Regulation of EZH2 protein stability: new mechanisms, roles in tumorigenesis, and roads to the clinic

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Summary

The importance of EZH2 as a key methyltransferase has been well documented theoretically. Practically, the first EZH2 inhibitor Tazemetostat (EPZ6438), was approved by FDA in 2020 and is used in clinic. However, for most solid tumors it is not as effective as desired and the scope of clinical indications is limited, suggesting that targeting its enzymatic activity may not be sufficient. Recent technologies focusing on the degradation of EZH2 protein have drawn attention due to their potential robust effects. This review focuses on the molecular mechanisms that regulate EZH2 protein stability via post-translational modifications (PTMs), mainly including ubiquitination, phosphorylation, and acetylation. In addition, we discuss recent advancements of multiple proteolysis targeting chimeras (PROTACs) strategies and the latest degraders that can downregulate EZH2 protein. We aim to highlight future directions to expand the application of novel EZH2 inhibitors by targeting both EZH2 enzymatic activity and protein stability.

Keywords: EZH2; Post-translational modification; Protein stability; Targeted cancer therapy

Introduction

EZH2 is the catalytic subunit of the epigenetic regulator polycomb repressive complex 2 (PRC2) and acts primarily as a gene transcription silencer by trimethylating lysine 27 of histone H3 (H3K27me3).[1] Numerous studies have shown that EZH2 influences a wide range of biological processes ever since its first report in 1996,[2] including regulation of the cell cycle, proliferation, differentiation, apoptosis, migration, senescence, DNA damage repair, immunity, and metabolic homeostasis.[3,4] Thus, it is not surprising that EZH2 dysregulation is present in multiple human diseases, including cancer. Activating mutations of EZH2 and abnormal expression have been shown to enhance tumor progression.[5]

Targeted therapy has shown promise in the treatment of cancer.[6] Based on the reported oncogenic role of EZH2, several EZH2 inhibitors (EZH2i) developed in recent years are currently used clinically or in clinical trials including Tazemetostat, GSK126, CPI-1205, SHR2554, and PF-06821497.[7] The FDA has approved Tazemetostat in 2020 for the treatment of epithelioid sarcoma (ES) and relapsed or refractory (R/R) follicular lymphoma (FL).[8] However, the EZH2 inhibitors have not been as effective as desired, especially in solid tumors.[5] One of the reasons for this, may be the fact that existing EZH2i only decrease EZH2 histone methyltransferase activity without affecting EZH2 noncanonical functions. The latter include transcriptional activation in a PRC2 independent mechanism and EZH2 mediated methylation of non-histone proteins during oncogenesis.[9] The discovery and investigation of EZH2 non-canonical functions in cancer highlight that targeting EZH2 expression may be a promising strategy to halt tumor progression.

Protein homeostasis is regulated through protein synthesis, folding and unfolding, protein degradation (e.g., ubiquitin-proteasome system, autophagic lysosomal pathway) and is essential for normal physiological processes.[10] Notably, PTMs play a crucial role in the regulation of protein stability. Among the numerous PTMs, ubiquitination is an essential mechanism of protein degradation. Thus, it is not surprising that abnormalities of the ubiquitin-proteasome system (UPS) can lead to a wide range of diseases, including cancer.[11] To date, studies have demonstrated that E3 ligases and deubiquitinases can mediate EZH2 protein degradation through UPS, paving the way to novel strategies to target EZH2 functions.[12] PROTAC, a novel technology for degrading a broad range of proteins, has been recently developed and garnered increased attention.[13] A

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PROTAC molecule comprises three parts: a linker, a protein of interest (POI), and an E3 ubiquitin ligase ligand, which together form a ternary complex capable of multiubiquitination and degradation of the protein target of interest. The use of PROTACs to degrade EZH2 may provide a practical and feasible approach and compensate for the limitations of targeting the enzymatic activity of EZH2 alone in cancer therapy.

EZH2 functions in cancer

There is ample evidence implicating EZH2 in the development and progression of various cancers, including breast cancer, colorectal cancer, lymphoma, ovarian cancer, liver cancer, etc.[5,14] The best studied function of EZH2 in normal tissue homeostasis and in tumorigenesis is its role as a histone methyltransferase catalyzing H3K27 methylation with resultant transcriptional silencing of specific target genes.[15] In addition to histones, EZH2 is involved in the methylation of non-histone proteins through a PRC2-dependent mechanism. One example is observed in the methylation of the cardiac transcription factor GATA4 at Lys299. This modification results in the reduction of p300-mediated GATA4 acetylation, consequently leading to the repression of GATA4 transcription.[16] In recent years, non-canonical PRC2 and H3K27me3-independent EZH2 functions have been reported in cancer.[9,17] For instance, invasive cancer differs in the expression levels of various PRC2 subunits, such as EZH2 overexpression relative to other subunits. It was reported that EZH2 interacts with cMyc and p300 through its trans-activation domain in MLL-r leukemia, activating genes associated to AML including cMyc. Upregulation of cMyc in cancer may amplify this difference, enhancing EZH2's non-classical function via the reciprocal loop.[18] In a prostate cancer investigation, it was shown that EZH2 activates its gene transcription by directly occupying the promoter of the androgen receptor (AR), a procedure that is independent of PRC2 and its methyltransferase activity. Enzymatic EZH2 inhibitors when used in combination with AR antagonists application can block the dual roles of EZH2 and suppressing prostate cancer progression in vitro and in vivo.[19] According to another research on prostate cancer, EZH2 has a dual influence on the expression of the mitotic regulator CDCA8 in PCa. In addition to unlocking the transcriptional suppression towards to CDCA8 mediated by let-7b via typical H3K27me3 tagging, EZH2 can also promote CDCA8 transcription driven by E2F1 via increasing E2F1 self-activation. This finding indicates that methylation-dependent and -independent roles of EZH2 synergize in CDCA8 activation in prostate cancer.[20] Besides the trans-activation effect mentioned above, EZH2 can also play a role in translational and post-translational procedures towards the targets. For instance, in 2021, researchers discovered that EZH2 can serve a favorable regulatory function in the translation process. In contrast to its usual action as a transcriptional suppressor, EZH2 may directly methylate rRNA 2′-O by binding to fibrillarin. This refreshes people's understanding of EZH2's function, which ranges from canonical transcriptional inhibition to positively translational initiation.[21] EZH2 can also maintain damage-specific DNA binding protein 2 (DDB2) stability by weakening its ubiquitination in order to facilitate nucleotide excision repair in cancer, a mechanism that is independent of PRC2 activity. This offers new insight into the treatment of cisplatin-resistant non-small cell lung cancer.[22] Thus, it is becoming clear that EZH2 activities in cancer may involve: (i) PRC2-dependent H3K27 methylation, (ii) PRC2-dependent non-histone protein methylation, and (iii) PRC2-independent gene transactivation.[3] The recent discovery of PRC2 and/or H3K27me3 independent EZH2 functions in tumorigenesis underscore the need for novel therapeutic strategies that target EZH2 expression.

Regulation of EZH2 protein stability

Ubiquitin proteasome system

The ubiquitin-proteasome system is the primary pathway of intracellular protein degradation, and the degradation of EZH2 is no exception. Ubiquitin-mediated degradation entails covalently attaching ubiquitin to the target substrate followed by recognition by the 26S proteasome and degradation of the ubiquitinated protein[11]

Ubiquitin (Ub), a 76-residue protein that contains seven lysine residues (K6, K11, K27, K29, K33, K48, and K63), is highly conserved in most eukaryotes. The different lysine residues can act as acceptor sites forming ubiquitin chains and then performing specific functions, including protein degradation.[23] Ub polymeric connects to its target substrate (ubiquitination) via a cascade action of three enzymes: E1 ubiquitin activating enzyme, E2 ubiquitin conjugating enzyme, and E3 ubiquitin ligase. During ubiquitination, these three enzymes act in a cascade to attach Ub to its target substrate. In this process, ubiquitin is ligated to E1 ubiquitin activating enzyme for activation; the carboxyl group at the glycine end of ubiquitin is attached to the sulfhydryl group of ubiquitin activating enzyme E1. This step requires ATP as energy and culminates in the formation of a thioester bond between ubiquitin and ubiquitin activating enzyme E1. Next, ubiquitin is bound to E2 ubiquitin conjugating enzyme; E1 hands over the activated ubiquitin to ubiquitin-binding enzyme E2 via a cross-esterification process. Finally, ubiquitin is attached to the target protein via E3 ubiquitin ligase; ubiquitin ligase E3 attaches the ubiquitin bound to E2 to the target protein, and when ubiquitin is already present on the protein, the ubiquitin bound to E2 can be attached directly to it without passing through

E3. In the end, the labeled protein is proteolytically broken down into smaller peptides, amino acids, and reusable ubiquitin.[23] Deubiquitinases (DUB) catalyze the removal of Ub from the substrate protein. DUBs can mediate the recycling and conversion of ubiquitin and rearrange the ubiquitin-linked protein, therefore maintaining the stability of the target protein.[24]

As a consequence, ubiquitin modifications tightly regulate protein stability, subcellular localization, protein interactions, degradation, and enzymatic activity, which in turn affect biological processes, including autophagy, apoptosis, metabolism, DNA repair, signal transduction, and other important cellular processes.[25,26]

E3 ubiquitin ligases

E3 ligases can be roughly classified into four types based on the different structures and functions, which are RING finger type, HECT type, U-box type, and RBR type.[27] Several E3 ubiquitin ligases participating in the degradation of EZH2 through UPS have been identified, which we summarize below.

Praja1-the first E3 identified for EZH2 polyubiquitination. In 2011, Zoabi, M et al. first reported that E3 ligase Praja Ring Finger Ubiquitin Ligase 1 (Praja1), a RING finger type E3 ubiquitin ligase, directly ubiquitinated EZH2 and led to its proteasomal degradation in MCF7 breast cancer cells.[28] Subsequently, another study found that Praja1 could promote EZH2 degradation via K48-linked protein polyubiquitination.[29] The study also illustrated that FOXP3 remarkably attenuated EZH2 protein level through the ubiquitin-dependent proteasome degradation pathway by directly promoting the mRNA transcription of E3 ligase Praja1, which suppressed breast cancer progression.[29] Intriguingly, in the above studies, Praja1 knockdown partly reversed EZH2 protein levels, highlighting that there may be other E3 ligases involved in EZH2 ubiquitination.

Smurf2. After the first E3 ubiquitin ligase for EZH2 was reported, other E3 ligases targeting EZH2 were reported. Smad ubiquitin regulatory factor 2 (Smurf2), a HECT type E3 ligase, is a common ubiquitinase that regulates protein homeostasis.[30] Yu et al. discovered in 2013 that Smurf2 acted as an E3 ubiquitin ligase to enhance the degradation of EZH2 via the polyubiquitination proteasome system during the process of neuron differentiation, and they also pointed out that K421 of EZH2 was the key amino acid for Smurf2-mediated EZH2 degradation.[31] In the years that followed, several other studies have also presented evidence that Smurf2 can regulate the stability of EZH2 in cancer.[32,33] A recent study unveiled that Smurf2 acts as a tumor suppressor to prevent human dermal fibroblasts malignancy by regulating the expression of RNF20 and EZH2.[33] Moreover, Kun Liao and his colleagues' work revealed that oncogenic genotoxins suppressed gluconeogenesis and tumorigenesis in hepatic and renal tumors by promoting EZH2 accumulation. At the molecular level, genotoxin treatments augmented EZH2 acetylation at K348, which interfered with SMURF2-mediated EZH2 K421 ubiquitination and increased the stability and expression of EZH2.[32]

c-Cbl. Aside from Praja1 and Smurf2, Casitas B lineage lymphoma (c-Cbl), a RING finger type E3 ligase, has been found to be responsible for EZH2 degradation.[34] Chang et al. discovered that YC-1 led to Tyr 731 and Tyr774 phosphorylation of c-Cbl followed by ERK activation, and the formation of a c-Cbl-ERK-EZH2 complex, resulting in EZH2 ubiquitination and proteasome degradation in breast cancer.[34]

β-TrCP. The SCF complex, which consists of three regular subunits, RBX2, CUL1, and SKP1, is a multiprotein RING E3 ubiquitin ligase whose substrate specificity is determined by the F-box proteins.[35] After being recruited by the F-box protein, its substrate becomes polyubiquitinated and then degraded by the 26S proteasome as a result.[35] Regarding the association between SCF ubiquitin E3 ligase and EZH2, recent studies reported that β-transducin repeat-containing protein (β-TrCP), one of the well-studied F-box proteins, constituted an active SCF β-TrCP E3 ligase, and had a critical role in interfering EZH2 stability in lymphomas.[36,37] Mechanistically, EZH2 was a newly discovered catalytic substrate of SCF β-TrCP E3 ubiquitin ligase, which could interact with β-TrCP and be ubiquitinated, and JAK2-mediated EZH2 Y641 phosphorylation promoted its degradation mediated by β-TrCP.[36]

Fbw7. Similar to the β-TrCP, the F-box protein Fbw7 (F-box and WD repeat domain-containing 7, also known as Fbxw7), as one of the substrate recognition subunits of SCF-type E3 ubiquitin ligase, can also mediate EZH2 ubiquitination.[38] Jin et al. discovered that FBW7 served as a novel E3 ligase of EZH2, which mediated its degradation and decreased its activity in pancreatic cancer cells, and inhibited tumor metastasis.[38] By blocking the activity of FBW7, EZH2 aided in the stability of MYCN in neuroblastoma and small cell lung carcinoma cells.[39] The discovery of Fbw7 as an EZH2 regulator has deepened our understanding of the role of SCF-type E3 ligases in EZH2 stability.

TRIM21. The tripartite motif (TRIM) family, the majority of which members exert E3 ubiquitin ligase activities, regulates multiple biological processes.[40] A study found that the E3 ubiquitin ligase TRIM21, a RING type E3 ligase, induced EZH2 ubiquitination and the activity of E3 ligase was augmented by phosphorylation of

EZH2 at threonine 487 in drug resistant acute myeloid leukemia cells.[41]

TRAF6. TNF Receptor Associated Factor 6 (TRAF6) is a cytoplasmic adaptor protein, which was originally identified as a signal transduction molecule.[42] Recently, the discovery of TRAF6 as an EZH2 stability regulator was confirmed by different studies.[43–47] In these studies, TRAF6 was first reported as the direct E3 ligase of EZH2 in 2017. In this study, Lu et al. confirmed that TRAF6 can regulate EZH2 protein stability through K63-linked polyubiquitination in prostate cancer.[43] Similar effect has also been described in breast cancer.[44] Additionally, DNMT1 and SASH1 have been discovered as positive regulators of EZH2 by diminishing TRAF6 transcription and it-mediated ubiquitination of EZH2 in prostate cancer and hemangioma progression, respectively.[45,46]

UBR4. Ubiquitin Protein Ligase E3 Component N-Recognin 4 (UBR4) is a RING-type E3 ubiquitin ligase that identifies the proteins with specific N-terminal residues, and subsequently binds to them and resulting in their ubiquitination and degradation.[48] Researchers found that UBR4, as the downstream of TGF-β, polyubiquitinated the N-terminal domain of EZH2 through K63-linked ubiquitin for subsequent proteasomal degradation in cholangiocytes to promote biliary fibrosis.[49] A recent study also confirmed that E3 ubiquitin ligase UBR4 bound to the N-terminal domain of EZH2 to boost its ubiquitination, whereas this process was inhibited by competitive binding of HAT1 to EZH2, thus stabilizing EZH2 protein to promote HAT1-mediated gemcitabine resistance in pancreatic cancer.[50]

CHIP, VHL and CRBN. E3 ubiquitin ligase carboxyl terminus of HSP70-interacting protein (CHIP) is the chaperone of heat shock protein 70 and 90, which connect HSP70/90 with proteasome systems.[51] Chen's group designed an EZH2 inhibitor in 2017, using gambogenic acid (GNA) as the skeleton connecting EZH2 and E3 ligase CHIP. In which GNA could covalently join the EZH2 SET domain in a covalent way and significantly suppress tumor development by degrading EZH2.[52]

E3 ligases Von Hippel-Lindau (VHL) and cereblon (CRBN) proteins are the two most common ubiquitin enzymes in biology. They are also the two most commonly used E3 ligases to recruit ubiquitin molecules and thus disrupt target proteins by PROTACs. To date, all the reported EZH2 PROTACs are designed by these two ubiquitin enzymes.[53]

The key findings from the data reviewed above are summarized in Table 1. From these studies, it is clear that diverse E3 ligases participate in the ubiquitination of the EZH2 protein, which is key to the regulation of EZH2 stability. Together, these studies suggest that the ubiquitin system might be a promising approach to the development of strategies to treat cancers.

Deubiquitination

Ubiquitination is a reversible process due to the presence of deubiquitinating enzymes that can cleave ubiquitin from modified proteins. It has been reported that several DUBs mediate the deubiquitination of EZH2, which are discussed in more detail below.

USP family. Research on EZH2-related DUBs has been mostly focused on the ubiquitin-specific proteases (USP) family, the biggest DUB subfamily with over 50 members discovered to date. The USP family members belong to cysteine protease DUBs, which have a classic USP domain involved in the interaction with ubiquitin.[66]

USP1. USP1 is a DUB containing an N-terminal Cys box and a C-terminal His box motif with catalytic residues (Cys90, His593, and Asp751). Aberrant overexpression of USP1 is linked to the incidence and recurrence of numerous cancers including cervical cancer, gastric cancer, and sarcoma.[67] In 2019, a study demonstrated that USP1 can be activated by β-Catenin/TCF4, and then USP1 directly interacts with EZH2 to deubiquitinate it. The stabilization of EZH2 mediated by USP1 represses the expression of downstream tumor suppressor genes to drive glioma tumorigenesis.[55]

USP7. USP7 is one of the best characterized DUBs, which has been associated with the progression of various tumors.[68] In 2020, studies from two independent laboratories confirmed the function of USP7 to maintain the stability of EZH2 via deubiquitination in prostate cancer.[56,69] USP7 may increase the quantity of EZH2 protein, which then controls TIMP2 expression and activates the NF-κB pathway, influencing the onset and progression of cervical cancer.[57] Gagarina and colleagues discovered that USP7 catalyzed the deubiquitination of EZH2 through combining the ^{489}PRKKKRK^{495} region of EZH2 with its C-terminal domain, thus regulating the stability and function of EZH2 in HCT116 carcinoma cells.[70] USP7 was also reported to sustain high expression of EZH2 through its deubiquitination activity, thus promoting cell proliferation and tumorigenesis in melanoma.[58] A recent study demonstrated that USP7 recruited by nuclear p85β stabilized EZH1/2 and enhanced H3K27me3 n, thus leading to the promotion of the PIK3CA helical domain mutant tumor progression.[71]

USP21. USP21, with a C-terminal USP domain, functions as a DUB to deubiquitinate numerous proteins.[59] Two studies showed that USP21 could act as an EZH2 DUB to deubiquitinate and stabilize EZH2

	Catalyzing enzyme	Site	Biological function	Cancer	Refs
E3 ligases	Praja1	N/A	Inhibit metastasis	Breast cancer	28,29
	Smurf2	K421	Inhibit tumorigenesis	Hepatic cancer Renal cancer	32
	c-Cbl	N/A	Inhibit tumorigenesis	Breast cancer	34
	β-TrCP	N/A	Inhibit tumorigenesis	Lymphoma	36,37
	Fbw7	N/A	Inhibit migration and invasion	Pancreatic cancer	38
	TRIM21	N/A	Induce drug resistance	Acute myeloid leukemia	41
	TRAF6	N/A	Inhibit tumorigenesis and metastasis	Breast cancer Prostate cancer	43,44,46
	UBR4	N-terminal domain	Suppress gemcitabine resistance	Pancreatic cancer	50
	VHL	N/A	Inhibit tumorigenesis	Breast cancer	53
	CHIP	SET domain (Cys668)	Inhibit tumorigenesis	Head and neck cancer	52
	CRBN	N/A	Inhibit tumorigenesis	Lymphoma	54
DUBs	USP1	N/A	Promote tumorigenesis	Glioma	55
	USP7	^{489}PRKKKRK^{495} region	Promote tumorigenesis, proliferation and metastasis	Prostate cancer Cervical cancer Hepatic cancer Melanoma	56-58
	USP21	N/A	Promote proliferation and metastasis	Bladder cancer Lymphoma	59,60
	UPS36	K222	Promote cell proliferation	NKTL	61
	USP44	N/A	Promote tumorigenesis and progression	Prostate cancer	62
	ZRANBI	N/A	Promote tumorigenesis and metastasis	Breast cancer Colorectal cancer	63,64
	UCHL1	SET domain	Promote tumorigenesis	Neuroblastoma	65

Table 1: **The summary of E3 ligases and DUBs in the regulation of EZH2 stability.**

protein in bladder carcinoma and diffuse large B-cell lymphoma. It was shown that the deubiquitinating activity contributed to maintaining EZH2 protein level leading to tumor proliferation and metastasis.[59,60]

USP36. The DUB USP36 has been implicated in a range of disease processes, including cancer.[72] Li et al. reported that USP36 served as a DUB to mediate EZH2 deubiquitination at K222 site in natural killer/T-cell lymphoma, leading to decreased sensitivity to bortezomib.[61]

USP44. USP44, as a novel EZH2 DUB, has a zinc-finger domain and conserved amino acid residues such as cysteine and histidine. USP44 can promote prostate cancer progression by stabilizing EZH2, including mutants in particular, considering that oncogenic mutations of EZH2 are repeatedly found in lymphomas.[62]

ZRANB1. The zinc finger RANBP2-type containing 1 (ZRANB1, also known as Trabid), a DUB associated with tumors, has also been reported to deubiquitinate and stabilize EZH2 in breast cancer.[63] The C-terminal OTU domain of ZRANB1 directly interacts with both the N-terminal region and the CXC domain of EZH2.[63] Another study confirmed these findings, showing that ZRANB1 bound to EZH2 resulting in its deubiquitination in colorectal cancer tumor cells.[64] Both of these studies suggested that ZRANB1 could promote tumor progression by stabilizing EZH2 and was a potential therapeutic target.

UCHL1. Ubiquitin carboxy-terminal hydrolase L1 (UCHL1) was identified as PGP9.5 protein in 1987.[73] Different from other DUBs, UCHL1 has hydrolase and ligase activities. Notably, instead of removing ubiquitin from proteins, UCHL1 functions as ligase and hydrolase in neurodegenerative diseases and breast cancer.[73] In NB cells, UCHL1 bound the SET domain of EZH2. Further research found that long non-coding RNA MEG3 could suppress EZH2 through UCHL1 inhibition.[65]

Taken together, these reports illustrate that several DUBs can promote cancer progression by stabilizing EZH2 through deubiquitination, providing viable therapeutic targets. DUB inhibitors, such as U7D-1, the first selective USP7-degrading Proteolysis Targeting Chimera (PROTAC) targeting MDM2 that promotes the proteasomal degradation of p53, has showed selective and effective USP7 degradation, and maintained potent cell growth inhibition in p53 mutant cancer cells.[24,74]

In summary, ubiquitination and deubiquitination system has been shown to regulate EZH2 protein levels

in multiple tissues and diseases, including in cancer. Similarly, the intracellular protein level of EZH2 is closely related to the functions of various E3 and DUBs enzymes. Further study and understanding of these EZH2-related E3 and DUB could help us better target EZH2 for anti-tumor therapy.

Phosphorylation

Phosphorylation is a well-studied PTM that plays an important part in cellular regulatory networks and governs important cellular activities. EZH2 can be phosphorylated at multiple distinct amino acid residue sites, such as serine (S) and threonine (T), which can then regulate tumor progression.[75,76] EZH2 phosphorylation at different sites performs distinct functions. So far, most tumor-related investigations on EZH2 phosphorylation have revealed distinct kinase-mediated phosphorylation of EZH2 modulates its methyltransferase activity, influencing its interaction with other PRC2 components and activating various signal transduction pathways.[76,77] Several studies have found that EZH2 stability can be influenced by its phosphorylation. The most studied protein kinases phosphorylating EZH2 are Cyclin-dependent kinases (CDKs), which are serine/threonine kinases that require a separate cyclin subunit for their enzymatic activity. In 2011, Wu et al. provided the first evidence that EZH2 protein stability can be linked to EZH2 phosphorylation.[78] The study showed that CDK1-mediated phosphorylation of EZH2 at threonine 345 and 487 promoted EZH2 ubiquitination and subsequent proteasome pathway degradation, which was important to regulate cell proliferation in human cervical cancer and prostate cancer.[78] Subsequently, different research groups found that CDK1 could mediate the phosphorylation of EZH2 at the Thr345 and Thr487 sites and influence its stability.[79,80] It is important and interesting to note that this phosphorylation sites resulted in different functions in different cancer types. For example, phosphorylation of EZH2 at Thr487 induced drug resistance in AML cells,[41] phosphorylation of EZH2 at Thr345 and Thr487 attenuated tumor progression in breast cancer[80] and inhibited tumor metastasis in hepatocellular carcinoma.[79] Further, CDK1-mediated EZH2 phosphorylation at four threonine sites, including T350, T372, T419, and T492 is critical for the association of EZH2 and Smurf2 for EZH2 degradation.[81] Then, a report focused on CDK-mediated EZH2 phosphorylation demonstrated that activated CDK5 phosphorylated EZH2 at Thr261 and induced its degradation through FBW7-mediated ubiquitination, which can inhibit pancreatic cancer cell migration and invasion.[38]

Other protein kinases, in addition to CDKs, have been found to phosphorylate EZH2 and influence EZH2 protein stability. For example, JAK2-mediated phosphorylation of EZH2 Y641 promoted the interaction between EZH2 and E3 ligase β-TrCP, resulting in EZH2 proteolysis.[36] In addition, MELK kinase phosphorylated EZH2 at the S220 site and resulted in loss of EZH2 K222 ubiquitination to escape proteasomal degradation in NKTL.[61] Mechanistically, the formation of NEK2 and EZH2 complex phosphorylated and reduced EZH2 degradation, thus strengthening EZH2 protein stability to promote GBM tumorigenesis. A recent study demonstrated that the phosphorylation of EZH2 at Thr367 catalyzed by DCAF1 can enhance EZH2 protein stability in colon cancer and facilitate tumor progression.[82]

EZH2 has been recently shown to undergo phosphorylation at Thr367 by p38 MAPK in triple negative breast cancer.[77] This specific phosphorylation resulted in cytoplasmic accumulation of EZH2 to promote cell migration and metastasis. Preliminary experiments did not reveal an effect of p38-mediated phosphorylation at Thr367 on EZH2 protein stability, although further investigations to test this are warranted.

Collectively, these studies demonstrate that phosphorylation of EZH2 at different amino acid sites by specific kinases is an important post-translational event that can regulate its stability and protein level in numerous cancer types.

Acetylation

Protein acetylation is an important and reversible PTM that is also engaged in a number of cellular processes catalyzed by histone acetyltransferases (HATs) and histone deacetylases (HDACs).[83] In 2011, Lu et al. found that inhibiting SIRT1, an NAD-dependent protein deacetylase, increased EZH2 protein stability.[84] Furthermore, P300/CBP-associated factor (PCAF) interacted with and acetylated EZH2 at lysine 348 (K348), which can be relieved by the deacetylase SIRT1.[85] Furthermore, the study found that EZH2-K348 acetylation reduced its Thr345 and 487 phosphorylation and increased its stability, therefore facilitating lung cancer cell metastasis. Further investigations on the potential role of acetylation on EZH2 protein stability are necessary.

Methylation

Protein methylation is a PTM process in which methyltransferases catalyze the transfer of methyl groups from S-adenosyl-L-methionine (SAM) to target protein residues. EZH2 methylation can also affect its stability and function. A study showed that the SET and MYND domain containing 2 (SMYD2) acted as a methyltransferase to directly methylate the lysine residue at position 307 of EZH2 and increase EZH2 protein stability by protecting EZH2 from being degraded.[86] EZH2 can be methylated and demethylated by the SMYD2 and histone H3K4 demethylase lysine-specific demethylase 1 (LSD1), respectively. Functionally, SMYD2 coordinated EZH2 to participate in the recruitment of EZH2 to chromatin and the epigenetic transcriptional

repression, which promoted breast cancer tumorigenesis and metastasis.[86] The research mentioned above also supported the idea that asymmetrical dimethylation at arginine 342 of EZH2 (meR342-EZH2) mediated by Protein Arginine Methyltransferase 1 (PRMT1) can increase EZH2 stability through reducing TRAF6-mediated EZH2 ubiquitination.[44] Then, researchers further reported that tumor-associated macrophages stimulate PRMT1-related meR342-EZH2 formation in order to increase its stability and enhance breast cancer cell metastasis by secreting interleukin-6 (IL-6) cytokine.[87] In addition, Yuan et al. demonstrated that SET Domain-Containing Protein 2 (SETD2) directly methylated EZH2 at K735 and promoted its degradation, which impeded prostate cancer metastasis.[88] The study also confirmed that SETD2-mediated EZH2-K735me1 promoted its degradation in a Smurf2-dependent manner and thus inhibited tumor progression by down-regulating EZH2.

O-GlcNAcylation

Unlike other PTMs regulated by multiple enzymes, O-GlcNAcylation is mediated by O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA), which recognize hundreds of protein substrates.[89] In 2014, Chu et al. found that OGT-mediated EZH2 O-GlcNAcylation at serine 75 was important for the protein stability and the OGT-EZH2 regulatory axis promoted tumor malignancy in breast cancer.[90] Besides, the team further investigated EZH2 glycosylation using a combination of fluorescence-based sugar labeling and mass spectrometry methods and identified four additional O-GlcNAcylation sites of EZH2, which are S73, S84, S87, T313 and S729.[91] The O-GlcNAcylation of EZH2 at residues mentioned above contributed to EZH2 protein stability without affecting its combination of PRC2 complex subunits. However, O-GlcNAcylation at the S729 site, which is located at the SET domain, the methyltransferase domain of EZH2, interfered with its catalytic activity.[91] These results showed that OGT-mediated EZH2-GlcNAcylation played diverse roles in breast cancer progression. Recently, a study demonstrated that OGT-mediated EZH2 O-GlcNAcylation stabilized the protein and facilitated the development of liver cancer.[92]

PARylation

Additionally, recent research has also demonstrated that EZH2 PARylation mediated by PARP1 interfered with PRC2 complex formation and enhanced EZH2 degradation.[93] PARylation induces EZH2 degradation through UPS pathway, but the specific E3 ligase has yet to be determined. Collectively, increasing evidence indicates that PTM modifications of EZH2 are important in the regulation of its stability. Different PTMs, which usually do not exist alone, can be combined to occur successively or simultaneously, thereby enhancing the biological activities of proteins and greatly expanding their function in signaling pathways.

Proteins can be simultaneously modified by various types of PTMs (Fig. 1), therefore, crosstalk between different PTMs is widespread and exhibits synergistic effects. Different PTMs can regulate the enzymatic activity and/or protein stability of EZH2 in both a PRC2-dependent and independent manner (Table 2). Currently, due to the diverse distribution and activity of enzymes responsible for PTM modifications, different tumor types exhibit distinct PTM landscape, suggesting a lack of unified regulation pattern. Therefore, one of the future directions is to characterize the PTM profile across different tumor lineages and explore the targets of specific PTMs. This exploration can facilitate to achieve dual regulation of EZH2 both enzymatic activity and protein stability directly or indirectly.

Therapeutic strategies towards targeting EZH2 for cancer therapy

Strategies targeting EZH2 enzymatic activity for cancer therapy

Inhibitors of EZH2 methyltransferase activity have received extensive attention in recent years. 3-deazaadenosine A (DZNep), the first commercial EZH2 inhibitor, inhibits EZH2 by interfering with Sadenosyl-L-homocysteine hydrolase (SAH).[7] Another class of inhibitors of EZH2 enzymatic activity are the highly selective and competitive small molecule inhibitors of S-adenosylmethionine, which include EZH2 inhibitors, including EPZ005678, GSK126 and Tazemetostat, and EZH1/2 dual inhibitors, such as UNC1999, DS-3201b and JQEZ5[5,7] (Fig. 2). Moreover, Tazemetostat was authorized by the FDA in 2020 to treat ES for adults and children 16 and older, as well as FL of adults.[8] A current Phase 1/2 clinical trial has discovered that CPI-0209, a second-generation, selective EZH2 inhibitor, has a longer residence duration than the first-generation EZH2 inhibitor and has more effective anti-tumor efficacy in preclinical cancer models.[94] So far, the drugs targeting EZH2 enzyme activity are basically applied in follicular lymphoma and diffuse large B cell lymphoma (DLBCL). Besides, increasing number of trials are investigating efficacy and safety of EZH2 inhibitors in targeting solid tumors (Supplementary Table S1). Among the ongoing clinical trials, it is worth noting that the EZH2 degrader AXT-1003 is currently in Phase I clinical trials for Relapsed or Refractory Non-Hodgkin's Lymphoma (NCT05965505) as a new type of trial. In addition, EZH2 inhibitors are generally prescribed in conjunction with immunotherapy, chemotherapy, or another treatment option for solid tumors to overcome the inefficacy of EZH2i alone.[7] Recently, new combination partners are under exploration. For instance, EZH2i promotes ferroptosis by increasing TfR-1

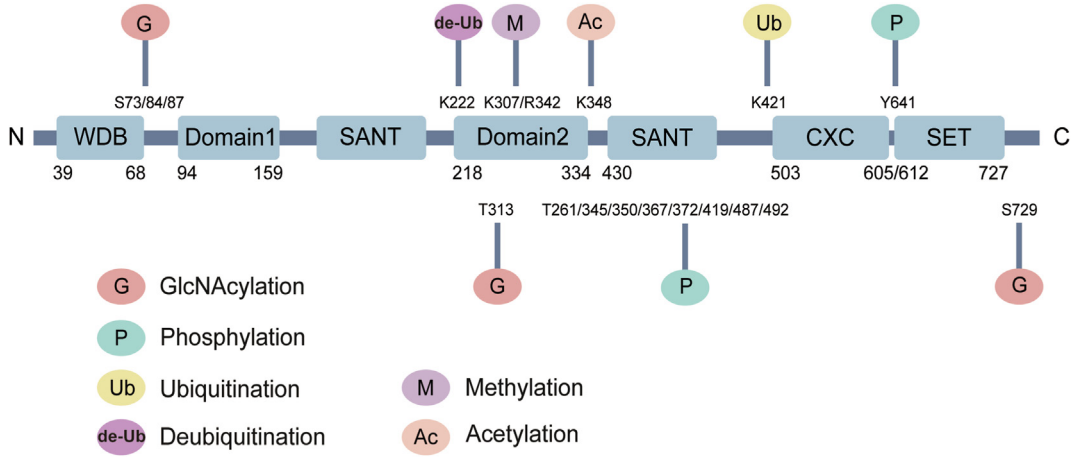


Fig. 1: Overview of PTMs related to EZH2 stability. The known modification sites of PTMs related to EZH2 stability are mapped, which are differentiated with different colors. (WDB, the WD-40 binding domain interacting with EED; Domain 1, interacting with PHF1; Domain 2, interacting with SUZ12; SANT, SWI3, ADA2, N-CoR and TFIIIB DNA binding domains; CXC, a cysteine-rich domain (CXC); SET, Su(var)3–9, Enhancer-of-zeste, Trithorax domain).

expression in cells and maintaining the iron death inhibitor glutathione peroxidase 4 (GPX4). According to the findings, co-treatment with the ferroptosis inducer erastin may effectively address the iron-dependent drug tolerance generated by EZH2i in DLBCL cells.[95]

Strategies targeting EZH2 stability for cancer therapy

Increasing evidence suggests that the oncogenic function of EZH2 is not entirely dependent on its enzymatic activity. Apart from catalyzing H3K27me3 and mediating the silencing of genes associated with various cellular processes, EZH2 also mediated the activation of genes in multiple cancers independent of the enzymatic function. Therefore, conventional small-molecule inhibitors directly targeting the catalytic functional domain of EZH2 are inadequate to fully obstruct its oncogenic activity, leading to limited clinical efficacy. Additionally, the enzymatic inhibitors operate through an "occupancy-driven" mechanism to block its catalytic function and generate therapeutic effects. However, this frequently demands higher concentrations to occupy the active sites, resulting in challenges associated with low selectivity, specificity, and efficacy. Tazemetostat, the first EZH2 inhibitor approved by FDA in 2020, has been utilized to treat advanced epithelioid sarcoma and follicular lymphoma, at a relatively high oral dosage of 800 mg twice daily. Specifically, the emergence of secondary mutations in EZH2 alleles cooperates in conferring resistance to EZH2 inhibitors. The emerging PROTAC technology offers the advantage of concurrently regulating both the enzymatic and non-enzymatic functions of EZH2 (Fig. 2). This presents a potential strategy to compensate for the limitations of inhibitors and to explore new therapeutic avenues through target degradation of EZH2. In recent years, a variety of EZH2 or PRC2 complex degraders have been discovered and developed with unprecedented efficacy against a multitude of tumor types. Remarkably, a recent study synthesized the EZH2 degrader Hyt-13 by linking norbornene to Tazemetostat, which exhibited a pronounced degradation effect on EZH2 and resulted in a greater than 12-fold increase in the anti-proliferative activity of MDA-MB-468 cells compared to Tazemetostat.[96] In contrast to traditional tumor targeted therapies, PROTACs attenuate systemic drug exposure, off-target effects and toxicity.[13] Currently, E3 ligases Von Hippel-Lindau (VHL) and Cereblon (CRBN) have been reported in EZH2-based PROTACs.

VHL-based PROTACs

VHL protein, a component of the E3 ligase Cul2-Rbx1-EloBC-VHL, has been widely utilized for the development of VHL-based PROTACs. Several VHL inhibitors, including VH032, VH101, and VH298, and their derivatives have been designed as suitable small-molecule VHL ligands with appropriate exit vectors for linker attachment.[97] In 2021, Wen's group designed and synthesized two series of EZH2 degraders based on VHL/CRBN ligands and EZH2 inhibitors EPZ6438 (Tazemetostat). By comparing the effects, E3 ligase VHL showed the superiority in degrading protein, besides VHL-based PROTAC EZH2 degraders YM181 and YM281 had higher antiproliferative activity than EZH2 inhibitor EPZ6438 in lymphoma cell lines.[98] The length and modification of PROTAC ligand led to protein degradation in an efficient way. In order to obtain higher degradation efficiency of EZH2, Jian Jin's lab developed an improved series of VHL-based EZH2

Type of PTMs	Catalyzing enzyme	Site	Related E3/DUBs	Biological functions	Cancer	Refs
Phosphorylation	CDK1	T345/T487	N/A	Cell proliferation and metastasis	Cervical cancer Prostate cancer Hepatic cancer	78–80
	CDK1	T350/T372/T419/T492	Smurf2	Erythropoiesis	Anemia	81
	CDK5	T261	FBW7	Cell migration and invasion	pancreatic cancer	38
	JAK2	Y641	β-TrCP	Lymphoma pathogenesis	Follicular lymphoma and aggressive diffuse large B-cell lymphoma	36
	MELK	S220	USP36	Chemo-sensitivity to bortezomib treatment	NKTL	61
	DCAF1	T367	N/A	Tumor progression	Colon cancer	82
	P38 (MAPK)	T367	N/A	Cell migration and metastasis	Triple negative breast cancer	92
Deacetylation	SIRT1	K348	N/A	Cell metastasis	Lung cancer	84
Methylation	SMYD2	K307	N/A	Tumorigenesis and metastasis	Breast cancer	86
	PRMT1	R342	TRAF6	Cell metastasis	Breast cancer	87
	SETD2	K735	Smurf2	Cell metastasis	Prostate cancer	88
O-GlcNAcylation	OGT	S75	N/A	Tumor malignancy	Breast cancer	89,90
PARylation	PARP1	N/A	N/A	PARPi sensitivity	Breast cancer	93

Table 2: **The summary of different PTMs involved in the regulation of EZH2 stability.**

PROTACs. In their study, they found that MS8815 with a longer linker could almost completely degrade EZH2 in time-dependent and concentration-dependent ways in triple negative breast cancer (TNBC) cell. In addition, MS8815 significantly inhibited cell proliferation with IC50 values ranging from 1.7 to 2.3 μM, demonstrating superior efficacy compared to EPZ-6438 and YM281, which exhibited relatively weaker inhibitory potency (IC50 > 10 μM and IC50 = 2.9–3.3 μM, respectively) in TNBC cell lines.[99]

CRBN-based PROTACs

CRBN, the substrate receptor of the $CRL4^{CRBN}$ E3 ligase, is one of the most typical E3 ligandin PROTACs. The design of CRBN ligands can be broadly divided into two main categories: chemical modifications of classical immunomodulatory drugs (IMiDs), such as thalidomide, lenalidomide, and pomalidomide, and non-thalidomide-like compounds.[100] In 2021, Luoting Yu's lab utilized 4-hydroxythalidomide, a CRBN ligand, to conjugate EZH2 inhibitors GSK126 and EPZ6438, thereby synthesizing a series of EZH2 PROTACs. Among them, compounds G12 (with GSK126 as the POI ligand) and E7 (with EPZ6438 as the POI ligand) demonstrated promising degradation effects on EZH2. In comparison, E7 exhibited superior degradation efficacy, which can degrade the PRC2 complex, including EZH2, EED, SUZ12 and RbAp48. Moreover, E7 fully blocked the oncogenic potential of EZH2 and demonstrated potent anti-proliferative effects in lymphoma cells reliant on both the catalytic and noncatalytic functions of EZH2.[54] Then, another research group also synthetized a CRBN ligand based PROTAC MS177, which was highly selective to degrade EZH2, PRC2 and cMyc. The anti-tumor effect of MS177 has been verified not only in tumor cell lines, but also in various tumor cell line xenografted and patient-derived xenografts.[18] In 2022, Xiaobo Wang used E3 ligase VHL and CRBN as the ligand to link GSK126 and EPZ6438 via different linkers. In their study, both CRBN-based U3i and G1c were found to markedly degrade the EZH2 protein and induce cancer cell apoptosis, with U3i exhibiting superior efficacy. Compared with the EZH2 inhibitor GSK126, the growth inhibition of TNBC cells increased 20- and 30-fold by the CRBN-based PROTAC U3i.[53]

EED-targeted PROTACs

Due to the fact that EED and EZH2 are both located within complex PRC2, some EED-targeted PROTACs, in addition to EZH2-PROTAC, might rapidly trigger EZH2 degradation. As the published reports, PROTACs targeting EED induce degradation of various constituents of the PRC2 complex, including EZH2, thereby offering novel therapeutic strategies for inhibiting EZH2 activity. In 2019, two research groups independently reported EED-targeted PROTAC. Both used the same triazolopyrimidine scaffold described by Novartis. Andrew Bloecher reported that two EED-targeted PROTACs, which combined the EED inhibitor MAK683 with a VHL ligand, effectively degraded EZH2 at a concentration of 1.0 μM within 24 h in Karpas422 cells. These PROTACs also demonstrated impressive inhibitory effects on the growth of Karpas422 cells, with IC50 values of 57 nM and 45 nM, respectively.[101] UNC6852, conjugating EED inhibitor EED226 with VHL ligand, is another EED-targeted PROTAC reported by Lindsey. It induced degradation of EED and EZH2 with DC50 values of 0.61 ± 0.18 and 0.67 ± 0.24 μM in DB cells respectively. The compound also exhibited potent inhibitory effects on cell proliferation, with IC50 values of 3.4 ± 0.77 μM in DB cells and 0.41 ± 0.066 μM in

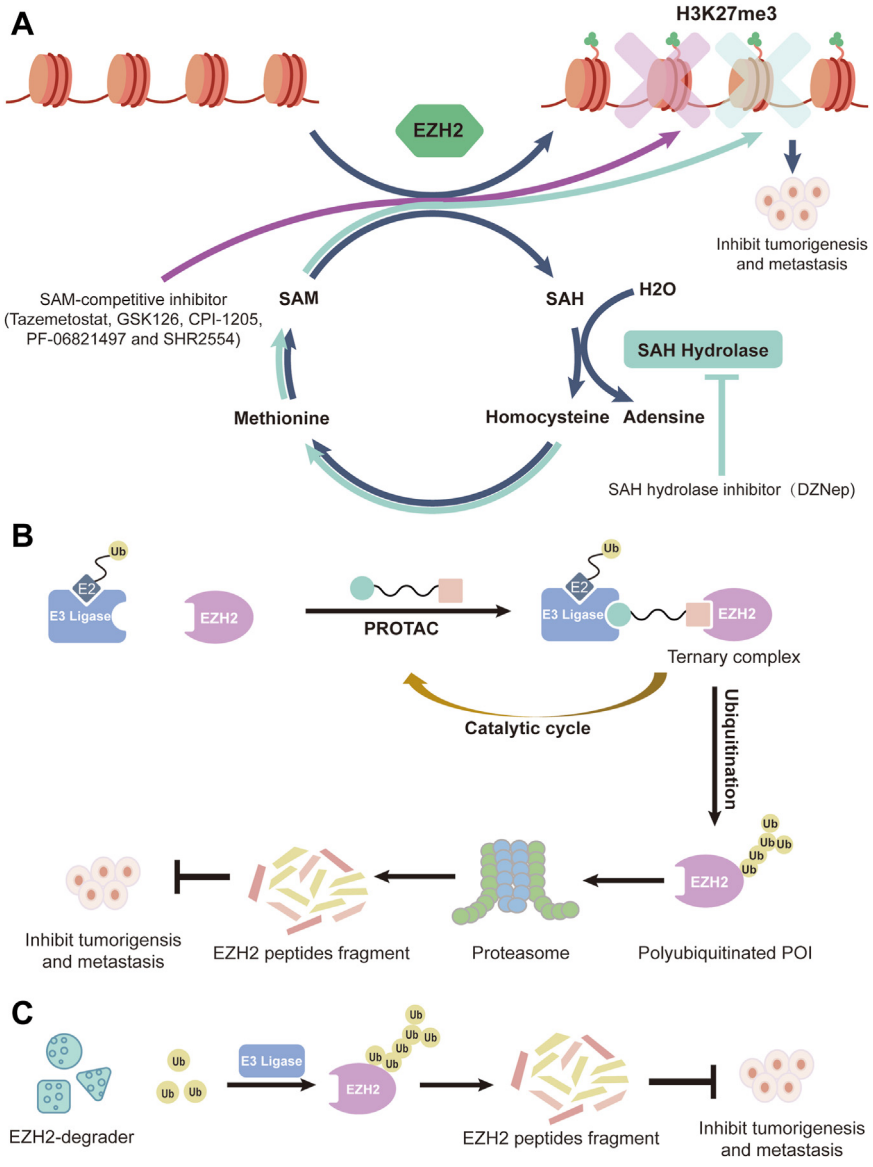
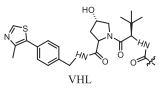
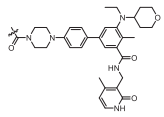
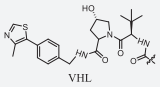
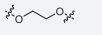
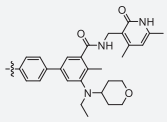
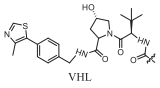
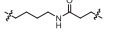
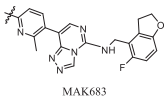
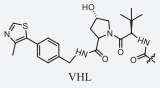
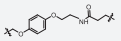
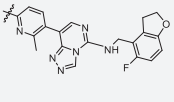
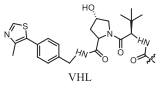
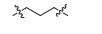
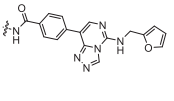
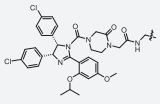
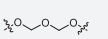
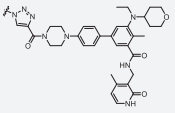


Fig. 2: Therapeutic strategies for targeting EZH2. a. Mechanism of EZH2 inhibitors targeting the methylation function. Current inhibitors targeting the methylation activity of EZH2 mainly fall into two categories (1) SAM competitive inhibitors, which partially occupy the site of the cofactor SAM in the EZH2 binding pocket, depriving EZH2 of the use of methyl groups, such as Tazemetostat, GSK126, CPI-1205, PF-06821497 and SHR2554 (2) SAH hydrolase inhibitors, which inhibit the hydrolysis of SAH and resulted in its accumulation, thereby blocking the production of methyl from the methionine cycle and indirectly inhibiting the enzymatic activity of EZH2. b. Mechanism of PROTAC-mediated EZH2 protein degradation. PROTAC comprises two small molecule ligands connected by a variable linker unit, one of which binds to an E3 ubiquitin ligase and the other to EZH2 protein. It induces the polyubiquitination and proteasome degradation of EZH2 in cells. c. Mechanism of EZH2 degrader-mediated protein degradation.

Pfeiffer cells.[102] Lindsey and colleagues reported the enhancement of UNC6852 and the development of a second-generation PRC2 degrader, UNC7700, with a cis-cyclobutane linker replacing the propyl linker in UNC6852. UNC7700 exhibited approximately 15-fold greater potency in degrading EED in DB cells compared to UNC6852. Moreover, in DLBCL cells, UNC7700 effectively degraded EZH2 ^{WT}/EZH2^{Y641N} with notable DC_{50} and D_{max} values (275 nM and 86%) in DB cells.[103]

In summary, PROTAC technology has shown promising potential for the targeted degradation of EZH2 (Table 3). It offers several advantages, such as, complementing the limitations of enzymatic inhibitors,

PROTAC	E3 ligand	Linker	POI ligand	POI	Degradation efficacy	Anti-tumor activity	Refs
MS8815	 VHL		 EPZ6438	EZH2	DC_{50}=140±56 nM (MDA-MB-453 cells)	EPZ6438: IC_{50} > 10 μM MS8815: IC_{50}=1.7 ± 0.07 μM (MDA-MB-468 cells) IC_{50}=2.0 ± 0.25 μM (BT549 cells) IC_{50}=2.0 ± 0.19 μM (SMM159 cells) IC_{50}=2.3 ± 0.30 μM (MDA-MB-453 cells)	98
YM281	 VHL		 EPZ6438	EZH2	80% degradation at 2 μM (22Rv1 and SU-DHL-2 cells)	EPZ6438: IC_{50} > 10 μM YM281: IC_{50}=3.2 ± 0.36 μM (MDA-MB-468 cells) IC_{50}=3.3 ± 0.04 μM (BT549 cells) IC_{50}=2.9 ± 0.23 μM (SMM159 cells) IC_{50}=2.9 ± 0.74 μM (MDA-MB-453 cells)	97, 98
EED-targeted PROTAC 1	 VHL		 MAK683	EED	Above 50% degradation of EZH2 at 1 μM (EZH2 mutant cells: Karpas422)	EED ligand: IC_{50}=14nM PROTAC 1: IC_{50}=57nM (EZH2 mutant cells: Karpas422)	101
EED-targeted PROTAC 2	 VHL		 MAK683	EED	Above 50% degradation of EZH2 at 1 μM (EZH2 mutant cells: Karpas422)	EED ligand: IC_{50}=14nM PROTAC 2: IC_{50}=45nM (EZH2 mutant cells: Karpas422)	101
UNC6852	 VHL		 EED226	EED	DC_{50}=0.3 ± 0.19 μM (Hela cells) EZH2 DC_{50}=0.67 ± 0.24 μM (DB $EZH2^{Y614N}$ cells)	IC_{50}=3.4 ± 0.77 μM (DB ($EZH2^{Y614N}$) cells) IC_{50}=0.41 ± 0.066 μM (Pfeiffer cells)	102
E-3P-MDM2	 MDM2		 EPZ6438	EZH2	Above 50% degradation of EZH2 efficacy at 1.11 μM (SU-DHL-6 ($EZH2^{Y614N}$) cells)	EPZ6438: IC_{50}=21.95±5.16 μM E-3P-MDM2: IC_{50}=3.39±0.69 μM (SU-DHL-6 ($EZH2^{Y614N}$) cells) EPZ6438: IC_{50} > 30 μM E-3P-MDM2: IC_{50}=17.48±0.50	104

(Table 3 continues on next page)

overcoming mutation-directed drug resistance and allowing for efficient protein degradation with a lower of DC_{50}. These advantages highlight the potential of EZH2 PROTACs for clinical application. However, there are also significant challenges to overcome for the successful development of PROTACs as clinically effective drugs. For example, due to their relatively large molecular weights, the ADME properties of PROTACs could differ from those of small-molecule drugs, which may impact their pharmacokinetics and distribution in the body. Additionally, the activity of a PROTAC is dependent on its associated E3 ligand, which may have variable expression across different cell types or tissues. With over 600 E3 ligases available, numerous options exist for designing PROTACs. Advancements in this area, coupled with enhanced knowledge regarding the functions and tissue-specific expression of these E3 ligases, will significantly expand the feasibility of PROTAC technology. In addition to VHL and CRBN ligands, the exploration of new E3 ligands for synthesizing EZH2 PROTACs is expected to be a significant research area in the future. For instance, a recent study successfully synthesized a PROTAC by combining the E3 ligase ligand MDM2 with EPZ6438, referred to as E-3P-MDM2. This novel conjugate demonstrated significantly enhanced antiproliferative activity compared to EPZ6438 alone, with IC50 values of 3.39 ± 0.69 μM in SU-DHL-6 ($EZH2^{Y614N}$) cells and 17.48 ± 0.50 μM in HBL-1 ($EZH2^{WT}$) cells, respectively.[104] Extensive research in the fields of medicinal chemistry and cell biology is required to optimize the linkage site, linker, and E3 ligase of PROTACs for their clinical application.

(Continued from previous page)

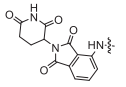
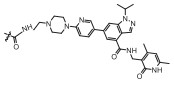
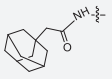
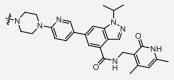
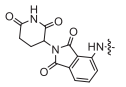
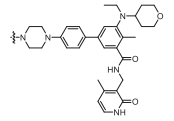
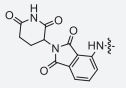
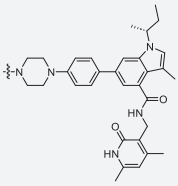
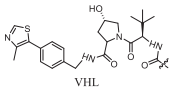
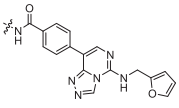
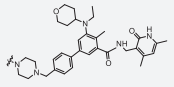
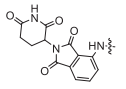
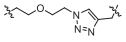
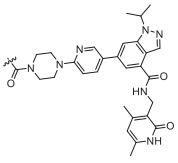
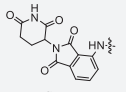
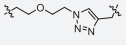
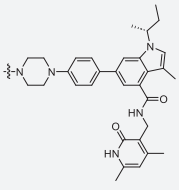
						μM (HBL-1 ($EZH2^{WT}$) cells)	
MS177	 CBRN		 C24	EZH2	DC_{50}=0.2± 0.1μM (EOL-1 cells) DC_{50}=1.5± 0.2μM (MV4;11 cells)	C24: IC_{50}=2 μM MS177: IC_{50}=0.01 μM (EOL-1 cells) C24: IC_{50}=7 μM MS177: IC_{50}=0.07 μM (MV4;11 cells) C24: IC_{50} > 100 μM MS177: IC_{50}=0.09 μM (MOML-13 cells) C24: IC_{50} > 100 μM MS177: IC_{50}=0.57 μM (THP-1 cells)	18
MS1943	 hydrophobic tagged		 C24	EZH2	Above 50% degradation of EZH2 at 5 μM (MDA-MB-468 cells)	C24: IC_{50} >8 μM MS1943: IC_{50}=2.2±0.5 μM (MDA-MB-468 cells)	105
E7	 CBRN	 n=4	 EPZ6438	EZH2	72% degradation of EZH2 at 1 μM (WSU-DLCL-2 cells)	IC_{50}=3.69-4.92 μM (WSU-DLCL-2 cells)	100
G12	 CBRN	 n=9	 GSK126	EZH2	Above 50% degradation of EZH2 at 2.5 μM (WSU-DLCL-2 cells)	N/A	100
UNC7700	 VHL		 EED226	EED	EED DC_{50} = 111 nM $EZH2^{WT}$/$EZH2^{Y641N}$ DC_{50} = 275 nM （DB cells）	IC_{50}=0.79±0.53 μM （DB cells）	103
Hyt-13	 Hyt		 EPZ6438	EZH2	Degradation of EZH2 at a concentration of 10 μM and thorough degradation at 40 μM. (MDA-MB-468 cells)	EPZ6438： IC_{50}=173.7±5.4 μM Hyt-13： IC_{50}=14.3±2.5 μM (MDA-MB-468 cells)	95
U3i	 CBRN		 UNC1999	EZH2	Above 50% degradation of EZH2 at 1 μM (MDA-MB-468 cells)	UNC1999: IC_{50}=14.69±1.08 μM U3i: IC_{50}=0.57±0.07 μM (MDA-MB-231 cells) UNC1999: IC_{50}=7.67±0.45 μM U3i: IC_{50}=0.38±0.05 μM (MDA-MB-468 cells)	53
G1c	 CBRN		 GSK126	EZH2	Above 50% degradation of EZH2 at 1 μM (MDA-MB-468 cells)	GSK126: IC_{50}=12.82±1.07 μM G1c: IC_{50}=0.64±0.16 μM (MDA-MB-231 cells) GSK126: IC_{50}=11.92±1.23 μM G1c: IC_{50}=0.42±0.06 μM (MDA-MB-468 cells)	53

Table 3: **Overview of E3 ligases, linker structures, and POI ligands: potency and efficacy against EZH2.**

Other EZH2 degraders

Inhibitors and degraders specifically targeting EZH2 have been developed over the past few years. In 2017, a novel EZH2 inhibitor GNA002 was proved to inhibit the expression of H3K27me3 and EZH2 protein. Furthermore, GNA002 manifested remarkable anti-tumor effects in the mouse xenograft model.[52] In 2020, Anqi Ma excavated a hydrophobic tagged EZH2 selective degrader MS1943. Compared to traditional EZH2 inhibitors, MS1943 selectively kills the proliferation of EZH2-dependent TNBC cells over normal cells through diminishing the expression of EZH2 protein. Meanwhile, the unfolded protein response (UPR) of EZH2 could also be suppressed by MS1943 in TNBC cell lines. Therefore, MS1943 may become an effective small-molecule for TNBC therapy.[105]

In 2018, researchers used a selective $FKBP12^{F36V}$ degrader to degrade multiple proteins, which consisted of CRBN E3 ligase, $FKBP12^{F36V}$ and dTAG tool molecule, and CRISPR–Cas9 system or lentiviral system were used to start protein degradation. In this process, an array of fusion chimeras including Brd 4, HDAC1, EZH2, Myc, PLK-1, and $KRAS^{G12V}$ were rapidly degraded in this method.[106]

Inhibitors and small molecules downregulate EZH2

Besides EZH2-based PROTACs and degraders, some inhibitors and drugs with other proven functions have been shown to facilitate the degradation of EZH2.

YC-1 prevented and cured vascular embolisms by activating platelet guanylate cyclase.[107] It has shown some potential as a tumor growth inhibitor in a variety of tumor types.[107] By inhibiting EZH2, YC-1 stimulated C-CBL and ERK in breast cancer cells, which induced cell apoptosis and tumor growth arrest.[34] Heat shock protein 90 inhibitor NVP-AUY922 could effectively inhibit various tumor growth and metastasis via cell apoptosis, cell cycle arrest, tumor invasion, and angiogenesis.[108] A 2017 study found that AUY922 could reduce the occurrence of graft versus host disease (GVHD) by targeting EZH2. Degradation of EZH2 was observed to lower the apoptosis of activated T cells as well as the production of IFNγ and TNF α via decreasing HSP90 expression in this investigation.[108] Another study showed that PRMT1-specific inhibitor AMI-1 promoted EZH2 degradation, reducing breast cancer cell metastasis.[44]

In addition to these small molecule inhibitors, some drugs also disturb EZH2 protein stability. Sorafenib is an effective first-line therapy for late-stage hepatocellular carcinoma. And recent evidence has shown that sorafenib-related EZH2 degradation has been linked to cell death and cell cycle arrest in hepatoma cells. Meanwhile, hepatocellular carcinoma cells with EZH2 mutations or low levels of expression are more sensitive to sorafenib therapy. This might aid in the development of combination therapy for HCC patients.[109] Astemizole, an FDA-approved anti-histamine drug used for allergy treatment, has been discovered as a small molecule inhibitor of the EZH2-EED PPI interaction within the PRC2 complex, thereby leading to the degradation of PRC2 core proteins and the decrease of global H3K27me3 levels in cancer cells.[110–112] Furthermore, another anti-histamine drug, Ebastine, has also been identified as an EZH2 inhibitor via targeting EZH2 transcription and subsequently downregulating its protein levels and H3K27me3 in multiple cancer cell lines.[113]

The monomer of natural anthraquinone emodin-NSC745885 can similarly impact EZH2 protein stability. It can selectively kill bladder cancer cells and slightly affect normal epithelial cells. Moreover, it showed the advantages in killing multidrug resistant tumor cells.[114]

Conclusion and perspective

As an important epigenetic regulator, EZH2 has become highly sought out for research and development (R&D) of tumor-target drugs. The majority of EZH2 inhibitors are still in clinical and preclinical testing. Excessive dosage and narrow scope of application are common problems of EZH2i.

Further investigations into EZH2 regulation and function have revealed novel, non-canonical functions of EZH2 in multiple human tumors. In a specific tumor microenvironment, the non-classical functions of EZH2 are crucial for tumor development. Some solid tumors (e.g., prostate cancer[17]) are resistant to EZH2i, which might be due to its non-canonical function. Further investigation of EZH2 non-canonical functions and mechanisms of EZH2 protein stability regulation may offer new therapeutic strategies for cancer therapy. As noted in this review, the main mechanism to regulate the stability of EZH2 protein is through post-translational modifications, which function by modifying different amino residue sites on EZH2. Indeed, it has been shown that various types of PTMs, including ubiquitination, phosphorylation, and glycosylation can regulate EZH2 stability. Moreover, the underlying mechanism of PTMs crosstalk is variable and complicated, which remains to be uncovered and requires further investigations.

As a result of PROTAC's development, EZH2 inhibitors are currently being investigated in a novel manner. To date, only E3 ligases CRBN and VHL have been utilized for EZH2 PROTAC. Several E3 ligases have been identified to interact with EZH2, but none of them have been developed as the ligand for PROTAC. Therefore, it is important for PROTAC to load more specific E3 ligases. Further development of PROTAC technologies may enhance the potential of target-EZH2 PROTAC as a cancer targeted treatment.

In summary, high selectivity and high efficiency of EZH2 inhibitors are the development direction in the

Searching strategy and selection criteria

Articles referred for this review were collected by searches of PubMed using the search terms "EZH2", "protein stability", "targeted cancer therapy", "post-translational modification", "PROTAC", and "EZH2 degrader". The majority of cited publications were from the past 5 years.

future. In this review, we summarize the mechanisms that regulate EZH2 protein stability as well as highlight the development of PROTACs and degraders targeting EZH2, which may provide a new outlook on EZH2i research and possibilities for clinical translation.

Outstanding questions

EZH2 is a well-recognized therapeutic target in cancer. The inhibitor targeting EZH2 enzymatic activity was approved by the FDA in 2020 and has been applied in clinical practice. However, the solely inhibition of EZH2 trimethylase activity is not enough to achieve ideal therapeutic effect.

Why is inhibition of EZH2 enzymatic activity alone limited in the clinical application? EZH2 plays a role in various cellular processes independently of its histone methyltransferase activity. Simply targeting its enzymatic function may not adequately address the diverse biological roles in cancer development and progression. Thus, there has been a growing interest in the protein stability of EZH2. And it is of great significance to further elucidate the regulatory mechanism of EZH2 protein stability.

So, what are the main mechanisms governing EZH2 protein stability in cancer? Notably, the post-translational modifications are closely related to protein stability. Fortunately, the post-translational modifications of EZH2, the effects of modifications on its stability, and the associated biological functions in cancer progression have been gradually understood over the past decade. However, the underlying mechanism of PTMs crosstalk is variable and complicated, which remains to be uncovered and requires further investigations.

Then, is it possible to develop effective tumor treatment strategies based on the regulation of protein stability, and further enhance its therapeutic efficacy to expand its clinical applications? Strategies aimed at regulating EZH2 protein stability can include the development of small molecules or drugs, such as, PROTACs. PROTACs utilize the ubiquitin-proteasome pathway to selectively degrade target proteins and have emerged as a promising approach to target EZH2 protein stability. Nevertheless, there are still challenging questions that need to be addressed in future studies. To date, only E3 ligases CRBN and VHL have been utilized for EZH2 PROTAC. Several E3 ligases have been identified to interact with EZH2, but none of them have been developed as the ligand for PROTAC. Therefore, it is important for PROTAC to load more specific E3 ligases. Further development of PROTAC technologies may enhance the potential of target-EZH2 as a cancer targeted treatment.

Contributors

L.X.X. and C.G.K. contributed to the conception of the paper. Y.Y.G., R.C., Y.L. and Y.Q.W. wrote the manuscript and drew the figures. M E. G. and H.S.Z edited and revised the manuscript. All authors reviewed the manuscript and provided suggestions for revision. All authors read and approved the final manuscript.

Declaration of interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2024.104972.

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