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VIEWPOINT

Progress and outlook in studying the substrate specificities of PARPs and related enzymes

Marcin J. Suskiewicz[1], Luca Palazzo[2], Rebecca Hughes[1] and Ivan Ahel[1]

1 Sir William Dunn School of Pathology, University of Oxford, UK
2 Institute for the Experimental Endocrinology and Oncology, National Research Council of Italy, Naples, Italy

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Correspondence
Ivan Ahel, Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, UK
Tel: +44 1865 285656
E-mail: ivan.ahel@path.ox.ac.uk

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Despite decades of research on ADP-ribosyltransferases (ARTs) from the poly(ADP-ribose) polymerase (PARP) family, one key aspect of these enzymes – their substrate specificity – has remained unclear. Here, we briefly discuss the history of this area and, more extensively, the recent breakthroughs, including the identification of protein serine residues as a major substrate of PARP1 and PARP2 in human cells and of cysteine and tyrosine as potential targets of specific PARPs. On the molecular level, the modification of serine residues requires a composite active site formed by PARP1 or PARP2 together with a specificity-determining factor, HPF1; this represents a new paradigm not only for PARPs but generally for post-translational modification (PTM) catalysis. Additionally, we discuss the identification of DNA as a substrate of PARP1, PARP2 and PARP3, and some bacterial ARTs and the discovery of noncanonical RNA capping by several PARP family members. Together, these recent findings shed new light on PARP-mediated catalysis and caution to 'expect the unexpected' when it comes to further potential substrates.

PARPs, ARTs and ADP-ribosylation

ADP-ribosylation is a widespread protein post-translational modification (PTM) that involves the enzymatic transfer of the ADP-ribosyl moiety from β-NAD$^+$ to a protein amino acid residue [1] (Fig. 1). The attachment involves an inversion of configuration at the ADP-ribose C1'' atom and a simultaneous release of nicotinamide. With as much as one third of the human nuclear proteome being subject to ADP-ribosylation according to recent studies [2], it emerges as a regulatory mechanism of major importance. The best-studied ADP-ribosylation 'writer' is poly(ADP-ribose) polymerase 1 (PARP1), the founding members of the PARP family of ADP-ribosyltransferases (ARTs) [3], which in humans comprises 17 core members encoded by separate genes (PARP1 to PARP16 including two tankyrases, PARP5a and PARP5b). PARP1, which is thought to account for most detectable ADP-ribosylation in human cells, is one of the most abundant nuclear proteins, a key player in DNA repair and many other cellular processes, and a target of anticancer drugs [4–6]. PARPs and more distantly related ARTs are widely distributed among living organisms; in bacteria, ARTs include the paradigmatic ADP-ribosylating toxins diphtheria toxin and cholera toxin [7]. Based on the closer relationship with the former, PARPs are also classified as diphtheria toxin-like

Abbreviations
ADP, adenine dinucleotide; ART, ADP-ribosyltransferase; ARTC, cholera toxin-like ART; ARTD, diphtheria toxin-like ART; DNA, deoxyribonucleic acid; DTX3L, deltex 3-like; HPF1, histone PARylation factor 1; MARylation, mono(ADP-ribosyl)ation; NAD$^+$, nicotinamide adenine dinucleotide; PAR, poly(ADP-ribose); PARP, poly(ADP-ribose) polymerase; PARylation, poly(ADP-ribosyl)ation; PTM, post-translational modification; RNA, ribonucleic acid.

ARTs or ARTDs [8]. In human cells, ADP-ribosylation can also be catalysed by more diverged cholera toxin-like ARTs (ARTCs) and unrelated sirtuins [9]. Like most other PTMs, ADP-ribosylation is reversible; in human cells, it can be removed by numerous 'eraser' enzymes that possess ADP-ribosylglycohydrolase activity [10,11].

In addition to modifying protein amino acid side chains, PARP1 can elongate protein-linked ADP-ribose to form long poly(ADP-ribose) (PAR) chains with occasional branching, a process known as poly (ADP-ribosyl)ation or PARylation [12]. Chain formation has also been observed for PARP2 and PARP5a/b (tankyrase) [9]. In the polymer, the succeeding ADP-ribose is attached to the ribose hydroxyl oxygen of the preceding unit. PAR chains were discovered even prior to their covalent attachment to proteins [13,14] and have attracted a lot of attention. The progress over the years gradually led to the idea of these polymers as both biophysical entities that engage in electrostatic repulsion and condensate formation and biochemical scaffolds that mediate recruitment of various PAR-binding 'readers', especially in the context of DNA repair [15,16]. Moreover, detached PAR chains have been implicated in triggering a specific form of cell death referred to as parthanatos [17].

Short history of PARP specificity research

The focus on PAR chains as functional agents in their own right might have contributed to a relative neglect of the question of the exact location and amino acid specificity of PARP-catalysed protein modifications – a question, which has additionally proven technically challenging. However, even from a PAR-centric point of view, it might be argued that the proteins to which the chains are attached will determine PAR cellular localisation and at least modulate other aspects of its function. Moreover, ADP-ribosylation can also exist as attachments of a single ADP-ribose unit (mono-ADP-ribosylation; MARylation), and in this case, it would likely function as a more conventional modulator of protein function. For these reasons, knowing PARP substrates and the exact modification sites is crucial for understanding ADP-ribose-dependent regulation (Fig. 2). This is as important for PARP1 as it is for other PARP family members, whose emerging specialised functions [18] might depend on distinct specificities.

The PAR chains were first seen to be associated with histones, but a diverse set of mostly nuclear substrates have been reported over the years, even prior to high-throughput proteomic analyses [12]. The identification of PARP1 itself as the main or at least a major PAR acceptor *in vivo* [19] was followed by the narrowing down of the modification sites to the 'automodification domain', a central region of the polymerase between the DNA-binding and catalytic segments [20]. For reasons that are explained below, PARP1 automodifies in cells at both glutamate/aspartate and serine residues, and major sites include glutamates 488 and 491 [21] and serines 499, 507 and 519 [22,23]. One key function of PARP automodification appears to be to release the enzyme from DNA lesions, presumably through electrostatic and steric repulsion [24–26]. Interference with this mechanism accounts in part for the ability of clinical PARP inhibitors, which are used in anticancer therapy, to trap PARP1 on chromatin [27], thus promoting cytotoxicity, especially in cells with defects in DNA repair [28,29]. Additionally, PAR formation, including on PARP1 itself, has been implicated in orchestrating DNA repair through recruitment of PAR-binding

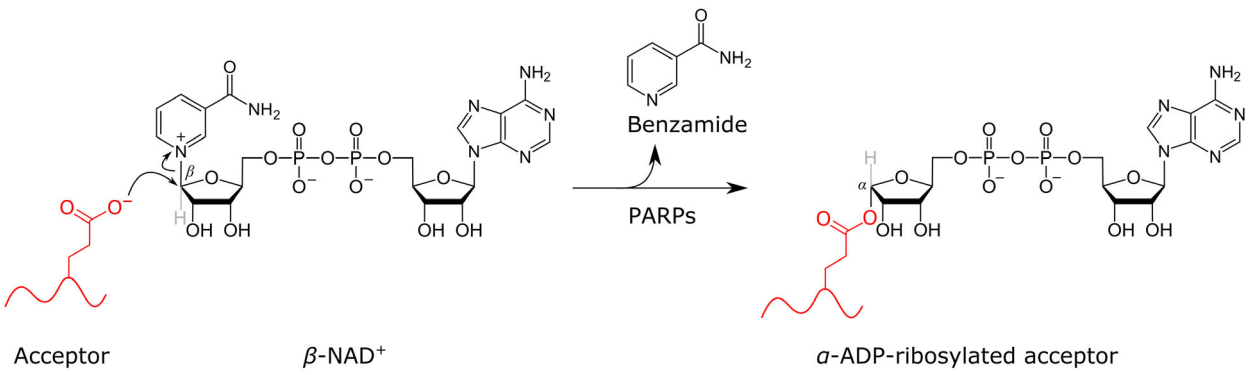

Fig. 1. A simplified scheme of the ADP-ribosylation reaction. A protein glutamate residue is shown as a sample acceptor. Configuration at the C1′′ atom (α or β) is indicated. The reaction might proceed through an oxocarbenium transition state or intermediate (not shown).

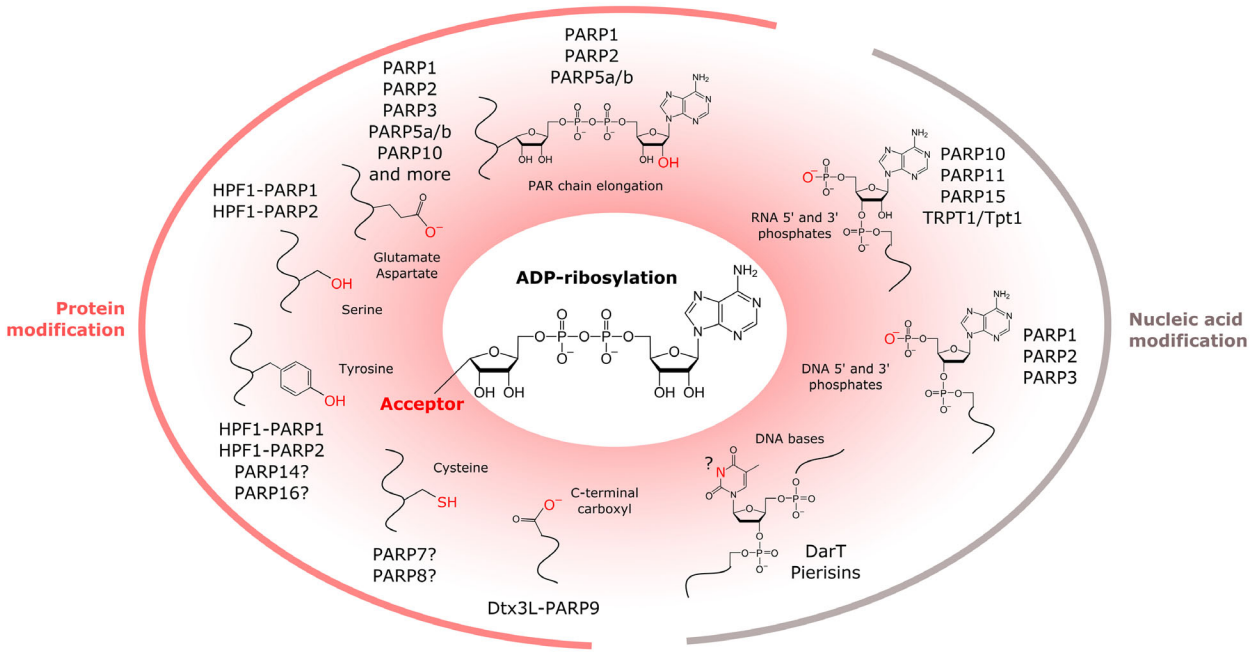

Fig. 2. Selected canonical and novel substrate specificities of mammalian poly(ADP-ribose) polymerase (PARP) family members and related bacterial enzymes. Substrate classes and corresponding enzymes are indicated. The atoms to which the ADP-ribosyl moiety is attached are highlighted in red. The details and supporting literature are provided in the main text.

factors and possibly direct effects on chromatin compaction [30].

Regarding amino acid specificity, while there has always been considerable ambiguity in the field, the majority of early biochemical experiments [12] and later mass spectrometric analyses [31–34] pointed to glutamate and aspartate residues as the main PARP target sites. Modifications at these residues can be erased *in vitro* by the human ADP-ribosylglycohydrolases MacroD1, MacroD2 and Targ1 [35–37], as well as some hydrolases from lower organisms [38,39], which would allow dynamic regulation of these marks *in vivo* and thus points to their physiological relevance. However, just as a relatively consistent picture was emerging, further mass spectrometric studies shook the field by pointing to the unexpected prevalence of serine residues as physiological PARP targets and suggesting further promising candidates including cysteine and tyrosine.

Recent advances in elucidating PARP protein amino acid specificity

A prelude to this breakthrough was the proposal of lysine as the main PARP target [40–42]. However, while some lysine sites might be physiologically relevant, their apparent predominance turned out to be largely a result of computational mislocalisation due to the failure to consider serine as a possible ADP-ribosyl acceptor *in vivo* [22,43–45]. Also, it is likely that a significant proportion of the correctly identified lysine sites are a consequence of nonenzymatic lysine ADP-ribosylation events [46].

Indeed, a broader approach to ADP-ribosylation localisation, accompanied by a more tailored mass spectrometry fragmentation procedure, allowed the identification of serine sites in the PARP1 automodification domain, histone tails and other known PARP substrates, both *in vivo* and *in vitro* [22,43,47]. Independent studies have confirmed serine as the most frequently detected ADP-ribose acceptor in mammalian cells, especially under genotoxic conditions [2,23,48–51]. The identified serine sites are enriched for Lys-Ser and, to a lesser extent, Arg-Ser motifs [22,47,50]. While it is possible that for technical reasons pertaining to sample preparation or stability during mass spectrometry measurements some forms of ADP-ribosylation cannot be detected or are underrepresented, serine does appear to be the major acceptor of ADP-ribosylation, at least upon DNA damage, as confirmed with an immunoblotting-based study [52]. Despite its recent discovery, serine ADP-ribosylation is already best understood mechanistically compared with other forms of ADP-ribosylation. As explained below in

more detail, it can be catalysed by PARP1 and PARP2 in the presence of a novel specificity-determining factor, HPF1 [22,53,54], and is reversed by the ADP-ribosylglycohydrolase ARH3 in a selective manner [55,56]. Further studies will have to address unresolved points, such as the question of the features of individual endogenous serine-linked modifications (mono *vs* poly, length and branching in the case of chains), and whether they are specifically recognised by particular reader domains.

The possible novel PARP acceptors do not stop at serine. Other amino acid residues that have been identified to be ADP-ribosylated in recent studies include – in addition to glutamate, aspartate and lysine – also arginine, cysteine, histidine, threonine and tyrosine residues [2,23,51,57,58]. It is not known to which ADP-ribosylation writers these sites can be attributed, and some of them might be, in part or completely, accounted for by non-PARP ARTs; this applies in particular to arginine, which is known to be modified by human cholera toxin-like ectoenzymes [9]. Generally, however, most detected sites – especially in cytoplasmic and nuclear proteins – are likely to be the products of various PARP family members.

Recently, complexes of PARP1 or PARP2 with HPF1 have been suggested to catalyse ADP-ribosylation at tyrosine residues in addition to serines [51,58], indicating that they could account for at least some of the detected tyrosine sites. Of particular interest is the family-wide analysis in which the automodification of GFP-PARP fusions overexpressed in, and immunoprecipitated from, human cells was used as a readout for PARP activity [57]. This study confirmed that PARP1, PARP2 and tankyrases (PARP5a/b) catalyse PARylation, while also demonstrating MARylation activity for PARP3, PARP4, PARP6, PARP7, PARP8, PARP10, PARP11, PARP12, PARP14, PARP15 and PARP16. Very little to no activity was observed for PARP9 and PARP13. These results are generally in line with previous reports available for some PARPs [59], while extending them to the whole family.

The mapping of automodification sites in seven PARPs (PARP3, PARP6, PARP8, PARP10, PARP11, PARP12 and PARP16) after incubation with NAD^+ *in vitro* yielded predominantly glutamate and aspartate residues as ADP-ribose acceptors, but cysteine and lysine sites have also been reported [57]. For PARP8 in particular, identified sites were exclusively at cysteine residues, all of which were located in a region reminiscent of the PARP1 automodification domain. This suggests that PARP8 might be a specific protein cysteine ART, a conclusion that is corroborated by recent *in vivo* identification of exclusively cysteine sites on PARP8 [23], most of them overlapping with the *in vitro* automodification sites. The same *in vivo* study showed exclusively tyrosine sites on PARP16 and tyrosine and histidine sites on PARP14. However, since it is not clear whether these originate from automodification or are synthesised by some other ARTs present in the cell, it is too early to draw conclusions about the specificity of these enzymes. A specific cysteine automodification site has been identified on PARP7 *in vitro* [60]. Generally speaking, however, glutamate/aspartate specificity might be the most common for PARPs, with best-supported examples including – in addition to PARP1 and PARP2 – also PARP3 [57,61] and tankyrases 1 and 2 (PARP5a and PARP5b) [62,63]. Finally, the absence of catalytic activity in PARP9 has since been revisited in a study that demonstrated ADP-ribosylation of ubiquitin at its C-terminal carboxyl group by the DTX3L-PARP9 complex [64]. Taken together, these studies strongly suggest that some PARPs might have 'noncanonical' amino acid specificities. Nevertheless, we are still far from certainty and – should any of these various specificities be confirmed – far from mechanistic understanding how they are achieved. A foretaste of what might lie ahead for different PARP family members is provided by the recent structural and mechanistic characterisation of HPF1-PARP1 and HPF1-PARP2 complexes.

HPF1-dependent regulation of the specificity of PARP1 and PARP2

In parallel to the identification of serine ADP-ribosylation by mass spectrometry, cell biology and biochemical analysis attributed this modification to PARP1 and PARP2 in complex with a novel accessory factor, HPF1 [22,52,53]. The recent crystal structure of the HPF1-PARP2 complex illustrates how HPF1 completes the PARP active site in a way that could explain the ability of PARP1/2 to modify serine residues and their preference for Lys/Arg-Ser motifs [54] (Fig. 3). The key elements provided by HPF1 include an additional catalytic residue, Glu284, that appears to be the determinant of serine specificity (see below for discussion of why it might be the case), as well as an adjacent negatively charged surface that likely mediates recognition of the Lys/Arg-Ser motifs. Additional insights into this process might be obtained by solving a structure of the HPF1-PARP1 or HPF1-PARP2 complex bound to a substrate-derived peptide. Of note, no such peptide-bound structure of any PARP family member is currently available.

The discovery of HPF1-dependent serine ADP-ribosylation, while dramatically changing our view of

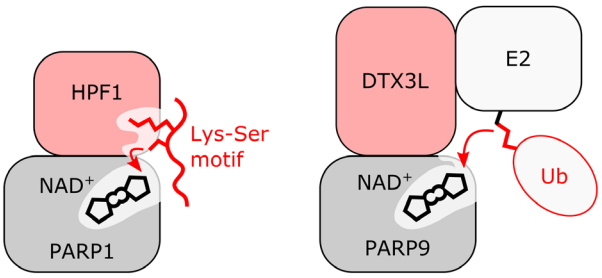

Fig. 3. Factors that regulate PARP specificity. *Left:* HPF1 completes the PARP1 (or PARP2) active site, providing both catalytic and substrate-binding elements needed for efficient ADP-ribosylation of serine residues in lysine-serine motifs in proteins. *Right:* DTX3L allows PARP9 to ADP-ribosylate ubiquitin, possibly by recruiting an E2-Ub conjugate to the PARP active site.

PARP1 and PARP2, does not necessarily contradict previous findings. Glutamate and aspartate residues are still likely to be the preferred target of PARP1 and PARP2 in the absence of HPF1 [52]. In effect, HPF1 causes a specificity switch within PARPs [45,54,65], something that is unprecedented in the field of post-translationally modifying enzymes. In PARP1, serine sites detected both *in vivo* and *in vitro* are located in the automodification domain, close to known glutamate sites, but – at least in *in vitro* assays on this isolated domain as a substrate – the serine residues are much more efficiently modified [22]. HPF1-dependent serine modification appears mutually exclusive with PAR chain formation due to structural incompatibility, but serine-linked mono-ADP-ribosylation can in principle be extended to form a chain [54]. If the initial attachment is rate-limiting and the reaction at serines more efficient than at glutamates, then serine ADP-ribosylation could actually promote PARylation. The details of the coordination between serine and glutamate modification on the one hand, and mono- and poly(ADP-ribosyl)ation on the other, are still unclear, but from what we know so far the relative abundance of these different forms of ADP-ribosylation would likely depend on the ratio of active free and HPF1-bound PARP particles in a given location and at a given time. In this regard, it is worth noting that while HPF1 is much less abundant than PARP1 in model human cells [53], its affinity for PARP1 and PARP2 increases when these are bound to DNA and become activated [54].

Terminal DNA and RNA phosphates as novel PARP targets

Even more surprising are recent studies that demonstrate the ability of certain PARPs to efficiently ADP-ribosylate DNA or RNA oligonucleotides *in vitro*. Specifically, the modification is attached to the 3′ or 5′ phosphate of a terminal deoxy- or ribonucleotide. Accordingly, ADP-ribosylation may turn out to be a post-replicative DNA modification and a post-transcriptional RNA modification in addition to being a protein PTM. PARP1, PARP2 and PARP3 have been shown to catalyse DNA ADP-ribosylation [66–70], while PARP10, PARP11 and PARP15 modify RNA, producing what can be described as a noncanonical RNA cap [71]. RNA is also efficiently ADP-ribosylated *in vitro* by TRPT1/Tpt1 [71,72], a highly diverged PARP-like protein that is sometimes referred to as the 18th member of the PARP/ARTD superfamily [8]. Although studies that demonstrate these activities were prevalently conducted *in vitro*, both the concentration of enzymes and of NAD^+, which are compatible with physiological values, as well as the features of nucleic acid substrates used in biochemical assays, strongly suggest that the same reactions may occur in cells [66–70]. Experiments with cell-free extracts and preliminary *in vivo* observations also point in that direction [68–70]. Finally, ADP-ribosylation of both DNA and RNA can be reversed *in vitro* by multiple ADP-ribosylglycohydrolases, including canonical (i.e. macrodomain-containing) and noncanonical erasers [11,66–70,73].

Of note, for the DNA damage-dependent PARPs PARP1, PARP2 and PARP3, DNA is not only a potential substrate, but also an established allosteric activator. Binding of single-strand DNA break (SSB) or double-strand DNA break (DSB) to the N-terminal DNA-interacting domains of these enzymes relieves their autoinhibited state, effectively stimulating the ADP-ribosylation reaction [74–78]. When DNA is also an acceptor of ADP-ribosylation, this mechanism might serve as a selectivity filter, whereby optimal DNA substrates would need to have the right spacing between two DNA breaks to simultaneously activate the enzyme at its N terminus and serve as its substrate at the catalytic C terminus. *In vitro* analyses of PARP1, PARP2 and PARP3 lend some support to this hypothesis [68–70]. From a functional perspective, DNA duplexes shown to be modified *in vitro* are compatible with potential *in vivo* substrates generated by reactive oxygen species, during DNA replication, or by different DNA repair pathways [66-70]. Moreover, initial studies suggest that DNA ADP-ribosylation by PARP3 may facilitate DNA break ligation [79]. It is also conceivable that DNA ADP-ribosylation, rather than serving a physiological role, is a detrimental side product of PARP activity, similar to DNA adenylation that occurs during DNA ligation [67,80]. If this

analogy held true, ADP-ribosylglycohydrolases that are capable of removing DNA ADP-ribosyl adducts would serve a similar role to that of DNA repair factor aprataxin in processing abortive DNA adenylates [81], that is one of keeping erroneous DNA modification under control. In such a scenario, any conditions or mutations that aggravate DNA ADP-ribosylation or prevent efficient 'proof-reading' by eraser enzymes could lead to increased endogenous DNA damage and contribute to disease.

The ADP-ribosylation of nucleic acids by PARPs opens new questions in the field, such as concerning PARP preference for protein *vs* nucleic acid substrates. This is particularly relevant for PARP1, PARP2 and PARP3, which would encounter DNA breaks as well as proteins while performing their function as DNA damage sensors. *In vitro* experiments show that these PARPs perform DNA modification more efficiently than simultaneous automodification [66,68–70]. However, it would be interesting to assess the results of competition between DNA and protein substrates, for instance histones, for the same enzymatic reaction.

Whereas the potential roles of DNA ADP-ribosylation are most likely connected with DNA replication or repair, RNA ADP-ribosylation could be part of antiviral immunity. Notably, the hydrolytic activity of certain macrodomain-containing proteins against ADP-ribosylated RNAs is conserved in the macrodomain-containing nsP3 protein of many viruses including – but not limited to – the causal agent of severe acute respiratory syndrome (SARS) and coronavirus disease 2019 (COVID-19) [11,67,71,82,83]. This activity could allow viruses to counteract RNA ADP-ribosylation-dependent host defensive mechanisms [71].

Lessons from bacterial ARTs – DNA bases as ADP-ribosylation targets

There has long been productive cross-fertilisation between research into human PARPs and research into related bacterial ADP-ribosylating toxins, for example with respect to the catalytic mechanism. This extends to the question of ADP-ribosylation substrate candidates. Indeed, recent advances suggest that both PARPs and bacterial toxins can modify a range of different protein amino acid residues. On the toxin side, known targets currently include arginine, asparagine, cysteine, diphthamide (modified histidine), glutamine and threonine [84,85], a list that is largely overlapping with that represented in the human ADP-ribosylome (with a notable exception of serine). In this context, it is interesting to note recent reports of toxins that ADP-ribosylate a novel substrate type: DNA bases. Of particular interest is DarT, an enzyme from the ARTD class, which also includes PARPs [86]. Its substrate is the thymidine base in single-stranded DNA, and the modification is sequence-specific. DarT – which is found in numerous bacteria including *Mycobacterium tuberculosis* and pathogenic *Escherichia coli* – is part of a toxin–antitoxin system with the essential DNA ADP-glycosylhydrolase DarG. DarTG represents the first well-characterised system for reversible ADP-ribosylation of DNA bases. While the physiological function of these two proteins might be related to controlling dormancy or the antiphage response, the essentiality of DarG makes its inhibition a potential novel antimicrobial strategy [87]. Bases are also modified by another ART group that belongs to the ARTC class. These are pierisins, found in some butterflies, shellfish and bacteria [88,89]. Pierisins irreversibly modify double-stranded DNA substrates on the N2 position of the guanine base [88].

These examples, although concerning distant 'cousins' of PARPs, suggest that we should keep an open mind with respect to possible PARP substrates, which might also include nucleic acid bases, or even other molecules such as lipids or small-molecule metabolites.

Towards a molecular basis for PARP specificities

PARPs and other related ARTs all share the same core structural elements that surround the NAD^+-binding site [90]. Evolutionary adaptation to various substrates is facilitated by the fact that the acceptor-binding site is lined by loops – including the so-called 'acceptor' and 'donor' loops – that can easily change without the core structure becoming destabilised. At the same time, however, certain features of these enzymes remain constant, including the unusual strained NAD^+ conformation [78,91] and the presence of negatively charged residues in the active site, both of which are thought to contribute to catalysis [1]. Comparing structures and sequences of ARTs that display various substrate specificities allows drawing some tentative but potentially useful generalisations, which we would like to present below. We focus on catalytic residues alone, not the substrate-binding loops that are much more variable in a way that makes structure–function associations less reliable.

Many ARTs including PARP1 and PARP2 contain a conserved active-site glutamate residue that has been implicated in catalysis [90]. The ADP-ribosylation reaction is thought to proceed via the oxocarbenium ion that develops upon dissociation of the ribose–

nicotinamide bond of NAD^+, and this transition state could be stabilised by the negatively charged glutamate (either directly or by polarising the ribose ring through interaction with 2'-OH); moreover, in the case of a protonated acceptor residue, the glutamate could activate it through general-base catalysis [1,92–95]. Of note, a reliance on a negatively charged glutamate seems to be also a conserved feature of another group of NAD^+-utilising enzymes, ADP-ribosyl cyclases [96].

The functions of the active-site glutamate residue in PARPs are illuminated by a seminal mutational analysis, which showed that, in the case of PARP1, this residue (Glu988) is dispensable for initial ADP-ribosylation of glutamate/aspartate residues, but is required for PAR chain elongation [94]. These observations can be explained by recourse to the concept of 'substrate-assisted catalysis', whereby the glutamates or aspartates that are being modified would contribute – through their negative charge – to NAD^+ activation, in effect replacing the active-site glutamate [59]. Furthermore, such a deprotonated acceptor would not itself require activation by deprotonation. The situation is different, however, during chain elongation, when the target of the modification is a ribose hydroxyl – a moiety that is not negatively charged and itself requires deprotonation, necessitating the catalytic glutamate [97]. Consistently, the comparison of sequences and specificities of various PARP family members, most of which lack the catalytic glutamate, shows that this absence predicts the lack of PARylation activity but is consistent with MARylation at glutamates/aspartates [57] or the C-terminal carboxyl [64]. Moreover, the catalytic glutamate appears dispensable for the ADP-ribosylation of terminal RNA phosphates [71], which could be explained by the negative charge of the phosphate moiety and its relative similarity to the carboxyl group. This, incidentally, raises the intriguing possibility that phosphorylated proteins could be ADP-ribosylated at their phospho-residues by some PARPs (even those lacking the catalytic glutamate), consistent with early reports suggesting ADP-ribosylation of histone proteins at phosphoserine residues *in vivo* [98,99].

In addition to PARylation, the catalytic glutamate appears to be required in the case of MARylation at targets other than glutamate, aspartate or phosphate – as in bacterial toxins that modify a variety of amino acids [84,85] and DNA bases [86,88]. One might conclude that the presence of the catalytic glutamate is predictive of a function that goes beyond modification of acidic residues: either PARylation or MARylation at targets other than glutamate, aspartate or phosphate.

A further insight into this topic is provided by the serine-specific HPF1-PARP1 and HPF1-PARP2 complexes, in which HPF1 provides an additional catalytic residue, Glu284 [54,65] (Fig. 3). Interestingly, serine mono-ADP-ribosylation is still catalysed, albeit less efficiently, by the Glu988Gln mutant of PARP1 in the presence of wild-type HPF1 [55], but it cannot be produced by the wild-type PARP1 when it is complemented by HPF1 harbouring the Glu284Ala mutation. This suggests that – unlike a ribose hydroxyl during chain elongation – a serine residue cannot be efficiently activated by Glu988 and this function seems to be taken over by Glu284 of HPF1. Both glutamates, however, are required for full catalytic efficiency, possibly because Glu988 still contributes to NAD^+ activation. Of note, a similar active site with two conserved catalytic glutamates occurs in arginine-specific cholera toxin and related enzymes [1]. Strikingly, Glu284 of HPF1 and Glu988 of PARP1 of the human serine ADP-ribosylation complex occupy similar positions in space to those of the cholera toxin glutamate dyad [54].

A seemingly similar case to HPF1-PARP1/2 is provided by the DTX3L-PARP9 complex in which both components are required for specific ubiquitin ADP-ribosylation at the C-terminal carboxyl group [64] (Fig. 3). Of note, PARP9 lacks the catalytic glutamate and on its own does not even automodify [57]. While it is possible that DTX3L complements PARP9 active site in a manner not dissimilar to the HPF1-PARP1/2 scenario, in the light of the above considerations we would expect that the modification of the C-terminal carboxyl group, like that of glutamate or aspartate residues, does not require dedicated catalytic residues and instead proceeds in a substrate-assisted manner. In this case, DTX3L could serve as a specificity-determining factor solely by binding ubiquitin and positioning it for the reaction. It must be stressed, however, that the authors observed ubiquitin ADP-ribosylation only when DTX3L (which has a ubiquitin E3 ligase activity) is accompanied by E1 and E2 ubiquitination pathway enzymes [64]; therefore, it is possible that the actual substrate that reacts with NAD^+ is a ubiquitin thioester and the reaction has mechanistic features of both ADP-ribosylation and ubiquitin ligation. A future structural and biochemical analysis will show whether these speculations are correct.

The two examples mentioned last represent new paradigms and thus prompt new questions. Are there any further factors that regulate PARP specificity by active site complementation and/or substrate recruitment? Or could, in some cases, other regions within a PARP protein – or even in a substrate – provide the

catalytic or substrate-binding elements that are missing from their catalytic domains?

Conclusions and outlook

PARP family members and related ARTs are found across all domains of life, where they play important roles in cellular physiology and pathology. In human cells, PARP1 is one of the most abundant nuclear proteins and a clinical cancer drug target. The functions of PARPs are related to their substrate specificities; these, however, have remained unclear. Recent years have brought new insights in this area, including the identification of serine as the major protein ADP-ribosylation acceptor in human cells and of HPF1 as the specificity-determining factor that allows PARP1 and PARP2 to catalyse this modification [22,52–54]. With thousands of sites in the human cell, serine ADP-ribosylation appears to be a major contributor to the proteome complexity. Further promising candidates include cysteine [23,57,60] and tyrosine [23,51,58], which might be the targets of specific PARP family members; several other amino acids are also possible as acceptors. Moreover, it has emerged that PARPs and related ARTs can modify not only proteins, but also nucleic acids. These breakthroughs, however, rather than answering all possible questions, suggest that we still know relatively little about PARP substrate specificities. We should be prepared for unexpected answers as we continue with detailed analyses of individual PARP family members and their substrates.

Future efforts should aim at identifying substrate specificities and explaining them in structural and mechanistic terms. In some cases, ADP-ribosylation synthesis might require unknown 'specificity-determining factors' that are waiting to be identified and characterised. The identification of new ADP-ribosylation targets will necessitate the search for erasers and readers that might be specific for a given modification. Lastly, understanding the functional importance of particular modification sites will require studying individual substrate proteins in their modified forms. In the end, a better understanding of PARP substrate specificities will contribute to our knowledge of fundamental biological processes and of pathological conditions induced by deficiency or malfunction of one or more of these enzymes [4,100].

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Author contributions

The authors jointly wrote the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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