Activities and Binding Partners of E3 Ubiquitin Ligase DTX3L and Its Roles in Cancer

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Abstract

Ubiquitination is a protein post-translational modification that affects protein localisation, stability and interactions. E3 ubiquitin ligases regulate the final step of the ubiquitination reaction by recognising target proteins and mediating the ubiquitin transfer from an E2 enzyme. DTX3L is a multidomain E3 ubiquitin ligase in which the N-terminus mediates protein oligomerisation, a middle D3 domain mediates the interaction with PARP9, a RING domain responsible for recognising E2~Ub and a DTC domain has the dual activity of ADP-ribosylating ubiquitin and mediating ubiquitination. The activity of DTX3L is known to be modulated by at least two different factors: the concentration of NAD^+, which dictates if the enzyme acts as a ligase or as an ADP-ribosyltransferase, and its binding partners, which affect DTX3L activity through yet unknown mechanisms. In light of recent findings it is possible that DTX3L could ubiquitinate ADP-ribose attached to proteins. Different DTX3L-protein complexes have been found to be part of multiple signalling pathways through which they promote the adhesion, proliferation, migration and chemoresistance of e.g. lymphoma, glioma, melanoma, and prostate cancer. In this review, we have covered the literature available for the molecular functions of DTX3L especially in the context of cancer biology, different pathways it regulates and how these relate to its function as an oncoprotein.

Introduction

In the early 90's, ubiquitin (Ub) was first described as a protein modifier that targets proteins for proteasomal degradation[1,2]. Ever since, our understanding of the so-called "ubiquitin code" has increased to the point that there are currently inhibitors of the proteasome system in clinical use[3–5]. Post-translational modification (PTM) of proteins by ubiquitination results in the covalent attachment of C-terminal glycine of an 8 kDa Ub to a lysine residue of the target protein. Subsequently, Ub can undergo further ubiquitination to form poly-Ub chains that may result in linear, branched, or mixed chain conformations. The conformation of the chain depends on the residue of the initial Ub to which the new moiety has been linked. With seven lysine residues and the initiator methionine as possible ubiquitination sites, there are countless conformations that poly-Ub chains can adopt, each of them acting as a distinct cellular signal.

The ubiquitination hierarchy is also reflected on the number of enzymes for every step of the reaction (**Text Box 1**). The human genome encodes for two E1s (UBA1 and UBA6)[6], 24 E2s[7], and approximately 1000 putative E3 ligases. Based on their Ub transfer mechanisms, E3 ligases are classified as homologous to E6AP carboxyl terminus (HECT), RING-between-RING (RBR) and really interesting new gene (RING) proteins. There are over 600 RING-type E3 ligases and only some of them, such as the homodimeric IDOL[8,9], the BRCA1/BARD1 heterodimer[10–12], and the anaphase promoting complex/cyclosome (APC/C)[13–15] have been well characterised and validated as potential drug targets[16,17]. These findings stress the importance that the functional consequences of ubiquitination mediated by RING-type E3 ligases have and that there is a need to systematically characterise E3 ligases in order to understand their roles in signalling pathways.

Like ubiquitination, ADP-ribosylation is a reversible PTM involved in several signalling pathways and different aspects of this versatile modification have been extensively reviewed recently in the literature. In humans, ADP-ribosylation is mainly catalysed by ADP-ribosyltransferases that use NAD^+ as a substrate and add an ADP-ribose (ADPr) moiety to a target macromolecule[18]. On proteins, ADP-ribosylation occurs on a range of polar sidechains but nucleic acids are also acceptors of this modification[19,20]. The founding member of the diphtheria-toxin like ARTD family PARP1, PARP2 and tankyrases perform poly-ADP-ribosylation (PARylation) by the subsequent addition of ADPr to generate branched or linear chains of poly-ADP-ribose (PAR)[21]. Most PARP enzymes however only attach a single ADPr unit to other macromolecules in a process known as mono-ADP-ribosylation (MARylation)[22,23]. ADP-ribosylation is detected by a variety of reader

domains such as macrodomains[24]. The modification can be removed by erasers for which different catalytic mechanisms and specificities have been identified[25,26].

Text Box 1: The mono- and polyubiquitination cascades of DTX3L require multiple enzymes.

In the presence of ATP, E1s catalyse adenylation of ubiquitin at its C-terminal glycine, which generates a high-energy intermediate[27]. Adenylated Ub, subsequently forms a thioester (~) bond with a catalytic Cys of the enzyme, while simultaneously, the E1 catalyses the adenylation of a second ubiquitin[6,28]. Subsequently, Ub conjugating enzymes (E2s) interact with the E1~Ub, thus promoting the transfer of Ub from the catalytic Cys of E1 to the catalytic Cys of E2. The final step of the ubiquitination cascade is regulated by Ub ligases (E3s), responsible for the recognition of a target protein and facilitating the transfer of Ub from E2~Ub to the ε-amino group of a lysine residue of a protein[29]. Although precise mechanism by which the transfer occurs remains obscure, it has been suggested that by juxtaposing the lysine residue of the substrate to the catalytic Cys of a "primed" E2, the pKa of the substrate lysine is reduced, promoting the ubiquitination of a given substrate[30].

RING-type Ub ligases are thus far the only ubiquitin ligases that can act as multi-subunit complexes as well as single multi-domain proteins[31–34]. While their architecture can be as diverse as the substrates they recognise and the E2s with which they cooperate, they all share the presence of a RING (or U-box) domain. The RING domain acts primarily as a scaffold between the primed E2 and the target through an allosteric activation of the E2, which promotes the nucleophilic attack from Ub to a lysine residue of the target[30,35].

Cell-based studies identified that RING-type DTX3L works in cooperation with E2 enzymes that belong to the Ube2D family with a strong preference towards Ube2D1[36]. E2 enzymes from the Ube2D family are non-specific and can arrange poly-Ub chains from all the possible conformations[37]. This indicates that DTX3L is responsible for the specificity of the generated Ub linkages, which have been identified to be through K6, K11, K48, and K63[38].

Human Deltex (DTX) proteins are RING-type E3 ligases that have increasingly gained attention as not only are they involved in the regulation of a myriad of signalling pathways, but they are also associated with multiple forms of cancer[39–42]. The five DTX proteins are characterised by diverse

N-terminal domains and a similar C-terminal part with RING and Deltex C-terminus (DTC) domains (**Fig. 1**). The DTX proteins can be grouped in two evolutionary clades based on the similarity of the DTC domains, with DTX1, 2 and 4 in one group, and DTX3 and Deltex3-like (DTX3L) in another[43]. The similarity between these groups goes beyond the DTC domain as DTX1, DTX2, and DTX4 share WWE domains that are widely associated with PAR binding activity (**Fig. 1**) and present also in other E3 ligases [44,45]. Furthermore, all the members of the family, except DTX3L, share a proline-rich central domain (D) that has been hypothesised to regulate protein-protein interactions[46]. Instead of the WWE domain tandem, DTX3L holds two small domains hitherto referred to as D1 and D2, which coordinate the formation of higher oligomers[38] and the proline-rich domain in DTX3L is replaced with a central D3 domain (**Fig. 1**). Taken all these characteristics into account, DTX3L is a unique member of the Deltex family.

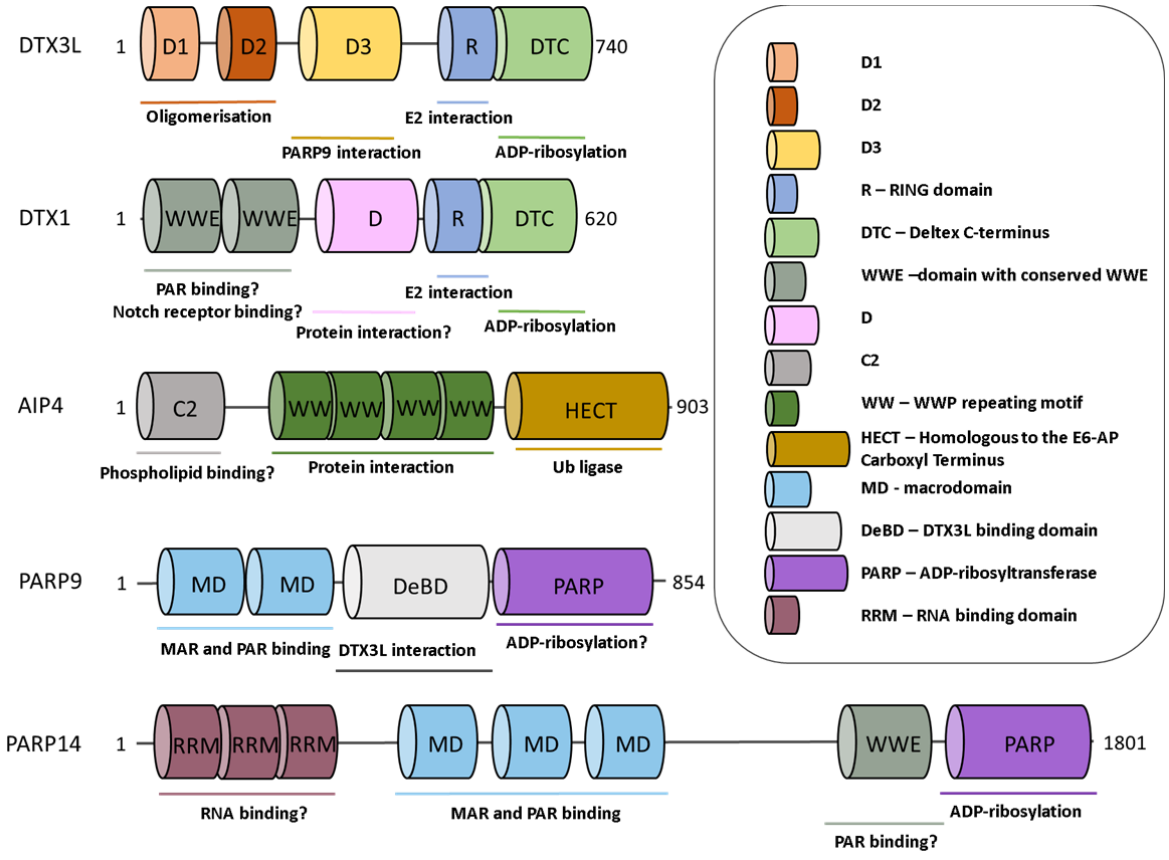

Figure 1. Domain structure of DTX3L and its verified binding partners drawn to an approximate scale.

The janus enzyme has both ubiquitination and ADP-ribosylation activities

DTX3L was initially discovered while looking for interacting partners of PARP9 with *in vivo* and *in vitro* data[36]. In the study it was also noticed that the DTX proteins undergo auto-modification as E3 ligases, but no substrate was identified for them. It was later shown that DTX3L can coordinate several conformations of poly-Ub chains (**Text Box 1**)[38] and mono-ubiquitinate histones, especially H2BJ, H3 and H4, and generate K48-linked Ub chains on a viral protease to enhance interferon (IFN) signalling upon viral infection[47–49].

In the context of ADP-ribosylation DTX3L was first thought to be an activator of PARP9[49], which has been long thought to be an inactive protein due to changes in the catalytic site[50]. Yang and collaborators described how the DTX3L-PARP9 complex could MARylate the C-terminus of Ub in an E2~Ub dependent manner[49]. The finding was initially attributed to PARP9, hypothesising that complex formation renders it active[49]. Surprisingly, it was later discovered that the DTC domain can alone catalyse this reaction[51,52].

The MARylation could be actually ubiquitination of ADP-ribose as a recent study showed that DTX2 can transfer Ub from an E2 to a hydroxyl group of ADPr via a mechanism involving a conserved histidine within the RING domain[53]. The results of this study might be specific to DTX2 as the authors note that they were unable to detect hydrolysis of Ub-ADPr with ADPr hydrolases, but they could detect it with deubiquitinases, consistent also with previous studies on DTX2[51,53]. It is noteworthy that the Ub-ADPr product generated by DTX3L was hydrolysed by a panel of enzymes not acting on the Ub-ADPr product formed by DTX2[38]. Both ADP-ribosylation and the recently discovered ubiquitination of ADP-ribose mediated by DTX2[53] should be assessed especially in the context of DTX3L and the complexes it forms with other enzyme.

The activity of DTX3L is regulated by its promiscuity

While DTX3L lacks canonical ADPr- or PAR-binding domains, most of its binding partners contain domains that can act as readers of this modification. Studies have pointed out that DTX3L can directly interact with PARP9, PARP14 and DTX1, all of them bearing either macrodomains (PARP9 and PARP14) or WWE domains (**Fig. 1**). However, despite the multiple binding partners associated with DTX3L, it has only been studied as a complex with PARP9[38]. As such, the precise interaction interface between DTX3L and its binding partners has not been yet identified.

Genes encoding DTX3L and PARP9 are both located in chromosome 3q21 and share a bidirectional promoter[54]. The DTX3L-PARP9 complex has been studied at the protein level, and PARP9 seems to co-exist in a heteromeric complex at least in the context of prostate cancer (PCa)[39,49]. The nanomolar affinity is mediated by DTX3L D3 domain and a domain preceding the PARP9 ART domain (DeBD; **Fig. 1**)[38]. PARP9 brings to the complex macrodomains that function as localisation modules by directing the complex towards PAR- or MARylated proteins[48]. Both Ub ADP-ribosylation and ubiquitination activities reside in DTX3L and are modulated by NAD^+ concentration as Ub ADP-ribosylation limits ubiquitination through Ub poisoning *in vitro*[38,49]. PARP9 has a key function in enhancing both ubiquitination and ADP-ribosylation activities in the complex[38]. The mechanism by which this regulation occurs remains to be revealed and the ubiquitination reaction is also affected to different extents depending on the recipient protein. For example, MARylation of Ub seems to abolish auto-modification of DTX3L[38] and mono-ubiquitination of histone H4, but the incorporation of NAD^+ to the reaction does not affect the conjugation of ubiquitin to histone H3[49]. The new proposed mechanism in which DTX proteins act as Ub ligases for protein MARylation *in vitro* adds to this complexity[53].

Co-immunoprecipitation studies in PCa cell lines point out that DTX3L also interacts with PARP14 [39], which contains three macrodomains and is found in the same conserved gene cluster as PARP9 and DTX3L[54]. Unlike PARP9, PARP14 was observed to be catalytically active[50]. The DTX3L-PARP14 interaction was deemed to be PAR-independent and the interface hub was narrowed down to the N-terminal region of both proteins[39], but there is no structural information that validates this.

In cellular context, DTX3L also forms heterodimers with two other ubiquitin E3 ligases, DTX1 and atrophin-1 interacting protein 4 (AIP4) with opposite regulatory effects (**Fig 1.**). The interaction between DTX3L and DTX1 was first described upon the discovery of the protein and it was established that complex formation between DTX1 and DTX3L has an enhancing effect on the ubiquitination activity of both ligases[36]. On the other hand, complex formation greatly reduces the activity of AIP4 but it does not affect the activity of DTX3L[55].

It can be stated that DTX3L is more than just a "writer" as its activities are modulated by the proteins with which it interacts and vice versa. These proteins bring additional functionality through e.g. PTM-binding domains (**Fig. 1**). As DTX3L forms complexes that include ubiquitination, and ADP-ribosylation writing and reading functions, new binding partners must be carefully validated

as they might not have a direct interaction with DTX3L, but the interaction could be mediated by a third component.

DTX3L functions in the IFN/JAK-STAT pathway in cell-dependent manner

The promoter for DTX3L and PARP9 is induced as a response to interferon gamma (IFNγ) in diffuse large B-cell lymphoma (DLB-CL). The resulting immune response induces expression of PARP9, which further enhances the expression of other IFN-stimulated genes[54]. It was later noted that IFN-induced expression is a cell-dependent event as IFN treatment did not affect the expression of DTX3L or PARP9 in PCa[39].

In PCa, the DTX3L-PARP9 complex downregulates the expression of IRF1, as in the aggressive metastatic PC3 and DU145 cells DTX3L-PARP9 are overexpressed and IRF1 is downregulated[39]. Conversely, in LNCaP cells IRF1 is upregulated while PARP9 is absent and DTX3L expression is low. DTX3L-PARP9-mediated downregulation of IRF1 could be at transcriptional level, likely through the ubiquitination of histone H4 on K91 (H4K91)[39]. It was observed that while PARP9 and PARP14 enhance phosphorylation of STAT1 by JAK (**Fig. 2**), DTX3L inhibits the phosphorylation in PC3 cells, providing an alternative mechanism for IRF1 downregulation as an active STAT is required for IRF1 expression[56].

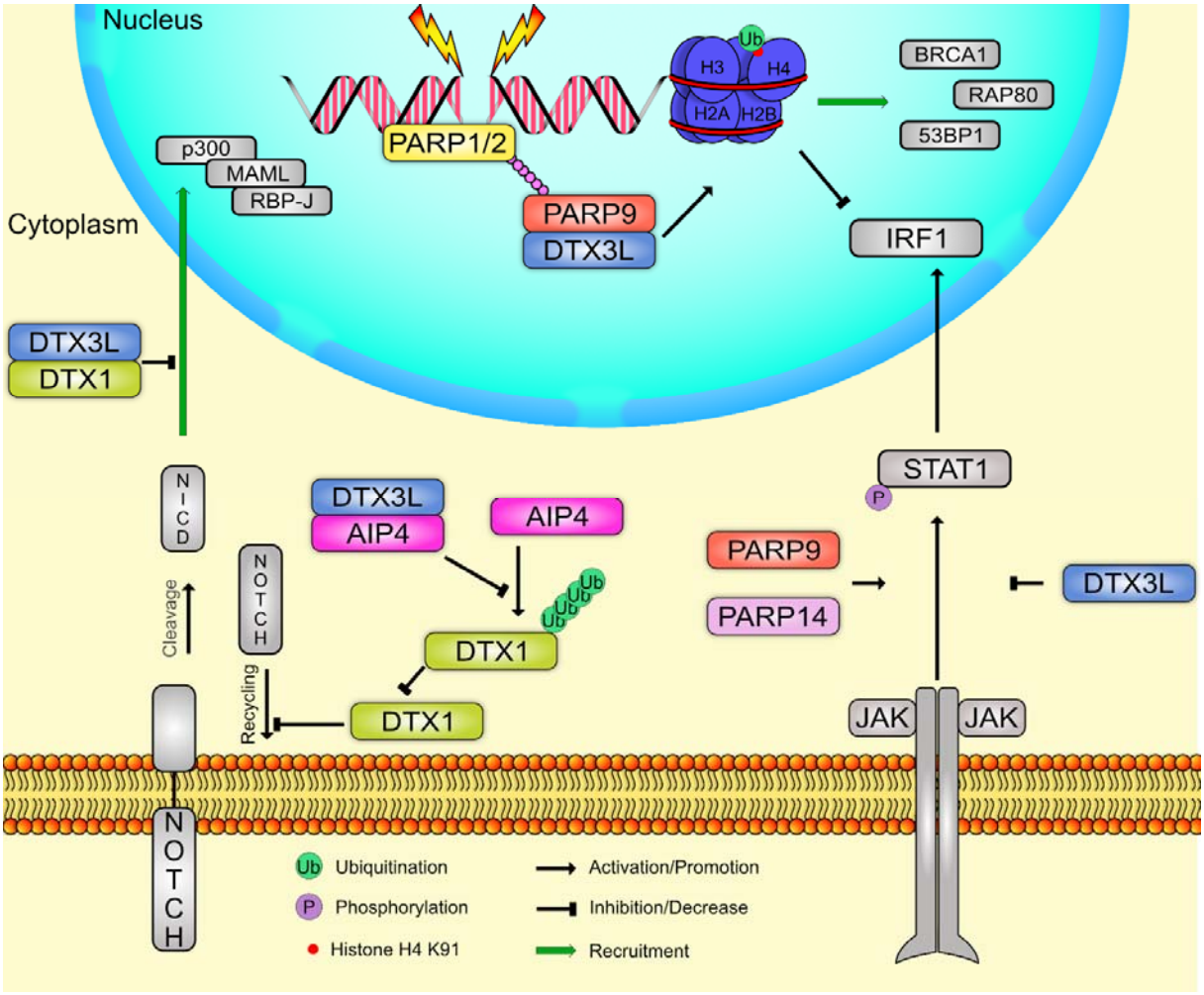

Figure 2. Overview of the roles of DTX3L in different signalling pathways.

DTX3L promotes DNA damage repair through histone ubiquitination

PARylation by PARP1 and PARP2 enzymes at the DNA damage sites is a key event resulting in the recruitment of the repair factor and chromatin remodelling (**Fig. 2**)[57,58]. The repair proteins are recruited through the PAR-binding modules, such as macrodomains, and it has been shown that macrodomains of PARP9 localise DTX3L-PARP9 to DNA damage sites[48]. DTX3L subsequently monoubiquitinates H4K91, causing a significant change in chromatin conformation[59].

Histone H4 monoubiquitination further acts as an early recruitment signal for 53BP1, RAP80, and BRCA1 at DNA damage sites[48,59], thus promoting non-homologous end joining (NHEJ) DNA repair[49]. It was also established that there is a dynamic regulation between HDAC1,2-mediated de-acetylation of H4K91 and DTX3L-mediated mono-ubiquitination on the same residue[60]. Inhibition

of HDAC1,2 reduces the ubiquitination, presumably by availability of K91. As a result, acetylation prevents the recruitment of 53BP to the DNA damage site and subsequent DNA damage repair leading to apoptosis[60].

DTX3L is a negative regulator of NOTCH pathway

The NOTCH signalling cascade is an evolutionarily conserved pathway involved in the regulation of developmental processes and that has been associated to tumour biology[61]. The cascade starts by one of the four NOTCH receptors recognising a ligand sent by a neighbouring cell, which results in the cleavage of the receptor and the release of a NOTCH intracellular domain (NICD). NICD is translocated to the nucleus where it activates other genes. Initially, DTX1 was identified as a negative regulator of the NOTCH signalling through direct ubiquitination of the NICD[62,63]. Later it was established that the DTX1-DTX3L heterodimer is involved[64], likely due to the enhanced activity of the heterodimer[36]. A more recent study suggests that the downregulation of the NOTCH signalling might not be mediated by direct NOTCH ubiquitination but rather by DTX1 activity on PI5P4Kγ, involved in NOTCH recycling[65].

The role of DTX3L in NOTCH signalling might also be linked to its interaction with AIP4[55], a HECT-type E3 ligase that acts as a negative regulator of DTX1[66]. In contrast to DTX1, DTX3L has the opposite effect on ITCH and reduces its ubiquitination activity[55]. There is no inter-modification between DTX3L and DTX1, and DTX3L and AIP4[36,55], which suggests that the regulation is independent of ubiquitination.

DTX3L plays a major role in cell proliferation, chemoresistance and adhesion in multiple cancers

DTX3L activity is highly modulated by its binding partners and there are probably multiple DTX3L complexes co-existing. As a result, distinguishing the activity of isolated DTX3L from DTX3L-PARP9, DTX3L-PARP14, DTX3L-DTX1, and DTX3L-AIP4 is not straightforward. We will cover the reports that directly indicate DTX3L to have functional consequences in cancer and disclose when the functionality is attributable to one of the complexes.

The first link between DTX3L and cancer came at the turn of the century when it was discovered to be an interacting partner of PARP9, the product of the B-aggressive lymphoma 1 (BAL1) gene, and

thus initially named B-lymphoma and BAL associated protein (BBAP)[36]. Individually, PARP9 has been identified as an overexpressed gene in chemoresistant DLB-CL[54,67] and established that it switches STAT1 from a tumour-suppressor to an oncogene by promoting the accumulation of phosphorylated STATβ (**Fig. 2**)[68]. The link between DTX3L and chemoresistance was further confirmed when it was shown that it was overexpressed in chemoresistant lymphoma cell lines Karpas-422 and Nalm6[60]. It is unclear, however, whether chemoresistance is attributable to the DTX3L-PARP9 complex as DTX3L prevents the JAK-mediated phosphorylation of STAT1 in PCa[39].

DTX3L has also been identified as a chemoresistance factor in myeloma, where it promotes cell adhesion-mediated drug resistance (CAM-DR)[69]. It was speculated that similarly to lymphoma, DTX3L-mediated H4K91 ubiquitination acts as a cell cycle arrest signal responsible for drug resistance[60]. DTX3L levels increase along the progression of myeloma cells and the expression is related to cell adhesion; DTX3L knock-down impedes cell proliferation and adhesion[69]. The promotion of cell adhesion seems to be mediated through the AKT pathway, although the exact mechanism by which this occurs is yet unknown[69].

DTX3L plays also an important role in the metastatic ability of melanoma through the action of DTX3L upon AKT, FAK and PI3K[70,71]. While DTX3L levels are low in normal human epithelial melanocytes (NHEM), expression levels increase in malignant melanoma cells, especially in the chemoresitant SKMEL28 cell line[70]. Consequently, DTX3L is a potential biomarker of malignant melanoma. Injection of murine B16F10 melanoma cells into the tail veins of mice led to the development of melanoma foci in the lungs, while this did not happen in DTX3L-depleted cells indicating that DTX3L could be a drug target in melanoma[71].

Glioma is another type of cancer in which DTX3L and PARP9 were found to be upregulated[40,72]. DTX3L was detected in cells and *ex vivo*[40], while PARP9 was identified through a retrospective *in silico* analysis from the TCGA and GEO databases[72]. Both studies seem to converge on the reasoning that DTX3L and PARP9 are more abundantly expressed in aggressive glioma. Additionally, in accordance to other types of cancer, silencing DTX3L expression sensitised cells to DNA-damaging agents, in this particular case temozolomide[40].

Perhaps the best understood cancer where the DTX3L has a role is PCa, where different studies have used multidisciplinary approaches to elucidate the relationship between DTX3L activity and

cancer proliferation. Initially, it was noted that DTX3L was not identifiable in human prostate luminal epithelial cell lines (HPE and RWPE1) but it was expressed in PCa cells and, significantly upregulated in castration resistant prostate cancer (CRPC) cell lines PC3 and DU145[39]. In addition to DTX3L, PARP9 and PARP14 are also expressed in the more metastatic cell lines[39]. In a recent study, it was noticed that the DTX3L-PARP9 complex recognises PARP7-mediated MARylated androgen receptor (AR)[73]. It has been proposed that PARP7 acts as a tumour suppressor and might be a biomarker for survival in PCa as its levels were higher in healthy cells than in metastatic cell lines[74]. Additionally, even though the exact mechanism is not yet understood, it has been proposed that both PARP14 and DTX3L promote PCa survival, and migration by acting on non-redundant pathways[39]. Therefore, targeting both proteins with small molecules might result in more effective therapies in the future.

Outlook

DTX3L has emerged a key regulatory enzyme in multiple cancers affecting adhesion, migration, proliferation and chemoresistance and in multiple cases it could act as a potential drug target to be validated. As a RING E3 ligase DTX3L is not a classical enzyme but rather facilitates its functions through protein-protein interactions and, although challenging to target with small molecules, could also provide specific ways to affect cellular pathways. DTX3L is also an ADP-ribosyltransferase or could ubiquitinate ADPr and it is not clear what the actual roles of these modifications are in the cells. The mixed Ub-ADPr chain has not been identified before and it is unclear if it can be recognised by Ub- or ADPr-readers or if there are specific readers for this modification. Furthermore, DTX3L forms complexes with multiple proteins that contain putative MAR and PAR binding motifs and as such the mechanism by which these two PTMs act in concert in different contexts will be important for future studies. Studies of distinct macromolecular complex of DTX3L and identification of new ones will be crucial in the future as it can help to provide a mechanistic way by which DTX3L activity is modulated.

Perspectives

- DTX3L has both ubiquitination, ADP-ribosylation, and potentially ADPr ubiquitination activities which are regulated by other macromolecules and DTX3L can also modulate the activity of its binding partners.

- DTX3L is an important enzyme in multiple cancers that promotes survival and proliferation contributing towards chemoresistance through several signalling pathways.
- Little is known in detail about how DTX3L interacts with multiple binding partners (possibly simultaneously), how these interactions co-regulate enzyme activity in the contexts of signalling pathways, how it structurally looks and what structural changes are needed in order to accommodate each binding partner. In the future these will be elucidated by integrative approaches of protein science and cell biology and help to understand its role as a cancer biomarker and as potential therapeutic target.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Author Contributions

C.V.R and L.L. designed and wrote the manuscript.

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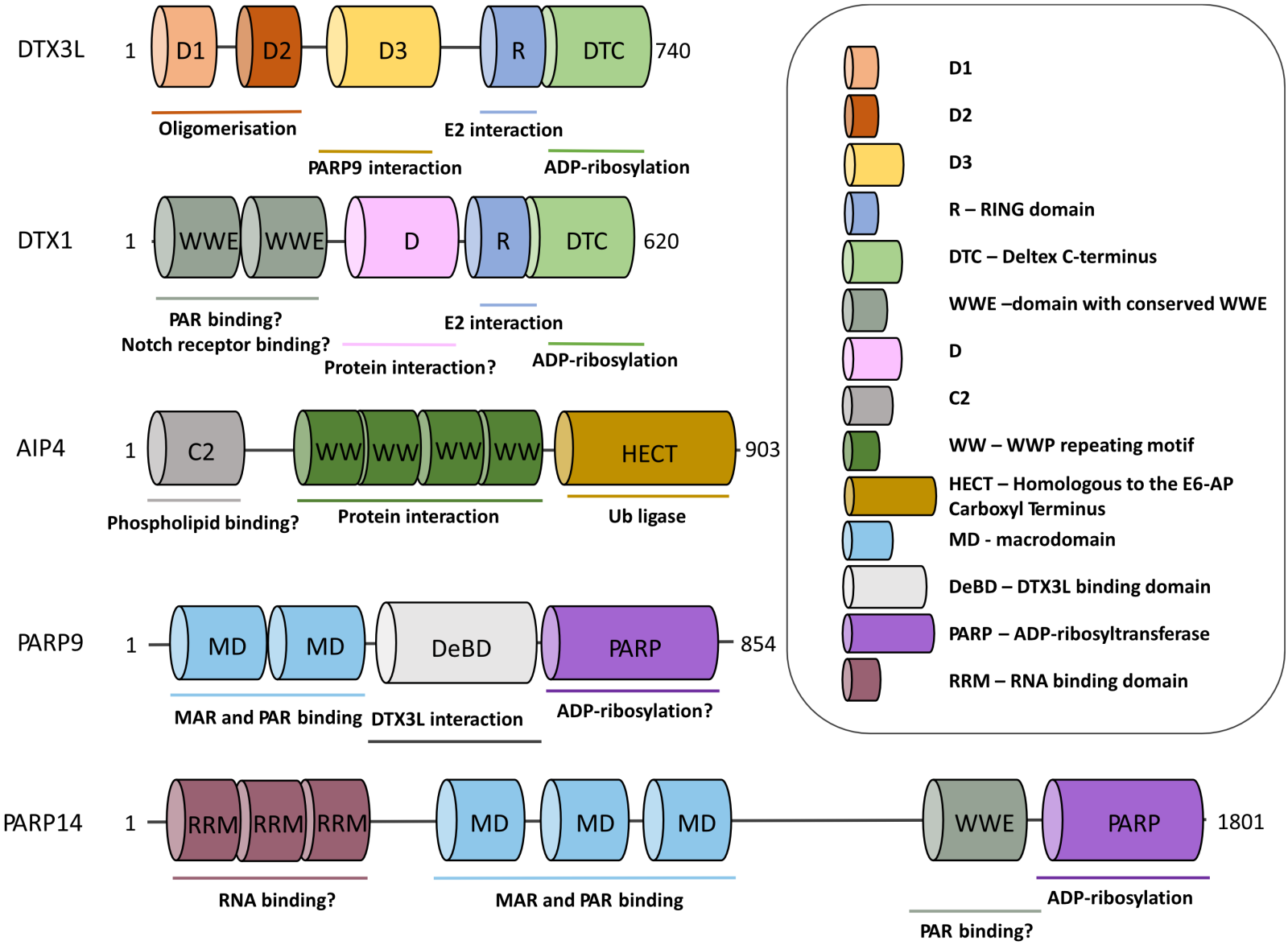
DTX3L
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Oligomerisation
E2 interaction
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1
WWE
WWE
D
R
DTC
620
PAR binding?
Notch receptor binding?
E2 interaction
Protein interaction?
ADP-ribosylation
AIP4
1
C2
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WW
WW
WW
HECT
903
Phospholipid binding?
Protein interaction
Ub ligase
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1
MD
MD
DeBD
PARP
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MAR and PAR binding
DTX3L interaction
ADP-ribosylation?
PARP14
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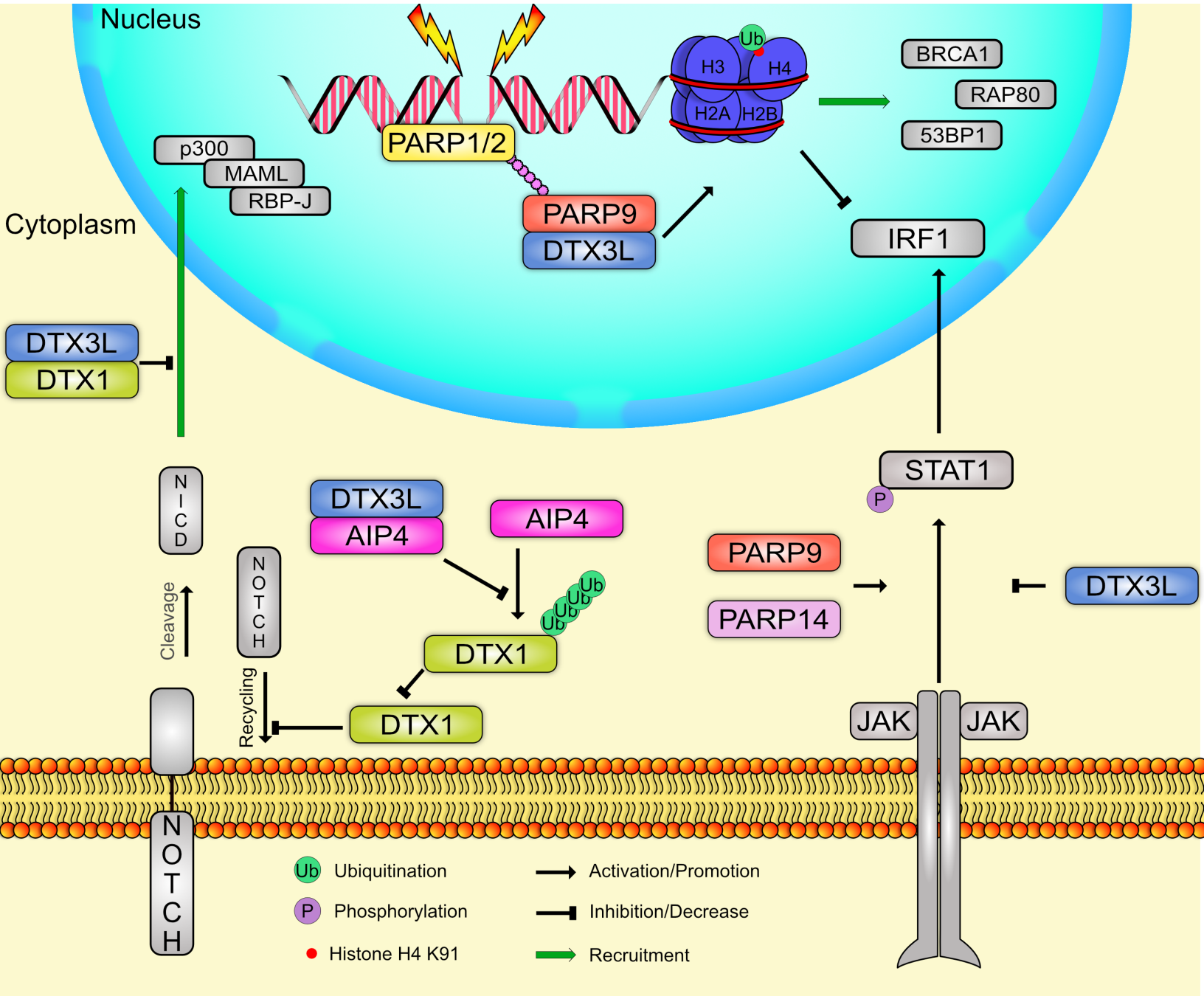
RRM
RRM
RRM
MD
MD
MD
WWE
PARP
1801
RNA binding?
MAR and PAR binding
ADP-ribosylation
PAR binding?
D1
D2
D3
R – RING domain
DTC – Deltex C-terminus
WWE –domain with conserved WWE
D
C2
WW – WWP repeating motif
HECT – Homologous to the E6-AP Carboxyl Terminus
MD - macrodomain
DeBD – DTX3L binding domain
PARP – ADP-ribosyltransferase
RRM – RNA binding domain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IRF1
DTX3L
DTX1
NICD
NOTCH
Cleavage
Recycling
DTX3L
AIP4
AIP4
Ub
DTX1
DTX1
STAT1
P
PARP9
PARP14
DTX3L
JAK
JAK
NOTCH
Ub Ubiquitination
P Phosphorylation
Histone H4 K91
Activation/Promotion
Inhibition/Decrease
Recruitment