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The role of deubiquitinases in breast cancer

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Abstract

Although growing numbers of oncoproteins and pro-metastatic proteins have been extensively characterized, many of these tumor-promoting proteins are not good drug targets, which represents a major barrier to curing breast cancer and other cancers. There is a need, therefore, for alternative therapeutic approaches to destroying cancer-promoting proteins. The human genome encodes approximately 100 deubiquitinating enzymes (DUBs, also called deubiquitinases), which are amenable to pharmacologic inhibition by small molecules. By removing monoubiquitin or polyubiquitin chains from the target protein, DUBs can modulate the degradation, localization, activity, trafficking, and recycling of the substrate, thereby contributing substantially to the regulation of cancer proteins and pathways. Targeting certain DUBs may lead to destabilization or functional inactivation of some key oncoproteins or prometastatic proteins, including non-druggable ones, which will provide therapeutic benefits to cancer patients. In breast cancer, growing numbers of DUBs are found to be aberrantly expressed. Depending on their substrates, specific DUBs can either promote or suppress mammary tumors. In this article, we review the role and mechanisms of action of DUBs in breast cancer, and discuss the potential of targeting DUBs for cancer treatment.

Keywords

breast cancer; deubiquitinase; ubiquitination; DUB inhibitor

1 Introduction

Protein ubiquitination is a multistep posttranslational modification process in which a highly conserved, 76-amino acid polypeptide, ubiquitin, is added to protein substrates through a cascade of reactions involving ubiquitin activation by a ubiquitin-activating enzyme (E1), followed by its transfer to a lysine residue on the substrate, which is catalyzed by ubiquitin-

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Conflict of interest

The authors declare no conflict of interest.

conjugating enzymes (E2) and ubiquitin ligases (E3) (Figure 1) [1,2]. In addition, another class of polyubiquitin ligases, E4, was identified as a ubiquitin chain elongation factor family required for polyubiquitin chain assembly for certain monoubiquitinated proteins [3,4]. Ubiquitin E3 ligases can act as oncoproteins or tumor suppressors in breast cancer. For instance, cancer-predisposing mutations of *BRCA1* inactivate its ubiquitin ligase activity, suggesting that the tumor suppressor role of BRCA1 is associated with its E3 ligase function [5,6]. On the other hand, SKP2, the E3 ligase of the SKP1-Cullin1-F-box protein (SCF) complex, targets the CDK inhibitor p27 for degradation and plays an oncogenic role in breast cancer [7-9].

Ubiquitination is reversed by deubiquitinases, or DUBs, a superfamily of cysteine proteases and metalloproteases that cleave ubiquitin-protein bonds (Figure 1) [10]. The human genome encodes approximately 100 DUBs, which can be classified into six families: ubiquitin-specific proteases (USPs), ubiquitin carboxy-terminal hydrolases (UCHs), ovarian tumor proteases (OTUs), Machado-Joseph disease protein domain proteases (MJDs), JAMM/MPN domain-associated metallopeptidases (JAMMs), and the monocyte chemotactic protein-induced protein (MCPIP) family [11]. The USP family is the largest and most diverse DUB family. Members of this family have a conserved catalytic domain that consists of three subdomains resembling the thumb, fingers, and palm of the right hand [12]. DUBs in the UCH family, the first structurally characterized DUB family, have six or seven β-sheets surrounded by eight α-helices, which act as a gate to preclude large substrates from getting access to the catalytic core located at the bottom of the DUB [13,14]. Thus, UCH family members can only target small peptides from the C terminus of ubiquitin. The OTU domain was initially identified in an ovarian tumor gene, which consists of five β-sheets interspersed between two helical domains [15,16]. The MJD family has four members, including the well characterized ATXN3 that is mutated in Machado-Joseph disease, and the other members are ATXN3L, JOSD1, and JOSD2 [17,18]. Unlike all other DUB families that are cysteine proteases, the JAMM family members are zinc metalloproteases [19,20]. Recent structural studies revealed that a JAMM family member, AMSH-LP (associated molecule with SH3 domain-like protease), specifically cleaves lysine 63-linked polyubiquitin from the substrate and regulates vesicle trafficking [21]. The MCPIP family has at least seven members, all of which consist of an N-terminal ubiquitin association domain, a central CCCH-type zinc-finger domain, and a C-terminal proline-rich domain [22].

DUBs regulate proteasome-dependent or lysosome-dependent degradation, localization, and recycling of substrate proteins (Figure 1), depending on the specific lysine (K) residue through which the ubiquitin chain is linked. Seven lysine residues, K6, K11, K27, K29, K33, K48, and K63, are present on the ubiquitin molecule, and K48- and K63-linked ubiquitination is best characterized. Polyubiquitin chains linked through K48, and likely K6, K11, K27, K29, and K33 mediate proteasomal degradation [23]. By removing these ubiquitin chains from target proteins, DUBs stabilize their substrates. For example, stabilization of NF-κB/RelA by USP48 [24], stabilization of MCL1 by USP9X [25], and stabilization of PTEN by OTUD3 [26] are associated with the cleavage of K48-linked polyubiquitin from the substrate. DUBs can also alter protein localization, which is usually mediated by the cleavage of K63-linked polyubiquitin from the target protein. For instance,

CYLD, a USP family member involved in cylindromatosis, antagonizes K63-linked ubiquitination of BCL3 and blocks its nuclear localization [27]. In addition, DUBs can also inhibit lysosomal degradation of proteins. A recent study suggested that ubiquitinated EGFR is internalized into early endosomes, where USP2a catalyzes deubiquitination of EGFR, leading to recycling of EGFR back to the plasma membrane [28]. Since DUBs modulate protein stability, signal transduction, and other non-proteasomal functions, they contribute substantially to the regulation of key cancer proteins and pathways. In this review, we focus on DUB-mediated regulation in breast cancer (Table 1).

2 Breast cancer-promoting DUBs

2.1 Deubiquitinases that regulate receptors involved in breast cancer

Approximately 70% of human breast tumors are positive for estrogen receptor (ER) α. Upon ligand binding, ERα translocates into the nucleus, recruits coactivator protein complexes to gene promoters, and activates the transcription of target genes [29,30]. Through mass spectrometric analysis of ERα-containing protein complexes, O'Malley and colleagues identified a member of the OTU domain family, OTUB1, as an ERα-interacting DUB. OTUB1 deubiquitinates ERα *in vitro* and *in vivo* and stabilizes ERα in the nucleus [31]. Additional substrates have also been found for this deubiquitinase. For instance, a recent study revealed that OTUB1 inhibits the ubiquitination and degradation of active SMAD2/3, thereby enhancing the activity of the TGFβ signaling pathway [32] (Figure 2A). Other studies have implicated OTUB1 in DNA damage response. It has been shown that OTUB1 inhibits DNA double-strand break-induced, RNF168-dependent polyubiquitination of histones [33]. Moreover, OTUB1 can deubiquitinate and stabilize the forkhead transcription factor FOXM1, which promotes DNA damage response and genotoxic drug resistance in breast cancer. Consistently, tissue microarray analysis demonstrated a positive correlation of OTUB1 expression with ERα expression and FOXM1 expression in human breast tumors [34].

Overexpression or activating mutation of EGFR, which promotes cell growth and survival, is associated with various human cancers including breast cancer [35]. A splicing isoform of USP2, USP2a, localizes to early endosomes and stabilizes internalized EGFR protein by antagonizing the ubiquitination and lysosomal degradation of EGFR [28]. Using microarray and tissue microarray analyses, Qu et al. found that USP2 was overexpressed in human breast tumors, especially in triple-negative breast cancer (TNBC), and that patients with USP2-positive breast tumors had worse clinical outcomes than patients with USP2-negative breast tumors [36]. This study also showed that USP2 upregulates MMP2 expression and induces the motility and invasiveness of TNBC cells [36]; however, it remains unknown whether MMP2 is a direct substrate of USP2. On the other hand, several USP2 substrates other than EGFR have been reported. For example, FASN (fatty acid synthase), which is overexpressed in many cancers including breast cancer and prostate cancer, is deubiquitinated and stabilized by USP2a [37]. In addition, USP2a binds and deubiquitinates Mdm2, leading to accumulation of Mdm2 and Mdm2-mediated degradation of p53 [38] (Figure 2B). Interestingly, knockdown of USP2a led to downregulation of Mdm2, upregulation of p53, and activation of p53-dependent gene transcription and cell death [38],

suggesting a strategy to restore p53 in tumor cells. However, it should be noted that ectopic expression of USP2a in the breast cancer cell line MCF7 caused apoptosis, which might be mediated by reversal of K63-linked polyubiquitination of RIP1 and TRAF2 by USP2a and subsequent inhibition of NF-κB signaling [39,40] (Figure 2C). These results suggest that the function of USP2 may depend on the cellular context.

In an RNA interference screen of 106 different genes related to deubiquitination, silencing of *USP18* reduced EGFR expression without affecting the levels of other receptor tyrosine kinases [41]. Mechanistic studies demonstrated that depletion of USP18 led to upregulation of microRNA miR-7, which in turn suppressed *EGFR* mRNA translation and induced apoptosis [41,42]. The USP18 substrate responsible for regulating miR-7 remains to be identified. Another RNA interference screen of 53 DUBs revealed that loss of USP18 enhanced apoptosis triggered by bortezomib or etoposide [43]. This was further corroborated by the finding that overexpression of USP18 suppressed IFN-α-, TRAIL-, or bortezomib-induced apoptotic signaling in MCF7 breast cancer cells; surprisingly, a catalytically inactive mutant of USP18 exhibited similar effects [43]. Taken together, the anti-apoptotic functions of USP18 may depend on its catalytic and non-catalytic activity.

2.2 USP9X

USP9X, an X-linked USP family member, was found to be overexpressed in human breast tumors relative to adjacent normal tissues through restriction fragment differential display polymerase chain reaction (RFDD-PCR) and proteomic analyses [44]. The Drosophila homolog of USP9X, FAM, interacts with and stabilizes β-catenin, an oncogenic protein in colon cancer, breast cancer, and other cancers [45]; however, whether FAM/USP9X directly deubiquitinates β-catenin has not been determined. By screening a DUB siRNA library, Piccolo and colleagues identified FAM/USP9X as a positive regulator of TGFβ signaling. FAM/USP9X is required for TGFβ-induced migration of MDA-MB-231 breast cancer cells; mechanistically, FAM/USP9X inhibits the monoubiquitination of SMAD4, a modification that blocks the association of SMAD4 with phospho-SMAD2 [46] (Figure 2A). USP9X has also been shown to antagonize polyubiquitination. For example, by removing K48-linked polyubiquitin chains from MCL1, USP9X inhibits the proteasomal degradation of MCL1, a pro-survival BCL2 family member that is overexpressed in multiple cancer types and contributes to chemoresistance and tumor recurrence [25]. Moreover, USP9X deubiquitinates and stabilizes SMURF1, a member of the NEDD4 family of ubiquitin ligases; silencing *USP9X* expression in MDA-MB-231 breast cancer cells destabilized SMURF1 and inhibited SMURF1-dependent cell migration [47]. Collectively, USP9X may promote breast cancer through deubiquitination of multiple substrates.

2.3 USP15

USP15 is a DUB associated with the COP9 signalosome (CSN), a conserved protein complex implicated in DNA damage response. Specific CSN subunits are upregulated in human breast cancer, positively correlate with Mdm2 expression, and antagonize p53-mediated tumor suppression [48]. Owing to the homology between CSN and the 19S lid complex of the proteasome, CSN is hypothesized to regulate the degradation of polyubiquitinated proteins [49]. Indeed, USP15 has been found to directly deubiquitinate

and stabilize Mdm2, which in turn inhibits the expression of p53 target genes, promotes tumor cell survival, and suppresses antitumor T cell responses [50] (Figure 2B). Moreover, Seoane and colleagues reported that the *USP15* gene is amplified in breast cancer, ovarian cancer, and glioblastoma, and that USP15 deubiquitinates and stabilizes type I TGFβ receptor, thereby enhancing TGFβ signaling and tumor growth [51] (Figure 2A). USP15 can also inhibit monoubiquitination. Recently, Piccolo and colleagues identified USP15 as a DUB for receptor-activated SMADs (R-SMADs) [52]. Upon ligand binding, TGFβ and BMP receptors phosphorylate R-SMADs, which then bind SMAD4 to form a transcriptional complex and regulate target gene transcription. USP15 is required for TGFβ and BMP responses, such as TGFβ-induced breast cancer cell migration. Mechanistically, USP15 reverses R-SMAD monoubiquitination, a posttranslational modification that blocks the DNA-binding ability of R-SMADs [52] (Figure 2A). Taken together, USP15 can enhance TGFβ signaling by opposing both TGFβ receptor polyubiquitination and R-SMAD monoubiquitination, which may contribute to tumor progression. Oncomine data revealed upregulation of *USP15* mRNA levels in human breast cancer, suggesting that USP15 may play a breast tumor-promoting role [53].

2.4 USP28

MYC is an oncogenic transcription factor that regulates cell growth, proliferation, and apoptosis. Amplifications or activating mutations of the *MYC* gene are associated with multiple types of human cancer [54]. MYC protein is normally subjected to degradation by the ubiquitin-proteasome pathway; however, the stability of MYC is elevated in human tumors as a result of mutations of the *MYC* gene or alterations in the regulators of MYC protein degradation [55-57]. Identified in a retroviral shRNA library screen, USP28 has been shown to decrease MYC polyubiquitination and increase MYC stability by antagonizing the activity of the SCF^{FBW7} ubiquitin ligase complex [58]. Silencing *USP28* expression in breast, colon, lung, and cervical cancer cells resulted in downregulation of MYC protein and inhibition of cell proliferation, which suggested a cancer-promoting role of USP28 [58]. USP28 also opposes the ubiquitination and degradation of other FBW7 substrates, such as HIF-1α, which in turn promotes angiogenesis [59]. In addition, USP28 confers stem cell-like traits on breast cancer cells by deubiquitinating and stabilizing LSD1, a chromatin modulator that demethylates H3K4me1/2 and is overexpressed in breast, bladder, lung, and colon tumors [60]. Knockdown of USP28 in human breast cancer cells inhibited tumorigenicity and cancer stem cell-like properties. In tumors from patients with breast cancer, USP28 protein is highly expressed and correlates with the level of LSD1 protein [58,60].

2.5 ATXN3L and BAP1: the KLF5 deubiquitinases

KLF5 (Krüppel-like factor 5) is a zinc finger transcription factor that is highly expressed in basal-like breast cancer and promotes cell proliferation, survival, migration, and tumorigenesis [61]. A recent study identified ATXN3L, a member of the MJD family, as a KLF5 DUB by siRNA screening [61]. ATXN3L directly binds, deubiquitinates, and stabilizes KLF5. Knockdown of ATXN3L decreased KLF5 protein levels and inhibited the proliferation of breast cancer cells [61]. Another KLF5 DUB that stood out in siRNA screening was BAP1 (BRCA1-associated protein 1), which also directly binds KLF5 and

stabilizes KLF5 through deubiquitination [62]. Knockdown of BAP1 in TNBC cells inhibited tumorigenesis and metastasis, which could be partially rescued by restoration of KLF5 expression, suggesting that KLF5 mediates, at least in part, the breast cancer-promoting function of BAP1 [62]. These studies shed light on DUBs that are potential antitumor targets in basal-like breast cancer.

2.6 Other DUBs upregulated in breast cancer

Some of the deubiquitinases involved in regulating immune responses are aberrantly expressed in breast cancer. *USP25* was first identified as a deubiquitinase gene located in the chromosomal region 21q11.2 [63]. Using RFDD-PCR and proteomic analyses, Deng et al. observed overexpression of USP25 in human breast tumors [44]; however, the function of USP25 in breast cancer remains unclear. Mutational analysis revealed that the ubiquitin-associated domain and ubiquitin-interacting motifs of USP25 regulate its ubiquitination state, and that the conjugation of ubiquitin or SUMO to the K99 residue of USP25 modulates substrate recognition [64,65]. A recent study showed that USP25 suppresses IL-17 signaling by reversing K63-linked ubiquitination of TRAF6 and TRAF5, and that IL-17-mediated inflammation was enhanced in *Usp25*$^{-/-}$ mice [66] (Figure 2C). Whether USP25 plays a functional role in breast cancer warrants further investigation.

Ubiquitination and deubiquitination play a pivotal role in DNA damage response. The BRCA1 ubiquitin ligase is implicated in many cellular processes, including DNA repair, cell cycle, and chromatin remodeling. Germline mutations in *BRCA1* and *BRCA2* predispose women to breast cancer and ovarian cancer [67]. BRCC36 (BRCA1/BRCA2-containing complex subunit 36), a member of the JAMM family of DUBs, is highly expressed in breast tumors [68]. Depletion of BRCC36 abrogated BRCA1 phosphorylation and nuclear foci formation in breast cancer cells exposed to ionizing radiation (IR), thereby sensitizing these tumor cells to IR-induced apoptosis [69]. On the other hand, BRCC36 is recruited to DNA lesions as a component of the RAP80 complex upon IR exposure and counteracts RNF8-UBC13-mediated ubiquitination of H2A-type histones [70]. The precise role of BRCC36 in BRCA1-associated breast and ovarian cancer risk remains to be determined.

Targeting specific DUBs may provide a strategy to overcome therapy resistance. UCH37 (also known as UCHL5) is a UCH family member that is upregulated in breast cancer [53]. Wicks et al. reported that UCH37 can deubiquitinate and stabilize type I TGFβ receptor and augment TGFβ signaling [71] (Figure 2A). b-AP15, an inhibitor of USP14 and UCH37, reverses resistance of tumor cells to the proteasome inhibitor bortezomib [72].

Cell cycle proteins are regulated by ubiquitination and deubiquitination. USP17L2 (also known as DUB3) is a deubiquitinase responsible for the stabilization of the Tyr/Thr phosphatase CDC25A, which activates cell-cycle progression and is overexpressed in many human cancers [73]. Overexpression of DUB3 induced oncogenic transformation, while knockdown of DUB3 promoted the ubiquitination and degradation of CDC25A, leading to the arrest of cells at G1/S and G2/M phases. In a subset of patients with breast cancer, DUB3 and CDC25A were found to be co-expressed at high levels in the tumors [73].

In addition, high expression levels of USP36 (a MYC DUB) [74], USP32 (a membrane-bound DUB) [75], and USP9Y (a Y-linked DUB) [44] have also been observed in human breast tumors. Their roles and mechanisms of action in breast cancer have yet to be elucidated.

3 Breast cancer-suppressing DUBs

3.1 CYLD

CYLD is a unique K63 linkage-specific deubiquitinase in the USP family that lacks the zinc finger domain responsible for distal ubiquitin interaction. The *CYLD* gene was first identified as a candidate tumor suppressor gene mutated in cylindromatosis, which is characterized by the development of multiple benign skin tumors [76]. A recent study revealed that CYLD is downregulated in human breast cancer and correlates with clinical outcomes [77].

CYLD regulates multiple pathways by deubiquitinating key signaling components, which has been best characterized for the NF-κB pathway. In canonical NF-κB signaling, TRAF2 and TAK1 are activated in response to ligand stimulation, leading to activation of the IKK complex consisting of two catalytic subunits IKKα and IKKβ and the regulatory protein IKKγ (also known as NEMO). Subsequently, IκB is phosphorylated by the IKK complex and degraded by the proteasome, releasing the p50-p65 dimer that enters the nucleus to activate gene transcription [78]. By screening an shRNA library targeting 50 human DUBs, Bernards and colleagues identified CYLD as a negative regulator of NF-κB signaling [79]. Mechanistically, CYLD binds to NEMO and TRAF2 and reverses non-K48-linked polyubiquitination of TRAF2, thereby blocking TRAF2-mediated activation of the IKK complex [79-81] (Figure 2C). Additional components of the NF-κB pathway, such as TRAF6, TRAF7, and TAK1, have also been reported to be regulated by CYLD [82]. Moreover, IKKε, a noncanonical IKK family member, phosphorylates CYLD at serine 418 and reduces its deubiquitinase activity, leading to induction of oncogenic transformation [83]. This implicates CYLD in breast cancer, since IKKε is over-expressed in more than 30% of breast tumors [84]. Notably, *Cyld*-deficient mice exhibited enhanced NF-κB activity and increased susceptibility to colonic inflammation and colitis-associated cancer [85], providing *in vivo* proof that CYLD is a *bona fide* suppressor of inflammation and tumorigenesis.

CYLD also has NF-κB-independent functions. For instance, CYLD interacts with Dishevelled (DVL) and counteracts its K63-linked ubiquitination, leading to inhibition of this cytoplasmic effector in the Wnt/β-catenin pathway (Figure 2D). Silencing *CYLD* expression resulted in accumulation of β-catenin and activation of its target genes [86]. Moreover, MIB2, an E3 ligase for the Notch ligand JAG2, is deubiquitinated and stabilized by CYLD. Depletion of CYLD led to upregulation of JAG2 expression and activation of Notch signaling, and *CYLD*-deficient tumor cells were sensitive to γ-secretase inhibitors that target the Notch pathway [87]. Therefore, targeting the DUB downstream pathways could be a useful therapeutic strategy for cancers that have lost a tumor-suppressing DUB.

3.2 The PTEN deubiquitinases

The tumor suppressor PTEN, a lipid phosphatase that antagonizes the PI3K-AKT pathway, is frequently lost in human cancer [88]. Whereas approximately 5% of sporadic breast tumors harbor *PTEN* mutations [89], loss of PTEN protein is found in a much higher percentage of tumors [90], suggesting that posttranscriptional and posttranslational regulation may contribute substantially to the absence of PTEN. Several E3 ligases target PTEN for proteasomal degradation. On the other hand, reversal of PTEN monoubiquitination by USP7 (also known as HAUSP) alters PTEN subcellular localization without affecting its protein level [91]. Unlike the great majority of DUB screening studies that rely on siRNA libraries, our laboratory used two alternative screening approaches to identify the PTEN DUB. In the first approach, we co-transfected a panel of triple epitope-tagged DUBs with MYC-tagged PTEN into 293T cells, and then pulled down the DUB with S-protein beads and used immunoblotting to detect PTEN. In the second approach, we performed tandem affinity purification and mass spectrometric analysis of PTEN-containing protein complexes. These two approaches identified the same set of five deubiquitinases that physically associate with PTEN, and only one of them, USP13, stabilizes PTEN protein via direct binding and deubiquitination of PTEN [92]. Depletion of USP13 in breast cancer cells promoted AKT phosphorylation, cell proliferation, glycolysis, and tumor growth through downregulation of PTEN. Conversely, overexpression of USP13 suppressed tumorigenesis in PTEN-positive but not PTEN-null breast cancer cells. Importantly, USP13 protein is downregulated in human breast cancer and correlates with PTEN protein levels [92]. This work identified USP13 as the first DUB that regulates PTEN polyubiquitination and protein stability, which has been independently confirmed by several recent studies [26,93]. Intriguingly, USP13 and a newly identified PTEN deubiquitinase, OTUD3, have a synergistic effect in regulating PTEN, phospho-AKT, and tumor growth in breast cancer [26].

3.3 The p53 deubiquitinases

The tumor suppressor protein p53 is regulated by Mdm2-mediated ubiquitination and degradation and has a short half-life (5-20 minutes) [94]. The first DUB for p53, USP7 (HAUSP), was identified by Gu and colleagues through mass spectrometric analysis of affinity-purified p53-interacting proteins. Wild-type USP7, but not its catalytically inactive mutant, deubiquitinates and stabilizes p53 [95] (Figure 2B). Interestingly, a biomarker of poor clinical outcome in breast cancer, TSPYL5, which is encoded by a gene located in a frequently amplified chromosomal region, binds to USP7 and suppresses its ability to deubiquitinate and stabilize p53, leading to increased p53 ubiquitination and oncogenic transformation [96]. Surprisingly, both the Gu group and the Vogelstein group reported that complete ablation of *USP7* resulted in stabilization of p53; this is because USP7 deubiquitinates and stabilizes Mdm2 more potently under physiologic conditions [97,98] (Figure 2B). However, under genotoxic stress conditions, binding of Mdm2 to USP7 is impaired as a result of ATM-mediated phosphorylation of Mdm2, shifting the balance towards stabilization of p53 [99,100]. Collectively, these findings reveal a dynamic role of USP7 (HAUSP) in regulating the Mdm2-p53 pathway.

Lou and colleagues then added another deubiquitinase, USP10, to the loop [101]. Unlike USP7, USP10 is a p53 DUB (Figure 2B), but not an Mdm2 DUB. In unstressed cells, USP10 is predominantly localized in the cytoplasm, which deubiquitinates cytoplasmic p53 and inhibits Mdm2-mediated p53 nuclear export and degradation. Following DNA damage, phosphorylation of USP10 by ATM leads to translocation of USP10 into the nucleus, where USP10 deubiquitinates and stabilizes nuclear p53 [101]. Because USP10 may stabilize both wild-type p53 and mutant p53, this deubiquitinase can potentially act as a tumor suppressor or an oncoprotein depending on p53 status, which warrants future investigation. It should be noted that somatic mutations in only three genes (*TP53, PIK3CA*, and *GATA3*) occurred at >10% incidence across all breast cancers [102], and that USP10 is overexpressed in human breast tumors compared with adjacent normal tissues [44].

4 Development of DUB inhibitors for cancer therapy

FDA approval of the proteasome inhibitor bortezomib for treating multiple myeloma validated the concept of targeting the proteasome for cancer treatment [103]. However, extended treatment with bortezomib is associated with drug resistance and toxicity [104]. Therapeutic strategies targeting specific DUBs, instead of the entire ubiquitin-proteasome system, might be better tolerated [105]. The rationale for targeting DUBs is that therapeutic inhibition of certain DUBs will inactivate some key oncoproteins or pro-metastatic proteins (including non-druggable ones), either by destabilizing these proteins or by changing their subcellular localization or activity; this will provide therapeutic benefits to patients whose cancers are driven by these tumor-promoting proteins. Although no DUB inhibitors have yet entered clinical trials, progress is being made in developing them as therapeutic strategies.

Similar to kinase inhibitors, DUB inhibitors range from broad pan-DUB inhibitors to specific inhibitors of single DUBs. Cyclopentenone prostaglandins, the first DUB active-site inhibitors, induced accumulation of polyubiquitinated proteins and caused apoptosis in colon cancer cells [106,107]. WP1130, a partially selective DUB inhibitor that inhibits the DUB activity of USP9X, USP5, USP14, and UCH37, triggered rapid accumulation of polyubiquitinated proteins in aggresomes and induced tumor cell apoptosis [108]. P5091, an inhibitor of USP7, induced apoptosis in multiple myeloma cells [109], while inhibitors of the USP1-UAF1 deubiquitinase complex, pimozide and GW7647, reversed the resistance of non-small cell lung cancer cells to cisplatin treatment [110]. Moreover, concurrent inhibition of DUBs (via b-AP15, an inhibitor of USP14 and UCH37) and autophagy (via vorinostat or chloroquine) led to synergistic killing of triple-negative breast cancer cells, providing a rationale for combination treatment of DUB-targeting agents with other drugs [111].

Several strategies have been developed to enable high-throughput screening (HTS) of DUB inhibitors. Ub-AMC (ubiquitin-7-amino-4-methylcoumarin), a fluorogenic substrate for a wide range of DUBs, is commonly used to measure DUB activity. Once the AMC fluorophore is cleaved from Ub-AMC by a DUB, fluorescence can be detected. Using HTS with Ub-AMC as the substrate, multiple compounds including HBX 41,108 were identified from a chemical library to inhibit the DUB activity of USP7 [112]. Similarly, 215 out of 63,052 compounds were found to inhibit USP14, and three of them showed selectivity for USP14. Further tests demonstrated that at least one of those three, IU1, inhibited USP14's

function and accelerated the degradation of several substrates that have been implicated in neurodegenerative disease [113]. However, screening for DUB inhibitors using Ub-AMC has two disadvantages. First, false positives are common, since AMC has an excitation wavelength in the UV range. Second, Ub-AMC is not hydrolyzed efficiently by the UBP/USP class of DUBs, weakening its application to some extent [114,115]. The FRET (fluorescence resonance energy transfer) assay, which selectively incorporates the FRET fluorescence donor (terbium) or acceptor (fluorochrome) onto ubiquitin, has been used to identify novel small-molecule inhibitors of AMSH [116]. Finally, the Ub-PLA_2 assay developed by Progenra consists of ubiquitin fused to the N-terminus of the reporter enzyme phospholipase A_2 (PLA_2). The basis of this assay is that PLA_2 requires a free N-terminus for its catalytic activity to cleave phospholipids into lysophospholipids and non-esterified fatty acids [117]. Therefore, when PLA_2 is fused to ubiquitin, it is catalytically inactive. When a DUB is present, it cleaves ubiquitin from PLA_2, freeing PLA_2 to act on its substrate. There are multiple commercial PLA_2 substrates, including the fluorescent phospholipid NBD C_6-HPC [115,118]. The Ub-PLA_2 assay has been used to identify P5091 and P22077 as USP7 inhibitors [109,119]. Compared with other strategies, the Ub-PLA_2 fusion proteins represent more physiologically relevant substrates while not as sensitive for detecting the activity of the UCH family [115].

In summary, while development of DUB inhibitors are still in the early phase, recent studies have provided the proof of principle that selective small-molecule inhibitors of cancer-promoting DUBs (e.g., USP7) can induce cancer cell death. DUB inhibitors with improved efficacy, specificity, and safety may emerge as new agents for the treatment of cancer.

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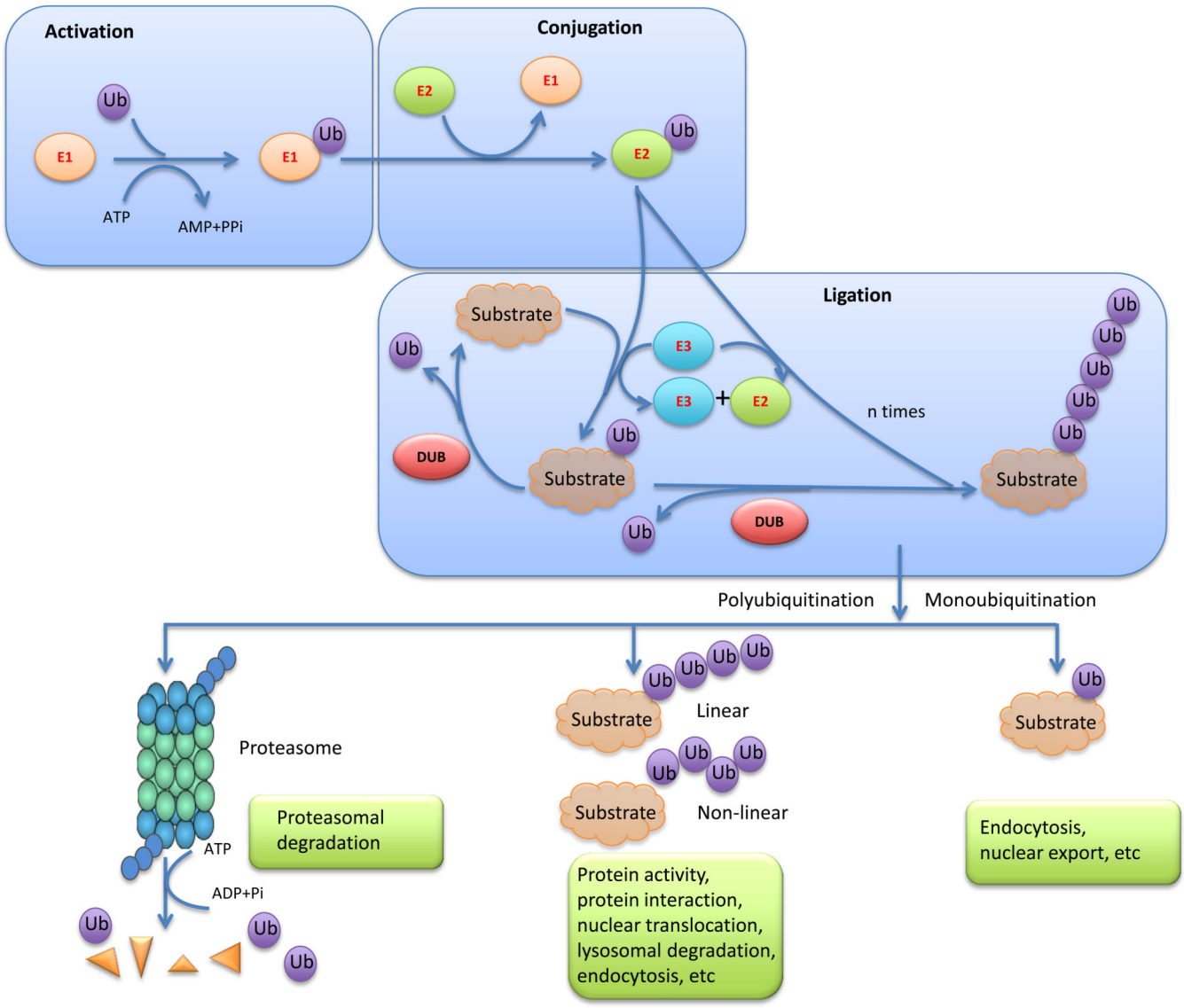

Figure 1. Ubiquitin ligases, deubiquitinating enzymes, and other components in the ubiquitination pathway

Ubiquitin is activated by a ubiquitin-activating enzyme (E1), followed by its transfer to a lysine residue on the substrate, which is catalyzed by ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3). DUBs reverse this process by removing polyubiquitin chains or monoubiquitin from target proteins, and thus rescue proteins from proteasome-dependent degradation or modulate non-proteasomal processes.

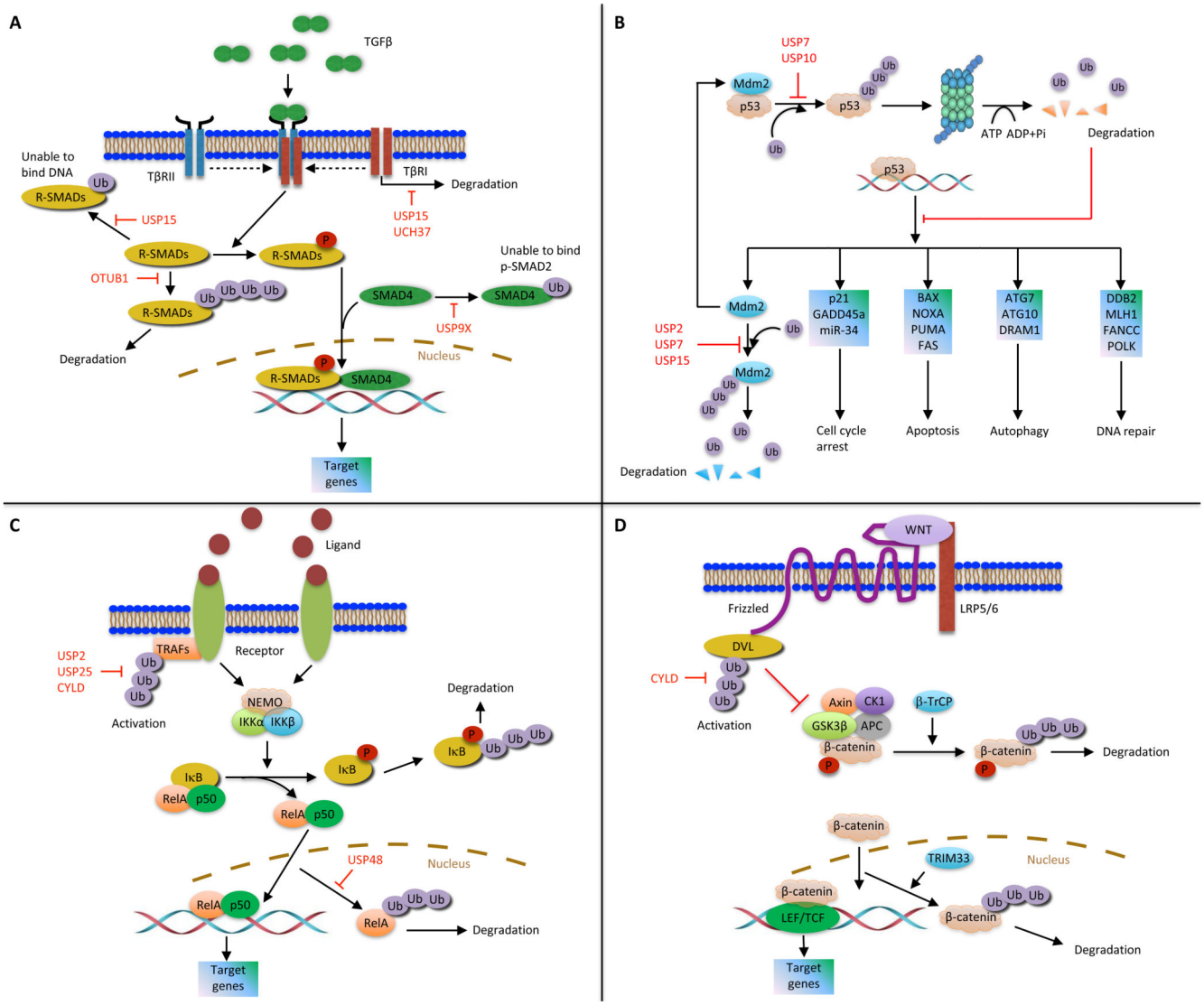

Figure 2. DUB regulation of several signaling pathways

(**A**) TGFβ pathway. (**B**) Mdm2-p53 pathway. (**C**) NF-κB pathway. (**D**) Wnt pathway.

Table 1

DUBs involved in breast cancer

DUB	Substrate	Deubiquitination-induced change	Potential role in breast cancer	References
USP2	EGFR, FASN, Mdm2, RIP1, TRAF2	Protein stability or activity	Promote or suppress	28, 37-40
USP7	PTEN, p53, Mdm2	Protein stability or localization	Promote or suppress	91, 95-100
USP9X	MCL1, SMAD4, SMURF1	Protein stability or interaction	Promote	25, 46, 47
USP10	p53	Protein stability and localization	Promote or suppress	100
USP13	PTEN	Protein stability	Suppress	26, 92
USP15	Mdm2, TβR-I, R-SMADs	Protein stability or activity	Promote	50-52
USP18	Not reported	Not reported	Promote	41-43
USP28	MYC, HIF-1α, LSD1	Protein stability	Promote	58-60
USP36	MYC	Protein stability	Promote	74
ATXN3L	KLF5	Protein stability	Promote	61
BAP1	KLF5	Protein stability	Promote	62
BRCC36	H2A-type histones	Protein interaction	Promote	68-70
CYLD	TRAF2, TRAF6, TRAF7, TAK1, DVL, MIB2	Protein stability or activity	Suppress	77, 79-87
DUB3	CDC25A	Protein stability	Promote	73
OTUB1	ERα, SMAD2/3, histones, FOXM1	Protein stability	Promote	31-34
OTUD3	PTEN	Protein stability	Suppress	26
UCH37	TβR-I	Protein stability	Promote	71