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Chapter

ADP-Ribosylation of the Ubiquitin C-Terminus by Dtx3L/Parp9

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Abstract

Ubiquitylation is a post-translational modification that regulates a wide range of cellular pathways including protein degradation, autophagy, mitophagy, cell signaling, DNA damage response, and protein trafficking. This post-translational modification is characterized by covalent attachment of ubiquitin to lysine residues on target proteins by E3 ubiquitin ligases. These enzymes can catalyze both mono- and polyubiquitylation of target substrates. Because of the presence of multiple ubiquitylation acceptor sites on ubiquitin, polyubiquitin chains differing by linkage type and branching patterns can be generated. Post-translational modifications on ubiquitin including glutamine deamidation, lysine SUMOylation, lysine acetylation, and serine, threonine, and tyrosine phosphorylation add to the range of ubiquitin structures that can be synthesized in cells. Recently, ADP-ribosylation was discovered as a new post-translational modification on ubiquitin in two different biological contexts. The bacterial SidE proteins ADP-ribosylate ubiquitin to activate it for a unique mode of ubiquitylation. The human Dtx3L (E3 ubiquitin ligase)/Parp9 (ADP-ribosyltransferase) complex ADP-ribosylates ubiquitin which inhibits conjugation. In this review, we describe the discovery of ubiquitin ADP-ribosylation in the bacterial context, provide an overview of the biological roles of Dtx3L/Parp9, and discuss how NAD^+ levels and ubiquitin ADP-ribosylation could regulate the E3 output of Dtx3L/Parp9.

Keywords: ubiquitin, Dtx3L, Parp9, ADP-ribosyltransferase, ADP-ribosylation, SidE, *Legionella pneumophila*, DNA damage, cancer

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1. Introduction

Ubiquitylation (also known as ubiquitination) is a major type of post-translational modification that plays diverse roles in cells and involves covalent attachment of the 76 amino acid protein ubiquitin to target substrates [1]. The process of ubiquitylation involves the sequential actions of three classes of enzymes: E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and E3 ubiquitin ligase [2, 3]. Activation of ubiquitin by E1 takes place in an ATP-dependent manner where an ubiquitin-adenylate intermediate is initially formed. This is followed by release of AMP and the formation of a thioester bond between the C-terminal carboxyl group of ubiquitin and the sulfhydryl group of a cysteine in the active site of E1. The activated ubiquitin is transferred from E1 to a cysteine in E2 in a *trans*-thioesterification step. Finally, E3 ubiquitin ligase catalyzes the transfer of ubiquitin from E2 onto a lysine residue in the target substrate, forming

an isopeptide bond between the C-terminal carboxyl group of ubiquitin and the lysine epsilon-amino group. In mammalian cells, there are over 600 E3 ubiquitin ligases which are subdivided into three groups: really interesting new gene (RING), homologous to E6-AP carboxyl terminus (HECT), and ring between ring (RBR) [2]. RING class E3 ubiquitin ligases act as scaffolds to bring together E2 and substrate to mediate ubiquitylation. In contrast, HECT class E3 ubiquitin ligases catalyze a two-step ubiquitylation reaction where the ubiquitin is first transferred from E2 onto a cysteine on HECT E3 ubiquitin ligase before it is transferred to the target substrate. RBR has characteristics of both RING and HECT E3 ubiquitin ligases because of the presence of RING domains as well as a key cysteine that acts as an acceptor for ubiquitin from E2, analogous to HECT E3 ubiquitin ligases [3].

E3 ubiquitin ligases can catalyze the attachment of a single ubiquitin to a substrate (monoubiquitylation) where the linkage is usually formed between the carboxyl group of the C-terminal glycine 76 from ubiquitin and the epsilon-amino group of a lysine from the substrate. Monoubiquitylation can occur on multiple lysine sites on a target protein, which is referred to as multi-monoubiquitylation [1]. In addition, E3 ubiquitin ligases can catalyze attachment of ubiquitin to a monoubiquitylated substrate and generate an ubiquitin chain through successive rounds of ubiquitylation (polyubiquitylation). The basis for chain formation is that ubiquitin contains seven lysines (residues 6, 11, 27, 29, 33, 48, 63) that can serve as acceptor sites for additional ubiquitin to form a series of covalently-linked ubiquitins. In addition, the N-terminal primary amine group from methionine 1 of ubiquitin can serve as an additional site of linkage with another ubiquitin [1, 4]. Thus, there are eight possible linkages for ubiquitin that can be utilized for polyubiquitylation. Homotypic polyubiquitin chains only contain a single type of ubiquitin linkage, while heterotypic polyubiquitin chains contain more than one type of ubiquitin linkage [1, 4, 5]. The latter can be further subdivided into mixed and branched types. In mixed chains, while different linkage types exist, each ubiquitin molecule is modified by ubiquitin at a single linkage site, resulting in an unbranched polyubiquitin chain. On the other hand, ubiquitin found in branched types may be modified by more than one ubiquitin, thereby serving as a branch point for chain formation. Overall, a range of polyubiquitin structures that differ in the type of linkage present within the polyubiquitin chain as well as branching pattern can exist within cells.

An additional layer of complexity to ubiquitylation is the discovery of post-translation modifications that can occur on ubiquitin. Based on mass spectrometry data from proteomic studies, ubiquitin can be modified by SUMOylation and acetylation of lysine residues, and phosphorylation of serine, threonine, and tyrosine residues [4, 5]. Some of these post-translational modifications have been shown to impact ubiquitin conjugation properties. One of the best characterized examples of ubiquitin post-translational modification is serine 65 phosphorylation catalyzed by the protein kinase PINK1 during mitophagy [4]. PINK1 can phosphorylate both ubiquitin monomers and polyubiquitin chains *in vitro*, making the latter more resistant to degradation by deubiquitinases [6], and polyubiquitin chains with serine 65 phosphorylation have been detected in cells [7]. In all 13 E2s tested, no major differences in terms of ubiquitin loading onto E2 by E1 was observed between unmodified ubiquitin and serine 65 phosphorylated ubiquitin, suggesting that in general, this modification on ubiquitin does not affect the initial E1 and E2 processing steps [6]. However, depending on the specific E3, the subsequent assembly of polyubiquitin chains can be inhibited by serine 65 phosphorylation on ubiquitin [6]. In terms of the effects that lysine acetylation has on ubiquitin conjugation properties, the most extensive characterization has been conducted for lysine 6 and 48 [8]. Using *E. coli* with an expanded genetic code, lysine 6 and lysine

48 acetylated ubiquitin was generated and used in *in vitro* experiments to show that ubiquitin charging of E1 and E2 as well as monoubiquitylation of histone H2B was unaffected by acetylation [8]. These two acetylation modifications were detected in cells by mass spectrometry from substrate-conjugated ubiquitin as well as ubiquitin monomers, suggesting that a pool of free acetylated ubiquitin is present in cells that can be utilized for acetyl-ubiquitylation of substrates. Potential substrates for acetylated ubiquitin are histone H2A and H2B, as enrichment of lysine 6 and lysine 48 acetylation marks were observed in monoubiquitylated histone H2A and H2B fraction versus total ubiquitin conjugate fraction [8]. Acetylation on ubiquitin can influence the linkage type present within polyubiquitin chains because acetylation and ubiquitylation compete for the same lysine sites. In support of this idea, acetylation of ubiquitin lysine 48 can inhibit the formation of lysine 48-linked polyubiquitin chains [8]. In addition, lysine 48 acetylation can repress polyubiquitylation at lysine 11 and 63, and lysine 6 acetylation can repress polyubiquitylation at lysine 11, 48, and 63. The lysine acetylation data show that modification at one lysine site can influence polyubiquitylation at another lysine, and suggest potential cross-talk between post-translational modification sites on ubiquitin that affect the ubiquitin conjugation properties. Deamidation of glutamine 40 on ubiquitin has been observed as an additional type of post-translational modification [9]. This is catalyzed by the bacterial effector Cif homolog from *Burkholderia pseudomallei* and blocks polyubiquitin formation. Based on the characterization of serine 65 phosphorylation, lysine 6 acetylation, lysine 48 acetylation, and glutamine 40 deamidation, it is clear that post-translational modifications can affect ubiquitin conjugation. For the majority of ubiquitin post-translational modifications, further studies are needed to understand the full impact that ubiquitin modifications have on conjugation properties as well as the functional significance. Follow-up studies would help address the question of whether these post-translational modifications occur on free ubiquitin monomers or conjugated ubiquitin (either mono- or polyubiquitylation).

The existence of post-translational modifications on ubiquitin leaves open the possibility of expanded diversity in terms of polyubiquitin chains containing a unique pattern of post-translational modifications on the constituent ubiquitins. Each of these polyubiquitin chains could promote specific biological outcomes for the modified substrate protein. This concept of post-translational modification patterns encoding biological information is reminiscent of the role of histone post-translational modifications in chromatin regulation. Histones, which are core components of nucleosomes, can undergo a large array of post-translational modifications including phosphorylation, methylation, ADP-ribosylation, acetylation, ubiquitylation, and SUMOylation [10–12]. The term 'histone code' has been put forth for the concept that various combinations of post-translational modifications on histones encode biological information that regulate underlying chromatin processes [13, 14]. Furthermore, there are specific recognition modules (e.g., bromodomain) and enzymes (e.g., histone acetyltransferases and histone deacetylases) that function as readers, writers, or erasers of histone modifications and allow cells to interpret and change this histone code [11, 12, 15]. Thus, histone modifications constitute an important regulatory mechanism for almost all chromatin-related processes. Analogous to the histone code, the term 'ubiquitin code' has been coined to reflect the rich biological information that could be encoded in polyubiquitin chains through different combinations of post-translational modifications, polyubiquitin linkage, and branching patterns [16].

The discovery of ADP-ribosylation of ubiquitin by bacterial proteins adds to the list of post-translational modifications for ubiquitin and further expands the ubiquitin code [17]. This was soon followed by the example of a heterodimeric

complex comprised of the human proteins Deltex-3-like (Dtx3L, an E3 ubiquitin ligase) and Parp9 (an ADP-ribosyltransferase (ART)) catalyzing ADP-ribosylation of ubiquitin [18], suggesting this type of ubiquitin post-translational modification could play broad roles in biology. In the following section, we discuss the initial observation of ADP-ribosylation of ubiquitin occurring in the setting of bacterial infection. We then describe the background on Dtx3L/Parp9 biological roles and the key data characterizing the ADP-ribosylation of ubiquitin by this complex. We highlight differences as well as common features between the two ways in which ADP-ribosylation of ubiquitin occurs. Finally, we pose some questions that remain to be resolved for this newly identified modification on ubiquitin.

2. ADP-ribosylated ubiquitin is generated by SidE effector proteins of *Legionella pneumophila*

ADP-ribosylation of ubiquitin was first discovered in the context of infection by the bacteria *L. pneumophila*, the causative agent for Legionnaires' disease [17]. During infection, the bacterial Dot/Icm type IV secretion system translocates into the host cell effector proteins that play a role in the formation of *Legionella*-containing vacuoles which support bacterial survival and replication [19]. Among these effectors are the SidE family of proteins consisting of four members (SdeA, SidE, SdeB and SdeC) which ubiquitylate ER-associated Rab small GTPases and Rtn4 [17, 20–25]. Investigation of Rab33b, one of the Rab small GTPases targeted by SidE proteins, showed that ubiquitylation causes modest decrease in both GTP loading and GTP hydrolysis [17]. It is not known whether these findings extend to other Rab small GTPases. Exactly how these biochemical changes in Rab33b, along with ubiquitylation of Rtn4, contribute to *Legionella*-containing vacuole formation remain to be determined. Notably, the ubiquitylation of Rab small GTPases and Rtn4 by SidE proteins does not involve E1 or E2, but requires NAD^+ in order to generate ADP-ribosylated ubiquitin as an activated form of ubiquitin. NAD^+ is a small molecule with diverse roles in biology including its role as a cofactor in redox reactions, as an ADP-ribose donor in ART-catalyzed processes, and as an enzyme substrate for the histone deacetylases, sirtuins [26]. SidE proteins use NAD^+ as an ADP-ribose donor to ADP-ribosylate and activate ubiquitin for subsequent conjugation. This stands in contrast to the canonical activation mechanism for ubiquitin that is mediated by E1 in an ATP-dependent manner. ADP-ribosylation of ubiquitin occurs on arginine 42 and is mediated by the mono-ART (mART) domain within SidE proteins [17, 20–25, 27]. Once ADP-ribosylated ubiquitin is generated by the mART domain, the modified ubiquitin is utilized by a phosphodiesterase (PDE) domain within SidE proteins to complete the ubiquitylation of target substrates on serine residues [20, 21, 23–25]. A catalytic mechanism has been proposed where AMP is initially hydrolyzed and the resulting phosphoribosylated ubiquitin is subsequently attached to serine residue of target substrates to complete the ubiquitylation process [20, 21, 23, 24]. In support of this mechanism, an enzyme reaction intermediate where the phosphoribosylated ubiquitin is covalently linked to a key catalytic histidine residue in the PDE domain of SdeA, a member of the SidE protein family, has been observed by mass spectrometry [23].

The mART and PDE domain active sites face away from each other and act independently to catalyze the two enzyme steps involved in ubiquitylation [20, 25]. Mixing together independent mART and PDE domains can recapitulate the ubiquitylation of substrate protein, although the efficiency is reduced because the separated domains cannot form proper inter-domain interactions that would be present in the intact protein [20, 25]. Supplying the PDE domain alone with

ADP-ribosylated ubiquitin recapitulates the ubiquitylation of substrate, supporting the notion that ADP-ribosylation of ubiquitin by mART and ubiquitylation of substrates by PDE using ADP-ribosylated ubiquitin are independent steps [20, 22, 23, 25]. Overall, ADP-ribosylation activates ubiquitin to allow ubiquitylation mediated by SidE proteins to take place in an E1- and E2-independent manner. Ubiquitylation of Rab small GTPases and Rtn4 by SidE proteins through a unique serine-phosphoribose linkage is important for mediating the formation of *Legionella*-containing vacuoles and supporting bacterial infection. ART mutants of SidE proteins displayed defects in terms of formation of *Legionella*-containing vacuoles within host cells, highlighting the essential role that ADP-ribosylation of ubiquitin plays in this novel mode of ubiquitylation [17, 24].

3. Dtx3L, an E3 ubiquitin ligase, forms a heterodimeric complex with Parp9, an ADP-ribosyltransferase

Deltex-3-like (Dtx3L, also known as B-lymphoma- and B aggressive lymphoma-associated protein (BBAP)) is a member of the Deltex family of E3 ubiquitin ligases [28]. Sequence analysis shows that Dtx3L shares amino acid sequence identity with other Deltex proteins in the C-terminal region containing a RING domain and the Deltex C-terminal domain, while the N-terminal region is distinct [28, 29]. Dtx3L was initially identified in a yeast two-hybrid screen as an interacting partner for Parp9 (also known as ART diphtheria toxin-like 9 (ARTD9), and B-aggressive lymphoma 1 (BAL1)), a key risk factor gene for an aggressive subset of diffuse large B cell lymphoma (DLBCL) [28]. Based on homology of the C-terminus to the Parp catalytic domain, Parp9 is classified as an ART, a class of enzymes that transfer ADP-ribose from NAD^+ onto target substrates [30–32]. In addition to its C-terminal Parp catalytic domain, Parp9 has two macrodomains in the N-terminus. Macrodomains are protein modules that bind to ADP-ribose [33, 34], and the Parp9 macrodomains have been shown to bind to poly-ADP-ribose (PAR) [18, 35, 36]. The interaction between Dtx3L and Parp9 is mediated through the N-terminus of Dtx3L and the C-terminus of Parp9 [28, 36]. Neither PAR-binding by Parp9 nor the E3 ubiquitin ligase activity of Dtx3L is required for formation of the heterodimeric complex [36].

4. Both Dtx3L and Parp9 are overexpressed in cancer

Both Dtx3L and Parp9 are overexpressed in an aggressive subset of DLBCL, and are coordinately expressed from a common, interferon (IFN) γ-inducible bidirectional promoter containing interferon regulatory factor (IRF) and signal transducer and activator of transcription (STAT) binding sites [37]. In addition, Dtx3L and Parp9 are highly overexpressed in metastatic prostate cancer cell lines that have increased IFNγ/STAT1 signaling activity [38]. Analysis of gene expression datasets from The Cancer Genome Atlas showed elevated levels of both Dtx3L and Parp9 in prostate cancer as well as breast cancer, bladder urothelial carcinoma, colorectal adenocarcinoma, head and neck squamous cell carcinoma, clear cell renal cell carcinoma, papillary renal cell carcinoma, lung adenocarcinoma, stomach adenocarcinoma, thyroid carcinoma, and uterine corpus endometrial carcinoma [18]. Dtx3L expression is increased in melanoma compared to benign melanocytic tumors [39], and in gliomas where Dtx3L expression level positively correlates with the grade of glioma [40]. In summary, these expression changes point toward Dtx3L/Parp9 having specific biological roles that support tumor behavior. Current evidence suggests

that the Dtx3L/Parp9 complex plays a role in DNA damage response [18, 35, 41] and the regulation of IFNγ/STAT1 signaling pathway [38, 43]. Dtx3L also may function independently of Parp9 in promoting cell migration and metastasis [38, 39, 40].

5. Dtx3L/Parp9 mediates DNA damage response

The Dtx3L/Parp9 complex is involved in DNA damage response. Dtx3L or Parp9 knockdown leads to increased sensitivity to the DNA damaging agent doxorubicin, suggesting the Dtx3L/Parp9 complex plays a role in DNA repair [35, 41]. Using a plasmid-based GFP reporter assay that measures non-homologous end joining (NHEJ) DNA repair, Yang et al. found that knockdown of either Dtx3L or Parp9 leads to an approximate two-fold reduction in NHEJ repair [18]. Conversely, overexpression of Dtx3L/Parp9 had increased efficiency of NHEJ. Recruitment of Dtx3L and Parp9 to laser micro-irradiated sites was observed in cells, supporting a role for this complex in DNA damage response [18, 35]. PAR synthesized by Parp1 and 2 at DNA damage sites serves as a scaffold for recognition by factors involved in DNA damage repair [42]. Because Parp9 contains two macrodomains that bind to PAR, heterodimerization with Dtx3L helps target this E3 ubiquitin ligase to sites of DNA damage. Consistent with this idea, depletion of Parp9 or treatment of cells with PJ-34, a Parp inhibitor that blocks PAR synthesis, prevented Dtx3L localization to sites of laser-induced DNA damage [35].

Once recruited to DNA damage sites, Dtx3L initiates an early wave of ubiquitylation to help coordinate recruitment of DNA damage response proteins. In cells exposed to DNA damage, early recruitment of DNA damage response proteins tumor suppressor p53-binding protein 1 (53BP1) and BRCA1 were reduced when Dtx3L or Parp9 was depleted via siRNA knockdown [35, 41]. Dtx3L knockdown caused decreased chromatin association of the histone methyltransferase SET8 as well as decreased histone H4 lysine 20 mono- and dimethylation [41]. The mechanism for Dtx3L-dependent recruitment of SET8 to chromatin remains unknown. Furthermore, histone H4 monoubiquitylation was reduced by Dtx3L knockdown, and lysine 91 was identified as the site of monoubiquitylation on histone H4 by Dtx3L [41]. Thus, histone H4 lysine 91 monoubiquitylation mediated by Dtx3L is a prerequisite for methylation of histone H4 lysine 20 and subsequent recruitment of 53BP1. In terms of BRCA1, recruitment to DNA damage sites depends on the adaptor protein RAP80 which contains ubiquitin interacting motifs that could recognize ubiquitylation marks generated by Dtx3L. Because of the DNA damage response role for this complex, Dtx3L/Parp9 overexpression in various cancers could have important clinical consequences in terms of therapy resistance to DNA damaging strategies such as chemotherapy and radiation.

6. IFN signaling regulation by Dtx3L/Parp9

In addition to the DNA damage response, the Dtx3L/Parp9 complex has protumorigenic functions through regulation of IFN signaling. In prostate cancer cells, both Dtx3L and Parp9 were shown to be critical factors for mediating cell proliferation and chemoresistance [38]. One mechanism for how Dtx3L/Parp9 supports tumor growth is the repression of IRF-1, an important transcription factor within the IFN signaling pathway that mediates anti-proliferative and pro-apoptotic responses. Expression of IRF-1 was negatively correlated with expression of Dtx3L and Parp9 in prostate cancer cell lines [38]. Consistent with this observation, knockdown of either protein led to increased expression of IRF-1,

and overexpression of Dtx3L or Parp9 had repressive effect on expression of a luciferase reporter under the control of a IRF-1 promoter [38]. The repression was further enhanced when STAT1β, a transcriptionally repressive isoform of STAT1, was co-expressed with Dtx3L and Parp9. This closely mirrors the result from a study in DLBCL where Parp9 together with STAT1β represses IRF-1 expression, thereby supporting cancer cell survival and proliferation [43]. Thus, overexpression of Dtx3L and Parp9 represents a strategy in DLBCL and prostate cancer to repress the expression of the tumor suppressor IRF-1, thereby blocking IFNγ signaling from acting in an anti-proliferative and pro-apoptotic manner.

7. Dtx3L promotes cell migration and metastasis

In addition to acting in a complex with Parp9 to promote tumor growth, Dtx3L may have Parp9-independent roles in mediating cancer metastasis. Knockdown of Dtx3L, but not Parp9, reduced prostate cancer cell migration [38]. Both STAT1 and STAT3 are involved in Dtx3L-mediate cell migration as co-knockdown of either STATs with Dtx3L did not lead to a further decrease in prostate cancer cell migration [38]. The role of Dtx3L in regulating cell migration may be relevant in other types of cancer as well. Analysis of the mouse B16 melanoma cell and its more invasive sublines showed that Dtx3L levels positively correlate with how invasive the melanoma cells are [39]. Knockdown of Dtx3L in human melanoma cells reduced their invasive properties, and decreased lung metastasis was observed when Dtx3L-depleted mouse melanoma cells were injected into tail veins of nude mice, suggesting Dtx3L promotes melanoma metastasis [39]. Additionally, depletion of Dtx3L inhibited migration of glioma cells in a transwell migration assay [40]. The role of Parp9 in cancer cell migration and metastasis was not investigated in the setting of melanoma and glioma. Overall, the available evidence supports the role of Dtx3L in cancer metastasis, and whether Dtx3L acts independently of Parp9 in settings other than prostate cancer remains an open question.

8. Enhancement of anti-viral response by Dtx3L/Parp9

Outside of the context of cancer, the Dtx3L/Parp9 complex promotes anti-viral response through regulation of the IFN/STAT1 signaling pathway. As shown in DLBCL and prostate cancer cells, both Dtx3L and Parp9 are coordinately expressed via IFN signaling pathway in order to allow infected cells to mount an effective anti-viral response [36]. Co-expression of Dtx3L and Parp9 augmented IFN/STAT1-mediated anti-viral response, resulting in reduced viral load for encephalomyocarditis virus, Sindbis virus, and influenza A virus [36]. Furthermore, Dtx3L and Parp9 co-expression enhanced luciferase reporter expression under the control of IFN-stimulated response element and IFNγ-activated site promoter and increased nuclear localization of STAT1 [36]. Both Dtx3L and Parp9 can interact with STAT1, suggesting an interplay between all three proteins to augment anti-viral IFN response and enable cells to better control viral replication [36]. Here, enhanced binding of STAT1 to the IRF-1 promoter by Dtx3L/Parp9 co-expression is observed, which contrasts with the repressive role against IRF-1 expression that this complex plays in cancer. The enhanced STAT1-dependent IFN-stimulated gene (ISG) expression requires Dtx3L-mediated histone H2BJ monoubiquitylation at the promoter. Deposition of this histone modification leads to a concomitant increase in chromatin accessibility and histone H3 lysine 4 trimethylation, an epigenetic mark for active transcription, at the ISG IFN-induced protein with tetratricopeptide repeats

1 (IFIT1) promoter. In addition to working with Parp9 to enhance ISG expression, Dtx3L also has a Parp9-independent role in directly suppressing viral replication by ubiquitylating and targeting the encephalomyocarditis viral 3C protease for degradation [36]. Thus, for effective viral defense, Dtx3L can function with Parp9 in a complex to enhance IFN/STAT1 signaling response, and also acts independently of Parp9 to target viral protease for degradation through ubiquitylation.

9. ADP-ribosylation of the ubiquitin C-terminal glycine 76 carboxyl group by Dtx3L/Parp9

The Dtx3L/Parp9 heterodimeric complex represents the first example of mammalian proteins that catalyze ADP-ribosylation of ubiquitin [18] (**Figure 1**). Parp9 was originally thought to be inactive in terms of ART activity based on previous studies where the ability of Parp9 to automodify itself was tested [30, 44]. However, Parp9 displayed comparable NADase activity to other ART family members when Dtx3L was also added to the *in vitro* reaction, suggesting Parp9 functions as an active enzyme when complexed with Dtx3L [18]. Furthermore, when ubiquitin was added to this

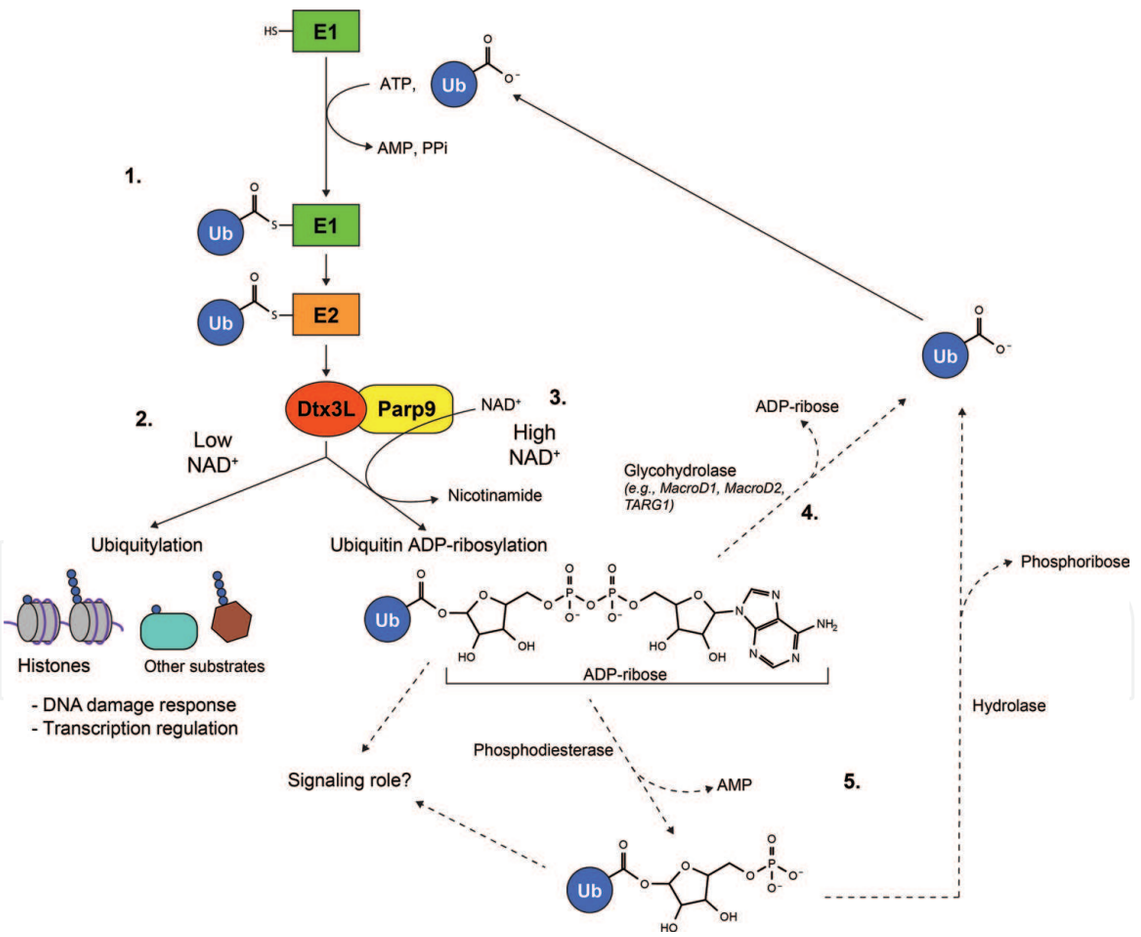


Figure 1.
ADP-ribosylation of ubiquitin by Dtx3L/Parp9 and proposed pathways for regeneration of free ubiquitin. (1) Ubiquitin (Ub, blue circle) processing by E1 and E2. (2) Under low NAD+, ubiquitylation by Dtx3L is favored that plays a role in DNA damage response and transcription regulation. (3) Under high NAD+, ADP-ribosylation of ubiquitin at the C-terminus by Parp9 is promoted, thereby blocking conjugation to substrate and restraining Dtx3L E3 ubiquitin ligase activity. ADP-ribosylated ubiquitin could undergo processing through two ways in order to regenerate free ubiquitin: a single step cleavage of ADP-ribose by a glycohydrolase (4), or a two-step process where a phosphodiesterase cleaves AMP to generate phosphoribosylated ubiquitin, followed by a hydrolase cleavage step to regenerate free ubiquitin (5). Both ADP-ribosylated and phosphoribosylated ubiquitin may play signaling roles. Dotted lines indicate speculative steps.

reaction, Parp9-mediated ADP-ribosylation of ubiquitin was detected. The ADP-ribosylation of ubiquitin was dependent on processing of ubiquitin by E1 and E2 as well as the RING domain in Dtx3L. Binding of an ubiquitin antibody that recognizes an epitope in the C-terminus was reduced when ubiquitin was ADP-ribosylated, indicating that the site of ADP-ribosylation was at the C-terminal portion of ubiquitin. ADP-ribosylation of proteins takes place on a variety of amino acids including glutamate, aspartate, serine, phosphoserine, threonine, lysine, arginine, asparagine, and cysteine [45, 46]. Arginine residues 72 and 74 at the C-terminus of ubiquitin were excluded as the sites of ADP-ribosylation because mutations targeting these residues did not prevent ubiquitin ADP-ribosylation by Dtx3L/Parp9 [18]. An independent approach for interrogating ADP-ribosylation sites takes advantage of the differential sensitivity to chemical-based ADP-ribose release, depending on the type of amino acid that is ADP-ribosylated [47]. Neutral hydroxylamine which removes ADP-ribose from acidic R group, efficiently removed ADP-ribose from ubiquitin, suggesting that the site of ADP-ribosylation on ubiquitin is an acidic R group [18]. Based on these observations, the site of ADP-ribosylation was narrowed down to the C-terminal carboxyl group of glycine 76, the only available acidic moiety that could serve as an acceptor for ADP-ribose in the C-terminus of ubiquitin (**Figure 1**).

10. Dtx3L E3 output is regulated by ADP-ribosylation of ubiquitin and PAR-binding to Parp9 macrodomains

Given that conjugation of ubiquitin onto target substrates occurs through its C-terminus, ADP-ribosylation of the C-terminus of ubiquitin would block ubiquitylation from taking place. Thus, modulation of Parp9-mediated ADP-ribosylation would be expected to change the ubiquitylation output by the heterodimer partner Dtx3L. This prediction was tested by adding NAD^+ to an *in vitro* ubiquitylation reaction, which would promote ADP-ribosylation of ubiquitin. As expected, increasing concentration of NAD^+ led to reduced ubiquitylation of histone H2A and histone H3 by Dtx3L [18]. This supports the idea that Parp9 ADP-ribosylation of ubiquitin effectively blocks ubiquitin from being utilized in Dtx3L-mediated ubiquitylation, and that Parp9 negative regulation of Dtx3L can be modulated through changes in free NAD^+ concentration (**Figure 1**). In addition to regulation of Dtx3L ubiquitylation by the Parp9 catalytic domain, Parp9 can also regulate Dtx3L through binding to PAR via its two macrodomains. Addition of PAR in an *in vitro* ubiquitylation reaction increased Dtx3L-generated polyubiquitylated product as well as monoubiquitylation of histone H2A [18]. This suggests PAR binding to the Parp9 macrodomains has a stimulatory role in terms of Dtx3L function and points toward a regulatory mechanism where a conformational change from PAR-binding to Parp9 macrodomains is transmitted to Dtx3L to enhance E3 ubiquitin ligase activity. A precedent for PAR activation of E3 function has been established where RNF146, an E3 ubiquitin ligase, is stimulated by PAR binding through its Trp-Glu-Glu (WWE) domain [48, 49]. On the other hand, PAR binding to Parp9 had no effect in terms of generation of ADP-ribosylated ubiquitin. As Dtx3L and Parp9 play a role in NHEJ DNA repair, the regulatory role of Parp9 ADP-ribosylating ubiquitin in this process was examined. Yang et al. found that co-expression of catalytically inactive Parp9 with Dtx3L had enhanced DNA damage repair, compared to co-expression of wild-type Parp9 and Dtx3L [18]. This is consistent with the model that Parp9 ADP-ribosylation of ubiquitin precludes ubiquitylation of substrates by Dtx3L that would be important for the recruitment of DNA damage repair proteins. Reducing the ART activity in Parp9 by mutation relieves this inhibitory effect, thereby promoting DNA damage repair dependent on Dtx3L E3 ubiquitin ligase activity.

Based on experimental evidence discussed earlier, we put forth the concept that in the context of DNA damage, NAD^+ functions as an important regulator of E3 output for Dtx3L/Parp9 via two distinct mechanisms. When cells are exposed to DNA damage, depletion of NAD^+ occurs because as part of the DNA damage response, Parp1 and 2 utilize NAD^+ as an ADP-ribose donor molecule for the synthesis of PAR at sites of DNA damage [42, 50]. As the NAD^+ level is low and PAR is present, the local environment at the DNA damage site would be optimal for Dtx3L E3 ubiquitin ligase activity that is critical for DNA damage response. Low NAD^+ means that ADP-ribosylation of ubiquitin by Parp9 is occurring minimally; hence, ubiquitin is available to participate in Dtx3L-mediated ubiquitylation (**Figure 1**). Moreover, PAR synthesized from NAD^+ helps recruit the Dtx3L/Parp9 complex to DNA damage sites and stimulates Dtx3L E3 ubiquitin ligase activity through recognition by the Parp9 macrodomains. As the repair of DNA damage proceeds, the balance shifts from synthesis of PAR toward disassembly of PAR and restoration of NAD^+ to basal level. The heterodimeric complex becomes disengaged from PAR, and hence, the stimulatory impact of PAR-binding to promote Dtx3L ubiquitylation is reduced. High NAD^+ levels would favor ADP-ribosylation of ubiquitin by Dtx3L/Parp9, and this attachment of ADP-ribose to the C-terminal glycine 76 would block utilization of ubiquitin in Dtx3L-mediated ubiquitylation (**Figure 1**). In this setting, the combination of high NAD^+ and low PAR levels would not be conducive for Dtx3L E3 ubiquitin ligase function, which is appropriate as DNA damage is resolved and Dtx3L-mediated ubiquitylation to coordinate early DNA damage response is no longer necessary. Thus, NAD^+ and its derived macromolecule PAR serve as critical regulatory mechanisms for controlling Dtx3L-mediated ubiquitylation in the context of DNA damage. As part of this regulation of Dtx3L, ADP-ribosylation of ubiquitin plays a restraining function for Dtx3L E3 ubiquitin ligase activity when DNA damage has been repaired.

11. Distinct biological properties and roles for ADP-ribosylated ubiquitin generated by Dtx3L/Parp9 and SidE proteins

While both the Dtx3L/Parp9 complex and the SidE proteins can mediate the formation of ADP-ribosylated ubiquitin, clear distinctions are evident between the two cases. The ADP-ribosylation of ubiquitin by Parp9 requires that ubiquitin undergo E1 and E2 processing as well as the presence of the E3 ubiquitin ligase Dtx3L. This requirement for strict coupling ensures that Parp9 ADP-ribosylation of ubiquitin is specifically tied to activated ubiquitin that is in the process of being handled by the heterodimer partner, Dtx3L, and ensures that ADP-ribosylation of ubiquitin does not take place promiscuously. In contrast, the mART domain of SidE proteins is sufficient to generate ADP-ribosylated ubiquitin in the absence of ubiquitin processing by E1 and E2. The roles of the generated ADP-ribosylated ubiquitin are different between Dtx3L/Parp9 and SidE proteins. For Dtx3L/Parp9, ADP-ribosylation of ubiquitin takes place to short-circuit the normal ubiquitylation process by Dtx3L, thereby acting in a negative regulatory manner. In essence, ADP-ribosylation at the C-terminus of ubiquitin prevents the modified ubiquitin from being conjugated onto target substrates, and thus appears to be a regulatory feature for Dtx3L E3 output in the context of DNA damage repair as discussed earlier. It is possible that the control of Dtx3L-mediated ubiquitylation by Parp9-mediated ADP-ribosylation of ubiquitin operates in the context of active IFN signaling and viral infection as well. Depletion of NAD^+ has been observed in cells treated with IFNγ [51], and this could further augment Dtx3L E3 output and downstream control of viral infection. Decreasing levels of NAD^+ would lead to less Parp9-mediated ADP-ribosylation of ubiquitin; hence, unmodified ubiquitin is available for Dtx3L-mediated ubiquitylation of histone

H2BJ which results in increased ISG expression. It would be interesting to test this hypothesis through co-expression of catalytically inactive Parp9 mutant and Dtx3L and examine how transcription of ISG is impacted. If our model is correct, then ISG transcription should be increased because the negative regulatory mechanism on Dtx3L E3 activity is relieved by inactivation of Parp9 catalytic activity. Similar to ubiquitin ADP-ribosylated at the C-terminal glycine 76, ubiquitin ADP-ribosylated on arginine 42 and its phosphoribosylated derivative are unable to be utilized in E1 and E2 processing, thereby disrupting host ubiquitylation processes [21]. However, the main role of ADP-ribosylated ubiquitin generated by SidE proteins is to serve as an activated intermediate for non-canonical serine ubiquitylation of host proteins that is important for supporting bacterial infection. Because Dtx3L/Parp9-mediated ADP-ribosylation of ubiquitin occurs on the C-terminus, this enzyme complex would not be able to ADP-ribosylate pre-existing polyubiquitin chains already conjugated to a target substrate, as no C-terminal carboxyl group from ubiquitin is available for the attachment of ADP-ribose. Unconjugated polyubiquitin chains are present in cells [4], so it is conceivable that the glycine 76 carboxyl group at the C-terminus of the polyubiquitin chain can be modified by Dtx3L/Parp9. Because of the tight coupling of Dtx3L/Parp9-mediated ADP-ribosylation of ubiquitin to E1 and E2 processing, this scenario would likely require that free polyubiquitin chains are first processed by E1 and E2 before ADP-ribosylation by Dtx3L/Parp9 could take place. However, to the best of our knowledge, handling of free polyubiquitin chains by E1 and E2 has not been observed, and thus, ADP-ribosylation of free polyubiquitin chains by Dtx3L/Parp9 remains highly speculative. On the other hand, SidE proteins can modify existing polyubiquitin chains. Incubation of lysine 48- or methionine 1-linked ubiquitin tetramers with SdeC led to ADP-ribosylation of the incorporated ubiquitin [24]. Further investigation showed that ADP-ribosylation and phosphoribosylation of lysine 63-, lysine 48-, lysine 11-, or methionine 1-linked diubiquitin chains could be catalyzed by SdeA [52]. No preference for modification of either ubiquitin within the diubiquitin was observed. In most cases, the ADP-ribosylated or phosphoribosylated diubiquitins were resistant to hydrolysis by deubiquitinases, suggesting SidE proteins could have broad effects in host cell by changing deubiquitinase susceptibility of polyubiquitin chains on various target substrates [52].

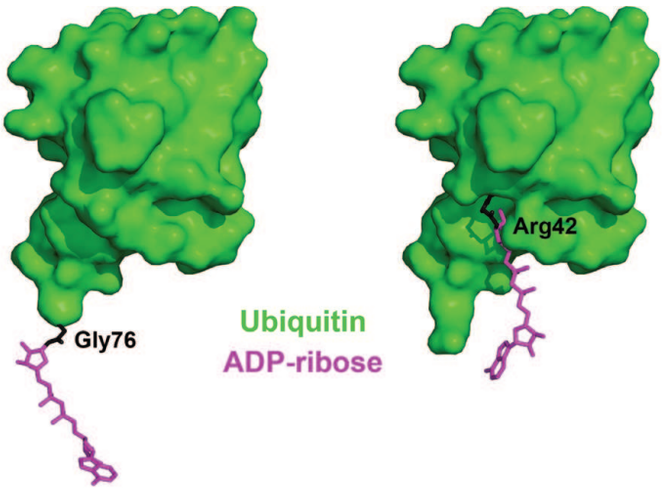
12. Some open questions for ADP-ribosylation of ubiquitin

While it is clear that ADP-ribosylation of ubiquitin by Dtx3L/Parp9 prevents substrate conjugation, the biological processes impacted by this form of regulation outside of Dtx3L-mediated NHEJ DNA repair remain to be identified. One can also speculate that ADP-ribosylated ubiquitin could be playing a signaling role as a second messenger molecule, similar to how free ubiquitin or a polyubiquitin chain could serve signaling roles [4] (**Figure 1**). Another major question is the fate of ADP-ribosylated ubiquitin after it is generated. It does appear that cells have enzymatic activity to reverse this post-translational modification on ubiquitin as treatment of ADP-ribosylated ubiquitin with cell lysate restores detection of the C-terminus by the C-terminal ubiquitin antibody [18]. There are at least two mechanisms by which cells can reverse the ADP-ribosylation and regenerate free ubiquitin (**Figure 1**). One is that a glycohydrolase cleaves the ADP-ribose from the C-terminus of ubiquitin in a single step reaction. MacroD1, macroD2, and terminal ADP-ribose protein glycohydrolase 1 (TARG1) are examples of glycohydrolases that remove ADP-ribose from acidic residues [45, 46], and are potential candidates for regenerating the C-terminal carboxyl group of ubiquitin. The other possibility is removal of ADP-ribose from the C-terminus of ubiquitin involves a

phosphodiesterase that releases AMP to generate phosphoribosylated ubiquitin, though regeneration of ubiquitin would still require a hydrolase to remove the phosphoribosyl group. It is conceivable that both mechanisms are operating within the cell. The latter mechanism where phosphoribosylated ubiquitin is generated opens up the question of whether this modified species of ubiquitin has biological functions, rather than it occurring as a recycling intermediate. As speculated earlier for ADP-ribosylated ubiquitin, the phosphoribosylated ubiquitin could have cell signaling roles. This idea is applicable to the phosphoribosylated ubiquitin generated by SidE proteins as well where it is currently unknown whether and how phosphoribosylated ubiquitin is processed by host cells. Although the sites of modification are different, there could be overlap in terms of the host cell mechanisms that handle the reversal of phosphoribosylated ubiquitin generated by SidE proteins versus phosphoribosylated ubiquitin generated as a result of breaking down ADP-ribosylated ubiquitin generated by the Dtx3L/Parp9 complex. Thus, we currently have little understanding of what other roles ADP-ribosylated ubiquitin may play aside from being a starting point for bacterial protein-catalyzed ubiquitylation or a regulatory mechanism against the Dtx3L E3 ubiquitin ligase, as well as how cells regenerate free ubiquitin from ADP-ribosylated or phosphoribosylated ubiquitin.

13. Concluding remarks

ADP-ribosylation of ubiquitin is an exciting new addition to the possible post-translational modifications for ubiquitin. To date, SidE proteins and the Dtx3L/Parp9 complex are the only proteins known to generate ADP-ribosylated ubiquitin. These two cases of ADP-ribosylation of ubiquitin represent distinct mechanisms by which ubiquitin conjugation activity is regulated (**Figure 2**). Dtx3L/Parp9-mediated ADP-ribosylation of the C-terminal glycine 76 of ubiquitin effectively prevents the modified ubiquitin from being utilized in ubiquitylation processes until the blocking ADP-ribose is removed. SidE-mediated ADP-ribosylation of ubiquitin occurs as an activation step for subsequent E1- and E2-independent ubiquitylation process. These are still early days in understanding the important roles ADP-ribosylation of ubiquitin plays in both the Dtx3L/Parp9 and SidE context and many questions remain. How exactly does abrogating the Parp9 ART activity enhance the NHEJ DNA repair mediated by Dtx3L? What are the Dtx3L target substrates involved in this process? While histone H4 is one of the important candidate substrates based on previous data [41], there may be other DNA damage response factors that are ubiquitylated by Dtx3L during NHEJ DNA repair. For SidE proteins, it would be interesting to see whether other bacterial effector proteins as well as eukaryotic counterparts exist that can catalyze the E1- and E2-independent ubiquitylation on serine. Furthermore, by utilizing ADP-ribosylated ubiquitin, SidE-mediated ubiquitylation results in a phosphoribose linkage between serine and ubiquitin that is distinct from the canonical isopeptide linkage between lysine and ubiquitin. How this novel ubiquitin linkage changes the biological properties of Rab and Rtn4, the targets of SidE proteins, is a future area of research. An intriguing possibility is that perhaps other bacterial effector proteins specifically recognize this unique serine-phosphoribose-linked ubiquitin and co-opt the modified Rab and Rtn4 for the generation of *Legionella*-containing vacuoles. Finally, much work remains to be done on characterizing the cellular mechanisms involved in processing and handling of ADP-ribosylated and phosphoribosylated ubiquitin after they are generated. Answering these questions regarding ADP-ribosylation of ubiquitin would provide additional insight into the ubiquitin code.



	Dtx3L (E3 ubiquitin ligase)/ Parp9 (ADP-ribosyltransferase)	SidE (ADP-ribosyltransferase)
Cofactor	NAD^+	NAD^+
ADP-ribosylation site	Glycine 76	Arginine 42
Effect on ubiquitin	Blocks conjugation	Alternative activation
Subsequent Processing	Hydrolase	SidE (phosphodiesterase)

Figure 2.
NAD^+ regulation of ubiquitin conjugation activity by two distinct mechanisms. Models of ADP-ribosylated ubiquitin were generated in Pymol by attaching ADP-ribose (magenta) to either arginine 42 (black) or C-terminal glycine 76 (black) in the solved ubiquitin crystal structure (PDB: 1UBQ, green).

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Conflict of interest

The authors report no conflict of interest.

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