Review

Regulation of HDAC6 Catalytic Activity in Cancer: The Role of Post-translational Modifications and Protein-Protein Interactions

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Review

Regulation of HDAC6 Catalytic Activity in Cancer: The Role of Post-Translational Modifications and Protein-Protein Interactions

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Abstract: Histone Deacetylase 6 (HDAC6) is a large multidomain protein that deacetylates lysine residues on cytoplasmic proteins, influencing a wide range of cellular processes. Both the catalytic and non-catalytic functions of HDAC6 are implicated in cancer development and progression. Over a decade of clinical trials with catalytic domain inhibitors, such as Ricolinostat (ACY1215), has shown that these drugs are well tolerated and can alleviate chemotherapy-induced peripheral neuropathies. However, their effectiveness in treating solid tumors remains uncertain due to the limited number of clinical trials and the small size of patient cohorts, leading to inconclusive results. HDAC6 activity is regulated by protein-protein interactions and post-translational modifications, which may allosterically influence its catalytic domains. Therefore, targeting HDAC6 in cancer therapy with small molecule inhibitors requires a more sophisticated understanding of its role within the tumor microenvironment, including whether its expression correlates with deacetylase activity. A comprehensive evaluation of both its enzymatic and non-enzymatic functions could be crucial in developing more effective strategies for targeting HDAC6 in cancer.

Keywords: HDAC6; Cancer; phosphorylation; acetylation; PPI

1. Introduction

The *histone deacetylase 6* (*HDAC6)* gene is located on Xp11.23 and consists of 1,255 amino acids giving rise to one of the largest human proteins described and largest of the histone deacetylase (HDAC) proteins [1]. HDAC6 is uniquely composed of two catalytic deacetylase domains (CD1, CD2) which are responsible for the removal of acetyl groups from lysine in their substrates. Both CD1 and CD2 domains are composed of similar amino acid sequences, conformation, and architectural structure. The CD2 domain is considered to be the main catalytic site bearing a wider substrate specificity [2]. The dynein motor binding region (DMB) links the CD1 and CD2 domains together. HDAC6 is exclusively located in the cytoplasm due the presence of a nuclear export signal (NES) that prevents its nuclear localisation and a SE14 Ser-Glu containing tetrapeptide region provides a secure cytoplasmic anchor. HDAC6 also possesses a C-terminal zinc finger ubiquitin binding domain (ZnF-UBP) and binds mono- and poly-ubiquinated proteins [1].

The catalytic dependent and independent functions of HDAC6 are involved in various cellular responses and appear to be at the crossroads between acetylation and ubiquitination respectively [3].

HDAC6 predominantly deacetylates cytoplasmic proteins such as α-tubulin, cortactin and heat shock protein HSP90 [1]. The deacetylation of -tubulin by HDAC6 is integral to microtubular integrity and stability mediating various cellular responses including cell motility and polarity, subcellular transport, cell proliferation, survival, adhesion, immune and inflammatory response [3,4]. The deacetylation of cortactin by HDAC6 connects HDAC6 activity to actin cytoskeleton dynamics and is a potential link between microtubule dynamics and the regulation of actin filament polymerisation [5,6]. Hyperacetylation of HSP90 in cells depleted of HDAC6 abolishes its protein

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chaperone function, preventing it from binding to pro-survival and pro-growth client proteins, leading to their depletion from cells [4].

The scaffold function of HDAC6 produces HDAC6 protein complexes, which are mainly involved in the regulation of protein aggregate accumulation, proteasomal degradation of misfolded proteins, and autophagy [7,8]. HDAC6 ubiquitin binding is a critical step which regulates the formation and dissociation of the HDCA6-HSP90-HSF1 (Heat Shock Factor 1 complex) [7,8]. HDAC6 senses the accumulated levels of ubiquitinated protein aggregates and consequently mediates the release of HSF1 from its repressive complex. This induces the activation of several cellular chaperones and stimulates the clearance of cytotoxic proteins [7,8]. Additionally, heat shock cognate protein 70 Hsc70 and dnaJ homolog subfamily A member 1 (DNAJA1) were identified as novel substrates of HDAC6. Hsc70/ DNAJA1 function together and are associated with correct protein folding [9]. The overexpression of HDAC6 deacetylation activity enhances Hac70/DNAJA1 interaction and its role in cell survival and cellular stress response where the hyperacetylation of HSC70/DNAJA1 causes its dissociation and loss of their chaperone activity [9]. Therefore, HDAC6 protein complexes provide an essential protective mechanisms against cellular stress [7,8].

The Role of HDAC6 in Cancer

Many of the cellular functions of HDAC6 are hallmarks of tumorigenesis and malignant transformation in which HDAC6 plays an important role in cancer survival [1,5,6,10–27]. Overexpression of HDAC6 has been reported in a variety of tumours [28] including low-grade and high-grade ovarian cancers [6,28–30], oral squamous cell carcinoma [31], hepatocellular carcinoma [32], pancreatic cancer [11] and breast cancer [33,34]. The deacetylase activity of HDAC6 is required for malignant growth in which HDAC6 null mice and HDAC6 deficient fibroblasts were shown to be more resistant to tumour formation (64). Loss of HDAC6 causes tumour cells to be less responsive to the oncogenic RAS pathway and activation of its signalling cascade [35]. The deacetylation of α-tubulin, cortactin, P53, HSP90 or oestrogen-related receptor ERRα have contributed to tumorigenic transformation by effects on cell proliferation, migration and through the regulation of several molecular signalling pathways. For example, the HDAC6/Hsp90 complex regulates tumour cell death by modulating several downstream targets and transcriptional factors [36]. HDAC6 affects intracellular ligand binding, nuclear translocation and transcription of various mutant cancer promoting proteins such as P53 observed in human leukaemia cells [28]. Furthermore, the ubiquitin-binding domain of HDAC6 binds to ubiquitinated proteins during cell stress to regulate the ubiquitination and aggresomal degradation of various tumour-promoting proteins [15,28].

Since microtubule dynamics are essential for cell division, motility, attachment and the maintenance of cell shape in addition to intracellular protein trafficking and modulation of several signal transduction pathways, the catalytic regulation of microtubule dynamics by HDAC6 is a primary underlying function of HDAC6 in cancer cells and a therapeutic target [21]. Furthermore, HDAC6 knockdown contributes to lysosomal trafficking and degradation of epidermal growth factor receptor (EGFR) as a result of increased α-tubulin acetylation [21]. This affects the microtubule-dependent transport of EGFR endosomal vesicles, in which the regulation of this pathway may be linked to the pathogenesis of various types of human cancer cells, such as prostate, pancreatic and lung cancer cells [21]. Notably, catalytic inhibition of HDAC6 attenuates tumour growth, increases apoptotic markers in lung cancer cells [37] and causes cell cycle arrest in skin tumours [22]. HDAC6 was explicitly shown to contribute to cell motility, migration and invasion of ovarian cancer cells [29], hepatocellular carcinoma [32] and bladder cancer [38]. Its expression was found to be localised in the leading edges of actively migrating ovarian cancers cells and targeting the ubiquitin proteasome system (UPS), in combination with HDCA6 inhibition, significantly reduced ovarian cancer cell migration and invasion [29]. The overexpression of HDAC6 resulted in a highly increased association of cortactin with F-actin filaments promoting cell migration and invasion [6]. Conversely, catalytic inhibition of HDAC6 induced cortactin hyperacetylation, blocked actin binding assembly in hepatocellular carcinoma cells and markedly reduced the migration and invasion capacity of these cells [32]. Cortactin may be considered a significant predictive marker of tumour progression and

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reduced survival in prostate cancer, and its regulation by HDAC6 may also contribute to its role in poorer patient outcomes [39].

Mass spectroscopy (MS) identified the HDAC6 acetylome of TNBC cells, revealing approximately 83 intracellular proteins displaying increased acetylation due to catalytic inhibition of HDAC6. Specific signalling pathway analysis showed significant alterations in metabolic cellular processes resulting from HDAC6 inhibition. Reduced aldolase and Gyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity occurred in the presence of HDAC6 inhibition and knockdown resulting in their increased acetylation and decreased glycolysis in TNBC cells highlighting the role of HDAC6 catalytical activity in regulating energy metabolism in cancer cells [40].

HDAC6 as a Target in Cancer

It is just over ten years since the discovery and application of HDAC6 inhibition with Ricolinostat (ACY1215) (NCT01323751), with the first clinical trial taking place in November of 2012 [41]. Ricolinostat is currently under investigation in clinical trials to treat various disease conditions, with a focus on investigating its efficacy and evaluating its safety as a cancer treatment [41–45]. Ricolinostat showed promising results regarding the safety, tolerability and efficacy in treating blood cancers such as leukaemia, multiple myeloma and lymphoma [41,44,46–48]. Results from existing phase I and II clinical trials of Ricolinostat showed a maximum tolerated dose of 160mg daily dosing for Ricolinostat used in combination with bortezomib or lenalidomide/Pomalidomide and dexamethasone for the treatment of multiple myeloma [41]. These clinical trials showed a higher therapy response, the least adverse effects and better disease stabilisation compared to standard treatment [49]. Using Ricolinostat as a single agent demonstrated a favourable efficacy and safety profile in patients with relapsed or refractory lymphoid malignancy [50]. These trials also showed a potentially meaningful impact of Ricolinostat in preventing taxane-induced neuropathy [43,51]. The efficacy of Ricolinostat has also been tested in solid tumours and showed synergetic anti-cancer properties in combination with several chemotherapies, proteasomal inhibitors and immunomodulatory drugs [42,45,50]. However, patient studies supporting its widespread use in solid tumours is still unclear and remains to be thoroughly investigated where limited number of clinical trials and small size of cohorts have contributed to inconclusive clinical results [42,45,52–54]. Furthermore, what makes a cancer cell sensitive to HDAC6 inhibition has not been clearly defined [55–57].

The effective acetylation of α-tubulin by HDAC6 inhibitors indicates that they are pharmacodynamically active and selective for HDAC6, whereas the acetylation of lysine residue 9 (K9) of Histone 3 demonstrates the inhibition of class I HDACs [58,59]. Several recently published studies recorded Ricolinostat to cause non-selective inhibition of class I HDACs in acute myeloid leukemic (AML) and high-grade serous ovarian cancer [60]. Moreover, the phenotypic responses associated with Ricolinostat in tumours have been reported at concentrations higher than the HDAC6 selective concentrations in which the reported toxicity of IC50 doses were found to be high enough to inhibit nuclear HDACs [60]. The biological toxic effects of most of the small molecule inhibitors of HDAC6 recorded in the literature were produced at nonselective concentrations [58,59,61] and several studies did not measure the effect of the HDAC6 inhibitor concentration on acetylated levels of histone 3 [59,62]. Therefore, many studies of HDAC6 inhibition in cancer do not specify its specific mode of action. HDAC6 mRNA or protein expression alone cannot stratify patients for HDAC6 inhibitory therapy whereby improved knowledge of HDAC6 activity modulation through post-translational modifications and protein interactions could inform better targeting strategies. For example, Zeleke et al. developed an approach to predict breast cancer sensitivity to HDAC6 inhibition using algorithmic reconstruction of HDAC6 transcriptional targets, calculating an HDAC6 score [63]. High HDAC6 score cancers are more sensitive to inhibition, while low score cancers show resistance. This study also presented an integrated analysis of cancer patient datasets and HDAC6 activity-affected gene expression profiles which indicated that the unfolded protein response (UPR) is the primary pathway associated with HDAC6 activity in cancer [63]. The catalytic activity of HDAC6 is modulated by protein-protein interactions and post-translational modifications, that may

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also allosterically control the catalytic domains of HDAC6 [64]. Thus, the inhibition of HDAC6 in cancer with small molecule inhibitors requires consideration of the function it performs in the cancerous environment and if it's expression in a tumour correlates with its deacetylase activity. Moreover, understanding the activation state of both HDAC6's enzymatic and non-enzymatic functions could further help in identifying the most effective strategy for targeting HDAC6 in cancer.

Regulation of HDAC6 Catalytic Activity by Post-Translational Modifications and Protein-Protein Interactions

Phosphorylation of HDAC6

The exact mechanisms regulating HDAC6 activity are still being examined to determine which kinases and phosphorylation sites contribute to its catalytic activity (Figure 1). Additionally, the regulation of HDAC6 activity by phosphorylation was found to have a direct impact on cell proliferation, migration, and motility [64]. The formation of a complex between atypical Protein kinase C zeta (aPKC ζ) and HDAC6 is considered to be a predominant phosphorylation response of PKCζ. Phosphorylation of serine and threonine residues by PKC ζ is conserved in both the CD1 and CD2 catalytic domains of HDAC6 in which increasing levels of PKCζ resulted in raised HDAC6 deacetylase activity and a dramatic reduction of acetylated -tubulin levels [65]. Immunofluorescence microscopy showed a considerable increase of -tubulin acetylation by 5-fold and 2-fold in HDAC6 inhibited cells compared to PKCζ inhibition, respectively, suggesting that there may be other protein kinases involved in the regulation of HDAC6 deacetylase activity [65]. In the induction of viral immunity, calcium-activated protein kinase C alpha (PKCα) was shown to phosphorylate and induce HDAC6 deacetylase activity towards β-catenin resulting in nuclear transcription and translocation in response to viral infection [65].

Phosphorylation of HDAC6 on sites other than its catalytic domains modulates the role of HDAC6 deacetylation activity in the regulation of transport and the clearance of misfolded proteins [66]. The phosphorylation of serine 458 in the dynein motor binding region of HDAC6, by protein casein kinase 2 (CK2), stimulates HDAC6 deacetylase activity and increases the binding of HDAC6 and misfolded proteins to dynein motor protein in response to stress. This indicates that HDAC6 itself is insufficient to deacetylate tubulin, and the phosphorylation of HDAC6 by CK2 may act as a regulator of HDAC6 deacetylase activity [66] (Figure 1).

Extracellular signal-regulated kinase (ERK) phosphorylates the C-terminal region of HDAC6 on threonine 1031 and serine 1035 [67]. The primary ERK1 phosphorylation site is the serine 1035 residue, in which the threonine 1031 phosphorylation site was found to be dependent on serine 1035 phosphorylation [67]. The suggested mechanism of HDAC6-mediated cell motility occurs through the EGFR-Ras-Raf-MEK-ERK phosphorylation of serine 1035 [67] and is associated with enhanced deacetylase activity towards -tubulin, leading to an increase in cell migration downstream of EGFR signalling activation [67]. MAP3K5 (ASK1) is a serine/threonine kinase which phosphorylates HDAC6 in hypoxia preventing the ubiquitination of HDAC6 via the VHL protein resulting in HDAC6 stabilisation and its increased expression [68].

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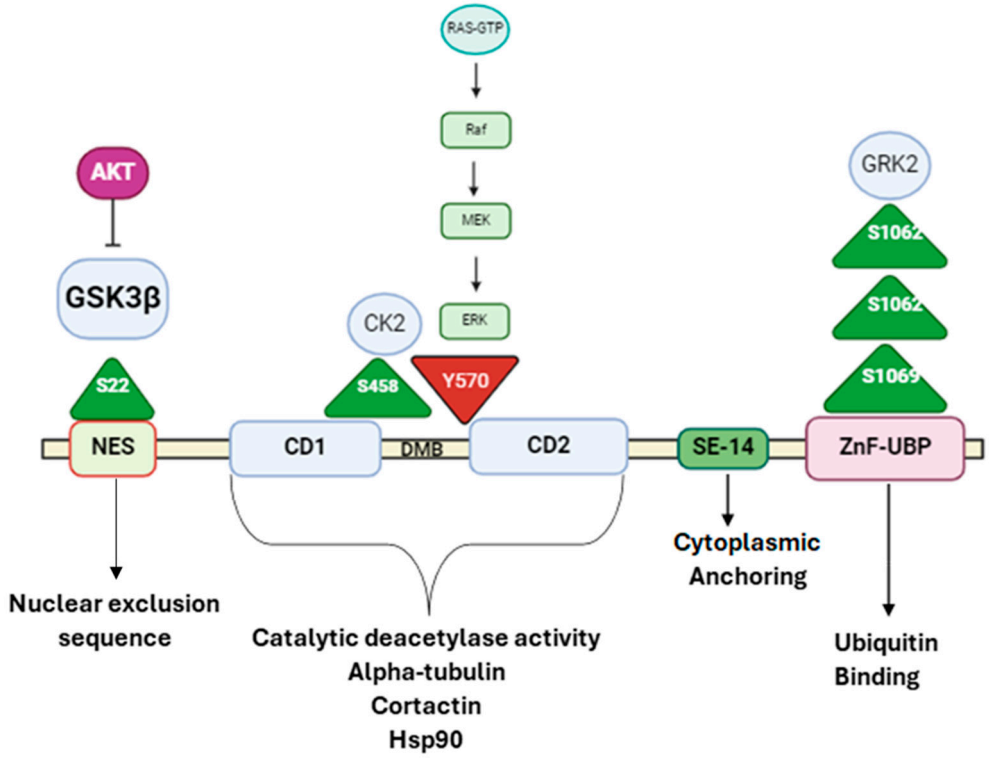

Figure 1. The major serine (S) and tyrosine (Y) phosphorylation sites affecting HDAC6 catalytic activity. HDAC6 catalytic domains (CD1, CD2) and Ubiquitin Binding domain (UBP), Nuclear export signals (NES), dynein motor binding region (DMB), a serine-glutamine-containing tetradecapeptide, cytoplasmic anchor (SE14) and nuclear localisation sequence (NLS). The protein kinases are glycogen synthase 3β (GSK3β), casein kinase 2 (CK2), Epidermal growth factor receptor kinase (EGFR), (Ras)-(Raf)-Mitogen-activated protein kinase-(MEK)-extracellular-signal-regulated kinase (ERK) G-protein coupled receptor kinase 2 (GRK2). Green and Red triangles indicate increased or decreased catalytic activity.

Glycogen synthase 3β (GSK3β) may directly regulate HDAC6 catalytic activity by phosphorylation at serine 22. The inhibition of GSK3β increases acetylated -tubulin and enhances neural mitochondrial trafficking as a result of decreased HDAC6 activity. GSK3β is a constitutively active kinase and its phosphorylation by the upstream signalling mediator, AKT, switches off its kinase activity. The inhibition of AKT prevents the phosphorylation of GSK3β leading to increased phosphorylation of HDAC6 at sereine 22 [69]. Significantly, HDAC6 is considered to be a key mediator of AKT dephosphorylation. The dissociation of HDAC6 and protein phosphatase 1 (PP1) protein complex facilitates the association of PP1 with AKT resulting in its dephosphorylation [70]. The inhibition of HDAC6 with TSA is suggested to occur by preventing the formation of the HDAC6-PP1 protein complex in a dose-dependent manner by promoting the dephosphorylation of AKT by PP1. The extensive range of PP1 interacting substrates suggests that HDAC6 may also modulate many other signalling pathways through binding to PP1 [70].

The G-protein coupled receptor kinase 2 (GRK2) directly binds to the CD1 and CD2 domain of HDAC6 without any intermediate regulator. Serine 670 phosphorylation by GRK2 is considered to be the main modulatory response of GRK2 regulation of HDAC6 activity. GRK2 predominantly phosphorylates the CD2 domain of HDAC6 and stimulates its tubulin deacetylase activity resulting in enhanced cell motility and metastasis. Serine residues 1060, 1062 and 1069 in the region between the CD2 catalytic domain and the ZnF-UBP domain are also considered to be critical phospho-acceptor sites of HDAC6 by GRK2 and essential for modulating tubulin deacetylase activity.

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Therefore, the allosteric conformational modification of HDAC6 by GRK2 phosphorylation affects the deacetylase activity towards specific HDAC6 substrates such as tubulin, but not cortactin since the acetylation level of cortactin is not altered by GRK2 levels [14]. This indicates that the post-translational modification of HDAC6, in addition to regulating its catalytic activity and binding to ubiquitinated misfolded proteins, is also regulated by HDAC6 substrate specificity. GRK2 binding and phosphorylation of HDAC6 selectively enhances cell migration through modulation of tubulin acetylation in which GRK2-HDAC6 complexes occur extensively at the leading edges of migrating cells [12,14].

In contrast, phosphorylation at tyrosine 570 residue of the CD2 domain of HDAC6 by epidermal growth factor receptor kinase (EGFR-K) results in reduced HDAC6 catalytic activity and increased -tubulin acetylation. Since the endocytic lysosomal degradation of EGFR mainly involves microtubules associated with dynamic motors, HDAC6 was found to be a significant regulator of EGFR trafficking and degradation. HDAC6 forms a protein complex with internalised EGFRs. In HDAC6 knockout cancer cells, EGFR undergoes premature degradation and trafficking, leading to diminished EGFR signalling activity [21]. The microtubules associated with the HDAC6 effect modulate the post-endocytic intracellular transport of activated EGFR . [21,71].

Acetylation of HDAC6

The acetylation of HDAC6 occurs at various sites of HDAC6 lysine clusters in the N-terminal nuclear localisation signal region, the CD2 domain and the link junction between CD2 domain and the SE region [72] in which the protein deacetylases and acetyltransferases affect each other's activities [73]. The P300 histone acetyltransferase (HAT) is a transcriptional co-activator found to acetylate HDAC6, downregulate its deacetylase activity and interact with HDAC6 catalytic domains with better binding affinity compared to the HDAC6 C-terminal ZnF-UBP domain [73]. In the presence of P300 mediated acetylation of HDAC6, the ability of HDAC6 to deacetylate -tubulin is almost abolished resulting in decreased cell motility [72,73]. Furthermore, the acetylation of the N-terminal nuclear signal localisation region of HDAC6, by the Creb binding protein (CBP) HAT inhibited its nuclear import and its interaction with nuclear import proteins [72,74]. The cytoplasmic retention of HDAC6 by its acetylation levels eventually affects its histone deacetylation activity [72]. While HDAC6 mainly localises in the cytoplasm, the presence of CBP acetyltransferase effects on the acetylation of survivin may stimulate nuclear trafficking of HDAC6 to regulate acetylated levels of nuclear survivin. Consequently, modulation of HDAC6 nuclear import by CBP and survivin deacetylation suggest that the cellular localisation of HDAC6 can be modified by acetyltransferase proteins and by regulating the interaction of HDAC6 binding with other proteins (Figure 2) [74].

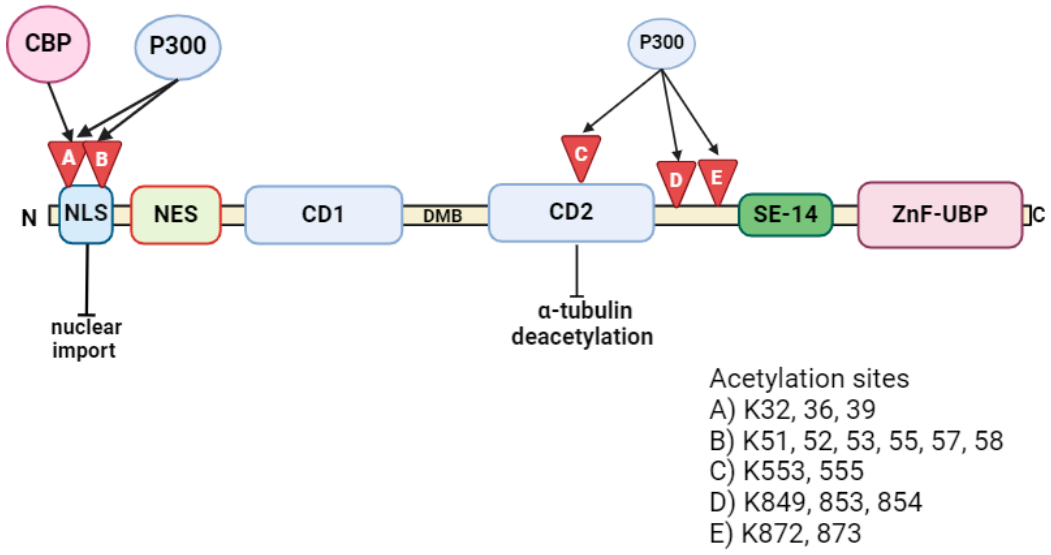

Figure 2. Schematic of the major acetylation sites of HDAC6 which affects its catalytic activity. HDAC6 catalytic domains (CD1, CD2) and Ubiquitin Binding domain (UBP), Nuclear export signals

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(NES), a serine-glutamine-containing tetradecapeptide, cytoplasmic anchor (SE14) and nuclear localisation sequence (NLS). Histone Acetyltransferases, CBP and P300. Red triangles indicate that the presence of the acetylation decreases the downstream function.

Protein-Protein Interactions

Several studies have also described the regulation of HDAC6 activity by protein-protein interactions (Figure 3). The acetylation of tubulin was found to be increased by the expression of the tubulin polymerisation promoting protein TPPP/P25. TPPP/P25 is an unstructured protein that was found to inhibit HDAC6 and increase tubulin acetylation. This interaction was shown to play a major role in microtubule network stability, bundling and rearrangement that controls cell differentiation, maturation, and motility. It was found that the TPPP/P25 inhibitory effect of HDAC6 was compromised in the presence of increasing tubulin concentrations, suggesting that both tubulin and HDAC6 compete for the same specific binding domain of TPPP/P25. Therefore, in the presence of higher tubulin levels, the inhibitor effect of TPPP/P25 on HDAC6 is removed and HDAC6 activity increases. Inhibition of HDAC6 with TSA in the presence of TPPP/P25 further increased tubulin acetylation, but was limited, suggesting that the active sites of the HDAC6 catalytic domains may become saturated [75]. The dynein light chain LC8 was also identified as a protein regulatory hub that mediates TPPP/P25 interaction with HDAC6. The association of TPPP/P25 with DYNLL/LC8 is attenuated in the presence of increasing tubulin concentrations which may suggest competition of tubulin and DYNLL/LC8 for binding to TPPP/P25. Once TPPP/P25 binding to DYNLL/LC8 is decreased, it is available to bind to HDAC6 and in the presence of DYNLL/LC8, tubulin acetylation decreases as a result of the inhibitory action of TPPP/P25 on HDAC6 [76]. LC8 alone has no effect on the degree of tubulin acetylation, but in the presence of TPPP/P25, tubulin acetylation decreased due to the impact of LC8 on the inhibitory potency of TPPP/P25 on HDAC6. Consequently, ternary and binary HDAC6 structure complexes modulate the intracellular localisation of HDAC6 [75,76].

Sequestosome 1(SQSTM1)/p62) is a scaffold protein involved in aggresomal formation and degradation. The downregulation of p62 significantly results in the hyperactivation of HDAC6-mediated deacetylation of -tubulin and cortactin. Higher HDAC6 activity increases the deacetylation of cortactin, leading to enhanced cortactin-dependent F-actin assembly with protein aggregates but causes defective F-actin remodelling and autophagosome-lysosome clearance in the absence of p62 [77]. The binding region of p62 on HDAC6 is localised to the 429–824 residues, which span the entire CD2 domain [77]. Accordingly, the catalytic activity of HDAC6 in aggresomal degradation is regulated by the extent of HDAC6 protein interactions [77]. HDAC6 interacting with p62 causes the association of HDAC6 with other interacting partners such as TRIM50 and parkin to be accumulated in aggresomes and transported to the microtubule organising centre (MTOC) for autophagic degradation [77]. Therefore, P62 binds to HDAC6 CD2 domain inhibiting its deacetylase activity [77].

The Rho effector mDia2, which modulates F-actin bundle assembly for cytokinesis and cell growth, was found to increase the deacetylase activity of HDAC6 by binding to its CD1 and CD2 domains and causing the deacetylation of microtubules [78]. The regulation of HDAC6 activity by mDia2 was also found to be a critical regulator of osteoclast maturation [78]. Cylindromatosis (CYLD), a tumour suppressor protein, has been found to regulate the level of -tubulin acetylation via HDAC6 inhibition [22]. HDAC6 binding to CYLD facilitates the localisation of CYLD in the perinuclear region causing cell cycle arrest [22]. A notable interaction between paxillin and HDAC6, in which the inhibition of HDAC6 by paxillin results in microtubules acetylation, was found to have a significant role in cell migration and invasion [79]. Paxillin knockdown in breast cancer cells disrupted Golgi complex localisation and cell reorganisation that was rescued by HDAC6 inhibition [79,80]. With the modulation of cell motility by HDAC6 being regulated by paxillin [79,80] the post-transitional modifications and protein interactions with HDAC6 affect the extent of its activity (Table 1)

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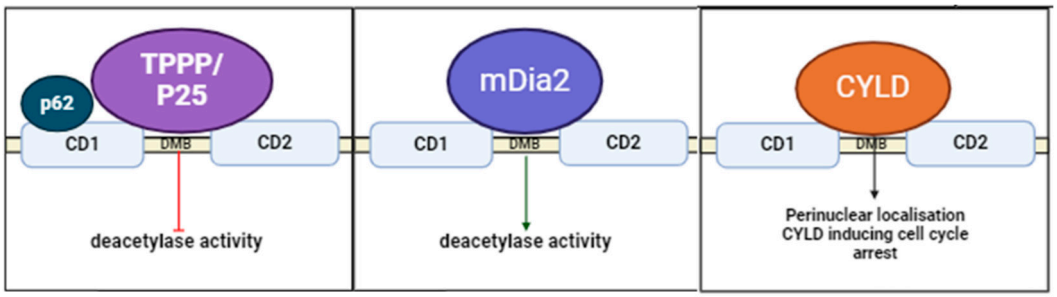

Figure 3. Schematic of major HDAC6-protein interactions which regulate the catalytic function of HDAC6.

The Protein Scaffold Function of HDAC6

The regulation of HDAC6 activity by protein ubiquitination is still not fully explained, however, the association of HDAC6 with ubiquitin, results in its dissociation from other protein complexes. The activity of the ZnF-UBP domain of HDAC6 is less active in full-length proteins compared to truncated C-terminal protein fragments suggesting that HDAC6 protein conformation or catalytic domain activity regulates the ubiquitin-binding function of HDAC6 [81]. The ZnF-UBP domain of HDAC6 is different to other known ubiquitin binding domains as it contains three zinc ions coordinated by 12 cysteine residues or 12 histidine residues [82]. Without the presence of these zinc ions it is thought that the stability of HDAC6s ZnF-UBP domain would be compromised [82]. Many ubiquitin binding domains bind to ubiquitin at low affinity, however, HDAC6 binds at a relatively high affinity (60 nM) [82]. HDAC6 is said to undergo two types of ubiquitin binding depending on the accessibility of the free arginine, glycine or glycine end of ubiquitin. This is unlike other interactions with ubiquitin binding where they interact with the hydrophobic core of HDAC6 ZnF-UBP domain. The binding of ubiquitin at high affinity to HDAC6 ZnF-UBP domain would decrease the catalytic activity of HDAC6 with polyubiquitinated proteins and thus regulate the turnover of proteins that are misfolded [82]. Due to the presence of this domain HDAC6 can be said to be linked to the ubiquitin system via conjugation of ubiquitin, polyubiquitin binding and co-purification with deubiquitinating enzymes [83]. HDAC6 will specifically bind free ubiquitin chains but not ubiquitinated proteins [84]. It was also shown that HDAC6 can disassociate freely from the aggresome rather than being degraded itself [85]. The inhibition of this non catalytic ZnF-UBPdomain could be an alternative strategy to prevent substrate binding to HDAC6 [86].

HDAC6 forms a cytoplasmic protein complex with P97. P97 is an endoplasmic reticulum ATPase enzyme also called valosin-containing protein VCP or CDC48 in yeast [81]. The interaction of the p97/VCP/CDC48 pathway with HDAC6 regulates the proteasomal segregation and degradation process [81]. The binding of HDAC6 with P97/VCP mediates the degradation of polyubiquitinated proteins by regulating the recruitment and sequestration of polyubiquitinated proteins into aggresomes [8,15]. The DBM region of HDAC6, which binds to dynein motors, promotes the loading of polyubiquitinylated misfolded proteins into dynein motors, forming complexes to facilitate its transport to the aggresomes in preparation for their degradation [15]. HDAC6 acts like a bridge between polyubiquitinylated misfolded proteins bounded to its ZnF-UBP domain and Dynein motors bounded to its DMB region [15]. HDAC6 was also found to be extensively colocalised with dynactin P150 which is also considered a component of dynein motor complexes [15]. The formation of HDAC6-dynein complexes increase as the misfolded protein levels increase which suggests that the association of dynein motors to misfolded proteins is significantly affected by HDAC6 levels [15]. The deacetylase domains of HDAC6 are also required for aggresomal formation suggesting that HDAC6 deacetylation of -tubulin or other microtubules and actin associated substrates modulate the binding of dynein motors to the DMB of HDAC6 with enhanced transport of misfolded proteins to the aggresomes [15]. Therefore, the deacetylation of microtubule-related proteins by HDAC6 may regulate the interactions between dynein, the DMB region of HDAC6 and the binding of polyubiquitinylated misfolded proteins to the HDAC6 ZnF-UBP domain (Figure 3) [15].

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The P62 protein is a multifunctional scaffolding protein called sequestosome 1(SQSTM1)/p62 which is involved in aggresomes formation, protein aggregation, and lysosomal and autophagic degradation of misfolded proteins [77] by the direct interaction with HDAC6 [77]. Moreover, HDAC6 interaction with TRIM50 cytoplasmic dynamic bodies, which are E3 ubiquitin ligase enzymes, is considered to be an aggresomal precursor that contributes to aggresomes clearance and regulates aggresomal accumulation [16]. It was found that HDAC6 is required for the correct localisation and recruitment of TRIM50 and ubiquitinated proteins into aggresomes [16]. HDAC6 interactions overlapped in the ubiquitin proteasome system and clearance of misfolded proteins [16]. The association of TRIM50 and P62 is enhanced by HDAC6, leading to subsequent protein mediated autophagy and aggresomal degradation and clearance [16,77].

Protein / Substrate	HDAC6 related interaction role and activity	Potential pathogenesis role	Domains involved	Reference
HDAC6 catalytic Substrates				
-tubulin	Deacetylation of -tubulin at Lys 40 residue, rearrangement of microtubule dynamics cell motility, migration and chemotaxis	Tumour cell metastasis Neurodegenerative disease (Parkinson's disease, Alzheimer's disease and others)	CD1 and CD2 Both domains are required for complete deacetylase activity with the predominant impact of deacetylation by CD2 CD1 diminished the HDAC6 deacetylase activity	[1,21,64,87,88]
Cortactin	Deacetylation of 9 Lys residues present in the repeated region between its terminals Involved in cell migration and F-actin based binding	Associated with actin-based cell motility disorders Tumour cell metastasis	CD1 and CD2 Both domains are required for the deacetylation interaction with cortactin	[6,64]
Heat Shock Protein HSP90	Deacetylation of HSP90 at Lys 294 Polyubiquitylation and proteasomal clearance of misfolded proteins	Neurodegenerative diseases Tumour cells metastasis	CD1, CD2 and ZN-UBP The catalytic domains alone have slight effect on HSP90 acetylation level and its chaperone function ability	[4,64,89]
Tau	Deacetylation of Tau on Lys 280 and 281 Which is within the microtubule binding domain HDAC6 inhibition result in tau acetylation and increased its phosphorylation Ubiquitin binding tau degradation either proteolytic activity of HDAC6 or enhancing Autophagy mechanisms	Tau aggregation and accumulation in neurodegenerative disease such as Parkinson's disease and Alzheimer's disease	CD1 and CD2 ZN-UBP	[64,90–92]
Ku70 / Bax	Deacetylation of Ku70 at Lys **539**/542 residues Block the pro-apoptotic effect of Bax Anti-apoptosis Pro-survival effect	In Neuroblastoma (paediatric solid tumour): - affect its survival but clinically not considered to be associated with aggressive tumour	Not identified yet	[64,93,94]

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		behaviour or poor patient outcome In Pulmonary Hypertension: - enhances the proliferation, vascular remodelling and survival of pulmonary arterial smooth muscle cells		
Survivin	Deacetylation at Lys 129 residue Anti-apoptotic and survival function	Tumour cells e.g Breast cancer	CD2	[64,74]
Peroxiredoxins (PrxI and PrxII)	Deacetylates PrxI at Lys 197 and PrxII at Lys 196 Oxidative stress induced cell death and Redox system modulation	Tumour cells and Neurodegenerative diseases Diabetes mellites induced myocardial infarction	CD2	[64,95,96]
β-catenin	Deacetylation of β-catenin at Lys 49 HDAC6 dependent Epidermal growth factor induced nuclear β-catenin localization and transcription Decreasing the expression of C-Myc oncogene	Tumour cell proliferation and metastasis	Not identified yet	[21,64,71]
Protein arginine methyl transferase 5 PRTM50	HDAC6 deacetylation of PRTM50 reduces its methyltransferase activity involved in proliferation and cellular response to stress and DNA damage.	---	CD1 and CD2	[97]
Myosin Heavy chain MHY9	HDAC6 deacetylation of MHY9 reducing its actin binding affinity Cell adhesion and migration	Tumour cell metastasis	CD1 and CD2	[9]
HSC70/DNAJA1	HDAC6 deacetylation enhances Hac70/DNAJA1 interaction and its role in cell survival in response to cellular stress and hormonal receptor maturation	---	CD1 and CD2	[9]
HDAC6 Ubiquitin Binding proteins				
Dynein	Binding to dynein motors and Misfolded Protein aggregation and degradation, Cargos formation and protein accumulation regulation	Neurodegenerative diseases e.g Parkinson's disease, Dementia with Lewy bodies DLB	DMB ZN-UBP CD1, CD2 required for functional activity	[15,64]
Ubiquitin	Protein ubiquitination, degradation and endocytosis	Neurodegenerative Diseases	ZN-UBP	[15,64,81]

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p97 VCP,p150Glued (dynactin]	AAA-ATPase in Endoplasmic reticulum dependent proteasomal degradation	Neurodegenerative Diseases	Not identified yet	[64,81]
TRIM50 E3-Ubiquitin ligases	Recruitment of aggresomes and protein degradation	Neurodegenerative disease e.g Alzheimer's disease and Parkinson's disease	Not identified yet	[16,64]
P62	The HDAC6 interacts with p62 by binding to its 164–225 residues Autophagic – lysosomal protein degradation regulation And promoting HDAC6 interaction with TRIM50	Neurodegenerative disease e.g Alzheimer's disease and Parkinson's disease	CD2 Mainly	[77]
HDAC6 Inhibitors				
P300 acetyltransferase	Interact and acetylate HDAC6 Downregulating its catalytic activities	Gene transcriptional dysregulation	Mainly CD1 and CD2	[64,73]
TPPP/P25 (Tubulin Polymerization-Promoting Protein/P25)	TPPP/P25 is -tubulin acetylation modulator mediated by HDAC6 inhibition	TPPP has role in the accumulation of α-Synuclein neurodegenerative disease such as Parkinson's disease Alzheimer's disease and Multiple System Atrophy	Not identified yet	[64,75,98,99]
DYNLL/LC8(dynein light chain LC8)	Regulatory hub modulator enhances the inhibitory effect of TPPP/P25-HDAC6 interaction	---	Not identified yet	[76]
CYLD	The interaction of the first two N-terminal CAP-Gly domains with HDAC6 causes its inhibition Increasing -tubulin acetylation Cell-cycle arrest and cell proliferation inhibition	Skin tumour cells Melanoma	CD1 and CD2	[22,64]
Paxillin	Focal adhesion component inhibits HDAC6 deacetylase activity Mediate cell polarization, directed migration and invasion	Tumour cell metastasis	Not identified yet	[80]
epidermal growth factor receptor kinase (EGFR-K)	HDAC6 phosphorylation at tyrosine 570 residue in CD2 domain Reducing HDAC6 deacetylase activity, regulation of EGFR trafficking and degradation	----	CD1 and CD2	[21,71,100]
HDAC6 Activators				

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Atypical Protein kinase C zeta (aPKC ζ)	HDAC6 phosphorylation of serein and threonine residues conserved in both catalytic domains CD1 and CD2 Increase HDAC6 tubulin deacetylase activity		Not identified yet	[65]
Calcium-activated protein kinase C α (PKCα)	PKCα induces HDAC6 deacetylase activity towards β-catenin and regulates its nuclear transcription and translocation	Viral infection and immunity	Not identified yet	[65]
protein casein kinase 2 CK2	HDAC6 phosphorylation of serein 458 residue in the dynein motor binding region increasing the binding of HDAC6 and misfolded proteins to dynein motor protein in response to stress to modulate autophagic degradation	Neurodegenerative disorders e.g. Alzheimer's disease	Not identified yet	[66]
Extracellular Signal-regulated kinase ERK	HDAC6 phosphorylation of serine 1035 residue HDAC6-mediated cell motility occurs through the EGFR-Ras-Raf-MEK-ERK signalling	----	Not identified yet	[67]
glycogen synthase 3β GSK3β	GSK3β phosphorylates HDAC6 Serine 22 residue to enhance tubulin deacetylase activity Enhance neural mitochondrial trafficking	Neurodegenerative disorders	Not identified yet	[69]
G-protein coupled receptor kinase 2 GRK2	HDAC6 phosphorylation serine residues 1060,1062 and 1069 in the region between the DD2 catalytic domain and the ZnF-UBP domain Improve HDAC6 tubulin deacetylase activity enhancing cell migration	----	CD1 and CD2	[12]
mDia2 Mammalian diaphanous forming	Form a protein complex with HDAC6 to increase its deacetylation activity Cell mitosis and maturation	Bone and calcium homeostasis regulation by Osteoclast maturation and bone resorption dysfunction	CD1 and CD2	[64,78]
Other HDAC6 Protein complex				
PP1 Protein Phosphatase1	HDAC6 formed protein complex with PP1 The inhibition of HDAC6 cause the dissociation of this complex	Anti-neoplastic effect Tumour cell growth and metastasis inhibition	Not identified yet	[64,70]

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	Increasing the AKT dephosphorylation Decrease cell growth and stimulate apoptosis	

5. Conclusions

The precise application of HDAC6 inhibitors to treat cancer is still unclear. Much debate centres around the selective nature of HDAC6 targeting compounds such as Ricolinostat where it is widely reported that the anti-proliferative response to this drug occurs at concentrations above the selective range for the drug. Cancer cells survive HDAC6 gene knock out which is inconsistent with the anti-cancer responses produced with catalytic domain inhibitor molecules suggesting that the anti-cancer effects of HDAC6 catalytic domain inhibition occur through off-target drug responses. Nonetheless, some cancer cells are sensitive to HDAC6 inhibition at selective drug concentrations and trying to understand this will help define the patient population who would gain most benefit from this therapy. As discussed in this review, the functional activity of HDAC6 is complex involving its many post-translational modifications and protein-protein interactions. Research of HDAC6 inhibition rarely describes the activation state of HDAC6 in the cells studied or its functional role, where HDAC6 mediates cell functions independent of protein deacetylation. The basal level of HDAC6 deacetylase activity is therefore likely to influence the efficacy of its inhibition and without considering this, research of its inhibition in cells is incomplete. Moreover, research also suggests that the phenotypic responses produced by HDAC6 inhibition is different to HDAC6 gene knockdown. Zeleke et al introduced the HDAC6 regulon score correlating HDAC6 inhibition with the expression of genes closely regulated by its activity [63], representing emerging research attempting to understand the molecular context in which HDAC6 inhibition would be successful. While further research will clarify the selectivity of HDAC6 inhibition in cancer, the selective nature of drugs like Ricolinostat may be less relevant if their off-target effects contribute to anti-cancer responses at their maximum tolerated clinical doses. Multiple clinical trials have consistently shown that Ricolinostat is safe and well tolerated, unlike pan-HDAC inhibitors, which often produce dose-limiting toxicities. Additionally, clinical trials of Ricolinostat have demonstrated its ability to protect against chemotherapy-induced peripheral neuropathies, thereby improving the quality of life for patients, regardless of its HDAC6-targeting anti-cancer activity. However, the synergistic effects of HDAC6 inhibition with other pathway inhibitors are well-documented, making it essential to identify which patients will benefit from HDAC6 inhibition to develop and optimise combinatorial drug therapies. In conclusion, ongoing research into the cancer-specific expression, functional activity, and activation state of HDAC6 will be crucial in refining the use of HDAC6 inhibition in cancer. This research will help define the patient populations most sensitive to HDAC6 inhibition, ultimately leading to more effective treatments and improved patient outcomes.

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