Commentary

Identifying ADP-ribosylation targets by chemical genetics

Aswin Mangerich[1], Matthias Altmeyer[2]

[1]Molecular Toxicology Group, Department of Biology, University of Konstanz, Germany; [2]Department of Molecular Mechanisms of Disease, University of Zurich, Zurich, Switzerland

Correspondence to: Aswin Mangerich. Molecular Toxicology Group, Department of Biology, University of Konstanz, Germany. Email: aswin.mangerich@uni-konstanz.de; Matthias Altmeyer. Department of Molecular Mechanisms of Disease, University of Zurich, Zurich, Switzerland. Email: matthias.altmeyer@uzh.ch.

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A posttranslational protein modification with increasing relevance for cancer biology is poly(ADP-ribosyl)ation (PARylation) (1). Inhibitors of PARylation show promising effects either as monotherapeutic agents or as chemo- or radiosensitizers to support classical DNA damaging cancer therapies. The targets of these inhibitors are enzymes of the family of poly(ADP-ribose) polymerases (PARPs, also known as ARTDs) (2). PARP inhibitors are analogs of NAD^+, which PARP enzymes use as substrate to synthesize poly(ADP-ribose) (PAR). PAR can be covalently attached to or interact non-covalently with target proteins (3,4). In humans, 17 genes encode for PARP/ARTD enzymes, of which 5 can generate PAR (PARP1/2/4 and TNKS1/2), while all others either catalyze mono-ADP-ribosylation (MARylation) or appear to be inactive. PARPs localize to various cellular compartments and participate in a multitude of cancer-relevant cellular functions, such as DNA damage responses, transcription, chromatin organization and regulation of cell death (1). As revealed by a series of recent mass spectrometry-based proteomics studies, these cellular functions correlate well with the PARylated proteome, which includes hundreds of proteins involved in DNA and RNA metabolism, stress responses and cell death pathways (5-9). Interestingly, PARP inhibitors are exquisitely toxic to cancer cells with defects in a DNA damage repair pathway called homologous recombination (10). This is the case for BRCA-deficient tumors, and both FDA and EMA have recently approved the PARP inhibitor olaparib for the treatment of such cancers. While various additional clinical trials with different PARP inhibitors are currently ongoing, an important unsolved issue is the polypharmacology associated with PARP inhibition, since so far truly homolog-specific PARP inhibitors do not exist (11). This has also hindered the identification of PARP homolog-specific ADP-ribosylation targets on a proteome-wide scale.

In a recent study, Gibson *et al.* developed an elegant chemical genetics approach to identify enzyme-specific ADP-ribosylation targets, preparing the ground for understanding their cellular functions and contributions to disease (12). They achieved this by exchanging large, so called 'gatekeeper' amino acids, which are buried in the catalytic centers of PARP/ARTD proteins, to generate mutant enzymes that are able to use non-natural, chemically modified NAD^+ analogs as substrates. First, by combinatorial screening of 20 PARP1 mutants versus 11 non-natural NAD^+ analogs, two amino acid exchanges in the PARP1 catalytic center were identified that allow usage of the bulky NAD^+ analog 8-butylthio-NAD^+. This substrate was then additional modified with an alkyne moiety resulting in 8-Bu(3-yene)T-NAD^+ to enable azide-alkyne cycloaddition (i.e., "click chemistry"). Thereby, attachment of fluorophores or other biochemical tags for downstream enrichment and purification of modified target proteins became possible. Next, similar analog-sensitive variants of PARP2 and PARP3 were designed, resulting in mutant enzymes that could be used for the identification of enzyme-specific target proteins.

Previous studies had already employed modified NAD^+ analogs to study PARP biology. For instance, Jiang *et al.* showed that N-6-alkyne-NAD^+, in contrast to C-8-alkyne-

NAD^+, can be used by wild-type PARP1 (13). Furthermore, NAD^+ analogs modified at the C-2 position of adenine are substrates for PARP1 (14,15). Such C-2 and N-6-modified molecules were also labeled with fluorophores to study PARylation dynamics by live-cell imaging and FLIM-FRET microscopy (16). However, since these molecules serve as substrates not only for PARP1, they are unsuitable to identify targets and assess functions of individual PARP homologs. A first approach to overcome this limitation was introduced by Carter-O'Connell I *et al*. who designed engineered PARP/NAD^+ analog pairs (17). This study employed NAD^+ analogs that were modified at the C-5 position of the nicotinamide moiety, which then served as a substrate for genetically engineered PARP1, PARP2, and TNKS2 (ARTD6) enzymes. The same group reported a similar approach to identify targets of PARP10/11 (ARTD10/11), further extending the spectrum of analog sensitive PARP/NAD^+ pairs (18). One caveat of these studies was, however, that only MARylation was catalyzed, and that PAR formation was inhibited in these reactions. Although this can be beneficial for certain downstream mass spectrometric approaches, it does not fully resemble the natural PARylation and MARylation reactions.

Gibson *et al*. now took chemical genetics of analog-sensitive PARPs (asPARPs) to the next level by presenting mutant asPARP1/2/3 enzymes that, unlike their wild-type counterparts, use 8-Bu(3-yene)T-NAD^+ as a substrate. By incubating extracts of HeLa cells and mouse embryonic fibroblasts (MEFs) with recombinant asPARPs in the presence of the NAD^+ analog they identified site-specific ADP-ribosylation of target proteins via mass spectrometry in a PARP homolog-specific manner. In line with previous studies analyzing the ADP-ribosylome (5-9), many targets identified by Gibson *et al*. were associated with DNA repair, transcriptional processes, and RNA processing. Interestingly, the targets identified include proteins unique to specific PARP/ARTD enzymes as well as shared targets, which are modified by all three enzymes. In HeLa cell extracts, 167 targets were identified for PARP1, 87 for PARP2 and 402 for PARP3. In this regard, it is particularly interesting to see that the mono-ADP-ribosyl transferase PARP3 (ARTD3) showed the widest spectrum of target proteins, indicating diverse and still largely uncharacterized biological functions for this enzyme.

Gibson *et al*. further combined their method with the ChIP-seq technology to map chromatin-associated ADP-ribosylation events on a genome-wide level. To this end, asPARP1 was expressed in MEFs, treated with the NAD^+ analog, nuclei were cross-linked with formaldehyde, the 8-Bu(3-yne)T-ADP-ribose clicked to biotin, and samples were subjected to chromatin immunoprecipitation and next generation sequencing (click chemistry-based chromatin isolation and precipitation with deep sequencing, Click-ChIP-seq). While a recently published method already analyzed chromatin-associated ADP-ribosylation events genome-wide (19), the method used by Gibson *et al*. identified PARylation in a PARP-homolog-specific manner.

Among the shared targets between PARP1 and PARP3 was the negative elongation factor (NELF) complex, which plays a profound role in the regulation of the pause release and transcriptional elongation of RNA Pol II (20). Consistent with the finding that NELF components are targets for PARP1-mediated ADP-ribosylation, by using the Click-ChIP-seq assay, Gibson *et al*. revealed asPARP1-specific ADP-ribosylation at gene promoters, in particular of actively transcribed genes. Furthermore, genome-wide correlation analyses showed a strong clustering between PARP1 and NELF components, cyclin-dependent kinase 9 (CDK9), and low levels of RNA Pol II pausing. Consistently, ADP-ribosylation of NELF was dependent on phosphorylation of CDK9, providing a functional link between PARP1-mediated ADP-ribosylation and transcription-related phosphorylation. The Click-ChIP-seq results were also correlated with global run on (GRO) sequencing to study the location of transcriptionally active RNA polymerases. This revealed a global increase in paused RNA Pol II at active promoters in cells with impaired PARP1 function. Consistently, Pol II transcriptional elongation in the gene body decreased. These results support the hypothesis that PARP1 activity inhibits NELF at sites of active promoters, thereby enhancing Pol II release and transcription elongation. The cellular events that regulate PARP1-mediated NELF inhibition to promote transcriptional elongation still remain to be defined. This will be particularly interesting in the context of genotoxic stress, since PARP1 is most strongly activated by genotoxic agents, which induce a transient transcriptional repression in the course of the DNA damage response.

A potential technical caveat, which may explain some of the differences compared to previous ADP-ribosylome studies, is given by the fact that Gibson *et al*. used a cell-free system, in which cell lysates were incubated with recombinant mutant enzymes and the non-natural NAD^+ analog. Therefore, concentrations and activation states of target proteins may not be fully comparable to the intracellular environment. In future studies, it would be

interesting to employ genome-editing techniques, such as CRISPR/Cas9, to introduce asPARP variants into the genome and combine this with carrier systems to allow NAD^+ analogs to enter the cell (16). Such an approach would further increase the physiological relevance of the PARP-homolog-specific ADP-ribosylome and allow target identification under more physiological conditions and in response to different stress stimuli. Moreover, this could be combined with the use of fluorescently labeled NAD^+ analogs to investigate the dynamics of PARylation and MARylation in their natural cellular environment and in an enzyme-specific manner.

In conclusion, Gibson *et al*. developed a system of an NAD^+ analog and matched asPARP1/2/3 mutants, which for the first time preserves the mono- and PARylation activities of these enzymes. They used this system to identify PARP-homolog-specific ADP-ribosylomes and to study the genome-wide distribution of ADP-ribosylation events mediated by specific PARP enzymes. They exemplify the potential of this approach by validating the PARP1-dependent regulation of NELF in the context of transcriptional activation. While validation and functional characterization of other targets still awaits future studies, we may expect to see additional sets of as PARP/ARTD variants, which will help to decipher the individual contributions of specific homologs to protein PARylation/MARylation. This in turn may contribute to the development and validation of homolog-specific PARP inhibitors to overcome some of the current limitations of these promising anti-cancer drugs (10,11).

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