Studies on the functional role of UFMylation in cells (Review)

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Abstract. Protein post-translational modifications (PTMs) play crucial roles in various life activities and aberrant protein modifications are closely associated with numerous major human diseases. Ubiquitination, the first identified protein modification system, involves the covalent attachment of ubiquitin molecules to lysine residues of target proteins. UFMylation, a recently discovered ubiquitin-like modification, shares similarities with ubiquitination. The precursor form of ubiquitin fold modifier 1 (UFM1) undergoes synthesis and cleavage by UFM1-specific protease 1 or UFM1-specific protease 2 to generate activated UFM1-G83. Subsequently, UFM1-G83 is activated by a specific E1-like activase, UFM1-activating enzyme 5. UFM1-conjugating enzyme 1 and an E3-like ligase, UFM1-specific ligase 1, recognize the target protein and facilitate UFMylation, leading to the degradation of the target protein. Current knowledge regarding UFMylation remains limited. Previous studies have demonstrated that defects in the UFMylation pathway can result in embryonic lethality in mice and various human diseases, highlighting the critical biological functions of UFMylation. However, the precise mechanisms underlying UFMylation remain elusive. This present review aimed to summarize recent research advances in UFMylation, with the aim of providing novel insights and perspectives for future investigations into this essential protein modification system.

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Contents

1. Introduction

Post-translational modifications (PTMs) refer to the covalent alterations of proteins that occur during or after their biosynthesis, typically catalyzed by enzymes (1). These modifications encompass the addition of functional groups (such as phosphorylation, methylation and acetylation), the conjugation of other proteins or peptides (such as ubiquitination), chemical modifications of amino acid (AA) residues (such as deamidation) and structural changes (such as disulfide bridge formation) (2,3). Ubiquitination, one of the most prevalent PTMs, involves a highly conserved protein composed of 76 amino acids with a molecular weight of approximately 8.5 kDa, which is ubiquitously present in all eukaryotic cells (4,5). Ubiquitin is conjugated to its target proteins through a complex enzymatic reaction cascade, consisting of an activating enzyme (E1), a conjugating enzyme (E2) and a specific ligase (E3), playing a pivotal role in the regulation of eukaryotic cellular processes (6,7).

Ubiquitin fold modifier 1 (UFM1), a member of the ubiquitin-like (UBL) protein family (8,9), is composed of

85 amino acids and has a molecular weight of 9.1 kDa. Despite limited sequence homology, UFM1 and ubiquitin exhibit analogous tertiary structures and conceptually similar enzymatic pathways, involving E1, E2 and E3 enzymes, which ultimately lead to the covalent attachment of UFM1 to the lysine residues of substrates through its C-terminal glycine. Akin to other UBLs, UFM1 is synthesized as a precursor protein that requires proteolytic cleavage to generate its active mature form (10).

UFM1 possesses the ability to form polymeric chains due to the presence of five lysine residues; however, these chains predominantly connect through lysine 69 (10). In contrast to ubiquitination, which involves numerous enzymes with overlapping functions, UFMylation is characterized by a more restricted set of cellular mechanisms, demonstrating high specificity. The maturation, activation, attachment and removal of UFM1 are facilitated by only a few ubiquitously expressed enzymes, specifically UBA5 (E1), UFC1 (E2), UFL1 (E3) and UFSP1 and UFSP2 (10-14).

Several proteins associated with UFMylation serve various scaffolding and targeting functions, including UFM1 binding protein-1 (UFBP1), cyclin-dependent kinase 5 regulatory subnit-associated protein 3 and retinal nerve fibre layer/ODR4. These proteins ensure that enzymatic activities are accurately directed and compartmentalized within relevant cellular environments (15). Compared with the more complex and redundant mechanisms governing ubiquitination, this unique regulatory framework underscores the specialized role of UFM1 in cellular processes (16).

UFMylation, a post-translational modification identified a decade ago (17), remains incompletely understood and its biological significance warrants further investigation. Initial studies on UFM1 substrates identified UFBP1 and ASC1 through *in vitro* assays (18,19); however, the majority of UFM1-modified proteins remain uncharacterized. With the advent of advanced screening technologies and purification methods, new UFM1 substrates have been identified, providing molecular-level insights into the structure and function of UFMylation (9,20,21). These discoveries have expanded its mechanistic roles in key cellular processes, including the DNA damage response, endoplasmic reticulum homeostasis, ribosomal quality control and hematopoietic differentiation, establishing UFMylation as a crucial regulator of cellular homeostasis (21,22). Its dysregulation has been linked to the development of various human diseases, for example, severe anemia (18), skeletal diseases such as Sohat spondyloepiphyseal dysplasia (SEMD) (23) and neurological diseases such as early-onset cerebellar atrophy and early-onset intractable epilepsy (24,25). The present review refined the current understanding of the enzymatic reactions underlying UFMylation, elucidated its catalytic mechanisms and explored newly emerging regulatory pathways that contribute to cellular homeostasis. Furthermore, it comprehensively elucidated the specific roles and novel molecular mechanisms of the UFM1 conjugation system in various cancers and antitumor immunity, for instance, the loss of UFL1 in T cells inhibits the UFMylation of programmed cell death protein 1 (PD-1), thereby promoting the production of effector cytokines in $CD8^+$ T cells and enhancing their antitumor efficacy. These findings may provide new strategies for identifying cancer diagnostic biomarkers and clinical therapeutic targets in the future.

2. Roles of different proteases in ubiquitin-like modifications

UFM1, like most UBL proteins, is expressed as a precursor that must be proteolytically cleaved to generate the active mature form (26). However, its precursor features a unique C-terminal Ser84-Cys85 dipeptide sequence, unlike the conserved C-terminal di-Gly (Gly-Gly) motif found in most UBLs. The proteases UFSP1 and UFSP2, purified from tissue extracts using His-GST-UFM1-Escontin as a substrate, have been identified as the enzymes responsible for processing the UFM1 precursor and facilitating the removal of UFM1 modifications (27).

UFSP1, an ~25 kDa family member protein, is present in flies, mice and humans but absent in plants and nematodes. By contrast, UFSP2, which is >40 kDa, is found in most multicellular organisms, including *Caenorhabditis elegans* and *Arabidopsis thaliana* (28). Despite sharing the same catalytic mechanism, UFSP1 and UFSP2 exhibit significant structural differences, particularly in the R-helix domain responsible for recognizing and binding the UFM1 precursor. In UFSP1, the R loop connecting β3 and β4, along with Trp98, is stabilized by interactions with water molecules and residues connecting the α6 helices and β7 strands. Conversely, in UFSP2, the R loop connecting β9, β10 and Trp342 shows no significant interactions apart from hydrophobic interactions between Trp342 and Val395 (29).

Thus, a crucial function of UFSPs is to proteolytically process the UFM1 precursor, producing mature UFM1. The removal of the C-terminal serine and cysteine residues exposes the C-terminal glycine of UFM1, enabling its maturation and subsequent binding to substrates (30,31).

Upon maturation, UFM1 undergoes activation through a trans-binding mechanism involving UBA5 dimerization. UBA5, the sole E1 enzyme for UFMylation, is primarily localized in the cytoplasm. Unbound UFM1-UBA5 dimers exhibit weak dimerization; however, UFM1 binding stabilizes the UBA5 dimer conformation and enhances its affinity for ATP (32). Specifically, two UFM1 molecules and two UBA5 molecules form an interlocking structure, with each UFM1 molecule interacting with one UFM1-interacting sequence (UIS) molecule at one end, while Gly83 at the other end forms a high-energy thioester bond with Cys250 of UBA5. ATP binding to the protomers in the UBA5 dimer enables the charging of UFM1, forming an activated complex carrying two UFM1 molecules (33). The dimeric UBA5 is essential not only for UFM1 activation but also for transferring UFM1 to UFC1. This highlights the critical interdependence of the two UBA5 monomers for adenylation and UIS domain structure (13,32).

Following activation, UFM1 is transferred from UBA5 to Cys116 of UFC1 through a trans-thiolation reaction, which involves interactions between the UBA5-UFM1 dimer and UFC1 (34). Unlike UBA5, UFC1 is primarily localized in the nucleus, with only partial cytoplasmic localization. Intriguingly, while the charging process of UBA5 requires ATP, the transfer of UFM1 from UBA5 to UFC1 does not (35). During this trans-thiolation reaction, one thioester bond (UBA5-UFM1) is cleaved and another (UFC1-UFM1) is formed, suggesting that energy transfer can occur bidirectionally, with both directions being energetically equivalent (36). The reaction direction is determined by the concentrations

of UBA5, UFC1 and UFM1; overexpression of UBA5 can reverse the energy transfer from activated UFM1 in UFC1 back to UBA5, indicating the reversibility of E2 transfer. In the normative direction (E1-E2), UFC1 binds to a tetrameric complex composed of two UFM1 molecules and two UBA5 molecules, employing a similar trans-binding mechanism that necessitates interaction with one UBA5 monomer and the receipt of activated UFM1 from another (37).

The final step involves the transfer of UFM1 from UFC1 to covalently link to the substrate's lysine residue, mediated by the E3 enzyme UFL1 (38). UFL1 deficiency results in the loss of UFMylation and mice lacking UFL1 exhibit hematopoietic failure and embryonic lethality, suggesting its role as the primary or sole E3 ligase. Although UFL1's role in UFMylation is well established, it does not directly mediate the process. Instead, it forms a functional heterodimeric E3 ligase complex with adapter proteins, such as UFBP1 and CDK5RAP3, classifying it as a scaffold-type E3 ligase (16,39). Scaffold-type E3 ligases recognize substrates and facilitate UFM1 transfer from UFC1 to the substrate. The E2 enzyme confers linkage specificity at K69 during dual UFM1 formation in the absence of E3 ligase, indicating that this specificity is determined by the E2 enzyme. The E2-E3 complex can exert various effects on the substrate, ranging from proteasome-dependent protein proteolysis to the regulation of protein function, structure, assembly, or localization (40,41). Following these processes, UFSP2 can recycle the UFM1 linked to the substrate, allowing it to enter the next UFMylation cycle (42) (Fig. 1).

3. The functions of UFMylation in cells

The UFMylation pathway has been demonstrated to regulate a wide range of cellular activities. These include DNA damage repair, endoplasmic reticulum stress response, ribosome modification, hematopoiesis, immune regulation (20) and neurodevelopment (43-47).

4. UFMylation and the maintenance of genomic integrity in the cell nucleus

The genome of the cell is continually exposed to attacks from both exogenous and endogenous DNA-damaging factors, such as radiation, carcinogens and reactive free radicals. To maintain genomic stability, cells have evolved a complex DNA damage response (DDR) system. This system is responsible for sensing DNA damage, halting the cell cycle and initiating repair processes. Failure to detect or repair DNA damage can lead to genomic instability, a hallmark of tumorigenesis (30).

Double-strand breaks (DSBs) represent the most toxic form of DNA damage and their repair is primarily initiated by ATM kinase (48). The activation of ATM is mediated through the meiotic recombination 11 homolog (MRE11)-radiation sensitive 50-Nijmegen breakage syndrome 1 (NBS1) (MRN) complex, which phosphorylates Ser1981 and acetylates Lys3106. Once activated, the ATM kinase rapidly phosphorylates local chromatin, providing a scaffold for the assembly of higher-order complexes that facilitate DNA repair. Subsequent studies have revealed that the activation of ATM also involves the UFMylation pathway. Following DNA damage, MRE11 undergoes UFMylation at Lys282, a modification that is crucial for the assembly of the MRN complex. This UFMylation event is essential for optimal ATM activation, homologous recombination-mediated repair and the maintenance of genomic integrity (49).

DNA damage induces the UFMylation of MRE11, which facilitates the recruitment of the MRN complex to the damage site. This process helps relieve the self-inhibition of ATM kinase at DSBs, thereby promoting DSB repair and enhancing chromosomal stability (45,46). In addition to MRE11, the UFMylation of histone H4 has also been implicated in ATM activation. The MRN complex recruits UFL1 to DNA DSBs, where UFL1 catalyzes the release of histone H4 at Lys31, enhancing the recruitment of the SUV39H1 complex to DSBs (50). SUV39H1 induces H3K9me3 modification at DSBs, which can spread over thousands of bases, forming a temporary repressive heterochromatic domain (39). Subsequently, Tip60 is recruited to bind H3K9me3 and this interaction enhances its acetyltransferase activity. This leads to the acetylation of Tip60 and subsequent activation of ATM at DSBs (51,52).

Related studies have identified the nuclear-localized UFMylated protein P53, with endogenous UFMylation being detectable in both human cancer cells and primary mouse embryonic fibroblasts. *In vitro* UFMylation assays have demonstrated that the UFMylation components UBA5, UFC1, UFL1, UFM1 and DDRGK1 (also known as UFM1-binding protein 1, UFBP1) are all necessary for the modification of P53 (53). The absence of the amino (N) terminal region of P53 prevents its binding to UFL1, indicating that this region is crucial for UFL1 interaction. Notably, this region can also bind to mouse double minute 2 homologue (MDM2), the primary E3 ligase responsible for P53 degradation. Consequently, UFL1 competes with MDM2 for binding to P53, thus contributing to its stability (54,55). Notably, UFMylated P53 can be detected in cells lacking UBA5 overexpression, provided that UFC1, UFL1 and DDRGK1 are overexpressed. Conversely, UFMylated P53 levels decrease in cells where UFC1, UFL1 and DDRGK1 have been knocked out, while knocking out UBA5 has no significant effect. This suggests that while UBA5 is essential for P53 UFMylation, it is not a limiting factor in the cellular context (47).

Furthermore, UFMylation has been reported to play an unanticipated role in maintaining telomere length. UFMylation-modified MRE11 promotes the recruitment of PP1-α to the MRN complex and facilitates the dephosphorylation of NBS1. This process aids in releasing the MRN complex from telomeres and allows Apollo to bind to TRF2. In the absence of UFMylation-modified MRE11, NBS1 remains phosphorylated, leading to reduced MRN recruitment at the telomeres (56,57). The absence of MRN at the telomeres favors the formation of the TRF2/SNM1 complex, ultimately resulting in the shortening of the telomeric leading strand length (44).

As a co-activator of nuclear receptors, the UFMylation modification of ASC1 is a pivotal step in the transactivation of estrogen receptor α (ERα) by 17β-estradiol (E2). In the absence of E2, the UFM1-specific protease UFSP2 binds to ASC1, maintaining it in a non-UFMylated state (43,58). However, in the presence of E2, ERα binds to ASC1, displacing UFSP2 and leading to ASC1 activation. The subsequent poly-UFMylation

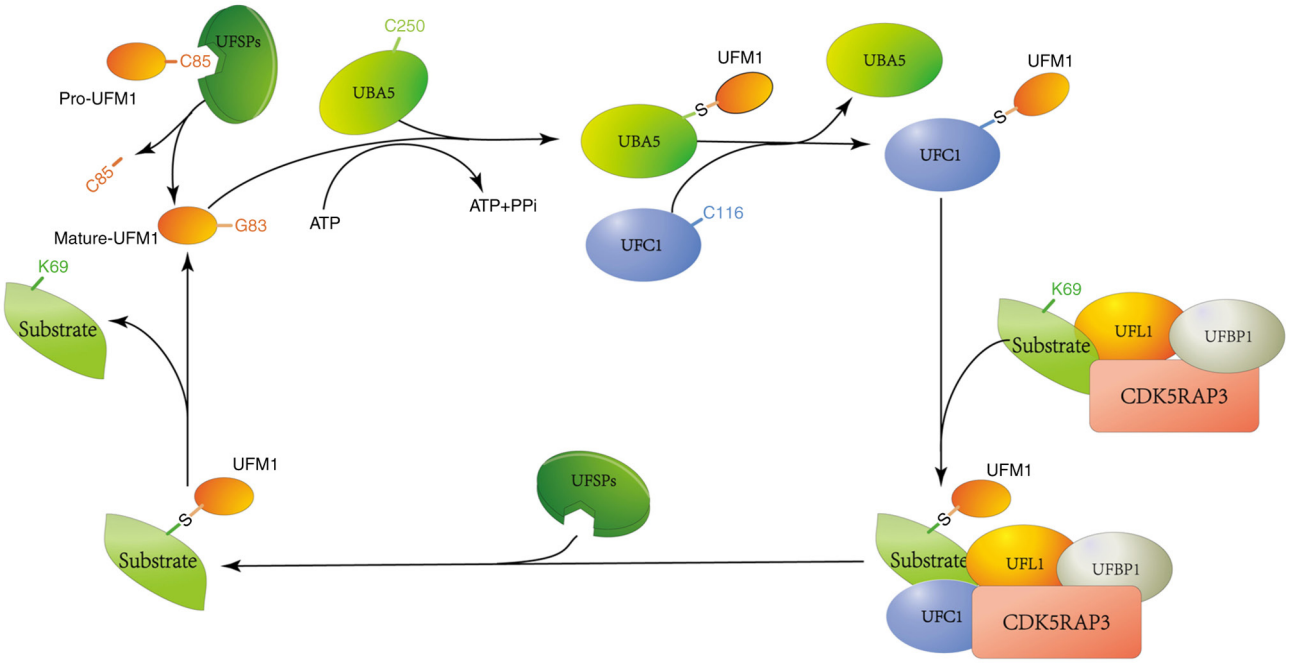

Figure 1. The role of different proteases in UFMylation. The UFM1 precursor undergoes proteolytic processing by UFSP1, which cleaves the Ser84-Cys85 dipeptide to expose Gly83, yielding mature UFM1. Subsequently, Gly83 of UFM1 forms a high-energy thioester bond with Cys250 of UBA5, stabilizing the dimeric conformation of UBA5 and enhancing its affinity for ATP. The UBA5-UFM1 dimer then interacts with the UFC1, facilitating the transfer of UFM1 from UBA5 to UFC1. This reaction involves the cleavage of one thioester bond (Gly83-Cys250) and the formation of another (Gly83-Cys116). Finally, UFM1 is covalently conjugated to a lysine residue (K69) on its substrate through an E3 ligase complex, comprising the ligase UFL1 and its adaptor protein. The cycle is completed when UFSP2 cleaves UFM1 from the substrate, allowing it to re-enter the UFMylation pathway. UFM1, ubiquitin fold modifier 1; UFSP1, UFM1 specific peptidase 1; UBA5, ubiquitin-activating enzyme 5; UFC1, ubiquitin fold modifier 1; UFSP2, UFM1 specific peptidase 2.

of ASC1 enhances the recruitment of P300, SRC1 and ASC1 to the promoters of ERα target genes. Overexpression of ASC1 or knockdown of UFSP2 promotes ERα-mediated tumor formation *in vivo*, which can be attenuated by treatment with the anti-breast cancer drug tamoxifen (18,43). These findings underscore the critical role of UFMylation in regulating genomic stability and its potential implications in breast cancer progression.

5. UFMylation and endoplasmic reticulum homeostasis

To maintain endoplasmic reticulum (ER) homeostasis, cells have evolved a sophisticated protein quality control system comprising various pathways. These pathways include ribosome-associated quality control (RQC), endoplasmic reticulum-associated degradation (ERAD), the unfolded protein response (UPR) and ER-phagy (59-61).

In eukaryotic cells, a primary cause of ribosome stalling due to erroneous translation is naturally occurring faulty mRNA. These aberrant translation products can interfere with functional proteins and are detrimental to the cell. The RQC pathway effectively eliminates these stalled products in the cytoplasm when ribosomes become inoperative (62,63). This pathway employs a series of coordinated factors that sense translation stalling, rescue stalled ribosomes and degrade abnormal nascent polypeptides. Specifically, ribosome stalling during protein translocation induces the attachment of the ubiquitin-like modifier UFM1 to two conserved lysine residues near the COOH terminus of the ER 60S ribosomal subunit RPL26 (uL24) (64,65). Notably, RPL26-UFMylation facilitates the degradation of stalled nascent chains. However, unlike the ERAD or previously established RQC mechanisms utilizing proteasomal degradation, ribosomal UFMylation directs translocation-stalled ER proteins for lysosomal degradation. In summary, UFMylation plays a critical role in regulating protein homeostasis within quality control pathways (66).

ERAD, a protein degradation system located in the ER, removes unfolded or misfolded proteins for proteasomal degradation (67). Proteins that fail to achieve their native conformation are targeted for degradation through the ERAD pathway via a series of closely coupled steps: substrate recognition, retrotranslocation to the cytosol and ubiquitination for proteasomal degradation. HRD1 (HMG-CoA reductase degradation protein, also known as SYVN1) serves as one of the key ubiquitin ligases for ERAD. HRD1 has been shown to target various proteins, including the tumor suppressor tumor protein p53, programmed death-ligand 1 (PD-L1), peroxisome proliferator-activated receptor 1β, inositol-requiring enzyme 1 alpha (IRE1α), cyclic amp-responsive element-binding, lipoprotein lipase, proopiomelanocortin, sigma non-opioid intracellular receptor 1, ATP citrate lyase, nuclear factor erythroid 2-related factor 2 and the rate-limiting acyltransferases glycerol-3-phosphate acyltransferase, monoacylglycerol and diacylglycerol O-acyltransferase 2 The regulation of these proteins by HRD1 affects cellular physiological and pathological processes through the degradation of misfolded proteins (68).

Under normal conditions, cells regulate the ERAD clearance of misfolded ER-resident proteins through the UFMylation modification of HRD1. However, during ER

stress, the accumulation of misfolded proteins within the ER triggers stress signals that cause the dissociation of the DDRGK1-UFL1 complex from HRD1. This dissociation diminishes HRD1 UFMylation, thereby inhibiting its function and initiating the UPR to mitigate ER stress. In this model, the removal of UFL1, HRD1, or the expression of HRD1-K610R in HRD1 knockout cells facilitates the activation of the UPR, particularly the IRE1α-XBP1 signaling pathway, which fine-tunes the folding capacity of the ER (68).

Although ER-phagy is a form of selective autophagy, it involves additional selectivity in the degradation of specific ER proteins and subdomains (60). Misfolded proteins in the ER are typically targeted for degradation via the ERAD pathway; however, some misfolded proteins cannot enter this pathway due to their inability to bind to the ERAD machinery or their tightly packed structure that prevents unfolding. These proteins ultimately accumulate in the ER and are delivered as cargo to isolation vesicles known as autophagosomes, which subsequently fuse with lysosomes in a process termed ER-phagy (69,70). In summary, ER subdomains containing these abnormal proteins exhibit affinity for ATG8 family proteins or FIP200, ensuring selectivity for the autophagic degradation of the ER. These proteins are expressed in specific tissues and localized to different ER subdomains, such as sheet-like ER, tubular ER and three-way junctions, facilitating the formation of autophagosomes and undergoing ER-phagy (71-73).

In macro-ER-phagy, isolation membranes or phagophores form along the ER marked for degradation (74). The localization of the E3 ligase complex at the ER is crucial for the autophagic degradation of the ER, with UFL1 and UFBP1 playing significant roles. The UFM1 substrate modification of NADH-cytochrome B5 reductase 3 (CYB5R3) is dependent on the E3 ligase complex UFL1 and UFBP1, which are located on the ER membrane (75,76). The UFM1 binding site, Lys214, is situated at the interface between the NADH and FAD domains of CYB5R3, resulting in the disruption of the FAD domain and rendering CYB5R3 inactive. UFMylated CYB5R3 is recognized by UFBP1, which is essential for further UFMylation of CYB5R3 and enhances the E3 ligase activity of the UFL1-UFBP1 complex towards CYB5R3. Ultimately, the interaction of the UFL1 complex with UFMylated CYB5R3 and UFBP1 leads to the autophagic degradation of ER subdomains (9,64,66,77).

In plasma cells, the IRE1α/XBP1 axis within the UPR pathway upregulates Ufbp1 expression, which is critical for the expansion of the ER network (75,78). Simultaneously, UFBP1 plays a pivotal role in suppressing the PERK pathway. This suppressive relationship is demonstrated by the observation that PERK deletion restores the defects in B-cell to plasma cell development caused by UFBP1 deficiency. Moreover, the expression of UFBP1 and other molecules involved in the UFMylation pathway in immature B cells is independent of IRE1α/XBP1 (75,79). Thus, UFBP1 markedly regulates different branches of the UPR pathway to promote plasma cell development and function. Specifically, the IRE1α/XBP1 axis upregulates UFBP1 and genes in the UFMylation pathway in plasma cells, while UFBP1 deficiency impairs ER expansion and hinders immunoglobulin production (53,80). Structural and functional analyses reveal that Lys267 in UFBP1 is a critical lysine residue. Although not essential for plasma cell development, it is vital for immunoglobulin production and stimulating ER expansion in IRE1α-deficient plasma cells. In summary, UFBP1 exerts differential impacts on the development and function of plasma cells by regulating distinct steps in the UPR pathway (79,81).

Relevant studies have combined genetic disruption of UFMylation and de-UFMylation with affinity capture liquid chromatography-tandem mass spectrometry (LC-MS/MS) techniques, applying three consecutive filtration steps: i) Low-confidence hits (<3 unique peptides in UFSP2 knockout), ii) proteins whose abundance did not increase after UFSP2 knockout and iii) proteins whose abundance increased following UBA5 knockout, to identify the conserved ribosomal protein RPL26. Subsequently, genetic manipulation using small interfering siRNA depletion or CRISPR-Cas9-mediated cell line models was employed to investigate the UFMylation process of RPL26 (65,77). Although the absence of UFL1 or its interaction with UFBP1 eliminates RPL26 UFMylation, the UFMylation of RPL26 at two distinct lysine residues (K132 and K134) is markedly increased in UFSP2-/- cell lines (77,82). This modification is continuous; overexpression of RPL26 K134R in UFSP2-/- cells abolishes both monomeric and dimeric RPL26, while monomeric RPL26 is still present in cells overexpressing RPL26 K132R. In conditions where UFMylation is not dominant, such as in UFSP haploinsufficient cell lines (UFSP1-/+/UFSP2-/-), only K134 modifications are observed, indicating that the modification of RPL26 is highly specific. Moreover, replacing endogenous RPL26 with a mutant allele lacking both residues (RPL26K132R/K134R) abolishes the modification of RPL26 (63,64,83). This further indicates that the ribosomal RPL26 undergoes a dynamic cycle of UFMylation and de-UFMylation catalyzed by enzymes attached to the cytoplasmic surface of the ER, providing a functional link between the UFMylation process and ER protein homeostasis (64,66).

In eukaryotes, secretory pathway proteins are primarily synthesized by ribosomes bound to the ER and inserted into the ER lumen or integrated into membranes via the Sec61 translocon (63,84,85). This process is highly sensitive to disturbances such as translation stalling or defects in protein modifications, folding and assembly, all of which can lead to defective polypeptides that clog the translocon. The clogging of translocons triggers ribosome filtration and activates transport-associated quality control (TAQC) to degrade the clogged substrates (86,87). Wang *et al* (88) identified a membrane protein named SAYSD1 through whole-genome CRISPR-Cas9 screening, which aids in the TAQC process by binding to SEC61. SAYSD1 also directly recognizes ribosomes and UFM1, associating with stalled nascent chains to ensure their transport to lysosomes for degradation via the TRAPP complex. Similar to UFM1 deficiency, depletion of SAYSD1 leads to the accumulation of proteins stalled in the ER, inducing ER stress. Notably, disrupting UFM1- and SAYSD1-dependent TAQC in *Drosophila* results in the accumulation of collagen that is stalled during translocation, leading to collagen deposition defects, abnormal basement membrane integrity and reduced stress tolerance (89). Thus, SAYSD1 functions as a sensor for UFM1, coordinating the ribosomal UFMylation of blocked translocon sites to maintain ER homeostasis during animal development (88) (Fig. 2).

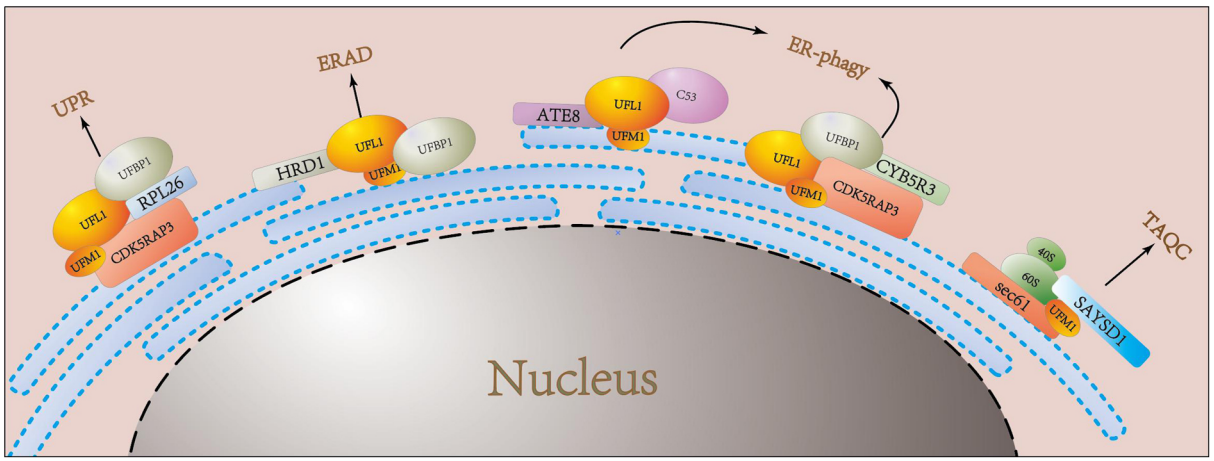

Figure 2. UFMylation regulates the process of the endoplasmic reticulum. In the UPR pathway, ribosomal protein RPL26 undergoes a dynamic cycle of UFMylation and de-UFMylation, catalyzed by Ufbp1 and UFL1 at the ER membrane. This regulatory cycle is essential for maintaining ER homeostasis. Under physiological conditions, cells modulate the ERAD of misfolded ER-resident proteins through the UFMylation of HRD1, a process mediated by the DDRGK1-UFL1 complex. During ER-phagy, the UFL1-UFBP1 complex facilitates the UFMylation of CYB5R3, promoting the autophagic degradation of specific ER subdomains. Additionally, these ER subdomains exhibit affinity for ATG8 family proteins or FIP200, ensuring the selective degradation of ER components. In the TAQC pathway, SAYSD1 acts as a UFM1 sensor, directly recognizing ribosomes and facilitating their transport to lysosomes for degradation via the TRAPP complex. ER, endoplasmic reticulum; UPR, unfolded protein response; RPL26, ribosomal protein L26; UFL1, UFM1 specific ligase 1; ERAD, ER-associated degradation; HRD1, HMG-CoA reductase degradation 1; DDRGK1, DDRGK domain containing 1; UFBP1, Ufm1 binding protein 1; CYB5R3, cytochrome B5 reductase 3; ATG8, autophagy-related protein 8; FIP200, focal adhesion kinase family interacting protein of 200kD; SAYSD1, SAYSVFN motif domain containing 1; TRAPP, trafficking protein particle complex.

6. UFMylation and hematopoiesis

Hematopoiesis, a complex process regulated by specific transcription factors and cytokines, supports the self-renewal, differentiation and survival of hematopoietic progenitor cells at various stages of maturation (90,91). The UFMylation system plays a pivotal role in modulating the expression levels and activation of transcription factors that facilitate erythropoiesis. It is crucial to identify UFM1 targets for understanding how UFM1 protein modification governs erythroid development, as defects in this process can lead to pathological conditions, such as leukemia and myelodysplastic syndromes.

Germline deletions of UBA5, UFL1, or UFBP1 in mice result in embryonic lethality and disrupted hematopoietic development (92,93). UBA5 and other genes involved in UFMylation are expressed in both primitive and definitive erythrocytes, with the highest levels of UBA5 found in primitive erythroid lineages. Mice deficient in UBA5 exhibit severe anemia due to defects in megakaryocyte and erythrocyte differentiation, ultimately resulting in death *in utero*. This deficiency impairs the differentiation of common bone marrow progenitors into megakaryocytes and erythroid progenitors. Notably, transgenic expression of UBA5 in the erythroid lineage can rescue embryos from anemia, extending their survival (92).

UFL1 is essential for embryonic development, hematopoietic stem cell (HSC) survival and erythroid differentiation. Knockout of UFL1 leads to reduced HSC survival rates and embryonic death due to severe anemia (94). UFL1 is considered to function as an E3 ligase for UFM1, promoting the UFMylation of DDRGK1 and ASC1. Notably, both UBA5 and UFL1 knockout mice display extensive phenotypic similarities, including defects in embryonic erythropoiesis and increased mortality (93). Specifically, UBA5 deficiency disrupts the development of megakaryocyte-erythroid progenitors (MEP) in the fetal liver without affecting granulocyte-monocyte progenitors (GMP). Conversely, conditional deletion of RCAD in adult mice causes defects in MEP progenitors but does not affect GMP. Additionally, a significant number of multinucleated erythroid cells are observed in the embryos of both UBA5 and RCAD-deficient mice, suggesting that UBA5 and RCAD may function in similar cellular processes or signaling pathways during erythroid development (90).

UFBP1 is also critical for embryonic development and hematopoiesis; its deficiency results in defects in erythroid development and embryonic death in mice. In adult mice, UFBP1 deficiency leads to impaired hematopoiesis, resulting in pancytopenia and increased mortality (18). At the cellular level, the absence of UFBP1 heightens endoplasmic reticulum stress and activates the UPR, causing cell death in hematopoietic progenitor/stem cells (19). Furthermore, UFBP1 deficiency suppresses the expression of erythroid transcription factors GATA-1 and KLF1, inhibiting the transition from colony-forming units-erythroid to proerythroblasts. Similar phenotypic defects are observed in UFBP1-deficient mice when compared with UFL1 and UBA5-deficient mice, indicating that UFBP1 serves as a crucial downstream effector in the regulation of hematopoietic development mediated by the UFM1 system through UFMylation. These findings underscore the significant role of UFMylation in erythroid development and differentiation (18) (Table I).

7. UFMylation and skeletal development

Clinical studies have identified diseases genetically linked to components of the UFM1 pathway, emphasizing their crucial roles in development and tissue homeostasis. The majority of mutations in UFM1 pathway components are associated with

Table I. Phenotypic comparison of Uba5, Ufl1 and Ufbp1 knockout mice.

First author, year	Gene	KO phenotype	CKO phenotype	Biochemical function	(Refs.)
Tatsumi *et al*, 2011	UBA5	Embryo death and impaired hematopoietic development. Fewer erythroid progenitor cells.		UFM1 E1enzyme	(92)
Zhang *et al*, 2015	UFL1	Embryo death and impaired hematopoietic development. Fewer erythroid progenitor cells.	Animal death around 3 weeks after initial injection of tamoxifen. Pancytopenia and severe anemia. Reduced number of erythroid progenitor cells in bone marrow. Increased percentage and number of GMPs in bone marrow. Impaired development from CFU-E to pro-erythroblast. Loss of HSC function.	UFM1 E3 ligase	(93)
Cai *et al*, 2015	UFBP1	Embryo death and impaired hematopoietic development. Impaired development of hemato-poietic progenitor cells.	Animal death around 3 weeks after initial injection of tamoxifen. Pancytopenia and severe anemia. Reduced number of erythroid progenitor cells in bone marrow. Increased percentage and number of GMPs in bone marrow. Impaired development from CFU-E to pro-erythroblast. Loss of HSC function.	UFM1 specific protease	(18)

UBA5, ubiquitin-like modifier activating enzyme 5; UFL1, UFM1 specific ligase 1; UFBP1, UFM1-binding and PCI domain-containing protein1; UFM1, ubiquitin-fold modifier 1; GMP, guanine monophosphate synthase; CFU-E, colony forming unit-erythrocyte; HSC, hemato-poietic stem cell.

a skeletal pathology known as SEMD (23,95). A clinical case study of affected Italian families reported a missense mutation, specifically UFSP2 D418A, in the UFSP2 gene. The patients exhibited systemic skeletal dysplasia with infantile onset, delayed skeletal development, demineralization, atrophy, restricted mobility and joint destruction (95). Subsequently, heterozygous variants of the UFSP2 gene, including Y290H, D426A and H428R, were identified through exome sequencing, with the SEMD phenotype observed in individuals harboring mutations in the catalytic histidine (H420R) and the tyrosine residue (Y290H) that form the oxocation pore (96). The Y282H mutation resulted in a markedly milder phenotype. This finding supports a new perspective that varying clinical outcomes may be associated with the loss of catalytic UFSP2 activity linked to different mutants, which has been confirmed *in vitro* (24).

Furthermore, loss-of-function mutations in DDRGK1 (UFBP1, C20orf116) have also been demonstrated to cause SEMD. DDRGK1-/- mice exhibit delayed mesenchymal condensation in limb buds and early embryonic lethality. To further investigate this, conditional knockout mice were generated by crossing Prx1-Cre transgenic mice with DDRGKfl/fl mice to delete DDRGK1 expression in limb mesenchymal cells. The mutant mice displayed progressively severe shortening of the limbs, joint abnormalities, disorganized growth plate tissue, reduced proliferation zones and enlarged hypertrophic zones. These data highlight the importance of DDRGK1 in the development of growth plates (97). By contrast, conditional knockout of DDRGK1 using osteoblast-specific osteocalcin-Cre and leptin receptor-Cre lines did not result in bone phenotypes, indicating that the effect on limb development is cartilage-specific. These findings suggest that DDRGK1 is necessary postnatally for the maintenance of normal growth plate morphology (98). Collectively, these discoveries underscore the physiological role of DDRGK1 in the development and maintenance of growth plate cartilage. Moreover, these genetic mouse models recapitulate the clinical phenotypes of short stature and joint abnormalities observed in patients with Sohat-type SEMD (98).

8. UFMylation and neurodevelopment

UFMylation plays a crucial role in neurodevelopment. Abnormalities in the UFMylation system can manifest as severe infantile-onset encephalopathy, with or without seizures, severe congenital neuropathies, or early-onset cerebellar atrophy accompanied by ataxia (17,99). As the disease progresses, non-specific findings such as mild myelination delay, hyperintense white matter signals, thinned thalami and corpus callosum, cerebellar hypoplasia, or generalized brain

atrophy can be observed (24). Individuals with the most severe forms of the disease may die shortly after birth or in infancy, while those with milder symptoms can survive >20 years. Whole exome sequencing analysis has identified rare autosomal recessive variants in UBA5 in five children from four unrelated families, all of whom exhibited similar severe intellectual disabilities, microcephaly, motor disorders, or early-onset refractory epilepsy (25,100). To date, 24 variants in UBA5 have been linked to neurological disorders. Of these variants, ~67% are located in the adenylylation domain of the UBA5 protein, 19% in the UFC1-binding domain and C-terminus and 14% in the N-terminus (101). Most missense mutations lead to mild impairment of UBA5 function; however, two homozygous inherited N-terminal variants have resulted in severe disease, leading to the mortality of affected individuals within 16 days and 16 weeks, respectively (102,103). In zebrafish, UBA5 gene knockout at early stages exhibits peripheral nerve and cerebellar axonal damage, with mitochondrial damage identified in peripheral and central nervous systems as well as in skeletal muscles (104). In *Caenorhabditis elegans*, knockout of UBA5 and its human ortholog in the UFM1 cascade alters cholinergic neurotransmission without affecting glutamatergic transmission (105).

NCAM, a molecule that plays a role in neuronal development and synaptic plasticity in the adult brain, has been shown to interact with the UFMylation system. NCAM140, a subtype of NCAM, interacts with UFC1 through its cytoplasmic domain and co-localizes with UFC1 at the surface of B35 neuroblastoma cells (101). Overexpression of UFM1 also leads to increased endocytosis of NCAM140. Whole exome sequencing analysis revealed rare autosomal recessive variants in UBA5 in five children from four unrelated families, presenting similar severe intellectual disabilities, microcephaly, motor disorders, or early-onset refractory epilepsy (106). Biochemical and cellular studies of UBA5 mutant proteins in fibroblasts from affected individuals indicate that pathogenic mutations impair UFMylation, resulting in abnormal endoplasmic reticulum structure. In *C. elegans*, knockout of UBA5 and its human ortholog in the UFM1 cascade affects cholinergic neurotransmission without affecting glutamatergic neurotransmission (107). Furthermore, silencing of UBA5 in zebrafish induced abnormal movements while reducing locomotor ability, suggesting a link between UFMylation and seizures (105,108). These clinical, biochemical and experimental findings demonstrate that UBA5 mutations can lead to early-onset encephalopathy due to abnormal protein UFMylation (105). Collectively, these studies highlight the significant role of UFMylation in neurodevelopment (Table II).

9. UFMylation and immune regulation

The UFM1 conjugation family has emerged as a promising therapeutic target for modulating immune responses, either by enhancing antiviral defense or mitigating excessive inflammation in autoimmune diseases. Studies have revealed that upon RNA virus infection (109,110), UFL1 is recruited to the membrane-associated scaffold protein 14-3-3ε, where it undergoes UFMylation. This process leads to the activation of retinoic acid-inducible gene I, which in turn triggers mitochondrial antiviral signaling protein (MAVS)-mediated signaling cascades. These cascades ultimately culminate in the induction of type I and III interferons (IFNs) (110). In response to herpes simplex virus type 1 infection, UFL1 expression is rapidly downregulated at both the mRNA and protein levels in peritoneal macrophages. The conditional deletion of UFL1 in macrophages results in increased viral loads in the serum and peripheral immune cells. This increase is accompanied by a marked reduction in proinflammatory cytokines, including interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and IFN-β. During Epstein-Barr virus (EBV) infection, the viral-encoded G protein-coupled receptor BILF1 hijacks UFL1 to mediate MAVS UFMylation. This process promotes MAVS mitochondrial mislocalization and subsequent lysosomal degradation, effectively suppressing EBV-induced activation of the NLRP3 inflammasome and thereby dampening host antiviral immune responses (109). These findings demonstrate that UFMylation plays a crucial role in the body's immune regulation.

10. The role of UFMylation in common malignancies

Numerous studies have reported the amplification, deletion, or mutation of genes encoding UFMylation factors (UBA5, UFC1, UFL1, UFSP2 and UFM1) in malignant tumors across various tissues. These findings indicate that UFMylation may either promote or inhibit tumorigenesis, depending on the cellular environment. The loss or mutation of components within the UFMylation pathway is associated with a range of diseases. Deepening our understanding of UFMylation's role in cancer may pave the way for the development of novel therapeutic strategies (43,53,103,107,111).

Breast cancer. Breast cancer ranks among the top three most common cancers worldwide (112,113). A significant body of literature indicates that estrogen receptor-positive (ER+) tumors constitute a large portion of cases. PTMs of ERα are critical in regulating its expression, subcellular localization and hormonal response sensitivity (114,115). UFMylation plays a crucial role in positively regulating ERα stability and transactivation, which is key in breast cancer development (58,116). By inhibiting ubiquitination, ERα stability is markedly enhanced, whereas silencing UBA5 decreases ERα stability. Lys171 and Lys180 of ERα have been identified as primary UFM1 receptor sites and substituting these lysine residues with arginine (2KR mutation) markedly reduces ERα stability (116). Furthermore, the 2KR mutation abolishes ERα's 17β-estradiol-induced transcriptional activity and the expression of downstream targets such as PS2, cyclin D1 and C-MYC, highlighting the essential role of ERα's transcriptional activation function. The 2KR mutation also prevents MCF7 cells from forming anchorage-independent colonies (48,116,117). Notably, UFM1 and its associated mechanisms (UBA5, UFC1, UFL1 and UFBP1) are markedly upregulated in ERα-positive breast cancer cell lines and tissues. In summary, these findings suggest that ERα enhances breast cancer development by stabilizing and promoting its transactivation function through UFMylation (116,118).

Metformin can induce ferroptosis in breast cancer cell lines, thereby inhibiting tumor growth independently of conventional

Table II. Summary of verified UFMylation substrates and enzymes.

First author, year	UFMylation substrate	Functional sites	Function	(Refs.)
Bakkenist and Kastan, 2003	MRE11	K282	Promotes MRN complex formation and recruitment, maintains telomere length.	(49)
Qin *et al*, 2020	Histone 4	K31	Amplifies ATM activation and maintains genomic. integrity	(50)
Liu *et al*, 2020	P53	K351, K357, K370, K373	Maintains P53 stability and suppresses tumor growth.	(47)
Wang *et al*, 2020	ASC1	K324, K325, K334, K367	Promotes ERα transactivation and breast cancer progression.	(165)
Luo *et al*, 2023	HRD1	K610	Regulating the degradation of endoplasmic reticulum related proteins and clearing misfolded ER resident proteins.	(68)
Ishimura *et al*, 2022	CYB5R3	K214	Promotes the formation of autophagosomes and initiate endoplasmic reticulum phagocytosis.	(76)
Zhu *et al*, 2019	UFBP1	K267	Maintains ER homeostasis, promotes self-renewal and differentiation of hematopoietic progenitor cells.	(75)
Liu *et al*, 2020	RPL26	K132, K134	Maintains ER homeostasis.	(47)
Wang *et al*, 2023	SAYSD1		Maintains ER homeostasis.	(88)
Tatsumi *et al*, 2011	UBA5		Promotes self-renewal and differentiation of hematopoietic progenitor cells, promotes neuronal development and synaptic plasticity in the brain.	(92)
Zhang *et al*, 2015	UFL1		Promotes self-renewal and differentiation of hematopoietic progenitor cells.	(93)
Di Rocco *et al*, 2018	UFSP2	Y290H, D426A, H428R	Maintains normal bone shape.	(95)

MRE11, meiotic recombination 11 homolog; ASC1, activating signal cointegrator 1; HRD1, HMG-CoA reductase degradation protein 1; CYB5R3, cytochrome b5 reductase 3; UFBP1, UFM1 binding protein1; RPL26, ribosomal protein L26; SAYSD1, saysvfn motif-containing domain 1; UBA5, ubiquitin like modifier activating enzyme 5; UFL1, UFM1 specific ligase 1; UFSP2, UFM1 specific peptidase 2; UFBP1, UFM1 binding protein 1; ATM, ataxia-telangiectasia mutated.

AMP-activated protein kinase (AMPK) signaling (119). Mechanistically, metformin increases intracellular Fe^{2+} and lipid ROS levels. Specifically, metformin suppresses the UFMylation process of SLC7A11, a key regulator of iron metabolism, leading to decreased protein stability of SLC7A11 and consequently inhibiting cancer proliferation (120).

Recently, two additional UFMylation substrates, PLAC8 and PD-L1, have been identified as playing significant roles in the pathogenesis of breast cancer (121-123). Mao *et al* (121) reported that PLAC8 is generally expressed at high levels in triple-negative breast cancer (TNBC). This is because it is modified by UFM1 UFMylation at Lys103, which maintains its protein stability. The stable PLAC8 interacts with glycosylated PD-L1, thereby upregulating PD-L1 levels, promoting cancer cell proliferation and inhibiting immune responses (123,124). Additionally, PD-L1 can be modified by various deubiquitinating and ubiquitinating proteins, such as USP22, CSN5 and ARIH1. Treatment of MDA-MB-231 and HCC-1937 cells with MG-132 and chloroquine demonstrated that MG-132 inhibited proteasome activity, while chloroquine inhibited lysosomal activity. Notably, chloroquine rescued the reduction of PD-L1 protein levels induced by PLAC8 knockout. Collectively, these data suggest that PLAC8 may also stabilize PD-L1 protein by regulating its ubiquitination (121).

Liver cancer. Hepatocellular carcinoma (HCC) is a leading cause of cancer-related deaths in a number of regions of the world. Risk factors for HCC include chronic hepatitis, alcohol addiction, metabolic liver diseases and exposure to dietary toxins such as aflatoxins and aristolochic acids. In most cases, acute hepatitis, chronic hepatitis and cirrhosis caused by chronic hepatitis B or C virus infections are significant contributors to the development of hepatocellular carcinoma (125). Reports indicate that the UFMylation system is associated with the occurrence and progression of HCC. In the livers of mice re-fed with dihydro-2,4,6-trimethyl-3,5-pyridinedicarboxylate (DDC) and in humans with alcoholic hepatitis (AH) and non-alcoholic steatohepatitis (NASH) characterized by the presence of Mallory-Denk bodies (MDB), the UFMylation pathway is downregulated, including protein quality control (126,127). Notably, feeding the methyl donor betaine alongside DDC markedly prevents the increase in UFMylation expression in DDC-pretreated mice (128,129). Betaine notably inhibited the transcriptional silencing of

UFM1, UBA5 and UFSP1 associated with MDB formation and prevented the increase in FAT10 and LMP7 expression induced by DDC re-feeding. A similar downregulation of UFMylation was observed in multiple biopsies from patients with AH and NASH. Compared with normal subjects, patients with AH and NASH exhibited markedly elevated levels of DNA methylation in the promoter CpG regions of UFM1, UFC1 and UFSP1 (130-132). The mRNA levels of DNA methyltransferase 1 (DNMT1) and DNA methyltransferase 3β (DNMT3B) were markedly upregulated in patients with AH and NASH, indicating that the maintenance of UFMylation methylation may be co-mediated by DNMT1 and DNMT3B (133,134).

It has been reported that an HCC suppressor known as B3GALT5-AS1 negatively regulates the proliferation, invasion and metastasis of HCC cells by modulating miR-934 and UFM1. This suppression of HCC cell proliferation, invasion and metastasis was observed when pGL3-UFM1-WT and pGL3-UFM1-MUT plasmids were co-transfected with miR-NC or miR-934 into HCC cell and compared with the co-transfection group with pGL3-UFM1-WT and NC (135), the co-transfection of the pGL3-UFM1-WT plasmid with miR-934 markedly reduced the luciferase activity of the reporter plasmid, thereby indirectly elucidating the interaction between UFM1 and miR-934 and confirming that UFM1 is a regulatory target of miR-934. Subsequently, the expression of UFM1 was measured in three groups (NC, miR-934 inhibitor and si-UFM1) after transfection, revealing that the UFM1 mRNA expression levels in the miR-934 inhibitor and si-UFM1 groups were markedly lower than those in the miR-934 inhibitor-only group. Importantly, it was determined that si-UFM1 could reverse the decreased cell proliferation and migration abilities induced by the miR-934 inhibitor (136).

Additionally, UFL1 has been identified as a tumor suppressor in HCC, playing a critical role in the pathogenesis of HCC by preventing cell invasion, inhibiting NF-κB signaling and increasing the stability of LZAP protein. However, the exact role of CDK5RAP3 in HCC remains controversial (39,137). One study reports that CDK5RAP3 may act as an oncogene, promoting the migratory and invasive characteristics of the SMMC-7721 and HEPG2 cell lines (138). By contrast, another group of studies indicated that CDK5RAP3 functions as a tumor suppressor in HEPG2 cells. The precise role, function and mechanism of CDK5RAP3 in HCC warrant further investigation (16,76).

Lung cancer. Lung cancer is one of the deadliest cancers and is becoming increasingly prevalent worldwide (139). Notably, the mortality rate of lung cancer exceeds that of other tumors. Lung cancer encompasses various subtypes, including lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), large cell lung carcinoma and small cell lung carcinoma (140,141). Recently, UBA5 has been identified as playing a significant role in the growth of LUAD cells, resistance to cisplatin and promoting immune evasion while also participating in the macrophage M2 polarization process, thereby altering the tumor microenvironment and facilitating tumor progression (142). In LUAD, both UBA5 mRNA and protein levels are highly expressed. The pharmacological inhibition of UBA5 using DKM 2-93 effectively inhibited LUAD growth, indicating that the suppression of UBA5 hinders LUAD cell proliferation (143).

Additionally, UBA5 positively correlates with macrophage M2 polarization in LUAD. The knockdown of UBA5 directly suppressed the *in vitro* polarization of macrophages to M2, reduced the *in vivo* infiltration of macrophage M2 and decreased lactate production in LUAD. These results suggest that UBA5 regulates macrophage M2 polarization through lactate secretion, thereby altering the immune microenvironment and facilitating the escape of LUAD cells from immune surveillance (144).

Despite its tumor-suppressive role in hepatocellular carcinoma, UFL1 may act as an oncogene in lung adenocarcinoma. UFL1 is upregulated in early lung adenocarcinoma tissues and its overexpression promotes the proliferation of H1299 lung adenocarcinoma cells. UFL1 can bind to the regulatory domain of P120-catenin, inhibiting the ubiquitin-mediated proteasomal degradation of P120-catenin. Subsequently, P120-catenin promotes the proliferation of lung adenocarcinoma through its interaction with NLBP (145).

Colorectal cancer. In relevant studies on colorectal cancer, Zhou *et al* (146) observed that the knockdown of UFSP2 markedly promoted the growth rate of colorectal cancer cells HT29 and HCT116 and their anchorage-independent growth. Notably, the knockdown of UFSP2 expression markedly enhanced the growth of xenograft tumors derived from UFSP2-depleted HT29 cells. These results demonstrate that UFSP2 is a potential tumor suppressor in colorectal cancer.

Gastric cancer. Despite a decline in the incidence and mortality rates of primary cancers in recent decades, gastric cancer remains one of the three most common cancers worldwide. Most gastric cancer patients exhibit nonspecific early symptoms and are often diagnosed at advanced stages, which severely affects their prognosis. Therefore, identifying new biomarkers is essential for early diagnosing and treating gastric cancer (147,148). DDRGK1 is a target protein for UFMylation and a key component of the UFMylation modification system, playing a crucial role in cancer development (149). Shiwaku *et al* (150) found that the amino acid sequence of CDK5RAP3 contains a ubiquitin-protein ligase binding domain. Wu *et al* (151) revealed that CDK5RAP3 can interact with DDRGK1 and UFL1 (RCAD), regulating the stability of CDK5RAP2 and DDRGK1. Notably, patients with low expressions of CDK5RAP3 and DDRGK1 had the worst prognoses, while those with high expressions of these proteins exhibited the best prognoses, with other patients falling in between. The predictive accuracy of the combined expression of CDK5RAP3 and DDRGK1 was higher than using either CDK5RAP3 or DDRGK1 alone and their combined expression demonstrated superior predictive capability for overall survival in cancer patients (152). Xi *et al* (153) discovered that DDRGK1 interacts with IkBa and regulates its stability, thereby modulating the transcriptional activity of NF-κB and the expression of its target genes. Conversely, Wu *et al* found that the downregulation of CDK5RAP3 increased cellular invasiveness and enhanced the transcriptional activity of NF-κB (151). CDK5RAP3 binds to RelA to inhibit its phosphorylation, increasing the association of HDAC with RERA and thereby suppressing NF-κB transcriptional activity.

In summary, CDK5RAP3 and DDRGK1 interact and their roles in the NF-κB pathway are similar. Additionally,

Table III. Function of UFMylation in various types of cancer.

First author, year	Cancer type	Gene	Function	(Refs.)
Yoo *et al*, 2022	Breast cancer	ERa	Promotes breast cancer development.	(116)
Yang *et al*, 2021	Breast cancer	SLC7A11	UFMylation stabilizes SALC7A11 and metformin reduces the protein stability of SLC7A11 by reducing UFM1.	(120)
Mao *et al*, 2022	Breast cancer	PLAC8	UFMylation of PLAC8 may influence tumor progression and immune response in triple negative breast cancer cells by reducing PD-L1 ubiquitination.	(121)
Lim *et al*, 2016	Breast cancer	PD-L1	UFMylation of PD-L1 destabilizes PD-L1 by acting synergistically to promote its ubiquitination.	(124)
Chen *et al*, 2021	Hepatocellular carcinoma	UFM1	B3GALT5-AS1 regulates miR-934 and UFM1 to achieve negative regulation of HCC cell proliferation, invasion, and metastasis.	(136)
Liu *et al*, 2014	Hepatocellular carcinoma	UFSP1	Alcoholic hepatitis and non-alcoholic steatohepatitis transcriptional down regulation of FATylation and UFMylation.	(133)
Yang *et al*, 2019	Hepatocellular carcinoma	UFL1	UFL1 act as gatekeepers to prevent liver fibrosis and subsequent steatohepatitis and Hepatocellular carcinoma development by inhibiting the mTOR pathway.	(16)
Zhou *et al*, 2021	Colon cancer	UfSP2	UFSP2 is a potential tumor suppressor in colon cancer.	(146)
Lin *et al*, 2018	Gastric cancer	CDK5RAP3 and DDRGK1	CDK5RAP3 interacting with DDRGK1 suppresses the development of gastric cancer by inhibiting the phosphorylation of AKT/GSK-3β and negatively regulating Wnt/β-catenin signaling.	(152)
Lin *et al*, 2019	Gastric cancer	UFM1	UFM1 has inhibitory effects on carcinogenicity, invasion, and migration of gastric cancer cells.	(154)
Liu *et al*, 2020	Renal cancer	P53	UFMylation stabilizes p53 by inhibiting its ubiquitination, which suppresses cell growth and tumor formation.	(47)

Era, estrogen receptor alpha; SLC7A11, solute carrier family member 11; PLAC8, placenta specific 8; PD-L1, programmed death ligand; UFM1, ubiquitin fold modifier 1; UFSP1, Ufm1 specific peptidase 1; UFL1, Ufm1 ligase 1; CDK5RAP3, cyclin-dependent kinase 5 regulatory subunit associated protein 3; DDRGK1, DNA damage-regulated autophagy modulator 1; UFM1, ubiquitin fold modifier 1.

Lin *et al* (154) found that UFM1 was downregulated in gastric cancer tissues. Patients with low UFM1 expression levels had poor prognoses, while UFM1 exhibits inhibitory effects on oncogenicity, invasion and migration of gastric cancer cells. Mechanistically, UFM1 suppresses gastric cancer cells' epithelial-mesenchymal transition (EMT) by negatively regulating the PI3K/AKT signaling pathway and increasing the ubiquitination of PDK1, thereby exerting its tumor-suppressive function (155).

Renal cell carcinoma. In renal cell carcinoma (RCC), the expressions of UFL1 and UFBP1 are also downregulated and positively correlate with P53 levels, a protein closely associated with various cancers. It has been reported that P53 interacts with UFL1 and UfBP1 and is modified by UFM1. UFMylation mediated by UFL1 and UFBP1 stabilizes P53 by antagonizing MDM2-mediated ubiquitination and proteasomal degradation, inhibiting cellular growth and tumor formation *in vivo* (155).

In addition to analyzing RCC tissue microarrays from 40 paired patient samples, studies using mouse xenograft models indicate that UFL1 and UfBP1 can function as tumor suppressors by regulating P53 stability. These results suggest that UFMylation is a key post-translational modification for maintaining P53 stability and tumor-suppressive function, implicating UFMylation as a promising therapeutic target in cancer (47) (Table III).

11. UFMylation in immunotherapy

Immune cells are central to antiviral defense and antitumor immunity, yet the intricate tumor microenvironment enables malignant cells to evade immune-mediated elimination through diverse mechanisms (156). Among these, the upregulation of inhibitory immune checkpoint receptors, such as PD-1, serves as a key strategy to suppress T cell activation and cytotoxic function. However, the limited clinical efficacy of anti-PD-1/PD-L1 immunotherapy underscores the urgent need for more effective therapeutic strategies (21). Recent findings highlight PD-L1 UFMylation as a crucial regulator of PD-1/PD-L1 axis homeostasis in both human

and murine tumor cells, with its dysregulation compromising immune evasion (157). Loss of UFL1 in T cells abrogates PD-1 UFMylation, thereby enhancing antitumor immunity. Specifically, UFL1 deletion in T cells diminishes PD-1 UFMylation, facilitates $CD8^+$T cell-mediated tumor rejection and promotes K48-linked ubiquitination and subsequent proteasomal degradation of PD-1. *In vitro* and *in vivo* analyses both demonstrate that UFL1 deficiency markedly reduces PD-1 protein abundance while augmenting the production of effector cytokines, including IFNγ, TNF and granzyme B, in $CD8^+$T cells.

Furthermore, AMPK phosphorylates UFL1 at T536, disrupting its interaction with PD-1 and attenuating PD-1 UFMylation (158). Notably, conditional T cell-specific knockout (cKO) of UFL1 enhances antitumor immunity. In murine models of Lewis lung carcinoma and MC38 colorectal cancer, UFL1 cKO markedly improves responsiveness to anti-CTLA-4 immunotherapy. However, UFL1 depletion in T cells diminishes the antitumor efficacy of AMPK activators, suggesting that the AMPK-UFL1 axis plays a pivotal role in regulating T cell-mediated antitumor immunity (159).

12. Other tumors

Beyond the aforementioned tumor types, the UFM1 conjugation system has also been implicated in other human malignancies. Sarcomas, a heterogeneous group of mesenchymal-derived malignant neoplasms of connective tissue, are broadly classified into soft tissue sarcomas and primary bone sarcomas. These categories encompass a wide array of subtypes, contributing to the remarkable diversity of this tumor group (160). Among them, osteosarcoma is the most prevalent primary bone malignancy in children, adolescents and young adults (161). Notably, studies have reported that UFBP1 suppresses the proliferation, migration and invasion of human osteosarcoma cells (15,16,162). Mechanistically, as a component of the E3 ligase complex in the UFM1 conjugation system, UFBP1 directly interacts with IκBα, regulating its stability and attenuating NF-κB transcriptional activity (16). Notably, UFBP1 primarily binds to the N-terminal domain of IκBα (1-106 aa), which harbors critical phosphorylation sites (Ser32 and Ser36) and ubiquitination sites (Lys21 and Lys22). By regulating the phosphorylation and stability of IκBα, UFBP1 modulates NF-κB transcriptional activity and the expression of its downstream target genes. Furthermore, UFBP1 depletion via siRNA markedly alters the expression of NF-κB target genes, including cytokines, chemokines, adhesion molecules and receptors, as well as enzymes involved in proliferation, differentiation, apoptosis and stress responses. In summary, UFBP1 may promote the UFMylation-mediated degradation of IκBα, thereby downregulating the expression of NF-κB pathway target genes and suppressing osteosarcoma cell proliferation, migration and invasion (153). In addition, RPL10 UFMylation has been identified in pancreatic cancer tissues and cell lines (163), with this modification being catalyzed by UFL1 at specific sites and reversed by UFSP2-mediated de-UFMylation (164). Reduced UFMylation of RPL10 inhibits pancreatic cancer cell proliferation and stemness. Moreover, transcription factor KLF4 positively regulates the relationship between RPL10 inactivation and cellular stemness. Loss of RPL10 is closely associated with tumorigenesis, primarily by enhancing the expression of stemness-associated surface markers and upregulating KLF4. Notably, mutations at key UFMylation sites in RPL10 markedly impair pancreatic cancer cell proliferation and stemness, further highlighting the functional relevance of this post-translational modification in tumor development (163).

13. Conclusion

Protein PTM is a standard physiological process in cells. Ubiquitination is a special modification of protein post-translational modification, which plays a vital role in cell life activities. UFM1 is a class ubiquitin modifier that attaches to lysine residues on substrates after translation by a dedicated enzyme system conserved in most eukaryotes. Despite structural similarities between UFM1 and ubiquitin, the UFMylation machinery employs unique mechanisms to ensure fidelity. Although the physiological triggers and consequences of UFMylation are not fully understood, its biological importance is reflected in frequent mutations in the UFMylation pathway in human pathophysiology, including musculoskeletal and neurodevelopmental disorders. Some of these diseases can be explained by the increased endoplasmic reticulum (ER) stress and disruption of translational homeostasis observed upon loss of UFMylation. The role of UFM1 in these processes may stem from its function in the ER, where ribosomes are UFM1-glycosylated due to translational arrest. In addition, UFMylation has been implicated in other cellular processes, including DNA damage response and telomere maintenance.

To date, certain studies have shown that UFMylation modification is also closely related to the development of tumors (43,146,164,165). UFMylation modification seems related to the stable expression of specific tumor suppressor genes. For example, in clear cell renal cell carcinoma, UFMylation of p53 promotes its stability, inhibiting tumor cell proliferation.

UFMylation also plays a pivotal role in antitumor immunity. Depletion of UFL1 in $CD8^+$ T cells suppresses PD-L1 expression, leading to the upregulation of downstream effector cytokines such as IFN-γ and enhancing T-cell cytotoxicity. As a potential therapeutic target, inhibition of UFMylation has shown promise in cancer treatment. Notably, metformin disrupts the UFMylation of ferroptosis-related proteases, destabilizing SLC7A11 and promoting ferroptosis, thereby suppressing tumor growth through a mechanism independent of conventional AMPK signaling. However, does UFMylation have a stabilizing effect on other tumor suppressor proteins? At the same time, does UFMylation also participate in the degradation of tumor-related genes and inhibit the occurrence and development of tumors? In addition, is UFMylation also associated with activation/inhibition of tumor-related signaling pathways? None of this has been reported in much research. The revelation of these mechanisms may expand the field of UFMylation and human diseases.

In summary, studies of the mechanisms and biological functions of UFMylation-related signaling pathways will reveal insights into basic cell biology and may provide new therapeutic opportunities for human health. However, this study

also has certain limitations, e.g. numerous tumor-related tumor suppressor proteins or oncoproteins may have UFMylation, which seriously affects the occurrence and development of various major human diseases; however, these proteins were not extensively described in this study. Therefore, such correlation studies should be further analyzed in the future.

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Authors' contributions

YT and CY designed and revised the article. RQ, JZ, YY, FM, XY and KZ wrote the first draft of this review. Data authentication is not applicable. All authors read and approved the final manuscript.

Ethics approval and consent to participate

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Competing interests

The authors declare that they have no competing interests.

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