(ADP-ribosyl)hydrolases: structure, function, and biology

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ADP-ribosylation is an intricate and versatile posttranslational modification involved in the regulation of a vast variety of cellular processes in all kingdoms of life. Its complexity derives from the varied range of different chemical linkages, including to several amino acid side chains as well as nucleic acids termini and bases, it can adopt. In this review, we provide an overview of the different families of (ADP-ribosyl)hydrolases. We discuss their molecular functions, physiological roles, and influence on human health and disease. Together, the accumulated data support the increasingly compelling view that (ADP-ribosyl)hydrolases are a vital element within ADP-ribosyl signaling pathways and they hold the potential for novel therapeutic approaches as well as a deeper understanding of ADP-ribosylation as a whole.

Posttranslational modifications [PTMs] of proteins provide efficient ways to fine-tune or repurpose protein functions by altering their activities, localization, stability, or interaction networks. PTMs thus allow organisms to adapt rapidly to changes in their environment, including nutrient availability or exposure to chemotoxins, or transition between environments, as in the case of a microbial pathogen entering a host body. Consequently, the function of PTMs can be conceived as expanding the limited genome-encoded proteome—typically only a few thousand proteins—to millions of distinct protein forms.

ADP-ribosylation—intricate and versatile

ADP-ribosylation is an ancient PTM and intrinsically links signaling with basic metabolism. The modification is established by the transfer of a single or multiple ADP-ribose (ADPγ) unit(s) from the redox cofactor β-nicotinamide adenine dinucleotide (β-NAD+) onto a variety of acceptor residues on the target protein [Table 1; Fig. 1].

Diversification of NAD+ signaling is particularly apparent in vertebrata, being linked to evolutionary optimization of NAD+ biosynthesis and increased [ADP-ribosyl] signaling [Bockwoldt et al. 2019]. ADP-ribosylation is used by organisms from all kingdoms of life and some viruses [Perina et al. 2014; Aravind et al. 2015] and controls a wide range of cellular processes such as DNA repair, transcription, cell division, protein degradation, and stress response to name a few [Bock and Chang 2016; Gupte et al. 2017; Palazzo et al. 2017; Rechkunova et al. 2019]. In addition to proteins, several in vitro observations strongly suggest that nucleic acids, both DNA and RNA, can be targets of ADP-ribosylation [Nakano et al. 2015; Jankevičius et al. 2016; Talhaoui et al. 2016; Munnur and Ahel 2017; Munnur et al. 2019].

The ADP-ribosylation reaction is catalyzed by a diverse range of [ADP-ribosyl]transferases [ARTs]. Phylogenetically, their catalytic domains are part of the ADP-ribosyl superfamily [Pfam clan CL0084] [Amé et al. 2004] and three main clades are generally distinguished based on their characteristic catalytic motif: [1] the H-H-Φ clade, containing TRPT1/KtpA [also termed Tpt1]; [2] the R-S-E clade, containing the cholera toxin-like ARTs [ARTCs]; and [3] the H-Y-[EDQ] clade, including the diphtheria toxin-like ARTs [ARTDs] [Aravind et al. 2015]. [Sequence motifs are given following the regular expression syntax of the ELM resource [http://www.elm.eu.org; Aasland et al. 2002; Gouw et al. 2018].] Functionally, the majority of ARTs catalyze the transfer of a single ADPr moiety onto an acceptor site, termed mono[ADP-ribosyl]ation [MARylation]. For example, ARTCs are mostly arginine-specific mono[ADP-ribosyl]transferases with the exception of a small group of guanine-specific ADP-ribosylating toxins found in some cabbage butterfly and shellfish species [Table 1; Takamura-Enya et al. 2001; Nakano et al. 2015; Crawford et al. 2018]. ARTDs [including the best characterized class poly[ADP-ribosyl]polymerases [PARPs]] appear to have a comparatively broad target range with acidic (glutamate/aspartate), thiol [cysteine], and hydroxyl (serine/tyrosine)-containing residues among others being described as acceptors [Table 1]. Lastly,
Table 1. Précis of the functional versatility of ADP-ribosylation

| Linkage type                  | Modification targets | Examples of known substrates | Transfases | Hydrolases                     |
|------------------------------|----------------------|------------------------------|------------|--------------------------------|
| **O-Glycosidic linkages**    |                      |                              |            |                                |
| Glutamic/aspartic acid^1     | β-TrCP^2, GSK3b^3,   | PARP1^6,8, PARP2^8,          | MacroD1^9,10,11, MacroD2^9,10,11, TARG1^12 |            |
|                              | LXRα/β^4, NXF1^5,    | PARP3^6, PARP5^8,           |            |                                |
|                              | PARP1^6,6,           | PARP5^9, PARP7^4,           |            |                                |
|                              | PARP2^6,3^6,         | PARP10^15,6,                |            |                                |
|                              | PARP5a^6,            | PARP14^7,                   |            |                                |
|                              | PARP5b^6             | PARP16^6                    |            |                                |
|                              | PARP11^1,8,7,        |                               |            |                                |
|                              | PARP13^7,            |                               |            |                                |
|                              | PARP16^5,6,          |                               |            |                                |
| Aspartic acid^1              | GevH-L^1,13,         | SirTM, PARP6^6,             | MacroD1^9,10,11, MacroD2^9,10,11, TARG1^12 |            |
|                              | PARP6^6,6,           | PARP12^8                    |            |                                |
|                              | PARP12^8             |                               |            |                                |
|                              | OAADPr^15            |                               |            |                                |
| C terminusb,14               | Ubiquitin^14         | PARP9^14                    | Unknown    |                                |
| Acylated lysine^6,15         | Ubiquitin^14         | PARP9^14                    | Unknown    |                                |
| Serine and tyrosine^1         | OAADPr^15            | PARP9^14                    | Unknown    |                                |
| ADPr                         | 2′-OH^25,26          | PARP1^6,25, PARP2^6,25,     |            |                                |
|                              | and 2″-OH^25,26      | PARP5a^6,26, PARP5b^6,26   |            |                                |
|                              | 3′/5′-phospho-tRNA^34,35 |                         |            |                                |
|                              | Arginine^1           | tRNA^34,35                  |            |                                |
|                              | integrin α^76,       | KptA/TRPT1^50,31,34         |            |                                |
|                              | hemopexin^37,        | PARP1^52, PARP3^43,         |            |                                |
|                              | GRP78/BiP^38,        | PARP10^100                  |            |                                |
|                              | Gsr^39               | PARP15^100                  |            |                                |
|                              |                       | hARTC1^1, hARTC5^1           |            |                                |
|                              |                       | cholera toxin^39             |            |                                |
|                              |                       | ARH1^40                     |            |                                |
|                              |                       |                               |            |                                |
| N-Glycosidic linkages        |                      |                              |            |                                |
| Arginine^1                   | PARP1^6,6            | PARP16^6                    | Unknown    |                                |
| Lysine^6                     | PARP16^6             | PARP16^6                    | Unknown    |                                |
| Diphtamide                   | EF2^41               | exotoxin A^41               | Irreversible^h |                |
| Guanine^42,43,44             | dsDNA^42,43,44       | Pierisin^42, CARP-1^43,     | Irreversible^h |                |
|                              |                       | ScARP^44                    |            |                                |

Continued
Beyond PTMs: ADP-ribosylation of nucleic acids

Despite their evolutionary separation, TRPT1/KptA-type transferases are sometimes classified as the 18th PARP. KptA was first characterized in yeast as a tRNA 2′-phosphotransferase that removes the 2′-phosphate at the splice junction generated by fungal tRNA ligase through a two-step reaction: [1] The internal RNA 2′-phosphate reacts with NAD⁺ to form an RNA-2′-phospho-ADP-ribosyl

Figure 1. The chemical structure of ADP ribose, including atom and substructure labels, as used in this review. Throughout the ADP-ribosylation cycle different moieties (R, red) are attached to the anomeric carbon (C1′); namely, the substrate β-NAD⁺ (nicotinamide is linked trans relative to the 2′OH), the formed reaction products (linked cis [a] relative to the 2′OH) and ADP (1′OH, anomeric mixture in aqueous solution). Linkage sites of consecutive ADP-ribose moieties within PAR are highlighted in blue ([2′] linear linkage; [2′′] branch point linkage).

| Modification | Examples of | Transferases | Hydrolases |
|--------------|-------------|--------------|------------|
| Cysteine⁴     | PARP8,⁶, ¹⁶,⁶ | PARP8,⁶, PARP16,⁶ | Unknown |
| Thymidine⁴⁵   | ssDNA-TNTC | DarT⁴⁵, DarG⁴⁵ |           |

Table 1. Continued

| Linkage type     | Modification targets | Examples of known substrates | Transferases | Hydrolases |
|------------------|----------------------|*****************************|--------------|------------|
| S-Glycosidic     | Cysteine⁴            | PARP8,⁶, ¹⁶,⁶              | PARP8,⁶, PARP16,⁶ | Unknown |

⁴Automodified in vitro.
⁵Gly76 of ubiquitin.
⁶The decylation reaction catalyzed by sirtuins can be viewed as acylic acid transfer from lysine residues onto ADPr.
⁷Branch point linkage highlighted in red.
⁸Forming linear 1′→2′ extensions
⁹Forming branch points with 1′→2′′ linkage.
¹⁰Cysteine linked lysine can undergo Amadori rearrangement leading to a ketoamine product [see Fig. 6A, below].
¹¹Irreversible has been suggested and so far no reversing activity has been described.

Table references: ¹Crawford et al. 2018; ²Guo et al. 2019; ³Feijs et al. 2013; ⁴Bindesbøll et al. 2016; ⁵Carter-O’Connell et al. 2016; ⁶Vyš et al. 2014; ⁷Carter-O’Connell et al. 2018; ⁸Zhang et al. 2013; ⁹Chen et al. 2011; ¹⁰Jankevicius et al. 2013; ¹¹Rosenthal et al. 2013; ¹²Sharifi et al. 2013; ¹³Rack et al. 2017; ¹⁴Yang et al. 2017; ¹⁵Sauve and Youn 2012; ¹⁶Peterson et al. 2011; ¹⁷Ono et al. 2006; ¹⁸Bondiglio et al. 2017; ¹⁹Leidecker et al. 2016; ²⁰Leslie Pedrioli et al. 2018; ²¹Bartlett et al. 2018; ²²Palazzo et al. 2018; ²³Fontana et al. 2017; ²⁴Abplanalp et al. 2017; ²⁵D’Amours et al. 1999; ²⁶Rippmann et al. 2002; ²⁷Brochu et al. 1994; ²⁸Barkauskaite et al. 2013; ²⁹Oka et al. 2006; ³⁰Munnur et al. 2019; ³¹Munir et al. 2018b; ³²Talhaoui et al. 2016; ³³Munir and Ahel 2017; ³⁴Munir et al. 2018a; ³⁵Banejee et al. 2019; ³⁶Zolkiewska and Moss 1993; ³⁷Leutert et al. 2018; ³⁸Fabrizio et al. 2015; ³⁹Vanden Broeck et al. 2007; ⁴⁰Moss et al. 1985; ⁴¹Honjo et al. 1968; ⁴²Takamura-Eya et al. 2001; ⁴³Nakano et al. 2006; ⁴⁴Nakano et al. 2013; ⁴⁵Jankevicius et al. 2016.
with internal tRNAs and/or no known pathways to generate RNAs. Surprisingly, TRPT1/KptA is evolutionary conserved in Archaea and Animalia, whose tRNA exon ligation does not result in a 2′-phosphate junction, as well as in bacterial species, which have no known intron-containing tRNAs and/or no known pathways to generate RNAs with internal 2′-phosphate modifications [Spinelli et al. 1998; Popow et al. 2012]. These observations suggested that TRPT1/KptA might catalyze additional enzymatic reactions other than RNA 2′-phosphate removal, for example, TRPT1/KptA from Aeropyrum pernix and humans catalyze the NAD′-dependent ADP-ribosylation of either RNA or DNA 5′-monophosphate termini [Munnur et al. 2018b; Munnur et al. 2019]. Moreover, several PARPs are capable of ADP-ribosylating DNA or RNA ends in vitro. Among them, DNA repair PARPs [PARP1–3] can modify terminal phosphate moieties at DNA breaks with diverse specificity, i.e., PARP2 and PARP3 preferentially act on 5′-phosphates in nicked duplex DNA, whereas PARP1 modifies 3′- and 5′-phosphates as well as the terminal 2′-OH groups in single-strand or double-strand DNA [Talhaoui et al. 2016, Munnur and Ahel 2017, Belousova et al. 2018; Zarkovic et al. 2018]. Beyond DNA, the antiviral PARPs 10, 11, and 15 have been shown to modify terminal phosphate moieties at DNA breaks. In contrast, the catalytic efficiency of PARG decreases for short polymers as well as the terminal serine linkage are the primary targets for hydrolysis of the ribose–ribose ether bond, but cannot act on the terminal protein–ribose bond [Slade et al. 2011]. Three vertebrate ARH homologs were identified with ARH1 and ARH3 being confirmed hydrolases, whereas ARH2 is suspected to be catalytically inactive [Table 1; Moss et al. 1985, Oka et al. 2006; Ono et al. 2006; Smith et al. 2016; Rack et al. 2018]. The available data indicate that ARH1 specifically reverses MARylation of arginine residues and appears to play a role in bacterial infections involving cholera exotoxins-like transferases [Moss et al. 1985, 1986; Kato et al. 2007]. In contrast, ARH3 has a broad target spectrum including OADDpr, modified serine residues as well as PAR [Oka et al. 2006; Ono et al. 2006; Fontana et al. 2017; Bartlett et al. 2018]. Both, ARH3 and PARG are recruited to DNA damage sites and are reported to play important parts in the DNA damage response [Mortusewicz et al. 2011; Palazzo et al. 2018; Wang et al. 2018]. As for PARP1/2, this overlap in ARH3 and PARG localization and activity is yet another indication for redundancy in the ADP-ribosylation system, but may also indicate a regulatory aspect. In vitro and in vivo data suggest that PARG is the primary cellular PAR hydrolase [Alvarez-Gonzalez and Althaus 1989, Brochu et al. 1994; Fontana et al. 2017; Drown et al. 2018]. However, the catalytic efficiency of PARG decreases for short polymers (less than four units) [Barkauskaite et al. 2013]; hence, it is tempting to speculate whether these oligomers as well as the terminal serine linkage are the primary substrates for ARH3. This idea is supported by the fact that ARH3 knockout (KO) cells have a dramatically increased level of persistent MARylation marks, especially on histones, even in the absence of exogenous DNA damage [Fontana et al. 2017; Palazzo et al. 2018].

In addition to this complete removal of the ADP-ribosyl modification, several noncanonical mechanisms of processing have been proposed. Members of the Legionella pneumophila SidE effector proteins use a cascade of arginine-ADP-ribosylation on ubiquitin, phosphodiester cleavage, and transfer of the phosphor-ribosyl-ubiquitin onto an acceptor protein as a novel ubiquitination mechanism [Bhogaraju et al. 2016; Puvar et al. 2019]. Similarly, it has been demonstrated in vitro that hydrolysis of the phosphodiester bond by NUDT16, ENPP1, or snake venom phosphodiesterases leaves phosphoribosyl-modified proteins [Matsubara et al. 1970; Palazzo et al. 2015, 2016]. It remains an open question whether NUDT16 and ENPP1 can process ADP-ribosylated proteins also in vivo and what the associated downstream processing or

**ADP-ribosylation reversal**

The chemical nature of the ADPr-protein linkage as well as the length and complexity of the modification can significantly affect the PTM’s half-life, the order in which downstream events occur, as well as the enzymes needed to reverse it [Alvarez-Gonzalez and Althaus 1989; Brochu et al. 1994]. The hydrolysis of ADP-ribosylation linkages is carried out by members of two evolutionary distinct protein families: the macrodomains and the (ADP-ribosyl)hydrolases [ARHs]. Macrodomains are both “readers” and “erasers” of ADP-ribosylation and can be evolutionarily subdivided into at least six phylogenetic classes. The hydrolytically active family members are associated with either the MacroD-type [MacroD1 and MacroD2 in humans], ALC1-like [human TARG1], or PARG-like class [human PARG] (Table 1; Rack et al. 2016). Of these enzymes, MacroD1, Macro2, and TARG1 break the O-glycosidic ester bond of modified aspartates, glutamates, and O-acetyl-ADPr (OADDpr), the reaction product of the NAD′-dependent sirtuin deacetylases, as well as phosphate ester at nucleic acid ends [Sauve and Youn 2012; Rack et al. 2016; Munnur et al. 2019]. PARG degrades polymers by hydrolysis of the ribose–ribose ether bond, but not on the terminal protein–ribose bond [Slade et al. 2011]. Three vertebrate ARH homologs were identified with ARH1 and ARH3 being confirmed hydrolases, whereas ARH2 is suspected to be catalytically inactive [Table 1; Moss et al. 1985; Oka et al. 2006; Ono et al. 2006; Smith et al. 2016; Rack et al. 2018]. The available data indicate that ARH1 specifically reverses MARylation of arginine residues and appears to play a role in bacterial infections involving cholera exotoxins-like transferases [Moss et al. 1985, 1986; Kato et al. 2007]. In contrast, ARH3 has a broad target spectrum including OADDpr, modified serine residues as well as PAR [Oka et al. 2006; Ono et al. 2006; Fontana et al. 2017; Bartlett et al. 2018]. Both, ARH3 and PARG are recruited to DNA damage sites and are reported to play important parts in the DNA damage response [Mortusewicz et al. 2011; Palazzo et al. 2018; Wang et al. 2018]. As for PARP1/2, this overlap in ARH3 and PARG localization and activity is yet another indication for redundancy in the ADP-ribosylation system, but may also indicate a regulatory aspect. In vitro and in vivo data suggest that PARG is the primary cellular PAR hydrolase [Alvarez-Gonzalez and Althaus 1989, Brochu et al. 1994; Fontana et al. 2017; Drown et al. 2018]. However, the catalytic efficiency of PARG decreases for short polymers (less than four units) [Barkauskaite et al. 2013]; hence, it is tempting to speculate whether these oligomers as well as the terminal serine linkage are the primary substrates for ARH3. This idea is supported by the fact that ARH3 knockout (KO) cells have a dramatically increased level of persistent MARylation marks, especially on histones, even in the absence of exogenous DNA damage [Fontana et al. 2017; Palazzo et al. 2018].

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functional consequences of the phosphoribosyl modification would be.

In recent years, attention in the community has increasingly shifted toward studying erasers of ADP-ribosylation: their molecular functions, physiological roles, and influence on human health and disease. Below, we discuss these new insights into ADP-ribosylation reversing enzymes and give an overview of the structural-functional features and biological roles.

Hydrolases of the macrodomain family

Macrodomains are evolutionarily conserved structural modules of ∼25 kDa with a typical length of 150–210 amino acids. The core motif of all macrodomains consists of a three-layer (α/β/α) sandwich architecture with a central six-stranded mixed β-sheet flanked by five α-helices [Allen et al. 2003; Till and Ladurner 2009]. Structurally, it belongs to the leucine aminopeptidase [subunit E, domain 1] superfamily [CATH classification 3.40.220.10], which characteristically consist of nucleotide and nucleic acid-binding domains [Dawson et al. 2017]. Macrodomains were shown to be binders of ADPr moieties as found in OAADPr, MAR-, and PARylated proteins [Karras et al. 2005]. The ADPr moiety binds in a deep cleft located on the crest of the domain. Within the macrodomain family, three classes have catalytically active hydrolases as members (for review, see Rack et al. 2016).

The PARG-like class

PARGs take a special place among the macrodomains as they are the only known members to possess PAR-degrading activity [Feng and Koh 2013]. In mammals, a single gene encodes alternative splice variants, which are believed to play a major role in its regulation, the subcellular distribution of de-PARylation activity as well as tissue specificity [Fig. 2; Meyer et al. 2003; Cortes et al. 2004; Meyer-Ficca et al. 2004; Cozzi et al. 2006; Niere et al. 2012]. For example, PARG11 [isoforms are designated by the molecular weight of the corresponding protein] is a primarily nuclear protein and responsible for the degradation of PARP1/2-derived PAR following genotoxic stress [Min et al. 2010], while PARG102 and PARG99 show cytoplasmic and perinuclear localization and are thought to act on the large fraction of PAR residing in the perinuclear region [Winstead et al. 1999; Gagné et al. 2001]. Furthermore, hydrolytic activity of the latter appears to be required for the regulation of PAR-induced cytoplasmic granules and protein aggregates [Grimaldi et al. 2019].

Dysfunctions in the hydrolysis of PAR chains induced by Parg inactivation are embryonically lethal in mice. Nevertheless, Parg−/− mouse trophoblast-derived stem cells are able to survive in the presence of chemical inhibitors of PARP1/2, suggesting that the accumulation of PAR chains, due to the absence of PARG activity, represents a cell death signal [Koh et al. 2004]. Importantly, PARG depletion leads to hypersensitivity to genotoxic and replication stress and, consequently, it was proposed as a novel target for modern chemotherapeutic approaches [James et al. 2016; Palazzo and Ahel 2018; Pillay et al. 2019]. In addition to its functions in DNA repair, PARG activity seems to be involved in the progression of replication forks and recovery from persistent replication stress [Illuzzi et al. 2014; Ray Chaudhuri et al. 2015]. These observations are in agreement with the interaction of PARG with the replication helicase PCNA and its localization to replication loci during S-phase [Fig. 2; Mortusewicz et al. 2011; Kaufmann et al. 2017].

Structure and function of PARG-like hydrolases

Evolutionarily, the PARG-like class can be subdivided into the canonical PARGs, found primarily in higher
organisms, and the microbial PARGs, often annotated as DUF2263 (Slade et al. 2011). While the latter resemble largely classical macrodomains, canonical PARGs occur together with a mainly α-helical accessory domain that extends the core motif into a typically 10-stranded β-sheet (Figs. 2, 3A). The ADPr-binding cleft, as in other macrodomains, is part of the canonical core fold and the physiological role of the accessory domain, beyond its effects on overall protein stability, remains elusive. Within the binding cleft, the adenine moiety of the ADPr lies parallel to the protein surface and is shielded from the aqueous environment by π–π-stacking with a conserved phenylalanine (Phe902 in humans) [Fig. 4]. Adenine binding is further stabilized by extensive protein and water-mediated contacts with the amino group on C6, as well as with the ring nitrogens N1 and N7 (Figs. 1, 4). These contacts convey ligand specificity as their disruption by an exchange of adenine by hypoxanthine, which substitutes the C6 amine, seriously ligand specificity as their disruption by an exchange of adenine by hypoxanthine, which substitutes the C6 amine, severely diminishes ADPr binding to PARG [Drown et al. 2018; Rack et al. 2018]. In canonical PARGs, ligand binding is further stabilized by a highly conserved tyrosine (Tyr795 in humans) that coordinates O5′ and edge stacks with the adenine moiety (Kim et al. 2012; Tucker et al. 2012; Lambrecht et al. 2015). Recently, these highly specific properties of the adenine-binding pocket were utilized for the development of a series of high-potency, competitive inhibitors [Waszkowycz et al. 2018]. Further along the ligand, the diphosphate-binding loop coordinates both the diphosphate and distal ribose and participates in forcing a strained conformation in this part of the molecule. The strained conformation is achieved via a hydrophobic patch [G[A,V][F,Y] motif] within the loop, which bends the distal ribose toward the catalytic loop and positions C1′′ and O1′′ in relative proximity to P3′. The conformation is further stabilized by a structural water molecule bridging the ribose and phosphate group [Fig. 1B]. In canonical PARGs, a highly conserved asparagine (aspartate in microbial PARGs) precedes the catalytic GGGx[6,8]QEE motif and interacts with the 3′OH group. Binding of the PAR substrate was suggested to increase the pKₐ of the catalytic glutamate [Glu756 in humans], which facilitates its protonation and allows it to act as the general base in the initial step of the reaction [Fig. 1C; Slade et al. 2011; Dunstan et al. 2012; Kim et al. 2012; Tucker et al. 2012; Barkauskaite et al. 2013]. The carboxyl hydrogen of Glu756 is transferred to the PAR-leaving group, while an oxocarbenium intermediate is formed on the bound distal ribose. The deprotonated Glu756 then assists in the activation of a water molecule, which reacts with the oxocarbenium intermediate and forms the ADPr product. It should be noted that due to the placement of Glu756 relative to the distal ribose, it is as yet unclear from which site the ADPr forming water attacks the ribose, and hence whether the α or β product is formed [Kim et al. 2012]. Interestingly, in microbial PARGs the proximal ribose is coordinated and shielded from the aqueous environment (Slade et al. 2011), which makes this subclass strict exo-hydrolases and the more open positioning of the proximal ribose within the binding cleft allows an endo-binding mode and congruously weak endo activity has been observed in vitro (Barkauskaite et al. 2013; Lambrecht et al. 2015). However, it remains to be elucidated whether this activity is of physiological relevance.

The MacroD-type class

Members of the MacroD-type hydrolases are widely distributed among all domains of life and several viruses (Chen et al. 2011; Rack et al. 2015, 2016; Fehr et al. 2018). In humans, the MacroD-type class has two members with highly similar catalytic domains, MacroD1 (also known as Leukaemia-Related Protein 16 [LRP16]) and MacroD2. Both enzymes are mono(ADP-ribosyl) hydrolases and active in vitro against protein substrates modified on acidic amino acids [Barkauskaite et al. 2013; Jankevicius et al. 2013; Rosenthal et al. 2013;
MacroD1 and MacroD2

Aberrant MacroD1 expression and gene fusions contribute to tumour pathology, e.g., in leukaemia, breast, gastric, liver, lung, and colorectal cancer (Imagama et al. 2007; Shao et al. 2015; Sakthianandeswaren et al. 2018). Several lines of evidence indicate that MacroD1 is involved in several important signalling pathways: In breast cancer-derived MCF-7 cells, MacroD1 expression is induced by estrogenic hormones in an estrogen receptor alpha (ERα)-dependent manner and subsequently acts as a cofactor for ERα and the androgen receptor (Han et al. 2007; Yang et al. 2009). In response to DNA double-strand breaks, MacroD1 is activated and enriched in the cytosol, which stimulates prosurvival and antiapoptotic functions of the dimeric [p65/p50] transcription factor NF-κB [Li et al. 2017]. MacroD1 stimulates the activity of NF-κB through the interaction with p65 and UXT, a transcription factor coregulator [Wu et al. 2011, 2015]. In hepatocytes, MacroD1 interacts and regulates liver X receptors α and β when these are MARylated by PARP7 [Bindesbøll et al. 2016]. Furthermore, MacroD1 was also proposed to act as a negative regulator of the insulin signaling pathway through the down-regulation of the insulin receptor substrate protein-1 (IRS-1) [Zang et al. 2013].

The MacroD2 gene locus is a hot spot for mutations and chromosome rearrangements that have been associated with several human disorders, such as autism diseases, schizophrenia, and several tumors (Anney et al. 2010; Mohseni et al. 2014; Fujimoto et al. 2016; Autism Spectrum Disorders Working Group of The Psychiatric Genomics Consortium 2017). These mostly pathoneurological phenotypes of the MacroD2 gene are associated with its loss of function, thus suggesting a physiological role in the central nervous system. This correlates with robust neuronal expression of MacroD2 during brain development [Ito et al. 2018].

Alterations of MacroD2 functions in the DNA damage response and signal transduction may also be linked to tumor formation and/or progression. Indeed, MacroD2 is phosphorylated by ATM in response to DNA double-strand breaks, as well as being involved in reversing the ADP-ribosylation of GSK3β, a key kinase involved in the WNT-mediated signal transduction pathway [Feijs et al. 2013; Golia et al. 2017].

Despite the phenotypic and clinical associations, as well as the in vitro studies discussed above, the precise physiological roles and detailed molecular functions of both MacroD1 and MacroD2 remain poorly understood. For example, the presence of several hydrolases, including MacroD1, within the mitochondrial matrix [Fig. 2; Niere et al. 2008, 2012; Agnew et al. 2018], together with unbiased mass spectrometric evidence for ADP-ribosyl-modified proteins within this compartment [Hendriks et al. 2019] raises the question of whether ADP-ribosylation signaling has a regulatory function in mitochondria.

Viral and microbial MacroDs

Beyond the human MacroD1 and MacroD2 proteins, viruses and bacteria encode MacroD-type hydrolases, too. MacroD-type macrodomains are encoded by a set of positive-strand RNA viruses, such as Coronavirus [Coronaviridae including severe acute respiratory syndrome [SARS-CoV] and Middle East respiratory-related coronavirus [MERS-CoV]], Togaviridae, and Hepeviridae, which all show [ADP-ribo-syl]hydrolase activity against MARylated aspartate and glutamate-modified substrates [Fehr et al. 2016, 2018; Li et al. 2016; Rack et al. 2016; Eckei et al. 2017; McPherson et al. 2017; Lei et al. 2018; Leung et al. 2018; Grunewald et al. 2019]. Although physiological substrates of viral MacroD-type hydrolases are not clear, they are known to be important for viral replication most likely due to their ability to counteract the host immune response by working against antiviral PARPs (PARP7, PARP9, PARP10, and PARP12–PARP15)
This was recently corroborated by the observation that VEEV and SARS macrodomain-containing proteins can efficiently reverse PARP10-derived RNA ADP-ribosylation in vitro (Munnur et al. 2019). Noteworthy, this aspect of viral-induced stress may create evolutionary pressure in vitro (Munnur et al. 2019). Noteworthy, this aspect of the studied microbial MacroD-type hydrolases are macrodomains associated with mono(ADP-ribosyl)transferases of the class M sirtuins (SirTMs) type that are found in bacteria (e.g., Clostridium, Treponema, and Lactobacillus species) and fungi (including Aspergillus, Candida, and Fusarium) (Chen et al. 2011; Rack et al. 2015). Extended operons containing a lipoyl-carrier protein (GcvH-L), a lipoyltransferase (LplA2), and the macrodomain-SirTM module are found almost exclusively in pathogenic bacteria, including Staphylococcus aureus and Streptococcus pyogenes. In this system, GcvH-L can be lipoylated by LplA2 and subsequently ADP-ribosylated by SirTM. The latter modification is reversible by the macrodomain. Interestingly, while the activity of the macrodomain is not dependent on the lipoylation, in vitro binding experiments indicate that the macrodomain interacts with GcvH-L in a lipoylation-dependent manner (Rack et al. 2015). SirTM operons in bacteria and fungi are induced by oxidative stress and it has been proposed that the lipoyl moiety acts as a reactive oxygen species (ROS) scavenger, while the ADP-ribosylation regulates its participation in the redox defence (Rack et al. 2015).

**Structure and function of MacroD-type hydrolases**

Members of the MacroD-type class partially resemble PARG proteins with respect to their ADPr-binding features [Figs. 4, 5A,B, Barkauskaite et al. 2013]. However, there are some key differences in the active site that result in very distinct catalytic mechanisms. The polymer substrate of PARG contains defined ether O-glycosidic bonds, whereas the linkage to acidic residues and OADPr, the preferred substrates of MacroD-type enzymes, are ester linkages. One important difference is that these esters undergo spontaneous transesterification, thus, glutamyl/
aspartyl of protein-linked ADPr or the acetyl moiety in OAAADPr migrate to the 2′ and 3′ positions and equilibrate between the three sites in a pH-dependent manner [Kasamatsu et al. 2011; Kistemaker et al. 2016]. Jankevicius et al. [2013] showed experimentally and in simulations that MacroD2 cleaves ADPr from the 1′ position [Jankevicius et al. 2013]. This can be attributed to the interaction between the carbonyl group and the conserved glycine within the catalytic loop, as well as shielding of the 2′OH-group from the environment by a conserved asparagine [Asn92 in human MacroD2] in a fashion similar to PARG [Fig. 5B]. Within the catalytic loop, the consecutive, catalytic glutamate residues are absent. Initial studies of human MacroD1 and MacroD2 identified a histidine and aspartate motif on helix α6 [MacroD2] coordinating the 2′OH, which were thought to constitute a catalytic dyad [Rosenthal et al. 2013]. However, a recent study demonstrated catalytic activity of viral MacroD homologs that lack the key aspartate residue [Li et al. 2016]. Taken together, these studies suggest a substrate-assisted reaction mechanism. It was proposed that P4 could act as a general base for activation of the structural water molecule, which would attack the C1′ position and hydrolyze the ADPr linkage. However, this mechanism was disputed as the low pKa of the phosphate group (~2) would disfavor this reaction [Barkauskaite et al. 2013, 2015]. Recent structural studies on Oceanobacillus iheyensis MacroD [OiMacroD] identified a well-defined water molecule above the structural water that interacts with a second glycine in the catalytic loop [Figs. 2, 5B; Zapata-Pérez et al. 2017]. Displacement of the water by a Gly > Val mutation reduced the catalytic efficiency of OiMacroD fourfold without affecting protein stability or ADPr binding [Fig. 5B; Zapata-Pérez et al. 2017]. The crystal structures of the SARS- and MERS-CoV macrodomains in complex with β-ADPr reveal occupation of the water binding site by the β-1′OH moiety, while the structural water remains bound in the same position [Fig. 5B; Egloff et al. 2006; Lei et al. 2018], thus suggesting that this newly described water is indeed the catalytic one. Furthermore, this arrangement makes it possible to transfer the proton from the water molecule onto the leaving group or the aqueous environment. A possible, substrate-assisted S2,2 reaction is depicted in Figure 5C, but further studies are needed to elucidate the exact nature of the transition state, mechanism, and the differences between enzymes in which the His/Asp dyad is present or absent, respectively. Comparison of MacroDs with available PARG structures revealed that the isoostructurally positioned catalytic MacroD glycine is not conserved but instead occupied by small aliphatic residues including alanine [tcPARG] or valine [hPARG] [Slade et al. 2011; Kim et al. 2012; Tucker et al. 2012]. Consistently, no water isostructural to the proposed catalytic one can be observed in PARGs. Whether this exchange contributes to the inability of PARG to hydrolyze the terminal ADPr moiety from proteins remains, however, an open question.

Interestingly, a diverse subclass of MacroD enzymes, which are found associated with SirTMs in bacteria and fungi [Fig. 2], contain an amino acid exchange in the catalytic loop. Instead of the typical glycine-rich stretch going into helix α6 [MacroD2], these macrodomains have an extended catalytic loop containing a zinc-binding motif [Fig. 2, Appel et al. 2016]. Positioning of the Zn2+ as part of the active site suggests a catalytic function of the ion and hence a diverged mechanism in comparison with the other members of this class.

The ALC1-like class

Defined by a similarity to the macrodomain of the chromatin remodeler ALC1 [Ahel et al. 2009; Gottschalk et al. 2009], the ALC1-like class contains both MARylation “readers” and “erasers.” ALC1 class macrodomain proteins can be readily found in Animalia and scattered examples can be also identified amongst bacterial species [Perina et al. 2014].

TARG1

TARG1 [also known as OARD1 and C6orf130] is the only hydrolitically active member of the ALC1-like class in Animalia. It was shown to interact with PARP1 and to possess hydrolytic activity against O-acyl-ADPr esters, ADPr-phosphoesters at nucleic acid termini, MARylated proteins, as well as the ability to release whole polymers from the target protein [Peterson et al. 2011; Rosenthal et al. 2013; Sharifi et al. 2013; Munur and Ahel 2017, Munur et al. 2019]. TARG1 is found in the nucleus and cytoplasm [Sharifi et al. 2013]. In particular, TARG1 has been observed to localize at the transcriptionally active nucleoli and binds strongly to ribosomes and proteins associated with rRNA processing and ribosomal assembly factors. In response to DNA damage, TARG1 relocates to the nucleoplasm, where it may contribute to reverse protein ADP-ribosylation [Bütepage et al. 2018].

A homozygous TARG1 gene mutation was described in a family with 11 individuals affected by a severe and progressive neurodegeneration and seizure disorder without dysmorphic features. In detail, a premature stop codon within the exon 4 of TARG1 locus results in the formation of a truncated and nonfunctional TARG1 protein [Sharifi et al. 2013]. In addition, a genome-wide association study revealed that the TARG1 gene could be associated with the loss of insulin sensitivity, a key factor contributing to metabolic disease. However, a functional link between TARG1 and the cellular insulin response has at yet not been established [Timmons et al. 2018].

DarG

DarG is a member of the ALC1-like macrodomains found strictly as a two-component toxin–antitoxin operon in a variety of bacteria, including pathogens like Mycobacterium tuberculosis, enteropathogenic E. coli, and Pseudomonas aeruginosa, as well as several hyperthermophiles such as Thermus aquaticus [Sberro et al. 2013; Jankevicius et al. 2016]. The toxin DarT, a Be4486-like member
of the PARP family [de Souza and Aravind 2012, Aravind et al. 2015], modifies ssDNA at thymine bases in a sequence-specific manner [Jankévicius et al. 2016]. The formation of the [ADP-ribosyl]-DNA adduct is reversed via the action of the antitoxin DarG, which shares some functional features with TARG1 [Jankévicius et al. 2016]. As such, DarTG represents the first characterized system for the reversible ADP-ribosylation of nucleic acids. While the exact physiological role of DarTG is unclear, it was shown that the toxin blocks DNA replication, and it has been speculated that the host bacteria may exploit this system in order to induce a persistence state to survive adverse environmental conditions including exposure to antibiotics [Jankévicius et al. 2016]. If true, resuming growth would require DarG antitoxin activity, which would be in line with *M. tuberculosis* transposon mutagenesis studies indicating that DarG is an essential gene [Sassetti et al. 2003; Griffin et al. 2011]. Taken together, the inhibition of DarG may present a new and promising therapeutic strategy to combat bacterial infections [Jankévicius et al. 2016].

**Structure and function of ALC1-like hydrolases**

In their overall structure, ALC1-like macrodomains are minimal without C- or N-terminal extensions and only five α-helices (Fig. 2). The most considerable divergence to other macrodomain hydrolases is, however, their catalytic mechanism. Crystal structures of the TARG1:ADPr complex showed that in crystallo Lys84 of TARG1 reacts with the distal ribose C1″ forming an open ring Amadori product [Fig. 6A,B, Sharifi et al. 2013]. Further functional analysis of this residue revealed that it is together with Glu125 part of a catalytic dyad. Interestingly, mutation of Glu125 leads in vitro to the formation of a covalent reaction intermediate, indicating that the hydrolytic mechanism also proceeds through a covalent intermediate, which is resolved by Glu125 [Sharifi et al. 2013]. Therefore, the authors suggested a reaction mechanism resembling that of 8-oxoguanine DNA glycosylase [OGG1] (Bruner et al. 2000). Such a mechanism would involve deprotonation of Lys84 by Glu125 and an attack of the nitrogen onto the anomeric carbon with liberation of the modified glutamate/aspartate (Fig. 6B,C). Subsequently, the ribose of the resulting N-glycosidic intermediate opens up to form a Schiff base, which is susceptible to a nucleophilic water attack. This step leads to the formation of a ring-opened ADPr and enables regeneration of the catalytic lysine. While the mechanism explains neatly that the hydrolysis could occur from the 2″ or 3″ position, and so far no experimental evidence is available to determine from which position the modification is cleaved.

**The [ADP-ribosyl]hydrolase family**

The ARH family is an evolutionary highly conserved structural module adopting a mainly α-orthogonal bundle architecture with a typical domain length of 290–360 residues. The first family member was identified as an activating factor, now termed DraG, which reverses the arginine-ADP-ribosylation-inhibiting dinitrogenase reductase [Fe-protein] in *Rhodospirillum rubrum* [Ludden and Burris 1976]. An enzyme with the same activity and comparable properties, now known as ARH1, was later identified in animal cells [Moss et al. 1985].

**ARH1**

ARH1 is a cytoplasmic protein, which is ubiquitously expressed in human and mouse tissues [Moss et al. 1992]. Its primary activity is the hydrolysis of the N-glycosidic arginine-ADPribosyl bond and has negligible activity against PAR and OAADPR [Oka et al. 2006; Ono et al. 2006; Mashimo et al. 2014; Rack et al. 2018]. Deficiency of Arh1 in mouse embryonic fibroblasts (MEFs) and tissues dramatically impairs the ability to hydrolyse endogenously produced arginine-modified substrates [Kato et al. 2007], suggesting that ARH1 is the main cytoplasmic enzyme carrying out this reaction. Although the physiological role of ARH1 is not well understood, phenotypic observation on Arh1<sup>−/−</sup> mice and derived Arh1<sup>−/−</sup> MEFs suggest a leading role of ARH1 in intracellular signal transduction and cell cycle regulation. Indeed, depletion of Arh1 in MEFs led to an abnormal proliferation rate characterized...
by a shortened G1 phase and rapid cell growth compared with wild-type MEFs [Kato et al. 2011]. Consequently, it was observed that Arh1−/− and Arh1+/− mice have an increased risk of developing several types of tumors, including carcinoma, sarcoma, and lymphoma [Kato et al. 2011]. Notably, estrogens play a key role in tumorigenesis observed in Arh1−/− mice and MEFs, thus showing a significant gender-specific phenotype [Shim et al. 2013]. The involvement of ARH1 in cancer progression is confirmed by the observation of frequent human somatic mutations in the ARH1 gene in lung, breast, and colon cancers [Kato et al. 2015]. Some of these mutations directly impact the catalytic activity; e.g., the p.D56N missense mutation affects Mg2⁺ coordination and inactivates ARH1 [Kato et al. 2015; Rack et al. 2018].

In addition, ARH1 plays a role in the protection from Vibrio cholera infections [Kato et al. 2007; Watanabe et al. 2018]. Cholera toxin, which is secreted during infection, inhibits the GTPase activity of the α subunit of the stimulatory guanine nucleotide-binding (Gₛα) protein by MARylation of an arginine residue, thus maintaining Gₛα’s active form. This results in accumulation of intracellular cAMP, ultimately leading to abnormalities in fluid and electrolyte transport that are the hallmark of Vibrio cholera pathogenesis [Vanden Broeck et al. 2007; Catara et al. 2019]. Arh1−/− mice exhibit enhanced sensitivity to the toxin with significantly increased fluid accumulation in the intestinal loops [Kato et al. 2007; Watanabe et al. 2018]. Moreover, a crosstalk between arginine- and serine-ADP-ribosylation has been recently reported. Specifically, exposure of cultured cells to cholera toxin caused formation of free arginine-ADPr (Arg-ADPr), as also demonstrated earlier in vitro [Oppenheimer 1978], which then specifically inhibits the ARH1 homolog ARH3 with nanomolar affinity [Drown et al. 2018; Rack et al. 2018]. ARH1 can degrade free Arg-ADPr in vitro [Moss et al. 1986], and congruously, withdrawal of the exotoxin from the culture media restores ARH3 activity [Drown et al. 2018]. Whether inhibition of ARH3 during infection involving cholera toxin-like enzymes is part of the bacterial virulence

Figure 6. ALC1-like hydrolases. [A] Reaction mechanism for the nonenzymatic formation of a Schiff base and the Amadori rearrangement. [B] Closeup of the active site of hTARG1 in complex with the Amadori product of ADPr (yellow) and Lys84. [Magenta] Catalytic residues; [red] structural water [w310]; [dashed lines] selected polar interaction. [C] Proposed reaction mechanism for TARG1 and related ALC1-like hydrolases. Residue numbering in accordance with hTARG1. [D] Electrostatic surface map of T. aquaticus DarG. [Red] Negative surface charge; [blue] positive surface charge; [white] neutral surface charge. Note that the prominent positively charged area, which runs perpendicular to the active site, was suggested as the DNA-binding surface. The cocrystallized ADPr is depicted in CPK coloring.
ARH3

ARH3 is a ubiquitous protein conserved in Animalia and Capnaspora (Oka et al. 2006). ARH3 localizes to the cytosol, mitochondria, and nucleus, and experimental data suggest that the precise subcellular distribution may depend on cell type as well as cellular requirement (Oka et al. 2006; Niere et al. 2012, Mashimo et al. 2013). For example, ARH3 was detected in the nuclei of mouse brain and MEFs, but was absent in the ones of HepG2 cells (Oka et al. 2006; Mashimo et al. 2013; Bonfiglio et al. 2017), which suggests that ARH3 may have cell type-specific functions.

ARH3 has a key role in the hydrolysis of serine-linked ADPPr that is used in regulation of numerous proteins controlling genome stability in higher organisms (Abplanalp et al. 2017; Bonfiglio et al. 2017; Fontana et al. 2017; Palazzo et al. 2018). In vitro studies with all known [ADP-ribo-syl]hydrolases indicate that for this function no backup pathway exists in mammalian cells (Fontana et al. 2017). In addition, hydrolysis of PAR chains as well as OAAADPR has been reported for ARH3 (Oka et al. 2006; Kasamatsu et al. 2011, Mashimo and Moss 2016; Fontana et al. 2017), but in this case, alternative hydrolases exist in the cells. PAR-removing activity of ARH3 has been linked to the regulation of parthanatos, a special type of apoptosis (Mashimo et al. 2013; Dawson et al. 2017; Robinson et al. 2019).

The partial redundancy between PARG and ARH3 and the preference for serine-linkages, the most prevalently modified residue in the DNA damage response, suggests a prominent role for those enzymes in the maintenance of genome stability (Mashimo et al. 2013, 2019; Tanuma et al. 2016; Fontana et al. 2017; Palazzo et al. 2018). The increased sensitivity of human and mouse ARH3-deficient cells to hydrogen peroxide-induced cell death supports this theory (Tanuma et al. 2016; Palazzo et al. 2018). Loss-of-function mutations in ARH3 were linked to the pathogenesis of a rare recessive autosomal neurodegenerative disorder (Danhauser et al. 2018; Ghosh et al. 2018), suggesting that ARH3 contributes to the protection of neurons from endogenous ROS. In contrast, the mitochondrial function of ARH3 remains elusive, but current observations support two possibilities: First, ARH3 can degrade ADP-ribosylation artificially targeted to the mitochondrial matrix, and hence may be responsible for potential endogenous ADP-ribosylation in this compartment (Niere et al. 2012). Second, the ability to degrade OAADPr suggests a role of ARH3 in metabolite salvage and NAD recycling (Dölle et al. 2013).

DraG

Several bacteria as well as a few archaea, collectively termed diazotrophs, have the ability to convert atmospheric, molecular nitrogen into ammonia, thus making it available for the biosphere. Due to the high energetic costs associated with this process, its tight regulation is crucial. Some diazotrophs control the pivotal nitrogenase complex by reversible ADP-ribosylation of the Fe-protein, also known as the dinitrogenase reductase component. Through dedicated investigation over the last decades, this system has become one of the best-studied reversible ADP-ribosylation signaling pathways. The Fe-protein homodimer is ADP-ribosylated at a single arginine residue [Arg101 in Rhodospirillum rubrum DraG [RruDraG]] by the ARTC family member DraT [Pope et al. 1985; Ma and Ludden 2001]. This prevents formation of the nitrogenase complex, which consequently reduces nitrogen fixation. The modification is reversed by [ADP-ribo-syl-dinitrogenase reductase]hydrolase DraG [Ludden and Burris 1976; Saari et al. 1984]. Furthermore, the system is controlled by members of the PII nitrogen regulatory protein family, which directly and indirectly sense a variety of negative stimuli, including high ammonia or glutamine, low cellular energy, or absence of light (Huergo et al. 2012; Nordlund and Högbom 2013). The cellular energy status is “read” by the PI proteins GlnB and GlnK (orthologous also called GlnZ), which competitively bind ATP and ADP in a cleft at the homotrimer interphase (Xu et al. 1998; Jiang and Ninfa 2007). In vitro studies have shown that in the ADP-bound state GlnB associates with DraT, which results in its activation. Concurrently, the PI protein GlnK:ADP complex associates with DraG, leading to its partial inhibition, and further full inactivation is achieved by association of this ternary complex with the ammonia transporter AtmB, hence sequestering DraG at the cellular membrane (Rajendran et al. 2011; Moure et al. 2019). Jointly, these processes lead to inactivation of the nitrogenase complex. Binding of ATP to GlnB and GlnK is synergistic with 2-oxoglutarate, a cellular signal of nitrogen and carbon status, (Jiang and Ninfa 2007) and leads to dissociation of DraT and DraG and activation of the nitrogen fixation pathway (Gerhardt et al. 2012; Nordlund and Högbom 2013). It is noteworthy that this represents only one aspect of nitrogen fixation regulation and the system can be further fine-tuned by uridylylation of the PI proteins as well as transcriptional regulation of components of the nitrogen fixation pathway (Huergo et al. 2012; Nordlund and Högbom 2013).

Structure and function of ARH enzymes

Structurally, ARH proteins are compact and globular with a central core motif consisting of 13 orthogonal a-helices and a variable number of auxiliary helices depending on the organism and type (e.g., total number of helices: 25 in hARH1 [PDB 6G28], 22 in hARH3 [PDB 2FOZ], and 18 in RnuDraG [2WOD]). The overall fold can be subdivided into four quasidomains with the ADP-ribose binding site as well as the catalytic binuclear metal center embedded into their interphase (Fig. 7A; Mueller-Dieckmann et al. 2006; Li et al. 2009, Rack et al. 2018). Coordination of the adenosine moiety differs greatly between the different ARH classes. In DraG, the adenosine moiety is coordinated parallel to the protein surface and stacks on top of a conserved tyrosine residue (Tyr212 in RruDraG). Exact
positioning is achieved by interaction of the C6 amine and N7 nitrogen with a conserved ExxA motif (Glu121 in RruDraG). The proximal ribose makes no contacts within the binding cleft and the 2′ and 3′ OH groups are orientated toward the aqueous environment. In ARH1, the human functional equivalent of DraG, the adenosine is likewise parallel to the protein surface; however, it is shielded from the environment by π–π stacking with a conserved tyrosine residue (Tyr263 in hARH1). While comparable coordination of the C6 amine N7 nitrogen can be observed in the hARH1 structure, the corresponding residues (Ser124 and Gly127 in hARH1) are not well conserved among ARH1’s (Rack et al. 2018). The 2′ and 3′ OH groups of the proximal ribose interact with an ARH1-specific loop region, termed the adenosine-binding loop (Rack et al. 2018). In ARH3, the adenine moiety is orientated perpendicular to the protein surface and stacked between two conserved aromatic residues (Phe143 and Tyr149 in hARH3). As in DraG, the hydroxyl groups of the proximal ribose are exposed to the environment. This orientation is compatible with both endo- and exo-PAR hydrolysis, yet ARH3 endo activity has not been demonstrated so far.

All ARH-type enzymes characterized so far are activated by divalent metal ions coordinated within a binuclear metal center (Nordlund and Norén 1984; Moss et al. 1985; Antharavally et al. 1998; Oka et al. 2006). The residues involved in metal coordination are highly similar, but subclass-specific motifs could be identified (Fig. 2; Mueller-Dieckmann et al. 2006; Berthold et al. 2009; Li et al. 2009; Rack et al. 2018). Dependence on the nature of the divalent-cation was investigated for DraG and ARH3: DraG primarily uses Mn2+, with its activity also supported by Fe2+ and, to a lesser extent, Co2+ and Mg2+ (Nordlund and Norén 1984; Ljungström et al. 1989). In contrast, ARH3 primarily uses Mg2+, but can also be activated by Mn2+ (Rack et al. 2018). No detailed investigation for ARH1 was so far carried out, but it is known that Mg2+ will support its activity (Moss et al. 1985). In the unligated state, the coordination spheres of the two divalent ions are connected by a syn–syn bridging aspartate (Asp316 in hARH3) as well as a μ-aqua ligand (Fig. 7B,C). The latter is displaced upon substrate binding by the 2′′ OH group of the distal ribose both in hARH1 and LchARH3 (Rack et al. 2018). In crystallo, hARH3 can coordinate ADP-ribose even in presence of the μ-aqua ligand albeit with unusually short coordination bonds (Pourfarjam et al. 2018; Wang et al. 2018). Therefore, details of the ligand binding under more physiological conditions remain to be elucidated. However, it is clear that the correct positioning of the substrate in the active site requires both metal ions to be present as well as the cis 2″′ and 3″′ OH groups of the distal ribose of the substrate (Pourfarjam et al. 2018; Rack et al. 2018; Wang et al. 2018). The observed arrangement of ligands in the active site also gives a structural explanation for the observed selectivity toward α-1″′-linkages by ARH1 and ARH3 (Moss et al. 1986; Voorneveld et al. 2018). Interestingly, ligand binding was also associated with conformational changes near the active site: One of

Figure 7. ARH structure and mechanism. (A) The left panel shows a ribbon representation of LchARH3 in complex with the ADP-ribose analog ADP-HPD (CPK coloring; PDB 6HH3). The conserved 13 α-helical core motif is colored according to quasidomain classification. (Red) A; (green) B; (yellow) C; (blue) D. The right panels show a close-up of the metal coordination of LchARH3 in complex with Mn2+ [dark gray] and RruDraG in complex with Mg2+ [mauve]. (B) Schematic representation of metal coordination defining the metal-to-metal distance. (C) Schematic representation of the dinuclear metal center. Both metals [dark gray] are octahedral coordinated. Ligands in the first coordination sphere are protein-derived monodentates [white], water [red], μ-aqua [purple], and syn-syn-bridging carboxyl [yellow]. Note that axial position 6 of MeII can be occupied by either water or glutamate, depending on the conformation of the Glu flap. (D) Potential reaction mechanisms for ARH3-type enzymes. Residue numbers according to hARH1.
the axial positions of the metal ion II (MeII) shows flexible occupation either by an \( \mu \)-aqua ligand or glutamic acid residue (Glu41 in hARH3) [Fig. 7C]. The loop containing the latter, termed the Glu flap, can undergo conformational changes and it was proposed that coordination of Mg\(^{2+}\) in ARH3 by Glu41 represents as a closed, self-inhibitory state, and that displacement of the loop is a prerequisite for substrate binding [Pourfarjam et al. 2018]. In addition to the conformational change, the glutamate residue is crucial for enzymatic activity of ARH3 [Mueller-Dieckmann et al. 2006; Apblanjal et al. 2017; Rack et al. 2018]. Beyond this common set of metal coordination features, DraG enzymes contain an additional, highly conserved aspartate [Asp97 in RtuDraG, absent in ARH1 and ARH3] in proximity of MeI. While direct contacts with a corecystallized Mn\(^{2+}\) ion could be observed in RtuDraG [Berthold et al. 2009], the interaction was absent in a structure of the Azospirillum brasilense homolog (AbrDraG) in complex with Mg\(^{2+}\) [Li et al. 2009].

So far only one structure of a DraG-type hydrolase in complex with ADPr is available [Berthold et al. 2009]. The electron density of the RtuDraG:ADPr complex showed an Amadori product similar to TARG1 [Fig. 6A]. However, in contrast to TARG1, the lysine reacting with the active site-bound ADPr is donated from a neighbouring protomer in the crystal packing rather than part of the active site itself. Secondly, it was shown that mutating Glu28 (RtuDraG), the structural homolog residue of the Glu flap glutamate, has only a minor effect on catalytic activity, while Asp97 is crucial [Berthold et al. 2009]. Comparison of the structures of RtuDraG and AbrDraG reveals stark differences in terms of metal coordination: While AbrDraG adopts a coordination similar to ARH1 and ARH3 [see above, Li et al. 2009], in the RtuDraG structure the geometry appears to be rotated by \( \sim 90^\circ \), which results in an axial positioning of the \( \mu \)-aqua ligand [Fig. 7A,C]. In this conformation, the \( \mu \)-aqua can act as a nucleophile attacking the Schiff base intermediate at C1′′ [Berthold et al. 2009]. However, the geometry observed in the RtuDraG crystal structure does not include a bridging carboxyl group as predicted from earlier electron spin resonance measurements and observed in all other ARH structures [Anthravally et al. 1998; Mueller-Dieckmann et al. 2006; Li et al. 2009; Pourfarjam et al. 2018; Rack et al. 2018, Wang et al. 2018]. While details of the DraG mechanism remain elusive, the data point to a prominent role of MeI in the reaction mechanism.

ARH1, the functional homolog of DraG, is mechanistically even less understood, but first structural insights have been gained recently [Rack et al. 2018]. Its structural features are a hybrid of DraG and ARH3 with the absence of the DraG-specific aspartate [Asp97 in RtuDraG; similar to ARH3] as well as increased constraints on the Glu flap flexibility [similar to DraG] [Pourfarjam et al. 2018; Rack et al. 2018]. Further studies are needed to understand the hydrolytic mechanism and reveal how far the similarities between ARH1 and the other ARH classes stretch.

The recently solved structures of ARH3 lead to the proposal of different catalytic mechanisms [Pourfarjam et al. 2018; Rack et al. 2018; Wang et al. 2018]. Absence of the \( \mu \)-aqua in ligand-substituted ARH3 structures indicates that it is dispensable for the catalytic mechanism [Rack et al. 2018]. However, the available data point toward a closer engagement of the substrate with MeI and a more structural role for MeII [Pourfarjam et al. 2018; Rack et al. 2018]. Furthermore, computational modeling and biochemical evidence suggest that the axial water in position 6 (Fig. 7C) is displaced with the C1′′ substituent, which is either the O-glycosidic serine linkage or 1′′ scissile bond of PAR. In this conformation, the \( \beta \) face of the distal ribose would be accessible for a nucleophilic attack of a Glu flap-activated water molecule. This would lead to an S\( _{\text{N2}} \)-type reaction intermediate and formation of an oxyanion [Fig. 7D]. Alternatively, direct protonation of the leaving group by Glu41 is possible and would result in the formation of an oxocarbenium intermediate [Fig. 7D]. Further studies focusing on the interaction with true substrates are needed to elucidate the details of the reaction mechanism.

Reversal of nucleic acid ADP-ribosylation

Within the realm of ADP-ribosylation signaling, modification of DNA and RNA phosphor-termini is a newly emerging field of study [Talhaoui et al. 2016, Munnur and Ahel 2017; Munir et al. 2018b, Munnur et al. 2019]. While the cellular functions are as yet elusive, the association of this modification with DNA repair as well as antiviral PARPs suggests functions in DNA damage repair and antiviral defence. One possibility is that DNA ADP-ribosylation may act as a reaction intermediate similar to DNA adenylation during DNA ligation [Lehnman 1974; Pascal 2008; Tanabe et al. 2015]. This hypothesis is particularly interesting, as a recent study suggests that human DNA ligase IV, involved in damage repair, can use NAD\(^+\) [Chen and Yu 2019]. Alternatively, capping of 5′ phosphates could have a protective function to preserve the phosphorylation until the required repair factors are assembled at the damage site. In contrast, presence of a 3′-phosphate can interfere with efficient repair and it has been suggested that \( E. \text{coli} \) primes such position for repair by attachment of a guanyl-cap [Chauveau et al. 2015]. As for RNA, ADP-ribosylation may contribute to the recognition and/or processing of exogenous and hazardous RNAs, e.g., transposon-derived noncoding or viral RNAs.

Regardless of the exact physiological role, the modification of 3′- and 5′-phosphor termini is reversible by the action of PARG, MacroD1/2, TARG1, and ARH3 [Munnur et al. 2019]. This diversity of enzymes capable of removal may be surprising given the diversity of hydrolytic mechanisms discussed above. However, this may at least partially be the result of the inherent properties of the enzymes and the substrate: 1) a high degree of accessibility of DNA/RNA ends relative to most modifications confined within a protein structure; 2) formation of the phosphate product is favorable in comparison with other reaction intermediates, thus supporting hydrolysis; and 3) ARH3 as well as macrodomains bind ADPr with high
affinity and hence are predicted to interact with ADPr ad- ducts readily as long as the linked group does not clash with the structure of the hydrolase. Together, the relative nonspecificity of ADPr hydrolysis from nucleic acid ter-
mini suggests that it is regulated through recruitment or exclusion of hydrolases from the cellular context in which this modification occurs, but further studies are needed to elucidate the exact similarities and differences in the hydrolysis catalyzed by the various enzymes as well as the exact nature of their regulation.

Conclusions and perspectives

The examples discussed in this review reflect the increasingly compelling view that [ADP-ribosyl]hydrolases deserve a more prominent role in the investigation of ADP-ribosyl signaling. Understanding their molecular function and substrate specificities will allow us to link them more conclusively to the specific ARTs and thus create a direct functional relationship between “readers” and “writers.” Beyond the immediate biochemical connection, it is our hope that future studies will use these links to elucidate the role of the hydrolases in their specific signaling pathways. In this context, it is important to note that the study of hydrolases should be extended beyond the human realm since many [ADP-ribosyl]hydrolases in plants, pathogenic organisms, and model systems among pathogens have still unclear functions (de Souza and Aravind 2012, Perina et al. 2014, Aravind et al. 2015, Zhang et al. 2015a, Gunn et al. 2016, Haikarainen and Lehtio 2016, Lalic et al. 2016, Zapata-Pérez et al. 2017).

Future efforts in the development of small molecule inhibi-
tors will hopefully produce new probes to study the [patho-]physiological roles of these fascinating enzymes as well as lead to new drugs with therapeutic applications. The potential of such an approach was highlighted over recent years with the development of PARG inhibitors finding their application in cancer therapy (James et al. 2016, Gravells et al. 2017, Waszkowycz et al. 2018).

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