas.1621485114 [PubMed: 28143925]
143. Leung AKL, Griffin DE, Bosch J, Fehr AR, & Feinstone WH (2022). The Conserved Macrodomain Is a Potential Therapeutic Target for Coronaviruses and Alphaviruses. *Pathogens*, 11, 94. 10.3390/pathogens11010094 [PubMed: 35056042]
144. Abraham R, Hauer D, McPherson RL, Utt A, Kirby IT, Cohen MS, Merits A, Leung AKL, & Griffin DE (2018). ADP-ribosyl-binding and hydrolase activities of the alphavirus nsP3 macrodomain are critical for initiation of virus replication. *Proc. Natl. Acad. Sci. USA*, 115, E10457–E10466. 10.1073/pnas.1812130115 [PubMed: 30322911]
145. Roy A, Alhammad YM, McDonald P, Johnson DK, Zhuo J, Wazir S, Ferraris D, Lehtiö L, Leung AKL, & Fehr AR (2022). Discovery of compounds that inhibit SARS-CoV-2 Mac1-ADP-ribose binding by high-throughput screening. *Antiviral Res.* 203, 105344. 10.1016/j.antiviral.2022.105344 [PubMed: 35598780]
146. Gahbauer S, Correy GJ, Schuller M, Ferla MP, Doruk YU, Rachman M, Wu T, Diolaiti M, Wang S, Neitz RJ, et al. (2023). Iterative computational design and crystallographic screening identifies potent inhibitors targeting the Nsp3 macrodomain of SARS-CoV-2. *Proc. Natl. Acad. Sci. USA*, 120, e2212931120. 10.1073/pnas.2212931120
147. Martello R, Mangerich A, Sass S, Dedon PC, & Bürkle A (2013). Quantification of cellular poly(ADP-ribosylation) by stable isotope dilution mass spectrometry reveals tissue- and drug-dependent stress response dynamics. *ACS Chem. Biol* 8, 1567–1575. 10.1021/cb400170b [PubMed: 23631432]
148. Aboul-Ela N, Jacobson EL, & Jacobson MK (1988). Labeling methods for the study of poly- and mono(ADP-ribose) metabolism in cultured cells. *Anal. Biochem* 174, 239–250. 10.1016/0003-2697(88)90541-6 [PubMed: 3218735]
149. Timinszky G, Till S, Hassa PO, Hothorn M, Kustatscher G, Nijmeijer B, Colombelli J, Altmeyer M, Stelzer EHK, Scheffzek K, et al. (2009). A macrodomain-containing histone rearranges chromatin upon sensing PARP1 activation. *Nat. Struct. Mol. Biol* 16, 923–929. 10.1038/nsmb.1664 [PubMed: 19680243]
150. Aguilera-Gomez A, van Oorschot MM, Veenendaal T, & Rabouille C (2016). In vivo visualization of mono-ADP-ribosylation by dPARP16 upon amino-acid starvation. *eLife*, 5, e21475. 10.7554/elife.21475 [PubMed: 27874829]
151. Koczor CA, Saville KM, Andrews JF, Clark J, Fang Q, Li J, Al-Rahahleh RQ, Ibrahim M, McClellan S, Makarov M. v., et al. (2021). Temporal dynamics of base excision/single-strand

break repair protein complex assembly/disassembly are modulated by the PARP/NAD⁺/SIRT6 axis. *Cell Rep.* 37, 109917. [PubMed: 34731617]

152. Furman JL, Mok PW, Shen S, Stains CI, & Ghosh I (2010). A turn-on split-luciferase sensor for the direct detection of poly(ADP-ribose) as a marker for DNA repair and cell death. *Chem. Commun* 47, 397–399. 10.1039/c0cc02229b
153. Krastev DB, Pettitt SJ, Campbell J, Song F, Tanos BE, Stoyanov SS, Ashworth A, & Lord CJ (2018). Coupling bimolecular PARylation biosensors with genetic screens to identify PARylation targets. *Nat. Commun* 9, 2016. 10.1038/s41467-018-04466-4 [PubMed: 29789535]
154. Challa S, Ryu KW, Whitaker AL, Abshier JC, Camacho CV, & Kraus WL (2022). Development and characterization of new tools for detecting poly(ADP-ribose) in vitro and in vivo. *eLife*, 11, e72464. 10.7554/elife.72464 [PubMed: 35476036]
155. Kuo C-W, & Khoo K-H (2020). Strategic Applications of Negative-Mode LC-MS/MS Analyses to Expedite Confident Mass Spectrometry-Based Identification of Multiple Glycosylated Peptides. *Anal. Chem* 92, 7612–7620. 10.1021/acs.analchem.0c00236 [PubMed: 32384234]
156. Fu H, Liu R, Jia Z, Li R, Zhu F, Zhu W, Shao Y, Jin Y, Xue Y, Huang J, et al. (2022). Poly(ADP-ribosylation) of P-TEFb by PARP1 disrupts phase separation to inhibit global transcription after DNA damage. *Nat. Cell Biol* 24, 513–525. 10.1038/s41556-022-00872-5 [PubMed: 35393539]
157. Haince JF, McDonald D, Rodrigue A, Déry U, Masson JY, Hendzel MJ, & Poirier GG (2008). PARP1-dependent kinetics of recruitment of MRE11 and NBS1 proteins to multiple DNA damage sites. *J. Biol. Chem* 283, 1197–1208. 10.1074/jbc.m706734200 [PubMed: 18025084]
158. Reber JM, & Mangerich A (2021). Why structure and chain length matter: on the biological significance underlying the structural heterogeneity of poly(ADP-ribose). *Nucleic Acid Res.* 49, 8432–8448. 10.1093/nar/gkab618 [PubMed: 34302489]
159. Krüger A, Stier A, Fischbach A, Bürkle A, Hauser K, & Mangerich A (2019). Interactions of p53 with poly(ADP-ribose) and DNA induce distinct changes in protein structure as revealed by ATR-FTIR spectroscopy. *Nucleic Acids Res.* 47, 4843–4858. 10.1093/nar/gkz175 [PubMed: 30892621]
160. Abraham R, McPherson RL, Dasovich M, Badiie M, Leung AKL, & Griffin DE (2020). Both ADP-ribosyl-binding and hydrolase activities of the alphavirus nsp3 macrodomain affect neurovirulence in mice. *mBio*, 11, e03253–19. 10.1128/mbio.03253-19 [PubMed: 32047134]
161. Rhine K, Dasovich M, Yoniles J, Badiie M, Skanchy S, Ganser LR, Ge Y, Fare CM, Shorter J, Leung AKL, & Myong S (2022). Poly(ADP-ribose) drives condensation of FUS via a transient interaction. *Mol. Cell*, 82, 969–985.e11. 10.1016/j.molcel.2022.01.018 [PubMed: 35182479]
162. Liu Q, Kistemaker HAV, Overkleeft HS, van der Marel GA, & Filippov DV (2017). Synthesis of ribosyl-ribosyl-adenosine-5',5'',5'''(triphosphate)-the naturally occurring branched fragment of poly(ADP ribose). *Chemical Commun.* 53, 10255–10258. 10.1039/c7cc05755e
163. Musheev MU, Schomacher L, Basu A, Han D, Krebs L, Scholz C & Niehrs C (2022). Mammalian N1-adenosine PARylation is a reversible DNA modification. *Nat. Commun* 13, 6138. 10.1038/s41467-022-33731-w [PubMed: 36253381]
164. Weixler L, Feijs KLH, & Zaja R (2022). ADP-ribosylation of RNA in mammalian cells is mediated by TRPT1 and multiple PARPs. *Nucleic Acids Res.* 50, 9426–9441. 10.1093/nar/gkac711 [PubMed: 36018800]
165. Zhu K, Suskiewicz MJ, Hloušek-Kasun A, Meudal H, Miko A, Aucagne V, Ahel D, & Ahel I (2022). DELTEX E3 ligases ubiquitylate ADP-ribosyl modification on protein substrates. *Sci. Adv* 8, 4253. 10.1126/sciadv.add4253

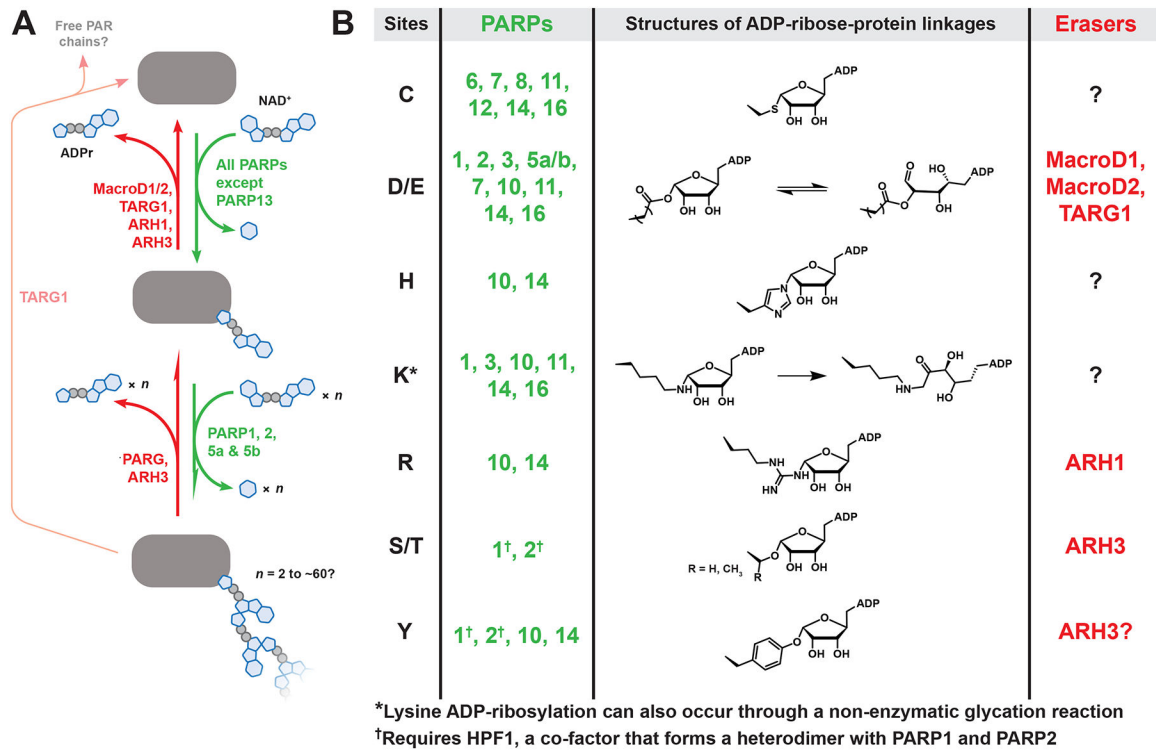


Figure 1. The chemical diversity of human PARP metabolism.

(A) PAR metabolism involves four steps: 1) transfer of a single ADP-ribose from NAD^+ to the target protein, resulting in mono-ADP-ribose (MAR). 2) Further ADPr transfers onto MAR, producing linear and branched poly(ADP-ribose) (PAR). 3) PAR degradation, which is primarily carried out by poly(ADP-ribose) glycohydrolase (PARG). Notably, human PARG does not remove the final ADP-ribose attached to the protein, thereby converting PAR to MAR. 4) Cleavage of the ADP-ribose-protein bond by various “eraser” enzymes, each with a preference for specific functional groups. Note: ARH3 can also degrade PAR, although it is less efficient than PARG and unable to remove branch points. (B) The chemical diversity of ADPr-protein bonds synthesized by human PARPs, with known site preferences listed. See Data S1 for related references.

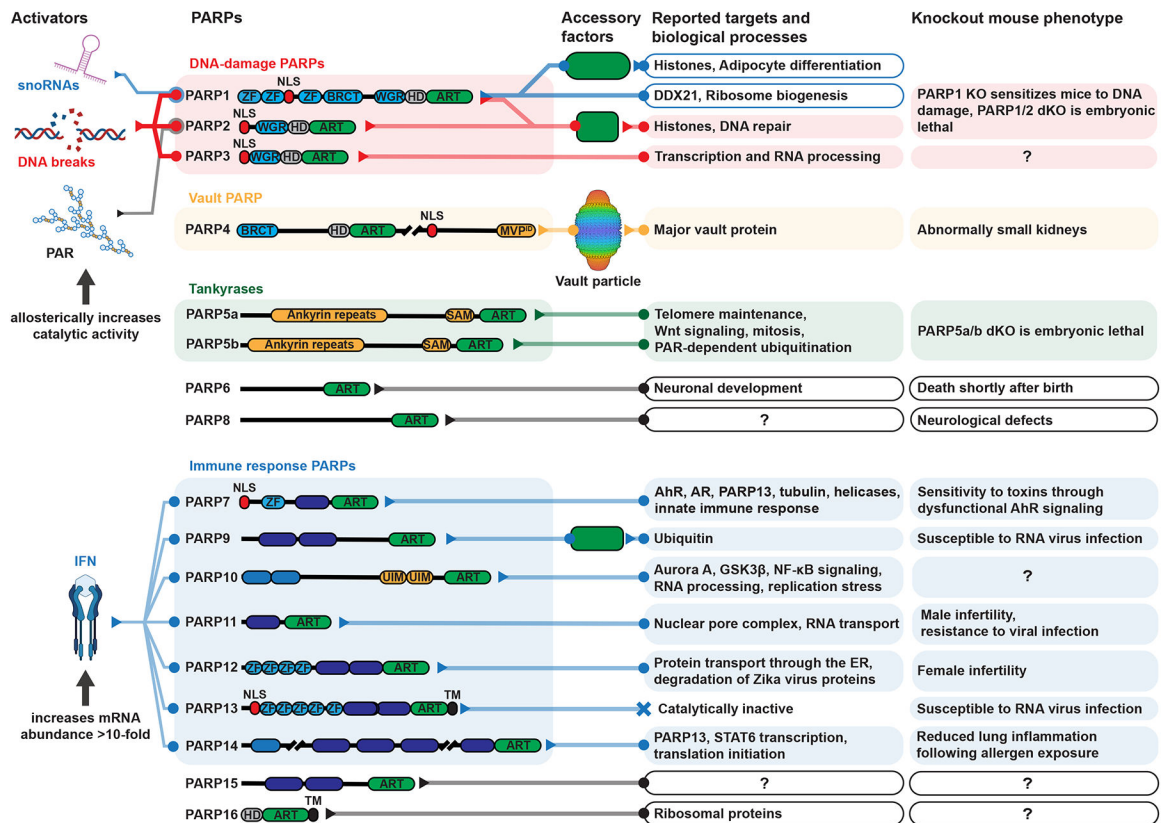


Figure 2. An overview of the PARP family, including domains, targets, and phenotypes.

Domain abbreviations: ZF = zinc finger, NLS = nuclear localization signal, BRCT = BRCA1 C-terminus, WGR = the three most conserved amino acids in this DNA-binding domain, HD = helical domain, ART = ADP-ribosyltransferase, MVP^{ID} = major vault protein interacting domain, SAM = sterile alpha motif, WWE = the three most conserved amino acids in this PAR-binding domain, Macro = macrodomain, RRM = RNA recognition motif, UIM = ubiquitin interacting motif, TM = transmembrane. See Data S1 for related references.

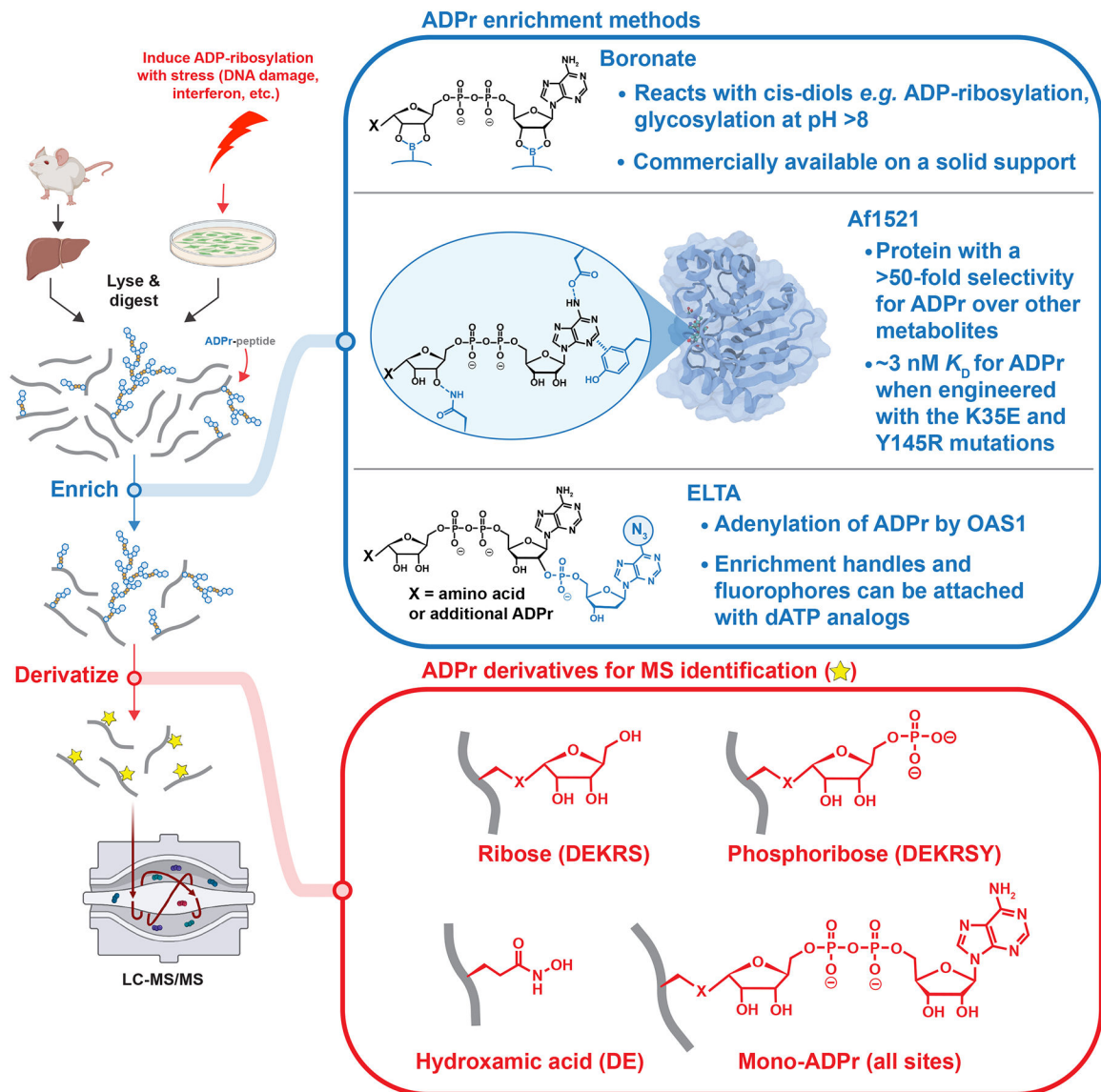


Figure 3. Key steps in ADP-ribosylomics workflows.

Following protein extraction from cultured cells or tissues and tryptic digestion, ADP-ribosylated peptides are enriched with a variety of methods (blue). Enriched peptides contain heterogeneous ADP-ribose modifications (*i.e.*, MAR and PAR), which are then derivatized to a single, homogenous mass (red) to simplify the interpretation of mass spectra. For each derivative, the amino acids searched in published experiments are in parentheses, though ribose and phosphoribose could be on any amino acid. The structure of Af1521 is from reference 50.

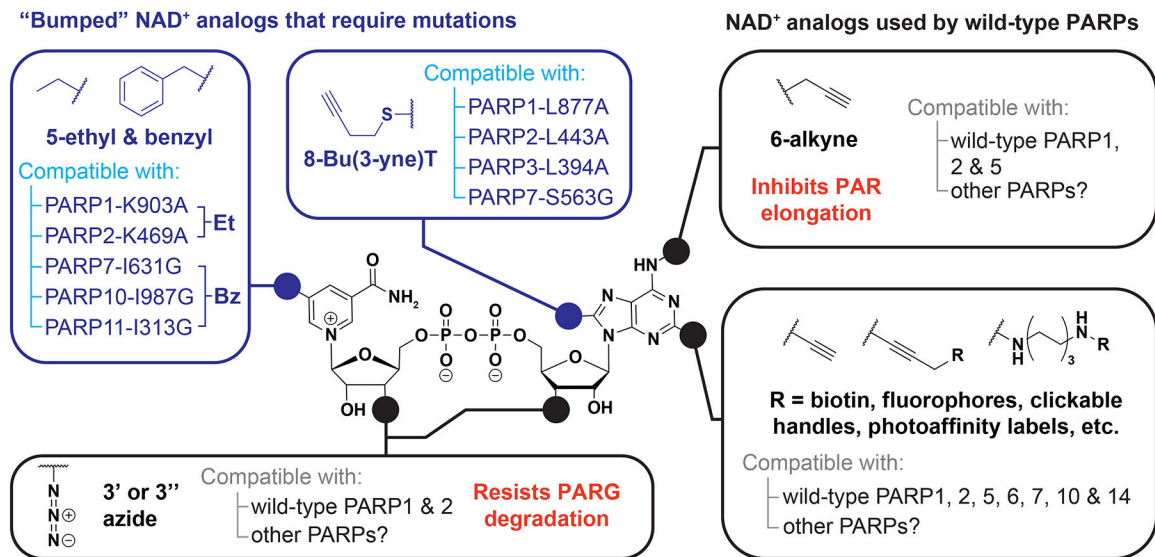


Figure 4. NAD⁺ analogs used to study ADP-ribosylation.

Analogues with substitutions to the N⁶, C², and 3'-OH positions can be used with wild-type PARP proteins, whereas substitutions to the C⁵ and C⁸ positions require mutations in the NAD⁺-binding pocket to confer analog sensitivity.

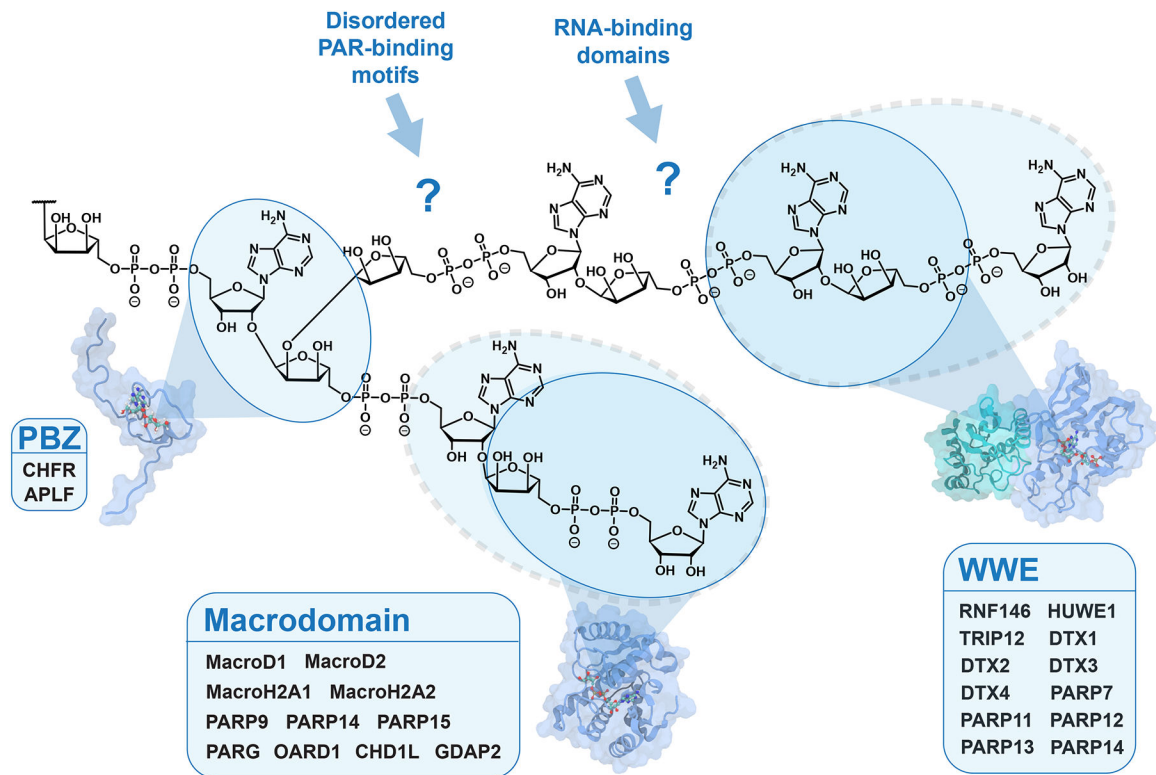


Figure 5. Protein domains that bind to ADP-ribosylated molecules.

The PAR-binding zinc finger (PBZ), WWE domain, and macrodomain are well-defined folds that recognize specific forms of ADP-ribosylation. The blue circles indicate the binding preferences for each domain based on structural data, with dashed circles indicating variations in binding preference within the family. For instance, most macrodomains bind ADP-ribose, MARylated proteins, and/or the terminal ADPr of PAR, except for CHD1L (ALC1), which specifically binds PAR.¹⁰⁹ Several RNA-binding domains and intrinsically disordered PAR-binding motifs are reported to bind PAR, but how these domains engage PAR is unclear due to a lack of structural data. The structures of Af1521, RNF146 RING-WWE, and APLF PBZ are from references 50, 99, and 107, respectively.

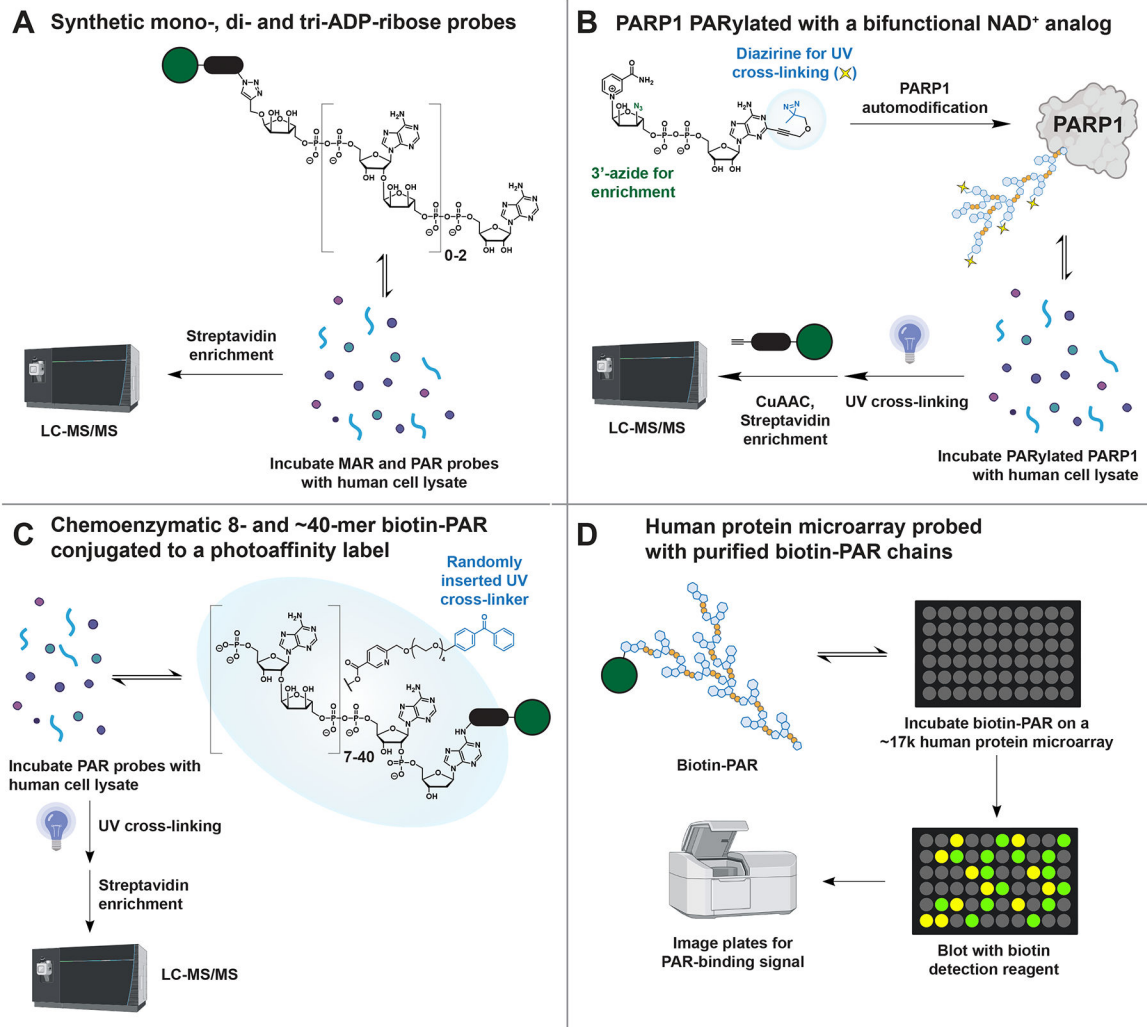


Figure 6. Techniques for identifying MAR and PAR readers with chemically and enzymatically synthesized probes.

(A) Kliza *et al.*,¹⁰⁰ (B) Lam *et al.*,⁹³ (C) Dasovich *et al.*,¹¹⁹ and (D) Kang *et al.*,¹²⁰

have developed complementary methods that together have identified 1,918 MAR- and PAR-binding candidate proteins.

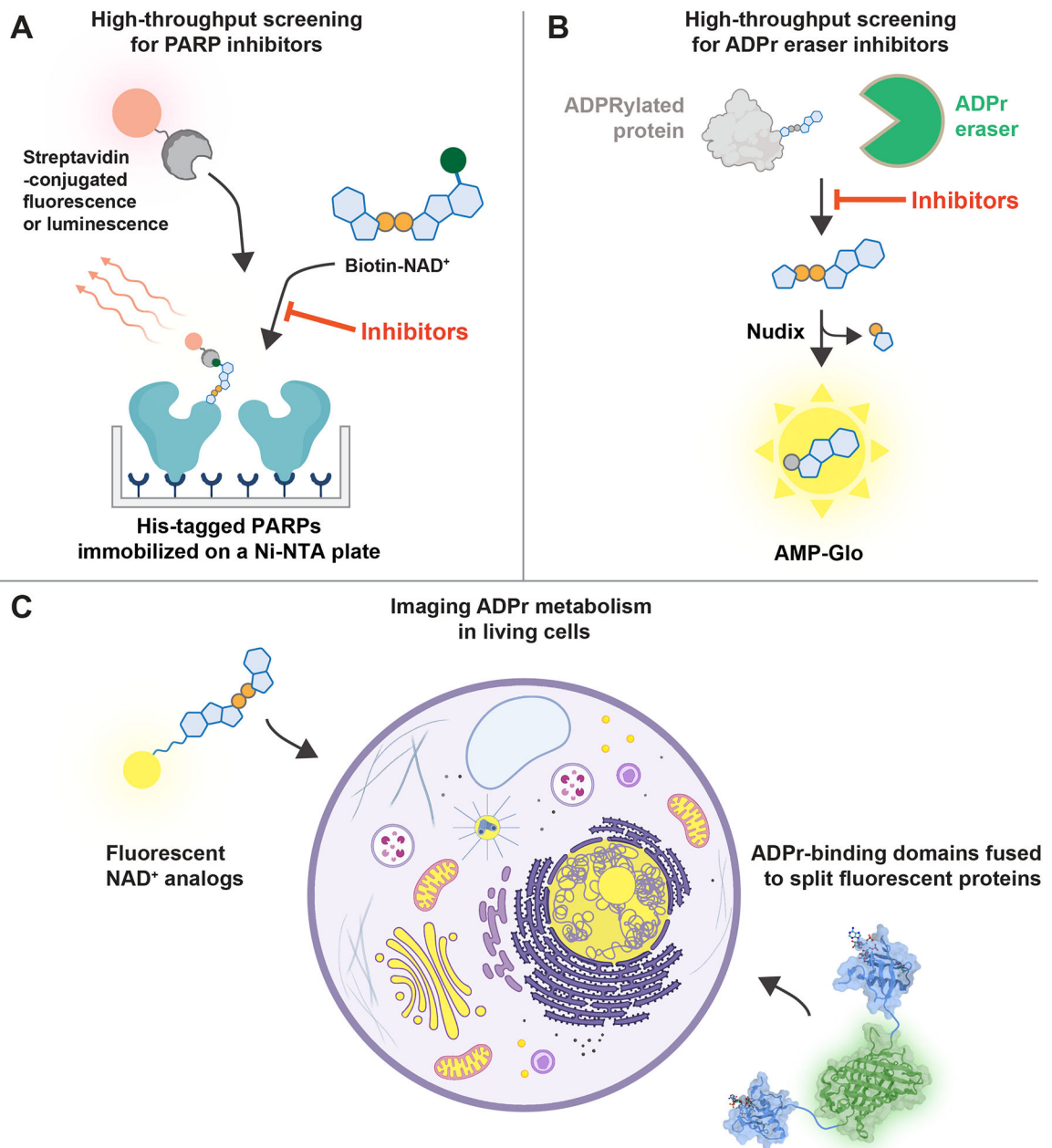


Figure 7. Methods for visualizing ADPr *in vitro* and *in vivo*.

(A) A high-throughput ADP-ribosylation assay identifies PARP inhibitors by measuring the number of biotin-ADPr modifications. (B) A high-throughput ADP-ribosylhydrolase assay identifies inhibitors by converting hydrolyzed ADP-ribose into luminescence. (C) Small molecule and genetically encoded methods for imaging ADP-ribosylation dynamics in living cells. The structures of RNF146 WWE and split GFP are from references 105 and 137, respectively.

Table 1 |

Functional characterization of ADP-ribosylation sites identified with proteomics

	Protein	Site(s)	Biological significance	Evidence	Ref. #
Aspartate & Glutamate	NELF-E	E121 E151 E152 E171 E172 E374	The negative elongation factor complex (NELF) inhibits transcription elongation by RNA polymerase II. PARP1 ADP-ribosylated NELF-E, which disrupted the complex and increased transcription at select promoters.	<i>In vitro</i> ADP-ribosylation assay, EMSA, ChIP-seq	68
	DDX21	E13 D15 E43 E67 E196	DDX21 is a nucleolar RNA helicase that localizes to rRNA loci and promotes rRNA transcription. PARP1 ADP-ribosylated DDX21, which disrupted DDX21-RNA interactions and prevented DDX21 from localizing to rRNA loci. This reduced rRNA levels, protein translation, and cell growth.	<i>In vitro</i> ADP-ribosylation assay, IP, RIP-qPCR, EMSA, ChIP-qPCR, RNA Bioanalyzer, puromycilation assay, cell proliferation assay	16
	Histone H2B	E35	PARP1 ADP-ribosylated Histone H2B at E35 in preadipocytes, which prevented differentiation of adipocytes by blocking AMPK-mediated phosphorylation of H2BS36.	<i>In vitro</i> ADP-ribosylation and phosphorylation assay, IP, RNA-seq, ChIP-qPCR, Oil Red O staining	18
	RPL24 RPS6	E4 E35	RPL24 & RPS6 are ribosome components at the interface of the 60S and 40S subunits. PARP16 ADP-ribosylated RPL24 & RPS6, which prevented ribosome assembly and reduced translation. This promoted cell survival by limiting the toxicity associated with excessive protein synthesis.	IP, IF, puromycilation assay, ribosome profiling, cell proliferation assay	33
	Tubulin	D69 E71 E79	PARP7 ADP-ribosylated α -tubulin, which contributed to microtubule depolymerization.	IP, IF	9
	STAT1 α	E393 E394 D721	STAT1 α is a key transcriptional regulator of pro-inflammatory gene expression mediated by interferon γ . PARP1 ADP-ribosylated the DNA-binding and transactivation domains of STAT1 α , both of which increased interferon-regulated gene expression by promoting STAT1 α interactions with proteins and DNA. STAT1 α ADP-ribosylation drove pro-inflammatory phenotypes in macrophages, increasing their ability to phagocytose bacteria.	IP, ChIP-seq, RT-qPCR, RNA-seq, phagocytosis assay, Seahorse assay, oligonucleotide-binding assay	69
	U2AF35 U2AF65	E162 E425	U2 snRNP auxiliary factor (U2AF) is an essential splicing factor that recognizes splice sites and initiates ribosome assembly. PARP1 ADP-ribosylated U2AF, which altered U2AF-RNA interactions and regulated RNA splicing events in stem cells.	<i>In vitro</i> ADP-ribosylation assay, IP, EMSA, splicing assay	70
Serine	Histone H1	S150 S188	The PARP1/HPF1 complex ADP-ribosylated histone H1 during the DNA damage response. ADP-ribosylation did not directly affect H1-DNA interactions but did decrease chromatin compaction.	<i>In vitro</i> ADP-ribosylation assay, IP, sedimentation assay, Native PAGE	32, 71
	Histone H2B	S6	The PARP1/HPF1 complex ADP-ribosylated histone H2B during the DNA damage response. H2B ADP-ribosylation reduced chromatin compaction and primed H2B for recognition and remodeling by the ADP-binding chromatin remodeler ALC1.	Sedimentation assay, fluorescence polarization, IP, nucleosome remodeling assay	37, 72
	Histone H3	S10	The PARP1/HPF1 complex ADP-ribosylated histone H3 during the DNA damage response. H3 ADP-ribosylation reduced chromatin compaction and primed H3 for recognition and remodeling by the ADPr-binding chromatin remodeler ALC1. ADP-ribosylation of H3S10 also blocked acetylation or methylation of H3K9. Conversely, acetylation or methylation of H3K9, phosphorylation of H3T11, and acetylation of H3K14 block H3S10ADPr.	<i>In vitro</i> ADP-ribosylation assay, western blotting, IP, sedimentation assay, fluorescence polarization, nucleosome remodeling assay, <i>in vitro</i> acetylation and methylation assays	19, 37, 72-74
	PARP1	S499 S507 S519	The PARP1/HPF1 complex auto-ADP-ribosylated PARP1 during the DNA damage response. ADP-ribosylation of PARP1 at these three serines promoted the dissociation of PARP1 from DNA breaks and decreases the toxicity of	Western blotting, live-cell microscopy, cell survival assay	32, 75

	Protein	Site(s)	Biological significance	Evidence	Ref. #
			PARP1 inhibitors by reducing the amount of PARP1 trapped on DNA.		
Cysteine	AR	C125 C131 C284 C290 C327 C406 C519 C596 C602 C620 C670	PARP7 ADP-ribosylated the androgen receptor (AR), which promoted interactions with the ADPr-binding PARP9/DTX3L complex and modulated androgen-dependent gene expression.	Western blotting, AR-dependent luciferase reporter assay	76
Arginine	HPX	R218	Hemopexin (HPX) is a heme-binding protein that transports heme to the liver for recycling and iron recovery. HPX is ADP-ribosylated by the ARTC1 ecto-ADP-ribosyltransferase. <i>In vitro</i> , HPX ADP-ribosylation by ARTC1 inhibited HPX-heme binding.	<i>In vitro</i> ADP-ribosylation assay, heme-binding assay	77

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Table 2 |

Overview of techniques used to study ADP-ribosylation

Technique	Summary	Advantages	Current challenges & future directions
PARP targets and sites	Boronate + hydroxylamine Enrich ADP-ribosylated peptides from cell lysates with boronate, then derivatize with hydroxylamine.	May improve the depth of ADPr detection at aspartate and glutamate. Identifies sites from living cells.	Only detects ADPr at aspartate and glutamate, and may be biased towards the detection of PARylation. Novel derivatization methods may allow identification of ADPr sites other than those at the acidic residues.
	PARG + Afl521 Trim ADP-ribosylated peptides to mono-ADPr with PARG, then enrich with Afl521.	Detects ADPr on all sites. Identifies sites from living cells and tissues.	Thousands of sites have been mapped, but only a handful have been functionally characterized. Most of the experiments involving ADPr site mapping have been done in cancer cell lines under genotoxic stress, indicating the need for further research in cells exposed to other stresses and specific mouse tissues.
	ELTA-MS Functionalize ADP-ribosylated peptides by labeling them with a clickable handle using oligoadenylate synthase I. Pull-down labeled peptides and derivatize them with phosphodiesterase.	Detects ADPr on all sites. Identifies sites from living cells.	ELTA may label other adenine-containing molecules in cells, thereby requiring the derivation for ADPr site identification. Novel derivatization methods that preserve the structure may allow for the site-specific characterization of different forms of ADP-ribosylation.
	Analog-sensitive PARPs Combine a “bumped” NAD ⁺ analog with a mutated PARP that has a “hole” in the binding pocket. ADP-ribosylated proteins are then enriched with an affinity tag on the analog.	Identifies proteins targeted by a specific PARG.	The membrane impermeability of NAD ⁺ analogs has limited experiments to cell lysates and intact nuclei. Recently reported NAD ⁺ transfection reagents may enable to use of bumped NAD ⁺ analogs in living cells.
ADPr readers	Synthetic ADPr probes Prepare ADP-ribosylated molecules with defined structures, then use them as baits to capture ADPr-binding proteins. Photo-cross-linking NAD⁺ analogs Synthesize photo-cross-linking NAD ⁺ , use it to modify PARPs in vitro, then use it as bait to capture binding proteins.	Can measure whether binding proteins prefer specific ADPr structures. The ADPr probe is protein conjugated.	The membrane impermeability of ADP-ribosylated molecules has limited experiments to cell lysates. Future experiments should focus on identifying the ADPr binding sites for mutagenesis to better understand their physiological roles in cells.
ADPr metabolism	ADPr biosensors Genetically encode ADPr-binding domains fused to fluorescent proteins and express in cells or tissues of interest.	Measures the highly dynamic, reversible nature of ADPr in living cells and animals.	While biosensors with PAR-binding domains have been largely successful, biosensors with MAR-binding domains have had limited success in cells. This may be due to off-target binding to free ADP-ribose and NAD ⁺ . The use of next-generation MAR detection reagents that specifically bind protein MARylation, without affinity for free ADP-ribose, may allow for the imaging of cellular MAR dynamics.