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Epigenetic abnormalities and therapies for hematological malignancies

Aberrant histone modifications induced by mutant ASXL1 in myeloid neoplasms

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Abstract
An epigenetic modulator *Additional sex combs-like 1* (*ASXL1*) is recurrently mutated in myeloid neoplasms such as myelodysplastic syndromes (MDS), acute myeloid leukemia (AML) and myeloproliferative neoplasms (MPNs). *ASXL1* mutations are also frequently detected in clonal hematopoiesis with indeterminate potential (CHIP), which is the clonal expansion of premalignant hematopoietic cells without any evidence of hematological malignancies. Thus, understanding the roles of ASXL1 in hematopoiesis and myeloid neoplasms is a clinically crucial issue. *ASXL1* mutations in hematological neoplasms are typically frameshift or nonsense mutations and occur near the 5′ end of the last exon, thereby the transcripts would escape from nonsense-mediated decay, Indeed, we identified the C-terminally truncated mutant protein of ASXL1 in several cell lines derived from patients with myeloid leukemia. In mouse models, expression of the mutant ASXL1 results in impaired hematopoiesis and promotes development of myeloid neoplasms. In addition, recent findings from biochemical analysis have demonstrated that the mutant ASXL1 protein gains new functions including enhancing catalytic activity of BRCA1-associated protein 1 (BAP1), resulting in reduction of H2AK119ub and aberrant gene expression essential for myeloid transformation. In this review, we will focus on the pivotal roles of the mutant ASXL1 on histone modifications and myeloid transformation.

Keywords ASXL1 · Histone modifications · H2AK119ub · BAP1 · Myeloid neoplasms

Introduction

Recent next-generation sequence studies have uncovered mutational landscape in various types of hematological neoplasms. *Additional sex combs-like 1* (*ASXL1*) is one of the most frequently mutated genes in myeloid malignancies including myelodysplastic syndromes (MDS) [1, 2], acute myeloid leukemia (AML) [3–5] and myeloproliferative neoplasms (MPNs) [6, 7]. *ASXL1* mutations are found in about a half of chronic myelomonocytic leukemia (CMML) [8–11]. Of note, *ASXL1* mutations are associated with poor prognosis [1, 3–5, 8, 10]. In addition, *ASXL1* mutations are recurrently found in healthy individuals with clonal hematopoiesis with indeterminate potential (CHIP) as with *DNA methyltransferase 3A* (*DNMT3A*) and *Ten–eleven-translocation 2* (*TET2*) [12–14]. CHIP is characterized by clonal expansion with somatic preleukemic mutations in absence of any evidence of hematological malignancies. CHIP is associated with an increased risk of development of hematological neoplasms [12–14]. A recent study demonstrated that CHIP is also an independent risk for coronary heart disease [15].

Therefore, understanding the roles of ASXL1 in hematopoiesis is much important. Recent studies have revealed that knockdown of Asxl1 accelerates N-ras induced myeloid transformation [16]. *Asxl1* knockout mice exhibited impaired hematopoiesis and development of MDS-like disease [17, 18]. These findings indicated that wildtype ASXL1 plays crucial roles as a tumor suppressor in hematopoiesis and suggested loss-of-function features of ASXL1 mutations. However, most ASXL1 mutations in hematological neoplasms occur near the last exon and its mutations are frameshift or nonsense mutations, thereby its transcripts would escape from nonsense-mediated decay. C-terminally

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truncated form of ASXL1 protein are detectable by Western blotting analysis and mTRAQ-based mass spectrometric analysis [19]. Biological findings and biochemical analysis of the mutant ASXL1 have demonstrated that the mutant ASXL1 accelerates myeloid neoplasms in a dominant-negative and gain-of function manner.

In this review, we will focus on the findings from recent biological studies how the mutant ASXL1 impairs normal hematopoiesis and promotes myeloid neoplasms. We will also introduce the potential therapeutic strategy of myeloid neoplasms harboring *ASXL1* mutations.

The structure of ASXL family proteins

Mammalian three ASXL family genes (*ASXL1, ASXL2* and *ASXL3*) are the mammalian homologs of Drosophila *Additional sex combs* (*Asx*) [20]. Drosophila Asx was originally identified as an enhancer of trithorax and polycomb (ETP) and required for both gene activation and repression [21, 22]. Drosophila Asx forms polycomb repressive deubiquitinase complex (PR-DUB) with a histone deubiquitinase Calypso [23]. The mammalian homolog of Drosophila Calypso is BRCA-1 associated protein 1 (BAP1). Mammalian ASXL1 and BAP1 also form a mammalian PR-DUB complex, which removes ubiquitination of H2A at lysine 119 (H2AK119ub). H2AK119ub modification is associated with a repressive chromatin status and catalyzed by an E3 ligase RING1A/RING1B, which is a main component of Polycomb repressive complex 1(PRC1) [24, 25].

Mammalian *ASXL1* and *ASXL2* are ubiquitously expressed in a variety of tissues, whereas the expression of *ASXL3* is low overall and restricted to lymph node, eye, lung, skin, brain, and pituitary gland [26]. Mammalian ASXL family proteins share highly conserved N-terminus ASXN domain, ASX homology (ASXH) domain and plant homeodomain (PHD) finger domain at C-terminal region (Fig. 1) [27, 28]. The ASXN domain is structurally similar to forkhead-box domain and predicted to be essential for DNA-binding ability of ASXL family proteins [29, 30]. ASXH domain (also referred to as DEUBAD, deubiquitinase adaptor) is essential for interacting with BAP1 [31]. PHD domain is a histone- or DNA-binding module and is reported to recognize different subtypes of histone modifications such as unmethylated H3K4 (H3K4me0) and trimethylated H3K4 (H3K4me3) [32]. What modifications does the PHD finger of ASXL1 recognize remains to be elucidated.

Human *ASXL1* gene is located on chromosome 20q11 and encodes ASXL1 protein [33]. In myeloid neoplasms, most of the *ASXL1* mutations are somatic ones, whereas germline mutations of ASXL1 are identified in patients with Bohring–Opitz syndrome, a developmental disorder [34]. Although *ASXL2* mutations are frequently found in RUNX1-ETO fusion leukemia [35, 36], the frequency of *ASXL2* mutations in other hematological neoplasms is much lower than that of *ASXL1* mutations [37, 38]. Unlike *ASXL1*

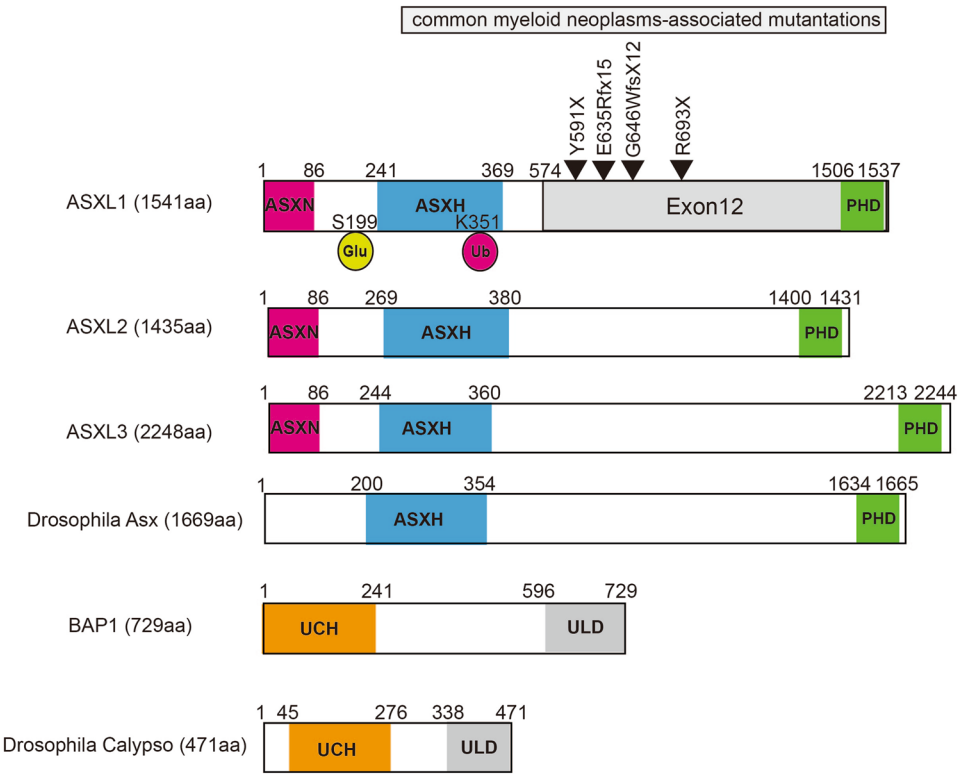

Fig. 1 The protein structure and main domains of mammalian ASXL family/Asx and BAP1/Calypso. Known posttranslational modifications and the most frequent somatic *ASXL1* mutations found in myeloid neoplasms are also shown. *ASXN* N-terminus ASXN domain, *ASXH* Asx homology domain, *PHD* PH domain, *UCH* Ubiquitin C-terminal hydrolase, *ULD* Uch37-like domain, *Glu* glycosylation, *Ub* ubiquitination

and *ASXL2, ASXL3* mutations are rarely found in myeloid neoplasms [39]. This reason remains unclear, but it might be caused by the differences of DNA-binding ability of ASXN domain and the modifications recognized by PHD finger domain among ASXL1, ASXL2 and ASXL3.

Altering histone modifications induced by ASXL1 loss or the mutant ASXL1 expression

To understand the roles of wildtype ASXL1 in histone modifications, ASXL1 knockdown and *Asxl1* knockout mouse studies have been performed. Abdel-wahab et al. demonstrated that knockdown of ASXL1 or *Asxl1* conditional knockout mice showed substantial reduction of trimethylated H3K27 (H3K27me3) due to loss of interaction with PRC2 complex, leading to development of MDS-like disease [16, 17]. Youn et al. revealed that Asxl1 disruption in mouse embryonic fibroblasts leads to dysregulation of *p16Ink4a* as result of the defect in Asxl1–Ezh2 interaction [40]. These data indicated that wildtype ASXL1 is essential for maintaining the level of H3K27me3 by interacting with PRC2 complex.

In addition, the relationship between wildtype ASXL1 and H3K4me3 modifications has been investigated. Wang et al. also revealed that constitutive *Asxl1* knockout mice showed global reduction not only of H3K27me3 but also of H3K4me3 [18]. Shi et al. also showed that depletion of ASXL1 resulted in impaired erythroid differentiation via reduction of the expression and H3K4me3 of *FOXO3, GCN5* and *MYB* [41]. Inoue et al. demonstrated that ASXL1 interacts with OGT and HCFC1, and knockdown of ASXL1, OGT, HCFC1 or MLL5 attenuates myeloid differentiation and decreases global level of H3K4me3 [42]. The precise mechanisms by which loss of ASXL1 lead to a decrease in the level of H3K4me3 remains unclear, while these data indicated that wildtype ASXL1 is also essential for maintaining the level of H3K4me3.

However, as mentioned above, *ASXL1* mutations typically occur near the last exon and its transcripts escape from nonsense-medicated decay (NMD) (Fig. 1). In patient-derived cell line harboring *ASXL1* mutations, the mutant ASXL1 protein was detected by Western blotting analysis and mTRAQ-based mass spectrometric analysis [19]. Therefore, it is essential to investigate how the mutant ASXL1 protein affects histone modifications and contributes to myeloid transformation. Inoue et al. demonstrated that the mutant ASXL1 binds EZH2 and overexpression of the mutant ASXL1 induced global reduction of H3K27me3 in a dominant-negative manner. The mutant ASXL1 overexpression induced derepression of *miR125a* and *Hoxa9* via reduction of H3K27me3, resulting in impaired myeloid differentiation and development of MDS/AML disease in mouse BMT model [43]. Combined expression of the mutant ASXL1 and SETBP1 mutations exhibited rapid development MDS/AML in mice [44]. To investigate the role of the mutant ASXL1 at physiological expression levels, Nagase et al. generated Rosa26 locus mutant *Asxl1* knock-in (*Asxl1*-MT KI) mice mimicking human *ASXL1* E635RfsX15 mutation derived from patients with MDS/AML [45]. *Asxl1*-MT KI mice displayed substantial reduction in H3K4me3 and H2AK119ub. Unlike Asxl1 knockout mice, global level of H3K27me3 was not significantly changed in *Asxl1*-MT KI mice. However, the level of H3K27me3 at *Hoxa* gene loci was decreased and the expression of *Hoxa* gene was also upregulated in *Asxl1*-MT KI mice. H3K4me3 reduction was observed especially at promoter loci of the genes associated with erythroid differentiations such as *Sox6, Id3, Tjp1* and *Hba*. Of note, overexpression of Id3 ameliorated erythroid colonogenicity in bone marrow cells derived from *Asxl1*-MT KI mice. Hsu et al. generated locus $Asxl1^{G643fs}$ mutant knock-in mice and reported that $Asxl1^{G643fs}$ mutant knock-in mice alone did not develop hematological malignancies within 18 months follow-up [46]. On the contrary, Uni et al. also generated locus $Asxl1^{G643fs}$ mutant knock-in mice and reported that a part of $Asxl1^{G643fs}$ mutant knock-in mice eventually developed MDS/MPN-like disease after a long latency, about 18–24 months. They demonstrated that wildtype ASXL1 but not the mutant ASXL1 can bind to BMI1, a key component of canonical PRC1 [47]. The level of H2AK119ub is decreased at *p16Ink4a* promoter locus, thus *Ink4a* expression is derepressed in $Asxl1^{G643fs}$ mutant knock-in mice. They also elucidated that *p16Ink4a* knockout rescues decreased HSC number and aberrant apoptosis in $Asxl1^{G643fs}$ mutant knock-in mice.

These findings indicated that expression of the mutant ASXL1 results in altering histone modifications and defects in hematopoiesis, leading to promoting myeloid transformation.

The monoubiquitinated mutant ASXL1 forms a hyperactive complex with BAP1

As mentioned above, BAP1 is a known interacting partner of ASXL1. BAP1 interacts with ASXL1 via DEUBAD domain [31]. BAP1 is a deubiquitinase which removes monoubiquitination of H2AK119ub. BAP1 also deubiquitinates and stabilizes non-histone targets such as IP3R3 [48], INO80 [49] and KLF5 [50]. Loss of function of *BAP1* mutations are recurrently detected in melanoma [51], malignant mesothelioma [52], and kidney renal cell carcinoma [53]. Therefore, BAP1 has been considered as a tumor suppressor. In hematological neoplasms, Bap1 conditional knockout mice represented MPN/MDS-like disease due to EZH2 dysregulation

[54, 55]. However, a large patient data set showed BAP1 is rarely mutated in myeloid malignancies [56]. In addition, individuals with BAP1 germline mutations rarely develop myeloid malignancies [57]. There is also growing experimental evidence showing BAP1 has a tumor-promoting role cooperating with the mutant ASXL1. Balasubramani et al. showed that cancer-associated *ASXL1* mutations enhanced the catalytic activity of BAP1 and profoundly reduced the global level of H2AK119ub [58]. Sahtoe et al. also demonstrated that ASXH domain is essential for recruitment of BAP1 on the chromatin [59].

Recently, we have reported that the mutant ASXL1 and BAP1 form a hyperactive complex to promote myeloid transformation (Fig. 2) [60]. As with wildtype ASXL1, the mutant ASXL1 also contains the ASXH domain (also referred to as DEUBAD), which is essential for interacting with BAP1. BAP1 expression resulted in stabilization and monoubiqutination at lysine 351 of the mutant ASXL1. The mutant ASXL1, but not wildtype ASXL1 in turn enhanced catalytic function of BAP1. Especially, the monoubiqutinated form of the mutant ASXL1 promoted nuclear localization of BAP1. As a result, the monoubiquitinated mutant ASXL1/BAP1 complex profoundly reduced the global level of H2AK119ub in hematopoietic cells. Functionally, the monoubiquitinated mutant ASXL1/BAP1 complex inhibited the differentiation of HSPCs towards multilineage except for immature monocytes. The monoubiquitinated mutant ASXL1/BAP1 complex also promoted myeloid leukemogenesis induced by RUNX1-ETO, an oncogenic fusion protein frequently coexisting with *ASXL1* mutations [35, 61]. Mechanistically, this complex directly bound to promoter loci of *HOXA* genes and *IRF8*, and decreases H2AK119 at these loci, resulting in dysregulation of posterior *HOXA* genes and *IRF8*. *HOXA* gene expression is essential for leukemic transformation [62] and *IRF8* plays a critical role in monopoiesis [63, 64]. We also revealed that endogenous depletion of BAP1 abrogated the mutant ASXL1-induced leukemogenesis. Interestingly, endogenous BAP1 also played essential roles in promoting survival of MLL-fusion leukemia cells, which depend on *HOXA* gene expression. Therefore, targeting enzymatic activity of BAP1 is a promising therapeutic strategy not only for ASXL1-mutated leukemia but also for a broad range of myeloid leukemia with *HOXA* gene dysregulation.

As mentioned above, we demonstrated that monoubiquitination of the mutant ASXL1 is important for enhancing BAP1 activity, thereby promoting myeloid leukemogenesis. Guo et al. also revealed that reducion of Bap1 activity reduced expression of posterior *Hoxa* genes and attenuated leukemogenesis in the mutant ASXL1-expressing transgenic mice [65]. Recently, Daou et al. also demonstrated that monoubiquitination of ASXL2 at lysine 370 catalyzed by UBE2E family is required for deubiquitinase activity

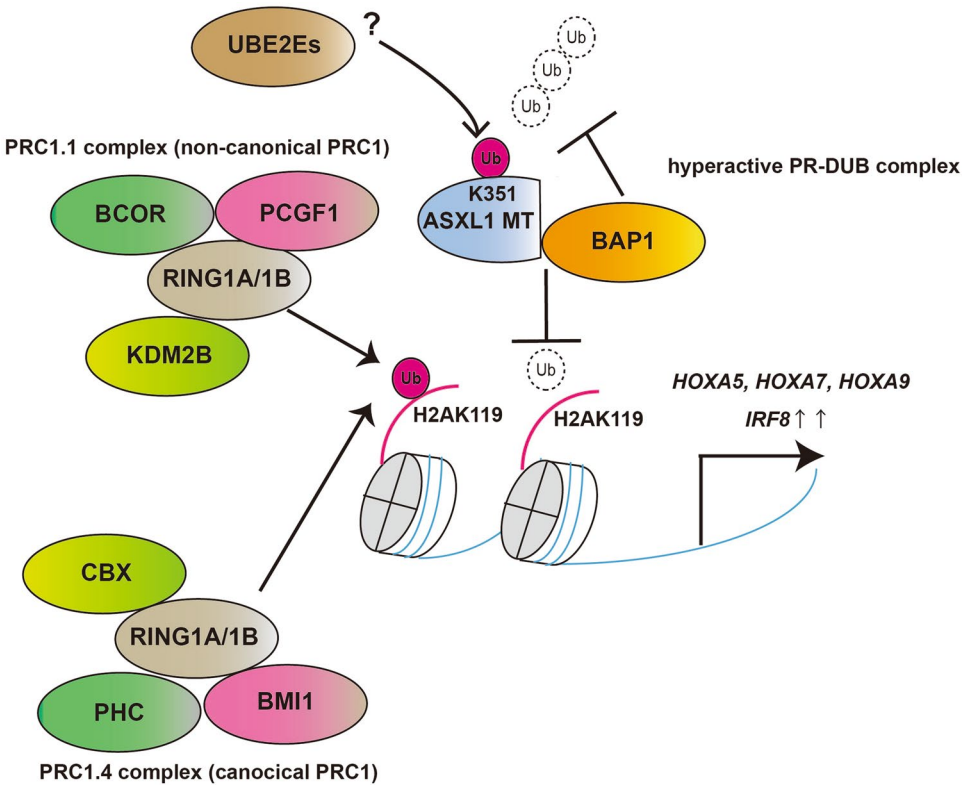

Fig. 2 Known and proposed components associated with H2AK119ub modifications. BAP1 stabilizes and induces monoubiquitination of the mutant ASXL1. The monoubiquitinated mutant ASXL1 in turn enhances catalytic activity of BAP1. The monoubiquitinated mutant ASXL1 and BAP1 form a hyperactive PR-DUB complex, which removes ubiquitination of H2AK119 catalyzed by canonical or non-canonical PRC1 complexes. Whether UBE2Es, which monoubiquitinate ASXL2, catalyzes monoubiquitination of the mutant ASXL1 remains unknown

of BAP1 [66]. Further studies are required to determine whether monoubiquitination of the mutant ASXL1 is catalyzed by UBE2E family.

Regulation of *HOXA* gene expression by H2AK119ub modification

H2AK119ub modifications are originally identified as a repressive mark which is catalyzed by canonical PRC1 complex containing BMI1 [24, 25]. PR-DUB complex removes ubiquitin of H2AK119ub [23]. Thus, it is expected that the target genes regulated by PRC1 complex and PR-DUB complex are largely overlapped. However, recent mass spectrometry and ChIP-seq analysis revealed that the targets of PR-DUB are distinct from that of canonical PRC1 complex [67]. Actually, mammalian PRC1 family is subdivided into canonical PRC1 (cPRC1) and non-canonical PRC1(ncPRC1) [68, 69]. Gao et al. defined six major groups of PRC1 complexes, each containing a distinct PCGF subunit, a RING1A/B ubiquitin ligase, and a unique set of associated polypeptides [70].

Recently, Tara et al. demonstrated that *Bcor* mutation which lacks interaction with non-canonical PRC1.1 containing PCGF1 and promotes MDS/MPN development in mouse model [71]. This *Bcor* mutant mouse showed substantial derepression of *Hoxa7* and *Hoxa9* gene via reduction of H2AK119ub at the *Hoxa7* and *Hoxa9* gene loci. These data indicate that PRC1.1 complex also regulates posterior *HOXA* gene silencing and has tumor suppressor roles in myeloid neoplasms. A cPRC1 complex containing BMI1 (PRC1.4) is known to regulate *HOXA* silencing via ubiquitination of H2AK119 [72]. Therefore, the balance among PRC1.1 complex, PRC1.4 complex and PR-DUB complex will be important to maintain healthy hematopoiesis and prevent transformation via regulating *HOXA* gene expression (Fig. 2).

In addition, crosstalk between H2K119ub and H3K27me3 at *HOXA* loci remains unclear. *Bap1*-depleted mice showed substantial retention of H2AK119ub and increased the level of H3K27me3 [55]. On the other hand, expression of the mutant ASXL1 results in dysregulation of *HOXA* gene and reduction of both H2AK119ub and H3K27me3 at *HOXA* loci [43, 45, 60, 65]. In classical view, CBX recognizes H3K27me3 catalyzed by PRC2 complex followed by recruiting of PRC1 complex to H3K27me3 and subsequent ubiquitination of H2AK119 [73]. However, a recent study showed that non-canonical PRC1.1 marks H2AK119ub at target loci and subsequently recruits PRC2 complex [69]. H2AK119ub also promotes H3K27me3 induced by Jarid2-Aebp2-containing PRC2 [74]. Further studies will shed light on precise gene regulation and histone modifications at *HOXA* loci.

Potential therapeutic strategy for ASXL1 mutated myeloid neoplasms

As mentioned above, BAP1 inhibition can be a promising therapeutic potential, while there are no clinically available agents inhibiting the activity of BAP1. To this date, several potential therapeutic agents including inhibitors for BRD4, OGA and HDAC have been investigated in *ASXL1*-mutated myeloid neoplasms (Fig. 3). Yang et al. generated C-terminal truncated mutant Asxl1 (Asxl1^{Y588X}) expressing transgenic mice and revealed that transgenic Asxl1^{Y588X} expression led to myeloid malignancies [75]. Mechanistically, they showed that the mutant ASXL1 protein, not wildtype ASXL1, binds to BRD4. BRD4 protein activates pTEFb complex and induces acetylation of H3 at lysine 122 (H3K122Ac), resulting in phosphorylation of RNA polymerase II and gene activation. In Asxl1^{Y588X} transgenic mice, the level of H3K122Ac at the promoter locus of *Prdm16* is increased, as a result, the expression of *Prdm16* is dysregulated. Bone marrow cells from Asxl1^{Y588X} transgenic mice exhibited higher sensitivity toward a BRD4 inhibitor than that from normal mice. Inoue et al. demonstrated that OGT stabilized wildtype ASXL1 via glycosylation of ASXL1 at serine 199 [42]. They also revealed that PUGNAc, an OGA (O-GLcNAcase) inhibitor stabilized wildtype ASXL1 protein and restored the expression of *Rara* and *Egr1* genes via increased level of H3K4me3 at these loci, thereby OGA inhibitor induced myeloid differentiation and showed antitumor effects on leukemia cells expressing the mutant ASXL1. Saika et al. also revealed that the mechanism by which leukemia cells combined expression with the mutant ASXL1 and mutant SETBP1 showed downregulation in TGFβ pathway was caused by reduction in acetylation of H3K14 and H4K5 at TGFβ gene loci [76]. They also demonstrated that vorinostat, a HDAC inhibitor showed substantial growth-inhibiting effects on MDS/AML cells expressing ASXL1 and/or SETBP1 mutation via restoring acetylation of H3K14 and H4K5 at TGFβ gene loci [76].

From these findings, the mutant ASXL1 altered various histone modifications and the novel agents against these alterations might be applied to clinical therapies for *ASXL1*-mutated myeloid malignancies.

Conclusion

Myeloid neoplasms harboring *ASXL1* mutations often have poor prognosis, thereby understanding the precise mechanisms by which the mutant ASXL1 induces myeloid neoplasms is clinically needed. Recent findings from the

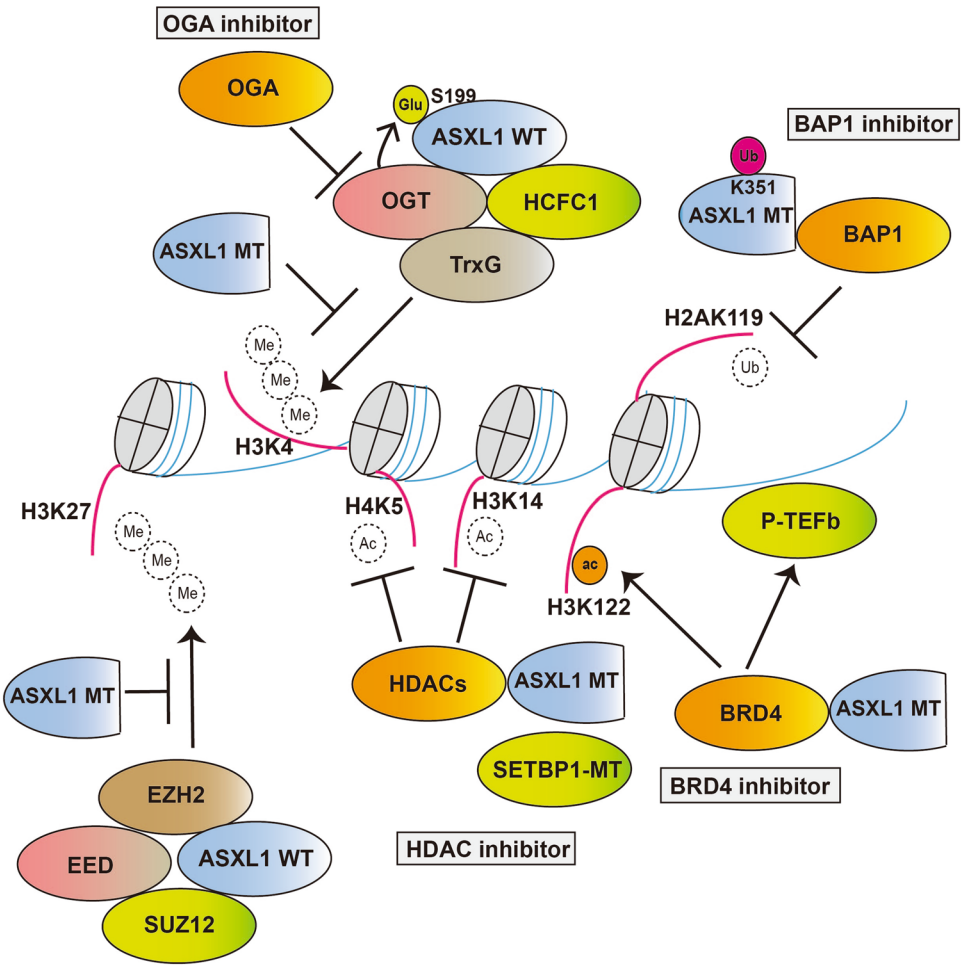

Fig. 3 Overview of altered histone modifications induced by the mutant ASXL1 and potential therapeutic strategies for *ASXL1*-mutated myeloid neoplasms

mutant ASXL1-expressing mouse studies have revealed that the mutant ASXL1 impairs hematopoiesis and promotes myeloid transformation via altering histone modifications. In addition, precise biochemical analyses have unveiled different molecular signatures between wildtype ASXL1 and the mutant ASXL1. These findings have also shed light on new potential therapeutic strategies such as BRD4 inhibition, OGA inhibition, HDAC inhibition and BAP1 inhibition. However, many unsolved riddles remain regarding functions of the mutant ASXL1 and wildtype ASXL1. For examples, the precise mechanism of how the mutant ASXL1 not wildtype ASXL1 gets stabilized in the presence of BAP1 is still unclear. In addition, hematopoietic cells expressing the mutant ASXL1 showed substantial reduction in both H2AK119ub and H3K4me3, which play opposite roles with each other in gene regulations. Because expression of individual genes must be regulated differentially, it would be important to investigate the molecular interactions among ASXL1, MLL and SET1/COMPASS complexes as well as PRC1 and PRC2 complexes. Further studies are also required to determine which therapeutic agents are more favorable to treat with myeloid neoplasms harboring *ASXL1* mutations.

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Compliance with ethical standards

Conflict of interest The authors have declared that no conflict of interest exists.

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