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Dysregulation of ubiquitin ligases in cancer

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Abstract

Ubiquitin ligases are critical components of the ubiquitin proteasome system (UPS), which governs fundamental processes regulating normal cellular homeostasis, metabolism, and cell cycle in response to external stress signals and DNA damage. Among multiple steps of the UPS system required to regulate protein ubiquitination and stability, UBLs define specificity, as they recognize and interact with substrates in a temporally- and spatially-regulated manner. Such interactions are required for substrate modification by ubiquitin chains, which marks proteins for recognition and degradation by the proteasome, or alters their subcellular localization or assembly into functional complexes. UBLs are often deregulated in cancer, altering substrate availability or activity in a manner that can promote cellular transformation. Such deregulation can occur at the epigenetic, genomic, or post-translational levels. Alterations in UBL can be used to predict their contributions, affecting tumor suppressors or oncogenes in select tumors. Better understanding of mechanisms underlying UBL expression and activities is expected to drive the development of next generation modulators that can serve as novel therapeutic modalities. This review summarizes our current understanding of UBL deregulation in cancer and highlights novel opportunities for therapeutic interventions.

Introduction

Clearance of functional proteins limits their availability and activity and is critical for their regulation. This process is carried out in a timely manner by the proteasome ubiquitin system (UPS), which consists of ubiquitin ligases and accessory adaptor and regulatory components, all of which act in a concerted manner to tag proteins for ubiquitination in a spatially- and temporally-regulated manner. As a result, the ubiquitinated protein is often degraded by proteasomes, multi-subunit complexes that recycle proteins to their amino acid components. This critical post-translational modification is recognized as a key regulator of every cellular process under normal homeostatic conditions or in response to stress such as DNA damage, cell cycling, altered mitochondrial dynamics or cellular metabolism. Processing of proteins through ubiquitination also governs cell fate decisions including

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senescence, autophagy or cell death, and controls cellular proliferation and differentiation. Thus, UPS perturbations either increase or reduce availability of cellular regulatory proteins and perturb normal cellular activity, possibly resulting in pathological conditions, including cancer.

A key regulatory step in this process is substrate recognition by ubiquitin ligases (UBLs), an interaction that determines a substrate's fate by modifying it with one or more ubiquitin moieties. Notably, not all ubiquitin-conjugation result in substrate degradation: that outcome is determined by ubiquitin chain topology, which in some cases governs a protein's subcellular localization or its ability to participate in a large signaling complex.

The covalent conjugation of ubiquitin occurs through the formation of an isopeptide bond between lysine residues in both ubiquitin and the substrate. Ubiquitin can be attached to substrates as a monomer (monoubiquitination) or as ubiquitin chains (polyubiquitination). The latter adopt different topologies defined based on the position of respective lysines in ubiquitin, which enable linking of one ubiquitin molecule to another to form polyubiquitin. Ubiquitin K48-linked and K63-linked chains are the best studied: the former are associated with substrate degradation by the proteasome, while the latter are implicated in formation of signaling complexes.

Ubiquitination is carried out by sequential activity of ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3s). Specifically, E3 ubiquitin ligases play a key role in this cascade by recruiting ubiquitin-loaded E2s, recognizing specific substrates, and then facilitating or directly catalyzing ubiquitin transfer to substrate lysine residues. E3 ligases can be classified into three families, of which only one (the HECT family) exhibits intrinsic enzymatic activity. The most abundant family includes a few hundred RING domain-containing E3 ligases, which structurally display a cysteine-histidine RING motif (the name is peculiarly derived from "really interesting new gene").

This group relies on enzymatic activity of E2s to ubiquitinate proteins bound by RING ligases. RING E3 ligase proteins act as either single-molecule E3 ligases or as part of multi-subunit ubiquitin ligase complexes. Somewhat similar to RING ligases are U-box (UFD2 homology) ubiquitin ligases, which function as a scaffold to facilitate ubiquitin transfer from E2 to target proteins. The third group consists of few dozen proteins that display a HECT domain (for "homologous to E6AP carboxyl terminus"). HECT ligases can catalyze transfer of ubiquitin to a target substrate independent of E2 catalytic activity.

Growing evidence suggests that deregulated E3 ubiquitin ligases play a crucial role in development, progression and response to therapy of human cancers and thus could serve as promising therapeutic targets for anti-tumor drugs. Depending on the substrates they ubiquitinate and degrade, E3 ubiquitin ligases themselves can play tumor-promoting or tumor-suppressing roles. Here, we summarize key changes that underlie the genetic and epigenetic deregulation of E3 ubiquitin ligases in human cancer, including mutations, deletions, gene amplification, and altered transcription, as well as the activity of microRNAs and effects of post-translational modifications of the E3 ligases themselves (Figure 1).

Genetic alterations: Mutations

Genetic mutations can affect ligase activity directly or indirectly. In terms of direct effects, mutations in the E3 ligases SPOP and FBXW7 (discussed below) attenuate their activity. Indirectly, mutations in upstream factors that regulate ligases (largely signal transduction components) can perturb the ability of an E3 to associate with a given substrate, which in turn alters substrate stability. Representative indirect regulators (discussed below) include the kinases GSK3 and BRAF.

Cullin-RING ubiquitin ligases (CRLs) are multi-subunit complexes that include a cullin scaffold protein, a RING domain protein (Rbx1 or Rbx2) that interacts with E2, and an adaptor protein that determines substrate specificity. Speckle-type POZ protein (SPOP) is the adaptor protein for the Cullin3/Rbx1 CRL and it selectively recruits substrates via its N-terminal MATH domain, while its BTB domain mediates dimerization and interaction with the scaffold CUL3 [1]. SPOP reportedly mediates degradation of several substrates including the steroid receptor co-activator SRC-3, androgen receptors (ARs), and estrogen receptors (ERs), among others [2,3,4]

SPOP is mutated in approximately 5–15% of patients with prostate cancer across ethnic and demographic backgrounds [5,6]. Prostate tumors with mutant SPOP show a distinct pattern of genomic alterations, suggesting that SPOP mutations could define a molecular subtype of prostate cancer [5]. SPOP mutations occur at regions encoding specific amino acid residues within the substrate-binding pocket and are predicted to attenuate substrate binding. In fact, mutated SPOP protein reportedly exhibits reduced binding to substrates such as SRC-3 [7] or AR [8]. Consistent with a proposed tumor suppressor role, expression of mutant SPOPs in prostate cancer cells promotes cell proliferation *in vitro* and tumor growth in immunodeficient mice [3]. SPOP is also one of the most frequently altered genes in endometrial cancer, based on somatic point mutations identified by large-scale exome sequencing [9, 10, 11, 12].

FBXW7 (F-box and WD repeat domain-containing 7) is an F-box family protein that functions as a substrate recognition component of the Skp-Cullin-F-box (SCF) E3 ubiquitin ligase [13]. It has a F-box domain, which associates with the SCF complex through interaction with Skp1 [14], and eight tandem repeats of the WD40 domain containing three critical arginine residues (R465, R479 and R505), which recognize a consensus phosphodegron motif within substrate proteins [15]. FBXW7 is generally viewed as a tumor suppressor in human cancers, and the SCF FBXW7 complex targets several well-known onco-proteins for ubiquitin-mediated, phosphorylation-dependent degradation, including c-Jun [16], c-Myc [17], Cyclin E [8], KLF5 [9], Mcl-1 [20], Notch [21] and mTOR [22]. Loss-of-function mutations in Fbw7 promote both hematopoietic and solid organ tumor formation in mice [23,24,25]. Furthermore, Fbw7 mutations have been identified in diverse human cancers, including cholangiocarcinoma [26], T cell acute lymphoblastic leukemia [27], cervical carcinoma [28], endometrial cancer [29], and colon cancer [30]. Notably, almost half of FBXW7 mutations found in cancers are missense mutations in regions encoding the arginine residues within the WD40 domain [31] critical for interaction with phosphorylated

substrates. Thus, FBXW7 mutations associated with cancer appear to disrupt substrate recognition.

Mutations in key signaling factors, which often occur in cancer, can enhance or decrease ligase binding to substrates. For example, Glycogen Synthase Kinase 3 (GSK3) is an enzyme functioning in inhibitory phosphorylation of proteins regulated by FBXW7. Hence, mutation of a GSK3 phosphorylation site in c-Myc is seen in lymphoma [32], and mutation of a phosphorylation site in v-JUN priming it for GSK3 phosphorylation allows v-JUN to escape FBXW7 recognition in cancer [16], resulting in greater oncogenic activity. In addition to mutations in the above E3 ligases or their regulators, other ubiquitin ligases have been found mutated in cancer. They are summarized in Table 1.

Genetic deletion

The von Hippel–Lindau protein (pVHL) forms a multiprotein ubiquitin ligase complex with elongins B and C, Cullin 2 and Rbx-1 (known collectively as the VBC-CR complex), and pVHL serves as the substrate recognition subunit in the complex. The best-known targets of ubiquitination by pVHL are the hypoxia-inducible factors (HIFs), the master transcription factors regulating the hypoxia response. HIF stabilization due to VHL loss or mutation is a key oncogenic event for VHL disease, a dominantly inherited familial cancer syndrome characterized by the development of hemangioblastomas, clear cell renal cell carcinoma (ccRCC), pheochromocytoma and pancreatic neuroendocrine tumors. Increased HIF stability also underlies development of certain sporadic tumors, including ccRCC and hemangioblastomas [33].

Patients with VHL disease or sporadic ccRCC harbor a single mutant allele of the VHL gene, and inactivation or loss of the wild-type allele is required for tumor development [34,35]. ccRCC is characterized by a very high frequency of biallelic VHL inactivation with a reported incidence up to 91% [33]. Over 90% of ccRCC cases show loss of heterozygosity (LOH) on chromosome 3p, where VHL is located [36, 37]. In one study of ccRCC, LOH at 3p was found in 94% of 240 tumor specimens [37]. ccRCC patients with LOH at 3p usually exhibit inactivation of the remaining VHL allele due to somatic mutations or promoter methylation. Among the somatic VHL mutations found in sporadic ccRCC, over 50% are frameshift or nonsense mutations [33] predicted to promote loss of function.

PARK2 encodes the ubiquitin ligase PARKIN, which consists of a C-terminal ubiquitin-like (UBL) domain and an N-terminal RING-IBR-RING motif [38]. PARK2 genetic alterations are common across many human cancers as well as in hereditary Parkinson’s disease [39, 40, 41]. PARK2 is mutated or deleted in cancers, with copy number loss being the primary genetic alteration [39, 40, 42]. PARK2 is a potential tumor suppressor gene at chromosome 6q25-q27 and is often lost in human cancers [43, 44, 45]. Analysis of approximately 5,000 tumor genomes shows that PARK2 deletions were the fourth most significant deletion among 70 significantly recurrent deleted regions across the entire data set [40]. Focal PARK2 deletions have been identified in 11% of tumors across all lineages, and loss of the entire chromosome arm occurred in 19% of samples, resulting in an overall 30% PARK2 deletion [40]. Specifically, PARK2 deletion occurs in various cancers, including serous

ovarian, bladder and breast cancer [40], colorectal cancer [46], glioblastoma [42], lung cancer [47], and ovarian cancer [45]. Other ubiquitin ligases reportedly deleted in various cancers include FBXW7, KEAP1, CBL, BIRC2/3 and DEAR1 and are listed in Table 2.

Promoter hypermethylation

DNA methylation of promoter regions is a key epigenetic event that underlies transcriptional deregulation of cancer-associated genes. Examples include deregulation of BRCA1, which is associated with susceptibility to breast and ovarian cancers. BRCA1 plays a key role in the DNA damage response, DNA repair, cell cycle regulation, chromatin remodeling, and transcriptional regulation. BRCA1-deficient cells display significant genomic instability and sensitivity to genotoxic agents, which are believed to underlie tumor development enhanced by BRCA1 deficiency.

BRCA1 is a RING finger ubiquitin ligase that forms a functional complex with BRCA1-associated RING domain protein 1 (BARD1) [48]. The BRCA1/BARD1 heterodimer can ubiquitinate substrates such as H2A, H2B, H3, H4, H2AX, CtIP, estrogen receptor alpha, RNAPII and TFIIE [49, 50, 51, 52, 53, 54]. How BRCA1's function as a tumor suppressor is linked to its ubiquitin ligase activity remains controversial. Cancer-predisposing mutations within its RING domain were shown to abolish its E3 ligase activity, and these mutants cannot rescue cell cycle checkpoint and γ-radiation hypersensitivity of the BRCA1-null human breast cancer line HCC1937 [55], suggesting a requirement for BRCA1 ubiquitin ligase activity in γ-radiation protection. In a mouse breast cancer model, mice harboring a known clinical mutant (C61G) of BRCA1 that disrupts its E3 activity and interaction with BARD1 showed the genomic instability and tumor development similar to the BRCA1-null mice, suggesting the essential role of BRCA1 ubiquitin ligase activity in tumor suppression [56]. In contrast, mice harboring a synthetic BRCA1 mutation (I26A) that abrogates E3 ligase activity do not develop tumors to a similar degree as do wild-type mice [57], suggesting that BRCA1 ligase activity is not required for tumor suppression in these tumor models. In addition to ubiquitin ligase activity, BRCA1 has been shown to function as a scaffold for multiple protein complexes that regulate diverse activities including DNA damage signaling, homologous recombination, cell cycle checkpoint control and transcriptional regulation. It is likely that both the protein-protein interaction and ubiquitin ligase activity of BRCA1 function in tumor suppression.

BRCA1 and BRCA2 are distinct tumor suppressor genes: both function in DNA damage repair, but they are structurally unrelated and BRCA2 has no RING domain. Carriers of BRCA1 or BRCA2 mutations have a significantly increased risk of developing breast cancer (up to 81%) and ovarian cancer (up to 39%) [58, 59, 60]. BRCA1 and BRCA2 mutations are seen in 20% of inherited breast cancer but are rare in sporadic breast and ovarian cancers. BRCA1 can also be lost by gene deletion or promoter hypermethylation [61]. The incidence of BRCA1 promoter methylation in breast cancer tissues is significantly higher (up to 82.1%) than in non-cancerous tissues [62, 63, 64, 65], and gene methylation status is inversely correlated with BRCA1 mRNA expression [63, 65, 66]. BRCA1 promoter methylation is also correlated with decreased overall survival and disease-free survival for triple-negative and basal-like breast cancer [62, 66]. Methylation of promoters of upstream UBLs or cooperating factors

governs expression of genes including HACE1, RNF180, CHIP, KEAP1 and PARK1. These are listed in Table 3.

Gene amplification

While methylation often silences genes that control expression or activity of oncogenes, gene amplification is often associated with ligases or ligase regulatory factors that regulate tumor suppressor genes, thereby limiting their availability.

One such case is relevant to MDM2 (murine double minute 2), a RING finger E3 originally discovered at a genomic locus amplified on double minute chromosomes in transformed mouse NIH-3T3 fibroblasts [67]. MDM2 consists of an N-terminal p53-binding domain, a central region that contains a nuclear localization sequence (NLS), a nuclear export sequence (NES), an acidic domain and zinc finger, followed by a C-terminal RING finger domain. MDM2 has a number of substrates, of which the best characterized is the tumor suppressor p53. MDM2 negatively regulates p53 degradation, affects its nuclear export, transcriptional activity or translation [68, 69, 70, 71, 72].

MDM2 overexpression is observed in a variety of human tumors of distinct tissue origin, such as sarcoma, glioma, leukemia, melanoma, lung cancer and breast cancer [73, 74]. High MDM2 expression levels decrease p53 protein levels and activity, thereby increasing cancer initiation and progression. The fact that MDM2 overexpression and p53 mutation in human cancers are usually mutually exclusive [74] highlights distinct mechanisms to limit p53 activity. MDM2 may also have oncogenic functions independent of p53 [75].

Increased MDM2 expression in human tumors is caused primarily by gene amplification. The human MDM2 gene is located on chromosome 12 (12q14–15), and its amplification is observed in colon cancer [76, 77], gastric cancer [78, 79], leukemia, breast cancer [80], glioblastoma [81], neuroblastoma [82], leukemia [83], and sarcoma [84]. Other UBLs subject to genetic amplification include SKP2, CUL-4A, SMURF1, WWP1 and are summarized in Table 4.

Gene polymorphisms

A common single nucleotide polymorphism (SNP) located in the second intronic promoter (P2) of MDM2 constitutes a T to G transversion, termed SNP309 (SNP309T>G; rs2279744) due to its position 309 bps downstream of MDM2 exon 1. The G allele (SNP309G) extends a binding site for the transcription factor Sp1, enhancing MDM2 transcription [85]. Transgenic mice carrying the SNP309G/G allele are more prone to tumor development than those carrying the SNP309T/T allele [86]. In addition, the SNP309G allele is associated with susceptibility to a variety of human cancers [87, 88, 89, 90].

A less common MDM2 polymorphism (SNP285G>C; rs117039649) is located 24 bps upstream of SNP309 in the same MDM2 intronic promoter (P2). Like SNP309, SNP285 is located within a predicted Sp1 binding site; however, the presence of the SNP285C-allele reduces Sp1 binding affinity for the MDM2 promoter, as compared to the SNP285G allele, and is thus associated with reduced cancer risk [91, 92].

The well-studied F-box protein β-TrCP (beta-transducin repeat-containing protein) contains an N-terminal F-box motif and C-terminal substrate-binding WD-40 repeats, which recognize substrates phosphorylated within the consensus DSGXXS degron [93]. β-TrCP targets degradation of many substrates including the tumor suppressor protein IkappaB [94], FOXO3 [95], and REST [96]. Targeted β-TrCP overexpression in mouse mammary gland promotes breast tumor development [97]. β-TrCP is upregulated in several types of cancers including colorectal cancer [98], pancreatic cancer [99] and hepatoblastoma [100]. A 9-bp (AACAGTGGA) ins/del polymorphism (rs16405) in the 3'-untranslated region (UTR) of β-TrCP is associated [101] with altered β-TrCP mRNA expression: β-TrCP transcript levels in hepatocellular carcinoma (HCC) tissues harboring homozygous 9N ins/ins were 4-fold and 7-fold higher, respectively, than tissues with heterozygous 9N ins/del and homozygous 9N del/del. The presence of the 9-bp insertion allele is proposed to disrupt miR-920 binding to the β-TrCP 3'-UTR, increasing β-TrCP mRNA levels and conferring susceptibility to HCC. However, the β-TrCP 9N ins/del polymorphism is not associated with susceptibility to ovarian [102] or breast cancer [103].

The rs6788895 SNP is located in an intronic region of the ubiquitin ligase SIAH2 and associated with estrogen receptor-positive breast cancer in Chinese [104] or Japanese [105] patients. SNPs rs2714805 and rs2255137 in FBXW7 intron 2 are associated with breast cancer risk [106]. It remains to be determined whether or how polymorphisms of SIAH2 or FBXW7 alter their expression or activity.

Transcriptional regulation

In addition to the above mechanisms, UBLs are also subject to transcriptional regulation. S-phase kinase-associated protein 2 (Skp2) belongs to the F-box protein family and is the substrate-recognizing subunit of the SCF Skp2 E3 ligase, which consists of Skp1, Cul-1 (Cullin-1), F-box protein Skp2, and Rbx1. The cell cycle inhibitor p27 is the most well-characterized Skp2 substrate and is a physiological Skp2 target [107, 108]. Other Skp2 substrates include p57 [109], and p21 [110], FOXO1 [109], among others. Skp2 is overexpressed in a variety of human cancers, including lymphomas, prostate cancer [111], colorectal cancer [112], melanoma [113], non-small cell lung cancer [114], gastric cancer [115], pancreatic cancer [116], and breast cancer [117]. Targeted overexpression of SKP2 in the mouse prostate induces hyperplasia, dysplasia, and low-grade carcinoma [118], whereas Skp2 knockout inhibits tumor development in a PTEN/P53 mouse prostate cancer model [119]. Thus, SKP2 is believed to function as an oncogenic protein.

Amplification of the Skp3 locus on chromosome 5p13 is reported in several tumor types including non-small cell lung cancer (NSCLC) [120], esophageal squamous cell carcinoma (ESCC) [121], myxofibrosarcoma [122], melanoma [113], and glioblastoma [123]. At a different regulatory layer, Skp2 transcription is regulated by other oncogenic factors including E2F1 [124], Sp1, Elk-1 [125], NF-κB [126], Myc [127, 128], and STAT3 [129]. Skp2 expression is also upregulated by phosphoinositol 3-kinase (PI3K)/Akt signaling [130, 131].

Transcriptional silencing of the gene that encodes the ubiquitin ligase RNF125 is associated with resistance of melanoma to vemurafenib [132], one of the lead BRAF inhibitors used in the

clinic. MITF and SOX10 have been identified as upstream regulators of RNF125 transcription, and downregulation of both in drug resistant tumors decreases RNF125 expression, in turn upregulating JAK1, a RNF125 substrate, and promoting concomitant upregulation of receptor tyrosine kinases. The latter include EGFR and AXL, which are also implicated in melanoma resistance to vemurafenib. Notably, RNF125 is deregulated genetically and transcriptionally in other tumor types, including pancreatic and colorectal cancers, indicating that diverse mechanisms govern its regulation and function in cancer.

Regulation by microRNAs

It is now appreciated that miRNAs play an important role in regulating gene expression [133]. miRNAs bind to the 3'UTR of mRNAs, destabilizing them or inhibiting their translation. FBXW7 expression is regulated by multiple miRNAs including miR-27a [134, 135], miR-223 [136, 137, 138], miR-25 [139], miR-92a [140], miR-182 and miR-503 [141]. An inverse correlation of miR-223 and FBXW7 expression is observed in several cancers including T-cell acute lymphoblastic leukemia [142], gastric cancer [138] and esophageal squamous cell carcinoma [137]. miR-223 overexpression downregulates FBXW7 expression [136, 137, 138, 143], whereas inhibition of miR-223 upregulates FBXW7 protein levels [136]. miR-223 represses activity of a luciferase reporter construct containing the FBXW7 3-UTR; in contrast, mutation of predicted miR-223 binding site within the FBXW7 3'-UTR relieves repression of reporter activity [136]. These results support direct targeting of the FBXW3 3'-UTR by miR-223.

Several microRNAs reportedly control UBL expression and activity. For example, Mir21 regulates expression of the F-box protein FBXO11 [144]. RNF8 is a RING-finger E3 ligase recruited to DNA damage sites and required for assembly of repair proteins [145, 146]. Mir214 regulates RNF8, and is therefore expected to impact chromosomal stability in ovarian cancer [147]. Notably, UBLs also regulate microRNA expression, as demonstrated for the ligase TIM65, which regulates miRs by ubiquitinating the protein known as trinucleotide repeat-containing 6 (TNRC6), a component of the RISC complex [148].

Regulation by alternative splicing

Alternative splicing includes or excludes specific exons from a gene transcript and generates multiple mRNAs and proteins from a single gene [149]. Like other proteins, ubiquitin ligases can also undergo alternative splicing to generate splice variants with diverse activities. For example, MDM2 splice variants have been detected with high frequency in several types of human cancers including invasive breast cancer [150], pediatric rhabdomyosarcoma [151], soft tissue sarcoma [152], ovarian carcinoma and bladder cancer [152]. MDM2 splice variants are associated with progression of glioma, ovarian and bladder cancer [152, 153]. Ectopic overexpression of some MDMD2 splice variants can transform NIH3T3 cells [152] and promote lymphomagenesis by HSC cells isolated from Eμ-myc mice [154]. Overexpression of MDM2-B (the most commonly detected MDM2 splice variant in human cancer) in transgenic mice induced formation of sarcoma and lymphoma [155]. More than 40 MDM2 splice variants have been identified, and many exhibit loss of the p53 binding domain or regions required to regulate p53 nuclear activity [156]. Therefore, most MDM2 splice variants

cannot directly bind p53 or regulate its expression and activity, and it remains unclear how these variants promote tumorigenesis. It is now recognized that many tumor-derived, mutant forms of p53 protein not only lose wild-type tumor suppressor function but gain oncogenic function [157]. The MDM2-B variant reportedly interacts with full-length MDM2 to inhibit the latter's ability to degrade mutant p53, leading to accumulation of oncogenic forms of mutant p53 and tumorigenesis [158], and MDM2-B overexpression is correlated with mutant p53 accumulation in human tumors. A MDM2 splice variant similar to human MDM2-B is overexpressed in tumors of mice harboring knock-in mutant (R172H) p53 (equivalent to human R175H) and is correlated with accumulation of mutant p53 in these tumors [158]. Furthermore, like full-length MDM2, MDM2 splice variants may also have p53-independent functions in tumorigenesis [155, 159].

FBXW7 can also undergo alternative splicing. For example, three FBXW7 splice variants (α, β, and γ) use distinct first exons, resulting in proteins with different N-terminal domains [160]. Differential localization of these variants has been observed: FBXW7-α, - β and - γ are largely localized to the nucleus, cytoplasm and nucleolus, respectively [161, 162]. Nucleolar localization of FBXW7-γ reportedly regulates expression of nucleolar c-Myc and cell size [161]. In addition, several forms of FBXW-α alternatively spliced at the 5'-UTR show significant differences in translational efficiency [163]. Transcript levels of splice variants with high translational efficiency are specifically lower in more than 80% of breast cancer cell lines and in more than 50% of various human cancers, suggesting that differential expression of FBXW7-α variants constitutes a novel mechanism underlying FBXW7 downregulation in human cancer [163].

Regulation by post-translational modifications

A central requirement for control of protein ubiquitination is the ability of an ubiquitin ligase to associate with its substrate. It is important to note that in all cases, substrate recognition by ubiquitin ligases depends on post-translational modifications of the substrate. Although less studied, post-translational modifications of the ligase also regulate its subcellular localization and activity. Below are examples of key post-translational modifications that control stability of both ligases and substrates.

The Jun oncogene is subject to several layers of regulation, which are altered by mutations observed in cancer. Mutations range from deletion of the N-terminal domain to mutations that increase Jun stability by interfering with its association with the UBL FBXW7. C-Jun association with its upstream stress kinase JNK reportedly limits its availability under non stress conditions, independent of JNK kinase activity, a mechanism required to limit availability of a several JNK substrates, including ATF2 and p53, under non-stress conditions [164, 165, 166]. Correspondingly, JNK upregulation is often seen in cancer and thought to contribute to increased c-Jun stability. Ras/MAPK signaling is also linked to c-Jun stability through the UBL Trim7 [167]. Tumor cells harboring increased RAS-MAPK signaling show increased TRIM7 phosphorylation and consequent activation, resulting in K63 ubiquitination and stabilization of RACO, a co-factor required for c-Jun activity. Although indirect, RACO control by Trim7 determines levels of c-Jun protein. It is noteworthy that FBXW7 mutations are also seen in tumors harboring Ras mutations,

although it remains to be determined whether stabilization of c-Jun by Ras/TRIM7/RACO is mutually exclusive with FBXW7 mutations.

BRAF mutations, which are seen in over 40% of human melanoma cases, result in amplification of MAPK signaling, with notable increases in ERK1/2 activity. Thus, expectedly, both substrates and UBLs that depend on ERK activity are subject to enhanced ubiquitination-dependent degradation. One example of this is BRAF-dependent regulation and consequent loss of the ligase TRIM7 [167], which enhances oncogenesis [167].

Increasing evidence suggests that post-translational regulation of deubiquitinating enzymes (DUBs) has a significant effect on the UPS system, as these factors determine the stability or activity of an ubiquitinated substrate. PKA-dependent phosphorylation of the DUB USP20 was recently shown to perturb post-endocytic trafficking of the β-andregenic receptor under cellular stress conditions. USP20 is implicated in control of the DNA damage response, cell cycling, and NF-κB activity [168, 169], and its regulation by PKA links this kinase to control of DNA damage and NF-κB activity.

The UBL Mdm2 is also regulated by post-translational phosphorylation by ATM. This activity was previously shown to affect Mdm2 localization and, consequently, its ability to associate with and ubiquitinate p53 [170]. Conversely, MDM2 phosphorylation by CAbl results in p53 activation and stabilization [171]. A more complex regulatory mechanism was recently reported by Batuello and colleagues who demonstrated that MDM2 phosphorylation by Src increased its stability and enhanced its association with UBC12, an E2 conjugating enzyme for NEDD8, enabling enhanced neddylation of p53, an activity that blocks p53 ability to activate transcriptional targets [172].

Control of stability of proteasome subunits regulates activity and function. Growing evidence suggests that diverse mechanisms, including post-translational modifications, underlie these activities. For example, phosphorylation of the proteasome subunit PSMA7 by cAbl kinase impacts its regulation by BRCA1, which controls PSMA7 ubiquitination and stability [173]. Both BRCA1 and cAbl are deregulated in cancer, suggesting that deregulated PSMA7 activity may function in oncogenesis.

Post-translational modifications of the ubiquitin ligase Siah2 ubiquitin ligase determine its activity in several ways. First, Siah2 phosphorylation by p38 kinase reportedly alters its activity by promoting its nuclear localization [174]. Furthermore, Siah2 regulation by the DUB USP13 limits its activity despite the fact that Siah2 expression levels increase [175]. Notably, USP13 also regulates PTEN activity [176]. Different forms of stress modulate these activities: hypoxia reduces USP13 expression enabling increased Siah2 activity, while cellular stress induces p38 activity and alters Siah localization, affecting its recognition and targeting of substrates [174]. Siah regulation by HIPK2 kinase also modulates its activity under hypoxia, rendering it more active and resulting in HIPK2 ubiquitination and degradation [177, 178]. These activities affect global transcriptional regulation mechanisms governed by HIPK2 under hypoxia.

Different post-translational modifications also control UBL function. HIF-1α, for example, is subject to prolyl hydroxylation by one of three prolyl hydroxylases (PHD1–3). Such

modification is required for HIF1a association with pVHL and subsequent HIF-1α ubiquitination and degradation [179]. Notably, PHD1 and PHD3 proteins themselves are regulated by the UBLs Siah1/2 [180], exemplifying multilayered regulation by UBLs.

Acetylation of the protein RASSF5 reportedly restricts its interaction with the UBL Itch, thereby increasing levels of the RASS5 protein. This activity affects the G1 transition in the cell cycle and induction of apoptosis [181].

Some UBLs are also glycosylated, among them A20, a UBL that also possesses DUB activity. Specifically, A20, which is implicated in control of NF-kB signaling, is regulated by post-translational *O*-glucosamine-N-acetylation (*O*-GlcNAcylation), which is required for A20 ubiquitination and subsequent proteasomal degradation [182].

ER stress activities and the UPR maintain protein homeostasis in normal cells and are perturbed in pathological conditions, including cancer. UPR deregulation is a common occurrence and a possible causative factor in numerous diseases, and numerous pathways, from metabolism to autophagy and cell death, are affected by its deregulation. UPR components are implicated in transcriptional regulation of several ubiquitin ligases. Among them are Siah1/2, which are controlled by ATF4 and sXBP1, two of the three major transducers of the UPR sensors PERK and URE1, respectively [183]. Accumulation of misfolded proteins, a common occurrence under deregulated UPR conditions or in tumor cells subjected to chemotherapies (such as taxanes), activates ER-associated degradation (ERAD), engaging ERAD-resident UBLs. This mechanism reportedly underlies the switch to glutamine metabolism seen in paclitaxel-treated breast cancer cells prompted by degradation of misfolded glutamine carrier proteins SLC1A5 and SLC38A2 by the ER resident UBL RNF5 [184].

Ubiquitin phosphorylation is a recent addition to layers of post-translational control of UBL signaling. PINK kinase is the first identified ubiquitin kinase, and it reportedly regulates activity of PARKIN ubiquitin ligase [185, 186]. Parkin activity is enhanced upon ubiquitin phosphorylation, a mechanism implicated in mitophagy. PARKIN, which was initially associated primarily with Parkinson's disease, is now known to be deregulated by genetic and epigenetic mechanisms in several cancers, including lung cancer [187] and glioma [188]. It is expected that ubiquitin phosphorylation will not be limited to single kinase and could affect activity of other ubiquitin ligases and their substrates.

Ubiquitin ligase expression or activity is also regulated by protein-protein interactions. For example, MDM2 ligase activity can be regulated by MDMX, an MDM2 homologue with no intrinsic E3 ubiquitin ligase activity despite sequence homology of MDMX and MDM2 RING finger domains [189]. MDMX can heterodimerize with MDM2 to inhibit MDM2 self-ubiquitination and enhance MDM2-dependent ubiquitination and degradation of p53 [190, 191]. Consistently, knock-in mice harboring a form of MDMX with a point mutation in the MDM2 binding site show embryonic lethality and elevated p53 levels; that embryonic lethality phenotype is fully rescued by concomitant p53 deletion [192].

The ubiquitin ligase Siah2 promotes transcriptional activity of the androgen receptor (AR) gene in prostate cancer cells by regulating the turnover of AR on select promoters [193],

enabling recycling of 15% of AR regulated genes associated with lipid and sterol metabolism. Among those, AKR1C3, a steroidogenic enzyme functioning in resistance of prostate cancer cells to androgen-deprivation therapy, can directly bind Siah2 and inhibit its self-ubiquitinaiton/degradation, thereby increasing its levels and ubiquitin ligase activity towards substrates such as AR or NCOR1 [194]. Consistently, Siah2 and AKR1C3 protein levels are positively correlated in human prostate cancer tissues [194].

Epilogue

Several mechanisms underlie regulation and activity of ubiquitin ligases; in all cases, alterations in these activities promote deleterious phenotypes. We have summarized major regulatory nodes that perturb UBL activity and are often deregulated in cancer, from genomic mutations at the DNA level, to transcriptional control and post-translational protein modifications. UBLs themselves are subject to modifications, and those activities are altered in cancer, although this area requires further study. Whether deregulation of a UBL occurs through multiple mechanisms is a question that merits further analysis. Lastly, as tumor cell plasticity enables survival of harsh environmental conditions, as exemplified by resistance to therapy or metastatic potential, it is expected that spatial and temporal deregulation of UBL activity will remain highly relevant to the development of therapies that antagonize these responses.

The critical role UBL play in fundamental processes and the fact that in cancer they are deregulated by a number of genetic, epigenetic including transcription, translation and post-translational modifications, points to opportunities for their targeting, as novel therapeutic modalities. Depending on their substrates, UBL can elicit tumor suppressor or tumor promoter, in a context-dependent manner. Thus, targeting UBL will require deep understanding of its activity in a tissue and tumor-dependent manner. For a tumor-suppressive UBL, the targeting compounds could include those that elevate its expression level or promote substrate recognition. In contrast, targeting a tumor-promoting UBL is likely to include modulators that can repress its expression, alter its subcellular localization, inhibit its interaction with substrate or its assembly in multi-subunit complex containing UBL. Developing modulators for UBL is not trivial given the need to modulate protein-protein interactions. Ongoing efforts include means to alter activity (ubiquitination capacity), alter conformation (based on structure or biophysical properties) or localization (based on temporal and spatial activities in a given tissue type). Currently, this remains among the more challenging aspects in drug development. Notably, advances are made and the notion of targeting UBL is gaining traction, exemplified by a growing number of examples [195, 196, 197, 198, 199, 200]. Knowledge of structural organization and defined modifications underlying spatial and temporal activity of UBL will likely guide future development of biologics and small molecule inhibitors for this class of proteins.

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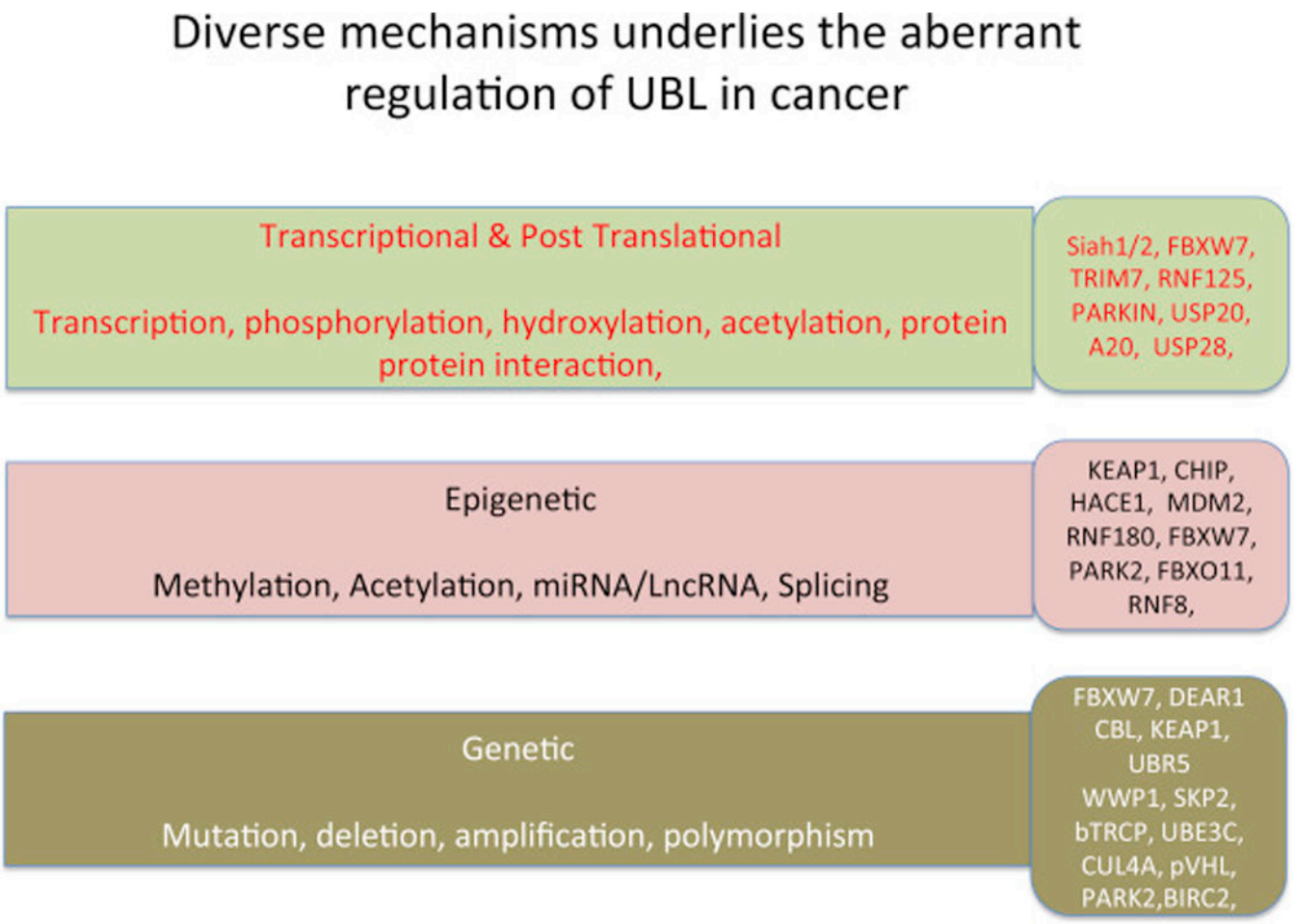

Fig. 1.

Outline of key steps involved in the regulation of ubiquitin ligases (UBL) which are deregulated in cancer.

Table 1

Mutations

RNF43	somatic mutation in 18% (35/185) of colorectal adenocarcinomas and endometrial carcinomas [201] somatic mutation in 14% (8/57) of intraductal papillary mucinous neoplasms of the pancreas [202] Somatin mutation in 4.8% of MSS (microsatellite stable) and 54.6% of MSI (microsatellite instable) group of gastric cancers [203] mutation in 4% (1 out of 23) pancreatic carcinomas [204] mutated in 13.3% (2 out of 15) mucinous ovarian carcinoma [205]; mutation in 9% (2/22) mucinous ovarian borderline tumors and 21% (6/29) mucinous ovarian carcinomas [206] mutation in 9.4% (5 out of 54) liver fluke–associated cholangiocarcinomas [207]
CBL	mutation in 10% of myeloid neoplasms [208, 209] mutation in Juvenile myelomonocytic leukemia [210]
BIRC2/BIRC3	BIRC2/BIRC3 mutation in multiple myeloma [211] BIRC3 mutation in the splenic marginal zone lymphoma (SMZL) [212] BIRC3 mutation in the mantle cell lymphoma (MCL) [213]
PARK2	mutation in the familial lung cancers [187] mutation in glioblastoma, lung cancer [42]
ZNRF3	deletion or mutation in adrenocortical carcinoma [214, 215]
UBE3C	mutation in hepatocellular carcinoma [216]
KEAP1	mutation in 12% lung squamous cell carcinoma [217, 218] mutation in clear-cell renal cell carcinoma [37] mutation in hepatocellular carcinoma [219] mutation in non-small cell lung carcinoma [220] mutation in 60% pulmonary papillary adenocarcinoma [221] mutation in 19% non-small cell lung carcinoma [222]
UBR5	mutated in 18% mantle cell lymphoma [223]

Table 2

Deletions

BIRC2/BIRC3	deletion in multiple myeloma [211]
DEAR1	deletion in breast cancer [224]
ZNRF3	deletion in adrenocortical carcinoma [214, 215]
KEAP1	deletion in non-small cell lung carcinoma [220]
CBL	deletion in non-small cell lung cancer [225]
FBXW7	deletion in uterine serous carcinoma [12] deletion in 4% Will's tumor [226] deletion in esophageal squamous cell carcinoma [227] deletion in gastric cancer [228] deletion in colorectal cancer [229]

Table 3

Promoter hypermethylation

KEAP1	promoter methylation in papillary thyroid carcinoma [230] promoter methylation in non-small cell lung carcinoma [220]
RNF180	promoter methylation in gastric cancer [231, 232]
CHIP	promoter methylation in colorectal cancer [233]
HACE1	promoter methylation in hepatocellular carcinoma [234] promoter methylation in sporadic Wilms' tumor [235] promoter methylation in colorectal cancer [236] promoter methylation in gastric cancer [237]
PARK2	promoter methylation in leukemia [238]

Table 4

Gene amplification

WWP1	WWP1 gene had copy number gain in 15 of 34 (44%) xenografts and cell lines from prostate cancer and 15 of 49 (31%) clinical prostate cancer samples [239] A copy number gain of WWP1 was found in 41% (17/41) of primary breast tumors. In a panel of cDNA from primary breast tumors and normal tissues, expression of WWP1 in tumors is significantly higher than that in normal tissues [240]
SMURF1	amplification in 4.2% pancreatic cancer [241] amplification in pancreatic adenocarcinoma [242]
CUL-4A	amplification in breast cancer [243] amplification in hepatocellular carcinoma [244] amplification in pleural mesothelioma [245] amplification in esophageal squamous cell carcinoma [246]
SKP2	amplification of SKP2 in 38% (31/82) of myxofibrosarcoma [122] amplification of SKP2 gene in 23 of 50 (46%) esophageal squamous cell carcinoma (ESCC) tumors [121] amplification in non-small cell lung cancer (NSCLC) [120] amplification in glioblastoma [247]