Poly(ADP-Ribose)-Dependent Ubiquitination and its Clinical Implications

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Abstract

ADP-ribosylation—the addition of one or multiple ADP-ribose units onto proteins—is a therapeutically important post-translational modification implicated in cancer, neurodegeneration, and infectious diseases. The protein modification regulates a broad range of biological processes, including DNA repair, transcription, RNA metabolism, and the structural integrity of nonmembranous structures. The polymeric form of ADP-ribose, poly(ADP-ribose), was recently identified as a signal for triggering protein degradation through the ubiquitin-proteasome system. Using informatics analyses, we found that these ubiquitinated substrates tend to be low abundance proteins, which may serve as rate-limiting factors within signaling networks or metabolic processes. In this review, we summarize the current literature on poly(ADP-ribose)-dependent ubiquitination (PARdU) regarding its biological mechanisms, substrates, and relevance to diseases.

Graphical Abstract

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PARdU Regulation of Rate-limiting factors
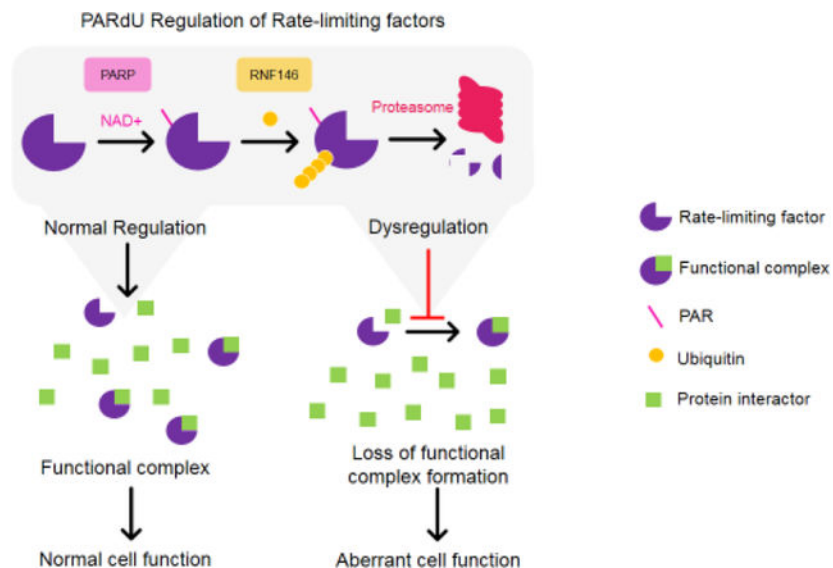
Keywords

ADP-ribosylation; Poly(ADP-ribose); Poly(ADP-ribose)-dependent ubiquitination; Ubiquitin-Proteasome System; Protein degradation; Rate-limiting factors; Tankyrase

1. Introduction

Post-translational modification regulates protein function through the addition of polypeptides or chemical groups. Post-translational modification enables a relatively limited number of genes to generate a far greater number of proteoforms with differential functionalities, localizations and stabilities compared to the unmodified form [1]. Here we focus on a protein degradation process that requires two post-translational modifications: ADP-ribosylation and ubiquitination.

1.1 ADP-ribosylation

ADP-ribosylation refers to the covalent addition of one or more ADP-ribose (ADPr) onto proteins, whereby the ADPr units are transferred from NAD^+ by ADP-ribosyltransferases, including a subset commonly known as Poly(ADP-ribose) Polymerases (PARPs) [2–7]. The modification exists as a monomeric [mono(ADP-ribosyl)ation or MARylation], or polymeric form [poly(ADP-ribosyl)ation or PARylation] (Figure 1). Amongst the 17 PARPs in humans, four synthesize poly(ADP-ribose) (PAR; PARPs 1, 2, 5a and 5b), two are catalytically dead (PARPs 9 and 13), and the rest synthesize mono(ADP-ribose) (MAR) based on automodification analyses [2]. While PARP5a only synthesizes linear PAR chains [3], the founding member PARP1 can also synthesize branched forms of poly(ADP-ribose) [4], further increasing the structural complexity of PARylation (Figure 1). PARylation has been implicated in a number of biological processes, including DNA damage-response [5], chromatin organization [6,7], liquid-liquid phase transition for the assembly of nonmembranous structures [8,9], as well as the focus of this review—protein degradation through ubiquitination.

1.2   Ubiquitination

Ubiquitin is a small protein of 8.5 kDa that is covalently attached to substrates at lysine residues [10,11]. Ubiquitin itself can also be modified on any of its own seven lysines by another ubiquitin molecule, resulting in a variety of different ubiquitin chains with unique linkages and functions [11]. For example, poly-ubiquitin chains linked at the lysine-48 (K48) position are targeted for proteasomal degradation while K63-linked chains play signaling roles, for example, during DNA repair. The conjugation of ubiquitin to target proteins requires an enzymatic cascade of several proteins [12]. An ubiquitin is activated by the ubiquitin-activating enzyme E1 with ATP, then the activated ubiquitin is transferred to an ubiquitin-conjugating enzyme (E2) before finally conjugating to the protein targets through an E3 ubiquitin ligase, which confers substrate specificity [13]. There are three main types of E3 ligases, which differ in their domain architecture and the mechanism of ubiquitin transfer [14]. Both HECT (Homologous to the E6AP Carboxyl Terminus) and RBR (RING-between-RING) E3 ligases first transfer ubiquitin to a catalytic cysteine on the enzyme before transferring ubiquitin to its substrate [15, 16]. RING (Really Interesting New Gene) E3 ligases position the E2 and the target substrate for direct ubiquitin transfer [17,18]. This review focuses on a RING E3 ligase that possesses a domain that binds PAR to trigger ubiquitination, a process recently coined PAR-dependent ubiquitination (PARdU) [19]. Here we will take a historical perspective on its discovery, explore the underlying biological mechanisms, and highlight contemporary strategies for substrate identification. The review will conclude with a discussion of the open questions regarding the mechanism of PARdU, its disease relevance, and the potential therapeutic opportunities.

2.   A brief history of PARdU

Current understandings of the mechanism of PARdU have been assembled from several independent studies (Figure 2). PARdU was first discovered through identifying the target of the *Wnt* signaling inhibitor XAV939, which inhibits PARP5a/b (commonly referred to as Tankyrase1/2) [20]. Inhibition of PARP5a/b increases the protein level of the rate-limiting factors within the *Wnt* signaling pathway—Axin1/2 [20]. Subsequent studies revealed that degradation of Axin1/2 was mediated by the E3 ligase RING finger protein 146 (RNF146), which is activated by binding of PAR [21–23]. The substrates of PARP5a/b, such as Axin1/2, possess an amino acid motif defined as the "Tankyrase binding motif" (TBM), which was later shown to be critical for PARdU [24–26]. Additional PARdU substrates, such as 3BP2 and PTEN, were then defined and characterized, with hundreds of candidate substrates identified in recent proteomics studies [21,26–29]. These studies provide the foundation to elucidate the mechanism of how PARylation leads to ubiquitination and protein degradation.

2.1   Identification of the WWE domain and PARP5a/b binding motif

In 2001, a PSI-BLAST search to investigate an 80 amino acid-stretch in the notch-signaling protein Deltex revealed a novel domain called WWE, named after its most conserved residues [30]. The WWE domain is found among several RING and HECT E3 ubiquitin ligases as well as five PARPs, leading to the authors' prediction that both enzyme classes share similar targets [30]. Subsequent biochemical studies revealed that the E3 ligase

RNF146 WWE domain binds to *iso*-ADP-ribose (*iso*-ADPr)—the shortest internal unit of PAR—with ~370 nM affinity (Figure 1; Figure 3A). The binding to PAR by RNF146 via the WWE domain later proved to be critical to activate the E3 ubiquitin ligase activity.

Around the same time in the early 2000s, an investigation of PARP5a/b binding to the protein IRAP revealed that IRAP binds an ankyrin repeat cluster (ARC), a conserved domain within PARP5a/b, through the specific hexapeptide motif: RQSPDG [24,25]. Mutagenesis studies of the known IRAP interacting motif revealed that the Q and S residues are less important for binding and that the RXXPDG motif is sufficient for a functional TBM. This notion was further confirmed in two other PARP5a/b binding proteins, TRF-1 and TAB182 [25]. Using a peptide library screen, Guettler and colleagues further defined an 8-residue consensus R-X-X-[small hydrophobic or G]-[D/E]-[G]-[no P]-[D/E] that binds to ARCs, resulting in a ranked list of thousands of proteins that match to this peptide motif [26]. In combination with structural studies of the PARP5a/b ARCs binding to peptides [31–33] (e.g., Figure 3B), models to predict PARP5a/b interactors were further improved [34], enabling the prediction of additional PARdU candidates.

2.2. Discovery of PARdU while characterizing the effect of PARP5a/b inhibitor in Wnt signaling

Fundamental to the discovery of PARdU was the identification of the PARP5a/b inhibitor XAV939 through a high-throughput screen of inhibitors of the *Wnt*/β-catenin signaling pathway [20]. XAV939 treatment increased the protein levels of the β-catenin destruction complex component Axin1/2 in cells. Using an immobilized XAV939 analog, an affinity-capture technique was used to identify molecular targets that bind to this small molecule from cell extracts. Several PARPs bound to XAV939, although with varying affinities: PARP1 (K_d = 1.2 μM), PARP5a (K_d = 0.099 μM) and PARP5b (K_d = 0.093 μM). Additionally, knockdown of PARP5a/b, but not PARP1, led to an increase in Axin1/2 protein level similar to XAV939 treatment. The use of IWR-1—a previously published inhibitor of the *Wnt* signaling pathway shown to increase Axin1/2 levels—also inhibited PARP5a/b activity, further supporting that the catalytic activity of PARP5a/b is critical for regulating Axin protein level [22,35]. Using Axin1 as an example, Huang and colleagues further showed that Axin1 interacted with PARP5a/b through a conserved TBM [20], and its disruption by mutagenesis resulted in high Axin1 protein levels. These data suggest that Axin1/2 regulation by PARP5a/b requires both PARylation activity and physical interactions. In addition, Axin1/2 regulation by PARP5a/b was identified to be mediated via the ubiquitin-proteasome system; treatment with the proteasome inhibitor MG132 increased levels of polyubiquitinated Axin1/2 [20,22]. The authors of these studies concluded that XAV939 may stabilize Axin1/2 by preventing its ubiquitination, consistent with later findings that PARylation of Axin1/2 leads to its ubiquitination and subsequent degradation [20–23]. However, it would take two years before the link between PAR and ubiquitination was uncovered through the discovery of the first known PAR-dependent E3 ubiquitin ligase, RNF146 [17–19,32].

2.3 RNF146 is the missing link of PARdU regulation of Axin1/2

RNF146 (also known as Iduna) was initially discovered as a gene induced in response to the N-methyl-D-aspartate (NMDA) glutamate receptor [36]. Later work revealed that RNF146 possesses two highly conserved domains: the WWE and RING finger domains [21,37–39]. Overexpression of RNF146 in neurons protected against NMDA-induced cell death caused by PARP1 activation and subsequent PAR synthesis [40–42]. In 2011, several groups independently identified RNF146 as the E3 ubiquitin ligase that ubiquitinates Axin1/2 [21–23]. Specifically, the binding of RNF146 to Axin1/2 and subsequent regulation of Axin1/2 protein levels was dependent upon the WWE domain of RNF146 [21]. Importantly, the interaction between RNF146 and Axin1/2 is abolished upon PARP5a/b inhibitor treatment and Axin1/2 PARylation in cells is reduced upon XAV939 treatment [21,22]. These findings suggest that (1) RNF146 regulation of Axin1/2 is mediated through PAR-binding ability of WWE, and (2) Axin1/2 PARylation is PARP5a/b-dependent, thereby linking PARP5a/b activity and PAR-binding ability of RNF146 in this novel PARdU pathway. A yeast two-hybrid screen for other PARP5a/b substrates in one of these pioneering studies further revealed two TBM-containing proteins (BLZF1 and MLN51) as potential PARdU candidates [21]. The protein levels of GFP-tagged BLZF1 and MLN51 proteins were increased upon PARP5a/b inhibitor treatment as well as knockdown of PARP5a/b or RNF146. Both GFP-tagged BLZ1 and MLN51 require a functional TBM for an increase in protein level to be observed upon these chemical or genetic perturbations, further suggesting that these proteins are likely PARdU substrates. Yet, it is unclear whether these two proteins are endogenous substrates of PARdU.

The PAR-binding ability of RNF146 and its roles in ubiquitination have also been explored in the context of DNA damage. RNF146 binds to many DNA damage-associated proteins, such as PARP1, XRCC6, DNA Polymerase III and KU70 in a PAR-dependent manner [38]. An in vitro ubiquitination assay showed that PAR-binding to RNF146 activates the ubiquitin ligase activity, and this PAR-dependent ligase activity protects cells from NMDA-induced cell death and DNA damage [38].

2.4. Structural Insights into PARdU

After the discovery was made that RNF146 requires PAR for its E3 ubiquitin ligase activity [21,23,38], efforts were focused on determining which structures of PAR were important for RNF146 activation. Biochemical analysis revealed that RNF146 does not bind to the ADPr monomer, but instead binds avidly to the smallest internal subunit of PAR, *iso*-ADPr (Figure 1) [37]. In particular, *iso*-ADPr binding is sufficient to activate E3 ubiquitin ligase activity [19]. X-ray crystallography studies showed that the adenine ring of *iso*-ADPr fits into the half-β-barrel pocket of the WWE domain structure, and the phosphate-ribose moieties on either side of the *iso*-ADPr structure make specific contacts with WWE domain residues [37], providing insights into why RNF146 binds *iso*-ADPr but not ADPr (Figure 3A). Without *iso*-ADPr or PAR, RNF146 is unable to transfer E2-conjugated ubiquitin to its lysine substrate [19]. RNF146 protein that contains a RING domain and a WWE domain binds *iso*-ADPr with ten-times higher affinity compared to the RNF146 WWE domain alone, suggesting a plausible role of the RING domain in *iso*-ADPr binding [19]. Comparisons between the apo-structure and *iso*-ADPr bound RNF146 revealed that the

RING domain adopts a conformation with a more available E2 binding surface upon ligand binding [19].

Additional binding domains of RNF146 and PARP5a/b play a role in PARdU. The Sterile Alpha Motif (SAM) domain of PARP5a/b enables PARP5a/b polymerization and facilitates its localization to cytoplasmic signaling complexes enriched with Axin1/2 [43,44]. Disruption of PARP5a/b polymerization via mutation of the SAM domain prevents PARP5a/b regulation of canonical *Wnt*/β-catenin signaling, suggesting that PARP5a/b polymerization promotes its catalytic activity and PARdU [43,44]. Further structural studies of the PARP5a/b ARC domain indicate that certain ARCs function together to aid in substrate binding [31]. ARCs vary in rigidity and flexibility, serving as a versatile binding platform for diverse PARP5a/b interactors [31]. RNF146 possesses one canonical (section 2.1) and four non-canonical TBMs [45]. The non-canonical TBMs of RNF146 are "extended" with additional amino acids between the conserved Arg and residue in position 4 (small hydrophobic or Gly residue) [19] (Figure 3B). The strongest binding non-canonical motif (Motif I), which is the most phylogenetically conserved, binds to PARP5a at 5.8 μM similar to canonical TBMs, which are in the range of 0.3–20 μM [15,22,41]. Individual RNF146 TBMs bind weakly to PARP5a but together facilitate strong multivalent binding [19,45]. Thus, in addition to the individual enzymatic activities, PARdU is mediated through multiple binding interactions of PARP5a/b and RNF146, as well as their interactions with the substrates.

3. Characterized PARdU substrates

Three methods have been commonly used to characterize PARdU substrates (Table 1): (1) PARP5a/b inhibitors, such as XAV939 and IWR-1, have been used to show that PARP5a/b catalytic activity regulates the protein level of substrates; (2) the PARdU substrate interacts with PARP5a/b and/or RNF146; (3) the PARdU substrate increases following RNAi-mediated knockdown of PARP5a/b and/or RNF146. Conversely, PARP5a/b or RNF146 overexpression may decrease substrate levels. Although mass spectrometry methods for identifying ADP-ribosylated sites on PARdU substrates are available (reviewed in [46]), only one study (PTEN) so far has identified the site of ADP-ribosylation necessary for ubiquitination and subsequent degradation [47]. Additionally, an in vitro ubiquitination protocol by RNF146 has been established [38] and could potentially be used to determine which site of ADP-ribosylation is necessary for binding and ubiquitination by RNF146. Below we will highlight several characterized PARdU substrates and their biological roles:

Axin1/2:

Axin1/2, the first well-defined substrate that was pivotal in uncovering PARdU [21–23,35], is integral to the β-catenin destruction complex and negatively regulates canonical *Wnt* signaling [48–50]. *Wnt* signaling plays a critical role in development by regulating cell proliferation, differentiation and survival [48–50]. Axin1/2 degradation by PARdU may prevent the formation of the β-catenin destruction complex, resulting in the constitutive activation of *Wnt* signaling and leading to aberrant cell proliferation observed in cancer [48–50]. Consistently, PARP5a/b and β-catenin are overexpressed in lung adenocarcinoma A549

cells, and PARP5a/b inhibition with inhibitor XAV939 reduces cell proliferation of the cell line [51]. In addition, the ubiquitin-specific protease USP25 antagonizes *Wnt* signaling by promoting deubiquitination and stabilization of PARP5a/b, and disruption of PARP5a interaction with USP25 destabilizes PARP5a/b, leading to Axin1/2 stabilization and subsequent attenuation of *Wnt* signaling [52]. However, PARdU regulation of Axin1/2 and *Wnt* signaling play diverse roles beyond cell proliferation. RNF146-mediated degradation of Axin1/2 is also required for proper embryonic development in *Xenopus* embryos, where depletion of RNF146 leads to downregulation of critical pattern-organization proteins [53].

3BP2:

Before 3BP2 was identified as possessing a TBM, it was known that mutations in the gene encoding for 3BP2 were the cause of the bone disease cherubism [54–56]. Further study found that the mutations in the 3BP2 gene are often in the region encoding the TBM [27]. When PARdU-regulated degradation of 3BP2 is disrupted, elevated 3BP2 protein levels persist, and osteoclast formation and function are promoted in vitro, consistent with the pathogenesis of cherubism [27]. 3BP2 has been further identified as an adaptor protein that plays a role in several signaling pathways important for osteoclast and immune-cell function, including the SRC, SYC, and VAV signaling pathways [27].

PTEN:

PTEN is a tumor suppressor, and knockdown of PARP5a/b has been found to stabilize PTEN, and slow cell proliferation [47]. Recent work by Li and colleagues have identified PTEN as a novel PARdU substrate that binds to both PARP5a (via a non-canonical RYQEDGFD motif) and RNF146, as shown through biochemical methods (Table 1) [47]. Importantly, the PARdU regulation was inhibited upon mutating ADP-ribosylated sites identified from recent proteomics studies, suggesting specific sites of ADP-ribosylation may signal for ubiquitination and subsequent degradation.

AMOT-family proteins:

After the initial observation that PARP5a/b inhibition or RNF146 knockdown perturbed the localization of proteins important for cellular tight junction integrity, a screen for interactors for both RNF146 and PARP5a/b identified the AMOT-family proteins, e.g., AMOTL2 [57]. AMOT proteins play a role in maintaining tight junctions and can affect the localization of other tight junction proteins if over-expressed. Therefore, controlling AMOT protein levels through PARdU is critical for preserving epithelial integrity [57]. Additionally, PARP5a/b inhibition suppresses the nuclear functions of the Hippo pathway effector and oncoprotein YAP through stabilization of AMOT-family proteins, suggesting PARdU of AMOT-family proteins may play a role in regulation of Hippo signaling [58].

PARP1:

PARP1, the founding PARP family member, was also identified as a PARdU substrate in one of the pioneering studies identifying RNF146 as a PAR-dependent E3 ubiquitin ligase [38]. PARP1 PARylation is required for RNF146-mediated ubiquitination of PARP1, and PAR binding ability of RNF146 is required to decrease PARP1 protein levels [38]. PARP1 plays a

crucial role in DNA damage repair and participates in the PAR-dependent cell death pathway, parthanatos [40,59]. PARP1 is auto-modified following recognition of DNA damage [60], resulting in recruitment of DNA damage repair proteins to the sites of damage [61,62]. Thus, PARdU may control the right amount of PARP1 for effective DNA repair or remove PARP1 from DNA damage sites.

RNF146 and PARP5a/b:

Importantly, RNF146 and PARP5a/b are substrates of one another [21,38]. Therefore, chemical inhibition or genetic knockdown of one leads to the increase in protein levels of the other, which may have other downstream effects that should be considered.

Notably many of these characterized PARdU substrates are rate-limiting factors of signaling pathways. Rate-limiting factors are proteins whose intracellular abundances must be maintained at particular levels to ensure proper cellular function. For example, Axin1/2 is critical to canonical *Wnt* signaling and formation of the β-catenin destruction complex [63]. In another example, genetic mutation of the TBM stabilizes the 3BP2 protein, and it is no longer regulated by PARdU. Elevated and stabilized 3BP2 contributes to downstream hyperactivation of SRC, SYC, and VAV signaling pathways, resulting in the disease pathogenesis of cherubism [27]. In these examples, PARdU regulation seems to be necessary for maintaining the abundance of these proteins at a critical level. To test whether this pattern holds true for all characterized PARdU substrates, we examined four datasets that quantitated the copy number of individual proteins in human cell lines [64–67]. Intriguingly, most characterized PARdU substrates are at least 10-fold lower in abundance than the median copy number of all proteins and   100-fold lower than the mean copy number across the four different studies (Figure 4). Similarly, two previously identified PARdU candidates BLZF1 and MLN51 are also low in abundance [21]. One possibility is that the observed low abundance could be because the protein level is suppressed by PARdU. However, given that these characterized substrates do not increase by 10–100 fold upon knockdown of PARP5a/b or RNF146, other factors must be at play in selecting which cellular proteins are PARdU substrates. We postulate here that PARdU may regulate low-abundance proteins that are rate- or concentration-limiting factors in their respective biological processes. Notably, the level of PARP5a protein in the cell is also similar to the protein levels of other characterized PARdU substrates. Therefore, PARP5a/b may be a rate-limiting factor in PARdU, which, in turn, influences the abundance of many substrate proteins.

4. Global analyses of the PARP5a/b interactome and potential PARdU substrates

Given that all characterized PARdU substrates interact with PARP5a/b, recent efforts have been focusing on identifying novel substrates through identifying these binding interactors. While in silico studies from Guettler and colleagues have generated a number of PARdU candidate substrates by identifying proteins possessing one or more TBM [26], two recent proteomics studies identified PARP5a/b interactors using immunoprecipitation [28] and determined which proteins change in abundance upon PARP5a/b knockout [29].

To identify PARP5a/b interacting proteins, Li and colleagues have generated 293T cells that stably express tagged PARP5a/b. After treatment of these cells with either DMSO (control) or PARP5a/b inhibitor XAV939, they immunoprecipitated these tagged constructs and performed mass spectrometry analysis to identify the PARP5a/b binding partners under these conditions [28]. All but one previously identified PARdU substrate (3BP2) was identified as a high confidence interactor. Significant overlap was observed between the PARP5a/b interactors identified in DMSO compared to the PARP5a/b inhibitor-treated condition, suggesting that the enzymatic activity of PARP5a/b is not critical for maintaining PARP5a/b interactions. Gene ontology revealed that PARP5a/b interactors participate in diverse signaling pathways, from *Wnt* and Hippo signaling, to hypoxia, autophagy, and Rho GTPase signaling. Consistent with the diverse roles in signaling pathways, PARP5a/b interactors localize throughout the cell, from the mitochondria to the Golgi and outwards to cellular membranes and the cytoskeleton. These observations paint a picture of PARP5a/b as a critical node linking diverse cellular processes through physical associations regardless of its enzymatic activity.

An additional proteomics screen by Bhardwaj and colleagues was performed to identify proteins whose abundance is altered in the absence of PARP5a/b [29]. 287 proteins increased in abundance following PARP5a/b double knockout, of which 74 possess TBMs. Amongst these 74 candidates, some of them have also been demonstrated to increase in abundance following PARP5a/b inhibition, suggesting these proteins are likely PARdU substrates. These proteins participate in diverse biological processes including *Wnt* signaling, microRNA processing, Notch signaling, and glucose transport. Li et al. and Bhardwaj et al. have each identified enrichment in Hippo signaling, suggesting a potential role of PARP5a/b in this pathway [28,29].

Upon comparison of these three studies, 13 proteins were identified to overlap in all studies (Figure 5A). They have three characteristics common for PARdU substrates: possession of a PARP5a/b binding motif, interaction with PARP5a/b, and increased abundance upon PARP5a/b knockout. Two established substrates (AMOTL2 and RNF146) appear in this set of 13 proteins, suggesting that this list may posit candidate PARdU substrates [21], that warrant future investigation. Intriguingly, we also found that most of these PARdU candidates are of low abundance, including multiple ones that cannot be detected by mass spectrometry in all studies analyzed (Figure 5B). Given that many of these substrates were curated in ADPriboDB with site information [68], it will be of interest to test whether any specific sites of ADP-ribosylation plays a role in PARdU.

5. Open questions

While it has been established that RNF146 binds PAR through its WWE domain and that binding leads to a conformational change that activates its E3 ligase activity [21,37,38], several questions remain regarding PARdU. Could other E3 ubiquitin ligases be activated upon PAR binding? Could other PARPs besides PARP5a/b synthesize the modification necessary for E3 ubiquitin ligase binding, activation and ubiquitination?

5.1   Other PARPs?

Binding of RNF146 to *iso*-ADPr requires linear PAR, which can be synthesized by PARP5a/b, and also by PARPs 1 and 2 [2,69,70]. Indeed, RNF146 is known to bind PARP1 and PARP2 [38,71,72], suggesting the possibility of the involvement of these PARPs in PARdU. Notably, a recent report suggests that PARP1 mediates PARdU of the E3 ubiquitin ligase Siah1, which in turn regulates the level of protein factors that are critical for HIV-1 transactivation [73]. The diversity of PARP localization presents a possibility that different pools of substrates can be targeted for PARdU by different PARPs (e.g., PARP1/2 in the nucleus versus PARP5a/b in the cytoplasm and nucleus [74]), adding an additional layer of specificity to the PARdU mechanism. In addition, it is possible that PARylation can be mediated through sequential actions of different PARPs, where one PARP adds MAR to its substrate, and then PARP5a/b or other PAR-synthesizing PARPs add additional ADPr units. The resultant *iso*-ADPr structure or PAR may then activate RNF146 for subsequent ubiquitination of the substrate. A recent study of auto-PARylation of PARP-1 suggests this possibility, where the initial ADPr was conjugated at a lysine residue by a non-PARP enzyme SIRT6 [75]. Notably, proteasomal degradation of Zika virus nonstructural proteins ns1 and ns3 depends on the catalytic activity of PARP12, which can only add single ADPr units [76]. It is unclear whether MARylation is sufficient for such degradation or an endogenous PARP synthesizes PAR on the substrates for PARdU.

5.2   Other E3 Ubiquitin Ligases?

Besides RNF146, multiple groups have noted that DTX1–4 (deltex homologs 1–4), HUWE1 (HECT, UBA, and WWE domain containing 1), and TRIP12 (thyroid hormone receptor interactor 12) are WWE-containing E3 ubiquitin ligases, which may regulate protein turnover in a PARylation-dependent manner [30,37]. Supporting this possibility, Callow and colleagues reported Axin1/2 can be stabilized upon silencing of PARP5a/b, but not RNF146, in a colorectal cancer cell line [22]. Notably, two classes of E3 ubiquitin ligases—HECT and RING E3 ubiquitin ligases—possess WWE domains, and they employ different catalytic mechanisms [77]. While RING E3 ligases (e.g., RNF146, DTX1–4) aid in the direct transfer of ubiquitin from an E2 ubiquitin carrier protein to protein substrates, HECT E3 ubiquitin ligases (e.g., HUWE1, TRIP12) form a covalent intermediate with ubiquitin before transferring the ubiquitin moiety to substrates (Section 1.2). If HECT E3 ligases are found to have functional WWE domains that bind *iso*-ADPr, it is worth questioning whether PAR binding is necessary for HECT E3 ligase activation? Or is binding to PAR enough to bring the E3 into proximity with its substrate? However, not all WWE domains bind *iso*-ADPr to the same extent, despite the fact that the domain is highly conserved [37,78]. For example, DDHD2 possesses a WWE domain that fails to bind PAR [37]. On the other hand, E3 ligases may possess non-WWE PAR-binding domains or motifs [79,80]. For example, RING-type E3 ligase CHFR possesses a PAR-binding zinc finger motif [81,82]. Future studies should determine whether other E3 ligases are PAR dependent, and if so, which pool of substrates these new PARdU players target.

5.3 Other PARdU Mechanisms?

While most current data support the model that RNF146 binds to and ubiquitinates a PARylated substrate, an alternative model is possible based on existing data (Figure 6). Kang and colleagues observed that RNF146 interacts with PARP1 only when PARP1 is PARylated, suggesting that the interaction between RNF146 and PAR, and not that between RNF146 and PARP1, determines whether PARP1 undergoes PARdU [38]. These data suggest that the activation of the E3 ligase does not require binding to PARylated substrates and is instead mediated by PAR. Perhaps, RNF146 binding to a PAR molecule is sufficient for the activation of its E3 ligase activity and ubiquitination of substrates, especially to those PAR binding proteins that are proximal to the ligase. Furthermore, autoubiquitination of RNF146 increases in a dose-dependent manner with the addition of free PAR polymers [38]. Taken together, these data suggest that free PAR polymer, if it exists in cells, or PAR conjugated to protein may play a role in activating RNF146 activity.

6. Considerations on exploiting PARdU to treat diseases

Several PARdU substrates, such as Axin1/2, 3BP2, and PTEN, are relevant to specific disease contexts, and dysregulation of PARdU of these factors leads to potentially deleterious effects (Section 3 and Table 2). Thus, the enzymes regulating PARdU may be attractive therapeutic targets. Inhibition of PARP5a/b has shown promising results in downregulating aberrant *Wnt*/β-catenin signaling and reducing growth in colorectal cancer cell lines and may, therefore, be used in the clinic to treat colorectal cancer, particularly those with overactive *Wnt* signaling [48,83]. Yet, given that PARP5a/b plays a role in a multitude of different biological functions, from mitosis and telomere maintenance to glucose metabolism [84], selective inhibition of PARP5a/b may lead to unwanted effects. Side effects such as intestinal toxicity have also been observed with various PARP5a/b inhibitors [85,86]. Instead, it may be more favorable to target the E3 ligase RNF146. Targeting RNF146 may, however, prove difficult since RING and WWE domains are highly conserved [19]. That said, several selective kinase inhibitors target highly-conserved ATP-binding pockets [87], which lends hope to prospective therapies targeting these conserved domains of RNF146. Finally, as discussed in Section 3, RNF146 and PARP5a/b regulate PARdU and degradation of one another [21,22]; therefore, perturbing the stability or activity of one enzyme may inevitably affect the stability and intracellular levels of the other.

Careful consideration must also be given to the context of PARdU regulation. Is the PARdU substrate a rate- or concentration-limiting factor? Is the protein an activator or suppressor in its biological pathway? For example, Axin1/2 is a rate-limiting factor and a negative regulator of the *Wnt* signaling pathway. PARdU of Axin1/2 limits the formation of the β-catenin destruction complex, affecting Wnt signaling and sensitizing cells to Wnt factors. In contrast, excessive PARdU of PTEN results in aberrant cell proliferation and potential tumorigenesis. Therefore, PARdU can either drive disease progression or serve as a safeguard against disease initiation. Thus, while PARdU is an attractive therapeutic target, inhibiting or activating this system in a targeted manner may be challenging, and several questions must be addressed prior to therapeutic considerations: in which contexts does

PARdU dysregulation lead to disease? What factors dictate the specificity and selection of PARdU targets? Does PARdU target selection vary among various cell types?

7. Concluding remarks

ADP-ribosylation is a functionally diverse post-translational modification with the ability to affect protein function, stability and activity. As a signal for ubiquitination and subsequent degradation, ADP- ribosylation seems to regulate the stability of proteins that are of low abundance. Perhaps, possession of two post-translational modifications adds an additional layer of specificity that facilitates tighter regulation—more than one enzymatic reaction is necessary for these low abundance substrates to be destined for proteasome degradation. Knowing that other post-translational modifications, like phosphorylation [88] and SUMOylation [89] can also target a protein for ubiquitin-mediated proteasomal degradation, one may wonder if these dual-modification degradation mechanisms also target proteins of low abundance, or if they target a specific pool of proteins that all share another common trait. The crosstalk between ADP-ribosylation and ubiquitination is not limited to PARdU. For example, ubiquitin itself is ADP-ribosylated, which has been shown to block subsequent attachment of ubiquitin to target proteins [90,91]. On the other hand, PARP5a/b ADP-ribosylates the proteasome regulator PI31, promoting the assembly of 26S proteasome [92]. Given the precedent set forth by PARdU, it is possible that ADP-ribosylation may also serve as a signal for regulating the synthesis of other post-translational modifications. It will, therefore, be of interest to see how many other modifying enzymes (such as kinases or SUMO E3 ligases) possess PAR-binding motifs.

With the availability of proteomics-based tools to globally identify the sites of ADP-ribosylation and ubiquitination, we may define better rules for PARdU substrates at the amino acid level, including its relationships with PARP-binding sites, such as the TBM, or specific motifs surrounding PTM sites. Through mutagenesis, we can rigorously test whether MARylation by a specific PARP (e.g., PARP12 [76]) is sufficient as a degradation signal for the ubiquitin-proteasome system. As novel methods to identify and characterize PARylation substrates are developed [93,94], we may identify additional degradation signals within PAR, such as its length and structure.

Acknowledgments

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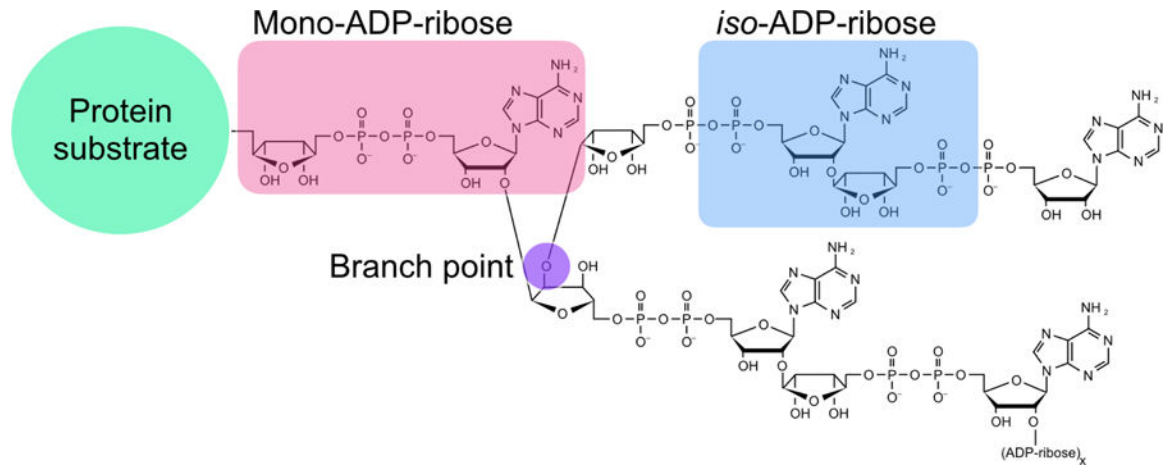
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Figure 1. ADP-ribosylation.

Proteins may be MARylated (pink) or PARylated. Branching of ADP- ribose can be synthesized by PARP1 and the branch point is shown in purple. The internal structure of poly(ADP-ribose) referred to as *iso*-ADP-ribose is highlighted in blue.
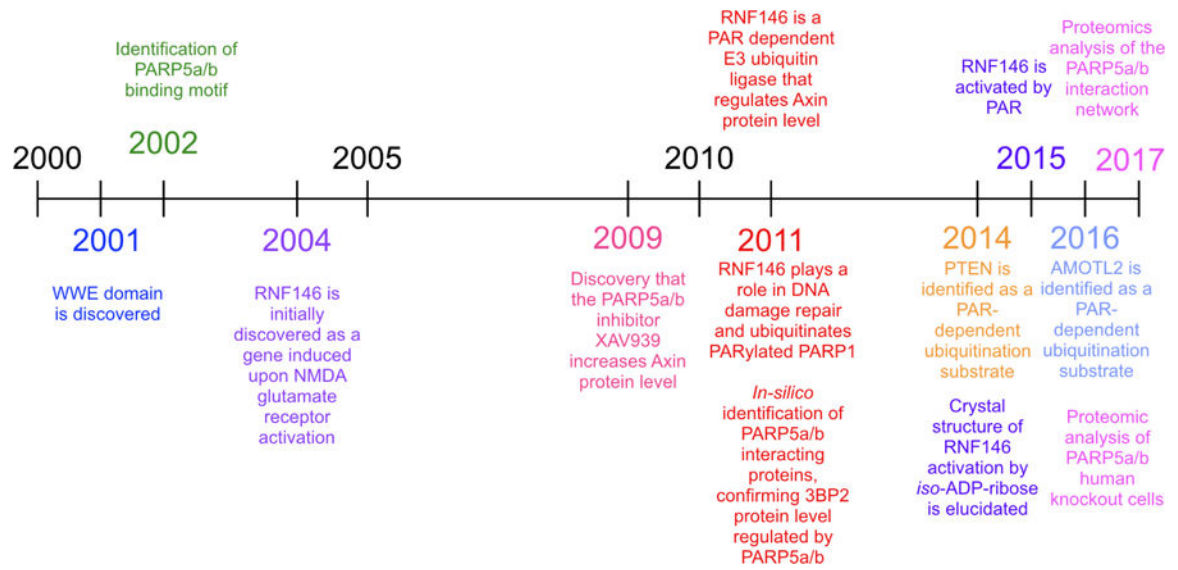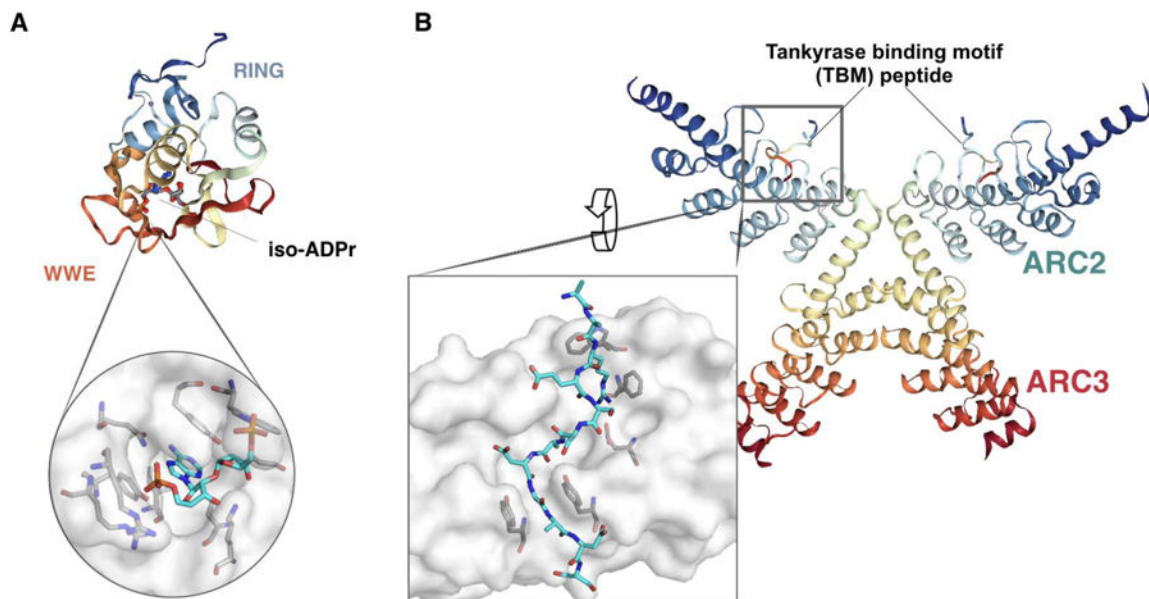
Figure 2.
A timeline of PARdU discoveries.

Figure 3. Structures of the RNF146-*iso*-ADPr complex and ankyrin repeat clusters (ARCs) of PARP5a binding to Tankyrase binding motif (TBM) of RNF146.

(A) Cartoon model of the RNF146- *iso*-ADPr complex highlighting the RING finger and WWE domain (PDB ID: 4QPL), with a close-up of the RNF146-*iso*-ADPr binding interface.

(B) Cartoon model of the complex between RNF146 TBM and PARP5a (PDB ID: 6CF6), with a close-up of the TBM binding interface at an ARC of PARP5a. Structures were generated via Protein Data Bank and PyMOL.
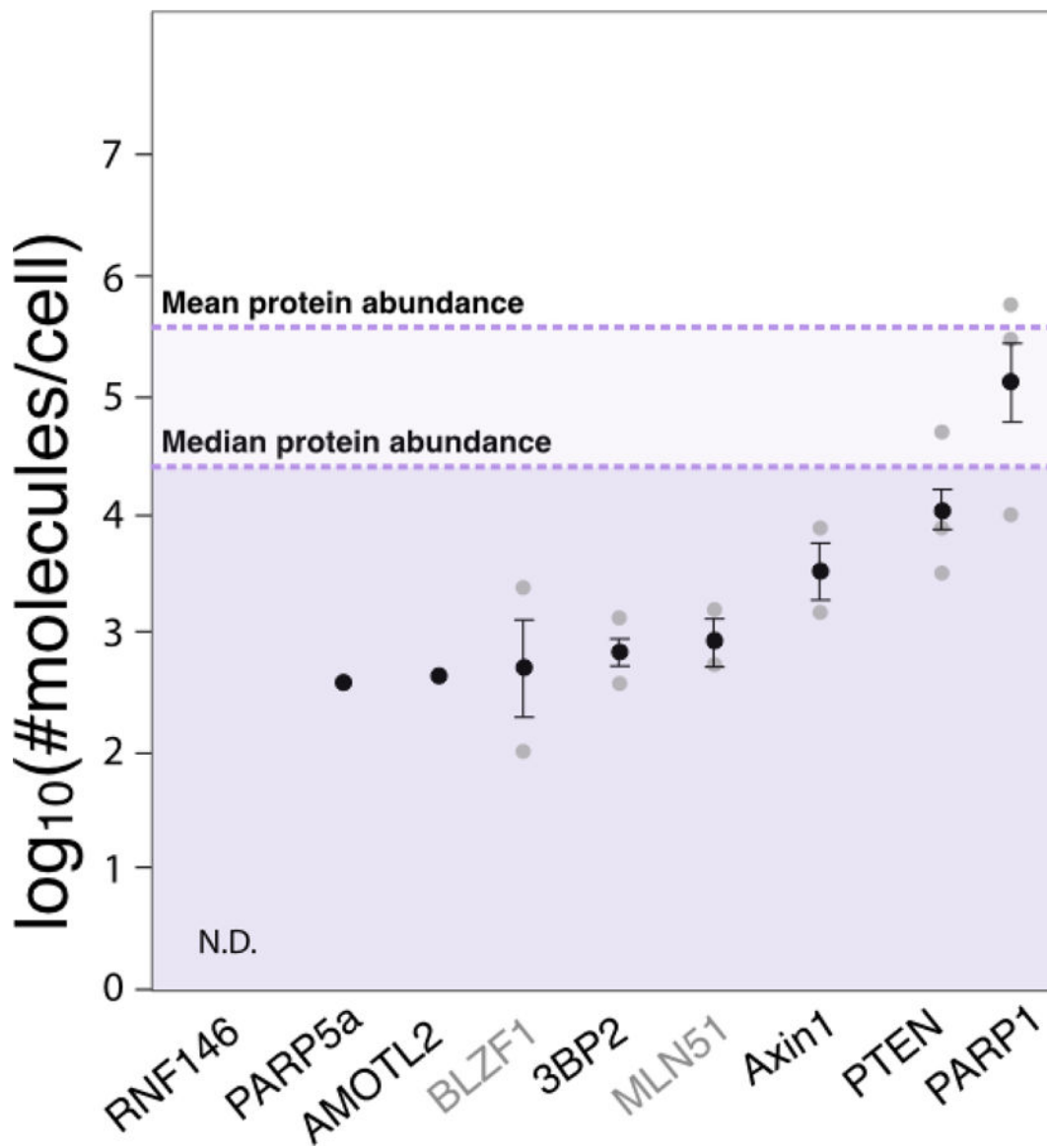
Figure 4. PARdU substrates are often low abundance proteins.
Protein levels measured in log10(#molecules/cell) from four human cell line studies are plotted as mean±standard error (in black) overlaid over individual data points (grey). All characterized PARdU substrates from Table 1 with protein copy number measured are plotted along with two potential PARdU candidates BLZF1 and MLN51. The mean and median protein abundance across all studies are noted. N.D., not detected by mass spectrometry across all studies.
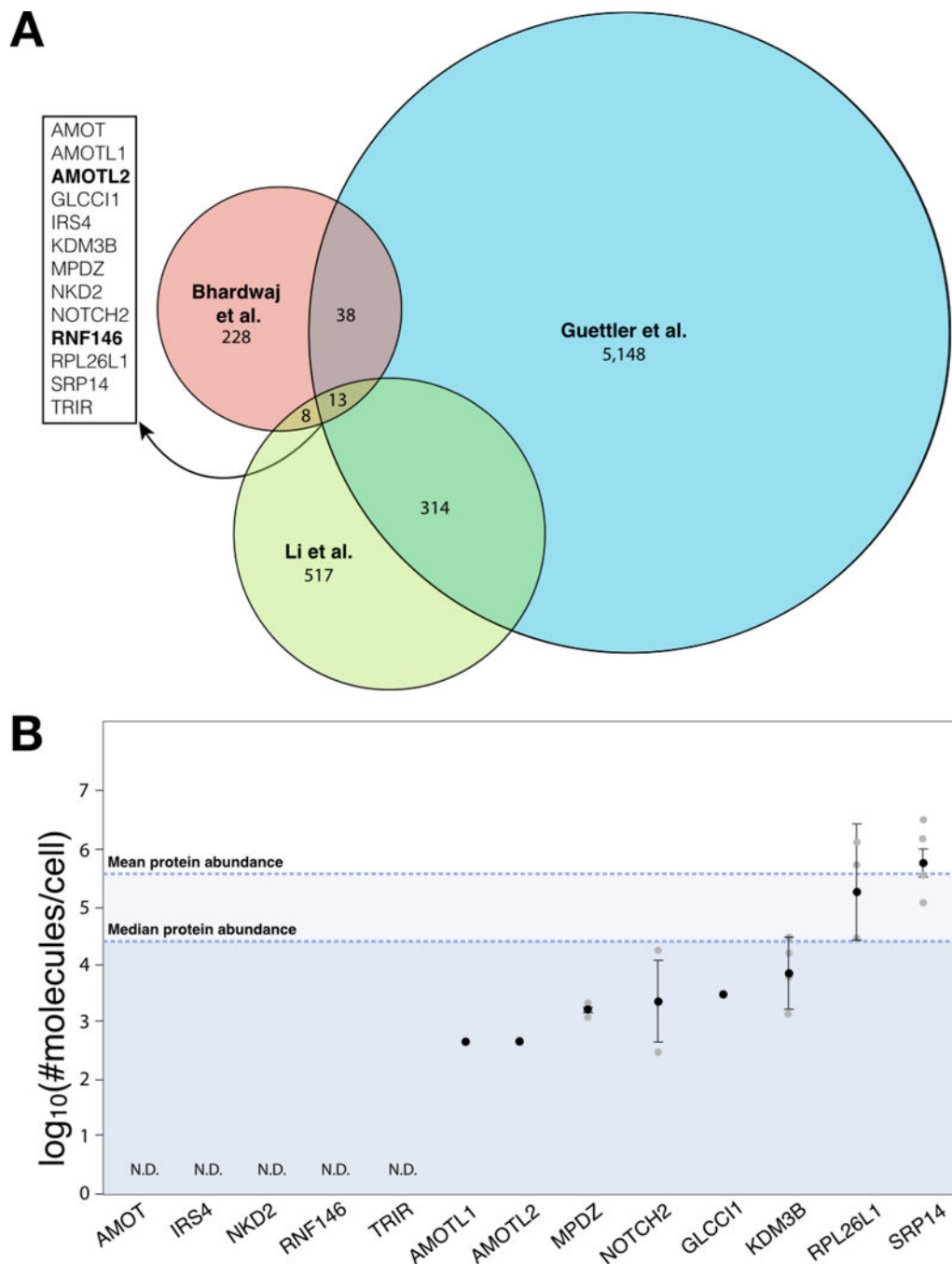
Figure 5. Comparison of three PARP5a/b interactome studies.
A. Venn diagram displaying the overlap of protein identifications among an in silico study identifying proteins with at least one Tankyrase- binding motif (5,513 proteins; Guettler), global analyses of proteins that increase in abundance upon PARP5a/b knockout (287 proteins; Bhardwaj), and proteomics analyses of PARP5a/b interactors (852 proteins; Li). For the Guettler study, only proteins with IUPred disorder score 0.45 are included for the analyses, which increases the stringency of the in silico prediction based on ref. [26]. Proteins identified in all three studies are listed on the left, with established PARdU

substrates in bold. B. As in Figure 4, protein levels measured in $\log_{10}$(#molecules/cell) from four human cell line studies are plotted as mean±standard error (in black) overlaid over individual data points (grey). The mean and median protein abundance across all studies are noted. N.D., not detected by mass spectrometry across all studies.
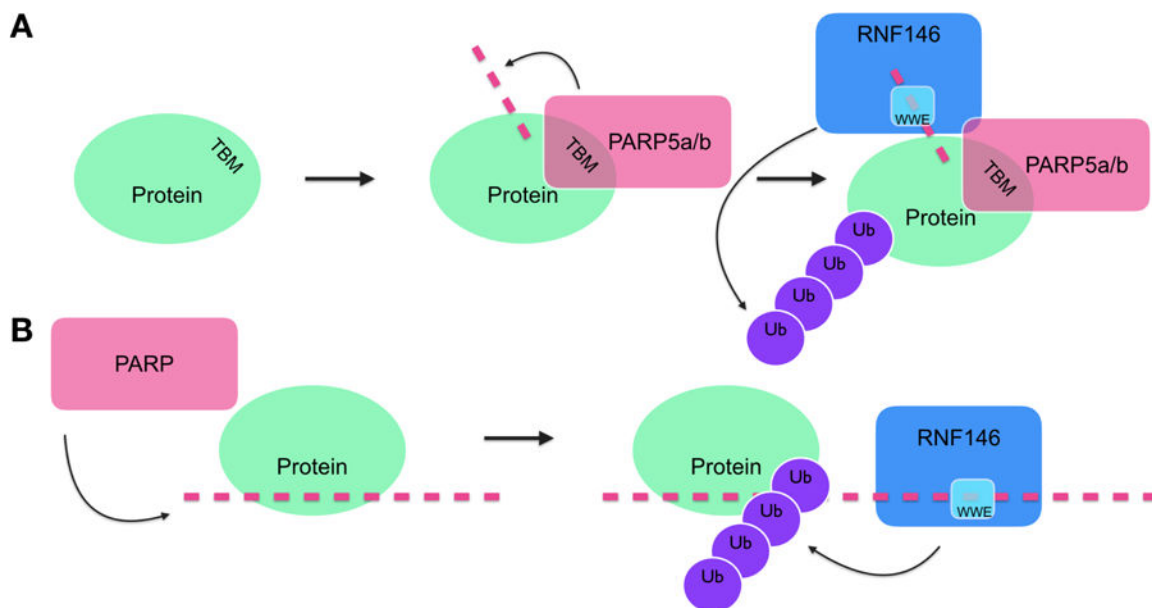
Figure 6. Working models of PARdU.

(A) PARP5a/b binds its substrate via Tankyrase Binding Motif (TBM) before RNF146 binds the *iso*-ADPr within PAR (pink dashed line) through its WWE domain and becomes catalytically active and poly-ubiquitinates the substrate to tag it for proteasomal degradation. (B) PARPs synthesize PAR, which is then bound by PAR-binding proteins. RNF146 binds PAR, becomes catalytically active, and ubiquitinates proximal PAR-binding proteins.

Table 1.

A summary of methods to characterize PARdU substrates.

	Axin1	Axin2	3BP2	PTEN	AMOTL2	PARP1	PARP5a	RNF146
PARP5a/b inhibition	●	●	●	●	●		●	
PARP5a/b knockdown/ overexpression	●	●	●	●				
RNF146 knockdown/ overexpression	●	●	●	●	●	●	●	
***In vitro* ubiquitination assay**				●		●	●	●
Ubiquitination sites identified				●		●		●
ADP-ribosylation sites identified to be critical for PARdU				●				

Table 2.

A summary of PARdU substrates, their associated biological processes and possible pathological outcomes upon PARdU dysregulation.

PARdU substrate	Biological Pathway	Pathology
Axin1/2	*Wnt* signaling	Cancer
3BP2	SRC, SYK, VAV signaling	Cherubism
PTEN	AKT signaling; tumor suppressor	Cancer
AMOTL2	Tight junctions	Cancer
PARP1	DNA damage, stress response	Cancer