



Lysine Demethylase KDM6A in Differentiation, Development, and Cancer

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ABSTRACT Lysine demethylase 6A (KDM6A), also known as UTX, belongs to the KDM6 family of histone H3 lysine 27 (H3K27) demethylases, which also includes UTY and KDM6B (JMJD3). The KDM6A protein contains six tetratricopeptide repeat (TPR) domains and an enzymatic Jumonji C (JmjC) domain that catalyzes the removal of di- and trimethylation on H3K27. KDM6A physically associates with histone H3 lysine 4 monomethyltransferases MLL3 (KMT2C) and MLL4 (KMT2D). Since its identification as an H3K27 demethylase in 2007, studies have reported KDM6A's critical roles in cell differentiation, development, and cancer. KDM6A is important for differentiation of embryonic stem cells and development of various tissues. Mutations of KDM6A cause Kabuki syndrome. KDM6A is frequently mutated in cancers and functions as a tumor suppressor. KDM6A is redundant with UTY and functions largely independently of its demethylase activity. It regulates gene expression, likely through the associated transcription factors and MLL3/4 on enhancers. However, KDM6A enzymatic activity is required in certain cellular contexts. Functional redundancy between H3K27 demethylase activities of KDM6A and KDM6B *in vivo* has yet to be determined. Further understanding of KDM6A functions and working mechanisms will provide more insights into enhancer regulation and may help generate novel therapeutic approaches to treat KDM6A-related diseases.

KEYWORDS KDM6A/UTX/H3K27 demethylase/enhancer regulation/gene expression, H3K27 demethylase, KDM6A, UTX, cancer, cell differentiation, development, enhancer regulation, gene expression

Chromatin modifications play a major role in regulating gene expression. The methylation of histone at certain lysine residues is one of the best-studied types of chromatin modification. Methylations catalyzed by site-specific histone methyltransferases correlate with gene activation or repression. In 2004, it was discovered that methylation on histone H3 lysine 4 (H3K4) could be reversed by a histone demethylase called lysine-specific demethylase 1 (LSD1 or KDM1A), suggesting that transcriptional regulation by histone methylation is a dynamic and intricate process (1). In 2007, several studies identified a group of histone H3 lysine 27 (H3K27) demethylases consisting of lysine demethylase 6A (KDM6A or UTX), lysine demethylase 6B (KDM6B or JMJD3), and ubiquitously transcribed tetratricopeptide repeat on chromosome Y (UTY) (2–5). KDM6A is an X-linked protein that, similar to KDM6B, contains a catalytic Jumonji C (JmjC) domain which facilitates the removal of the methyl group on di- and trimethylated H3K27 (H3K27me_{2/3}). While UTY is the Y-linked homolog of KDM6A and shares many structural similarities, it does not exert significant demethylase activity (2, 5).

Since its identification as an H3K27 demethylase in 2007, an increasing number of studies are being carried out every year to examine the functions of KDM6A in different biological contexts, with about 100 articles published in 2019 (Fig. 1). Many studies have shown that KDM6A regulates gene expression and enhancer activation indepen-

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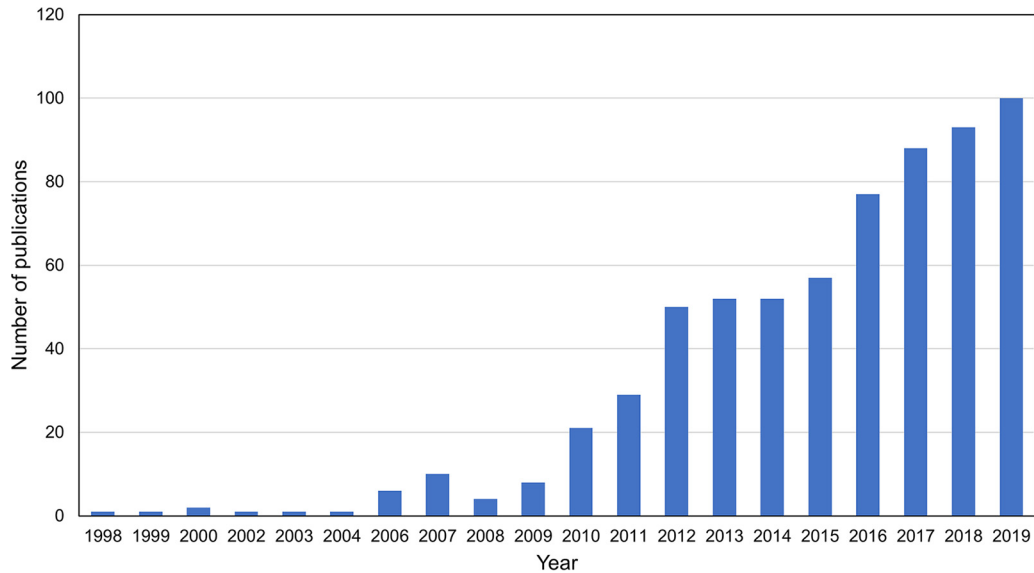


FIG 1 Trajectory of publication of KDM6A papers since the identification of the KDM6A gene in 1998. PubMed searches using keywords “kdm6a” and “utx” yielded more than 600 articles published from 1998 to 2019.

dently of its demethylase activity. KDM6A physically and/or functionally interacts with transcription factors (TFs), chromatin-modifying enzymes, including H3K4 methyltransferases MLL3/MLL4 and H3K27 acetyltransferases CBP/p300, and chromatin-remodeling complex SWI/SNF (6–11). KDM6A plays important roles in embryonic stem cell (ESC) differentiation, early embryonic development, and tissue-specific development, including cardiac, mammary, and immune development. KDM6A was also found to be mutated in many types of cancer and to function as a tumor suppressor in mouse cancer models. In this review, we summarize the general features of the KDM6A protein, its interactions with other chromatin regulators, and its roles in enhancer regulation. Current literature on the roles of KDM6A in cell differentiation, embryonic and tissue-specific development, developmental disease Kabuki syndrome (KS), and tumor suppression is discussed. Lastly, we speculate on several future directions to extend the understanding of KDM6A mechanisms and functions.

THE KDM6A PROTEIN AND ASSOCIATED FACTORS

Basic properties of the KDM6A gene/protein. The gene encoding KDM6A or UTX is located on the X chromosome in both mice, at XA1.2-1.3, and humans, at Xp11.3. *KDM6A* escapes X inactivation in both mice and humans (12). In mice, the *Kdm6a* transcript is 5,918 bp long and contains 29 exons, while the human ortholog is 5,438 bp long and contains 29 exons. In both mice and humans, the KDM6A protein comprises 1,401 amino acids and weighs around 154 kDa, though alternative splicing can produce isoforms with slight variations (Table 1). Sequence alignment between human and mouse KDM6A proteins reveals that they are 97% identical. In developing

TABLE 1 Basic properties of the KDM6A gene/protein

Property	Human version	Mouse version
Gene	<i>KDM6A, UTX</i>	<i>Kdm6a, Utx</i>
Chromosome	Xp11.3	X A1.2-A1.3; X 13.45 cM
Ensembl identifier	ENSG00000147050	ENSMUSG000000037369
Transcript length (bp)	5,438	5,918
No. of exons/no. of introns	29/28	29/28
UniProt identifier	O15550	O70546
Protein length (amino acids)	1,401	1,401
Protein mol wt (Da)	154,176.54	154,354.83

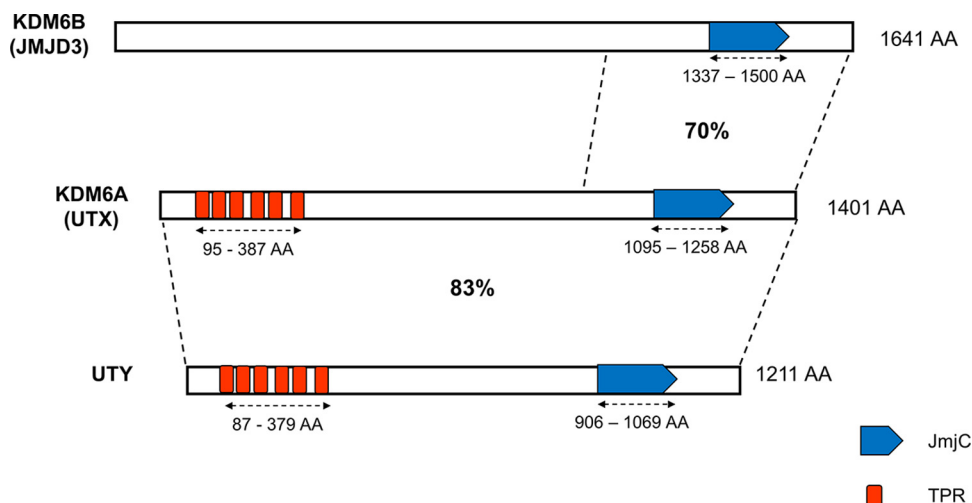


FIG 2 Schematic representation of the mouse KDM6 family of H3K27 demethylases, including KDM6A, KDM6B, and UTY. The catalytic JmjC domains and the TPR domains are indicated. KDM6A shares 83% protein sequence identity with its Y-linked homolog UTY. The C-terminal domain (amino acids [aa] 1174 to 1636) of KDM6B shares 70% protein sequence identity with KDM6A's C-terminal domain (aa 931 to 1394).

mouse embryos, KDM6A protein is widely expressed and highly enriched in the heart and neural tissues (8).

The KDM6A protein contains six tetratricopeptide repeat (TPR) domains at the N terminus and a Jumonji C (JmjC) domain at the C terminus. The catalytic JmjC domain allows KDM6A to demethylate di- or trimethylated lysine specifically at H3K27 without any considerable effect on methylated H3K4, K9, K36, or H4K20 (2, 5). Although KDM6A demethylase activity is not favorable on H3K27me1, it is still capable of demethylating H3K27me1 at high KDM6A dosage (5) or by using histone peptides as substrates (2). Knockdown of KDM6A leads to increased H3K27me3 levels in HeLa cells (3). The 6 TPR domains are dispensable for KDM6A H3K27me2/3 demethylase activity but appear to be important for H3K27me1 demethylation, at least *in vitro* (5).

KDM6A demethylase activity requires a catalytically active JmjC domain, which has conserved binding sites for cofactors Fe(II) ion and α -ketoglutarate (13). The Fe(II)-binding site is particularly crucial, as point mutations at this binding motif (H1146A and E1148A) can cause a total loss in KDM6A demethylase activity (4, 5). Through hydrogen bonds and hydrophobic interactions, the JmjC domain interacts extensively with residues immediately surrounding the H3K27me2/3 targets, including H3R26, H3A29, H3P30, and H3T32. KDM6A interactions with H3 across multiple regions collectively contribute to its conserved functions in specific binding and demethylation of H3K27me2/3 and not other methylated lysine residues, such as H3K9me3 or H3K36me3 (14).

The JmjC domain-containing KDM6 family. KDM6A is a member of the KDM6 family of H3K27 demethylases, which also contains KDM6B (JMJD3) and UTY (KDM6C) (Fig. 2). The KDM6B protein contains 1,643 and 1,641 amino acids in humans and mice, respectively, and has 70% protein sequence identity with KDM6A in the C-terminal region (5). KDM6B has broad expression during mouse embryonic development, with particularly high expression levels in neural tissues (15). Like KDM6A, KDM6B is capable of H3K27 demethylation. UTY is located on the Y chromosome, contains 1,347 and 1,211 amino acids in humans and mice, respectively, and has 83% protein sequence identity with KDM6A (5). During embryonic development, UTY has expression patterns similar to those of KDM6A, with an enrichment in the neural tube (16). Interestingly, in male embryonic stem cells (ESCs), KDM6A, but not its demethylase activity, positively controls *UTY* expression. However, in male embryos, *UTY* expression is not affected by the deletion of *Kdm6a* (KDM6A knockout [KO]) (7). Unlike KDM6A and KDM6B, UTY shows little to no H3K27 demethylase activity *in vivo* and *in vitro*, which can possibly be

attributed to some variants of critical amino acids as well as unidentified structural changes of the JmjC domain (2, 5, 16).

KDM6A is associated with the H3K4 methyltransferase MLL3/4 complex. KDM6A physically associates with the Set1-like H3K4 methyltransferases and epigenomic writers MLL3 (KMT2C) and MLL4 (KMT2D) in a protein complex that also contains PTIP, PA1, WDR5, RbBP5, ASH2L, DPY30, and NCOA6 and is in the nucleus in mammalian cells (4, 6, 17). KDM6A does not interact with other Set1-like H3K4 methyltransferase complexes, such as Set1A/B or MLL1/2, indicating that KDM6A is a unique subunit of the MLL3/4 complex (6). KDM6A protein stability also depends on the associated MLL3/4 proteins (18). More recent studies found that the TPR domains were necessary to facilitate KDM6A's association with the MLL3/4 complex. In particular, either the deletion of TPR domains or point mutations (G137V and D336G) in the TPR domains significantly reduce KDM6A's physical interactions with the MLL3/4 complex (19, 20).

KDM6A REGULATES ENHANCER ACTIVATION LARGELY INDEPENDENTLY OF ITS ENZYMATIC ACTIVITY

KDM6A function is largely independent of its enzymatic activity. While initial studies identified KDM6A as an H3K27me_{2/3} demethylase *in vitro*, follow-up findings have revealed that the roles of KDM6A in differentiation and normal development are independent of its enzymatic activity. In ESCs, KDM6A is required for differentiation along the mesoderm and ectoderm lineages. However, ESCs containing catalytically inactive KDM6A show normal differentiation (7). Meanwhile, catalytically inactive KDM6A is able to rescue the phenotype in KDM6A KO cells (21). *In vivo*, KDM6A demethylase activity appears to be dispensable for normal development, as homozygous KDM6A enzymatically dead knock-in (KI) female mice were found to be viable and fertile (22). In other organisms, such as *Caenorhabditis elegans*, KDM6A protein, but not its enzymatic activity, was found to play a critical role in posterior and gonadal development (23). KDM6A function as a tumor suppressor is also independent of its demethylase activity (11, 24).

KDM6A regulates enhancer activation through the MLL3/4 complex. Since KDM6A is a unique subunit of the MLL3/4 complex that is critical for enhancer activation (18, 25), studies have been done to elucidate the potential role of KDM6A in enhancer regulation. It was shown that KDM6A, as a component of the MLL3/4 complex, induced MLL4/p300-regulated enhancer activation and controls the synergy between H3K4me₁ and H3K27ac (10). KDM6A recruits the MLL4 complex to target genes by establishing interaction between MLL4 and the retinoic acid receptor (RAR)/retinoid X receptor (RXR) heterodimer. Moreover, KDM6A oversees the cross talk between MLL4 and p300 to synergistically facilitate MLL4-stimulated p300 recruitment and H3K27 acetylation. This establishes a feed-forward regulatory loop involving KDM6A, MLL4, and p300 that gives rise to a cooperative enhancer landscape. On enhancers regulated by this loop, deletion of KDM6A reduces H3K4me₁, H3K27ac, and target gene expression but does not affect H3K27me₃, suggesting that KDM6A regulates enhancer activation independently of its demethylase activity (Fig. 3) (10). Another study demonstrates that KDM6A loss is associated with repression of some superenhancers (SEs) and activation of others, as marked by H3K27ac and H3K4me₁. These effects correlate with the changes in MLL4 occupancy and H3K4me₁ signals over SEs in KDM6A KO cells (24).

KDM6A interacts with histone acetyltransferase and chromatin-remodeling complex. In addition to the MLL3/4 complex, KDM6A has also been reported to physically associate with the H3K27 acetyltransferase CBP and the enzymatic BRG1 (SMARCA4) subunit of the SWI/SNF chromatin-remodeling complex. In *Drosophila*, KDM6A was found to physically associate with CBP through its TPR domains. KDM6A colocalizes with CBP on many genomic regions marked by H3K27ac. Knockdown of KDM6A in *Drosophila* S2 cells moderately reduces the global H3K27ac level, suggesting that KDM6A regulates CBP-mediated H3K27ac (9). KDM6A was also found to physically associate with CBP in the breast cancer cell line MCF-7 as well as *in vitro* (26). Using

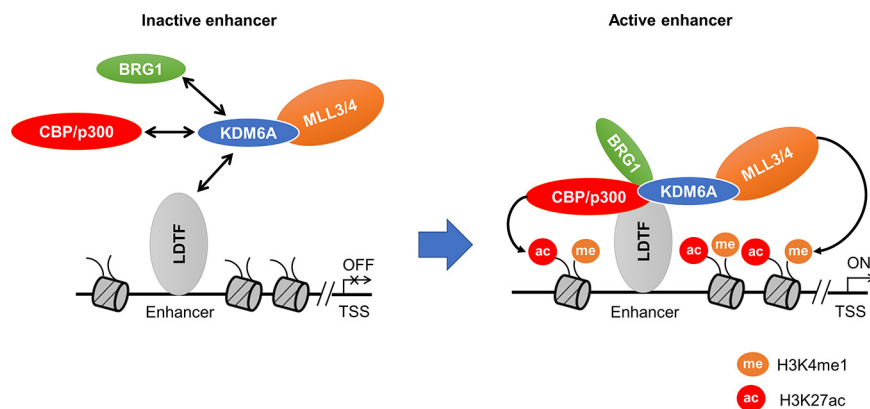


FIG 3 The role of KDM6A in facilitating enhancer activation. KDM6A is recruited to the enhancer region by lineage-determining transcription factor (LDTF). KDM6A then facilitates the recruitment of the associated H3K4 methyltransferase MLL3/4 and other chromatin modifiers, including H3K27 acetyltransferase CBP/p300 and the SWI/SNF-dependent chromatin remodeler BRG1. This process promotes histone methylation, acetylation, and chromatin opening, which subsequently activates enhancers and turns on gene transcription.

coimmunoprecipitation followed by Western blotting analysis, Miller et al. provided the initial evidence that KDM6A physically associates with BRG1 in T cells (27). Another study found that KDM6A promotes the interaction between BRG1 and TBX5, a T-box family transcription factor, and plays an enzymatically independent role in the recruitment of the BRG1-containing SWI/SNF complex to the enhancers of target genes (8). A recent study by Gozdecka et al. confirmed the physical association between KDM6A and BRG1 in mouse myeloid cells. They further showed that KDM6A loss in hematopoietic stem and progenitor cells (HSPCs) causes simultaneous downregulation of SWI/SNF-dependent chromatin accessibility and H3K27ac in certain pathways (such as the GATA transcriptional program) and upregulation in other pathways (such as the ETS program) (Fig. 3) (11).

KDM6A IN DIFFERENTIATION, DEVELOPMENT, AND REGENERATION

ESC maintenance and differentiation. (i) KDM6A is dispensable for ESC maintenance. Several studies have examined the role of KDM6A in the maintenance and differentiation of ESCs. It was consistently reported that in male ESCs, KDM6A KO had no effect on ESC proliferation, survival, or self-renewal. Expression of key ESC identity markers, including *Nanog*, *Oct4*, *Sox2*, and alkaline phosphatase, a marker for undifferentiated ESCs, is unaffected by KDM6A loss (7, 8, 21). UTY expression is also diminished in male KDM6A KO ESCs, thereby eliminating the potential compensatory function by UTY in the absence of KDM6A (7). These results indicate that KDM6A and UTY are dispensable for maintaining ESC identity.

(ii) KDM6A is necessary for differentiation of ESCs. While KDM6A is dispensable for ESC maintenance, ESC differentiation toward mesoderm and ectoderm requires KDM6A protein but not its demethylase activity. Meanwhile, KDM6A appears to be largely dispensable for endoderm differentiation in mouse ESCs (7, 21). Using an embryoid body (EB) differentiation model, Wang et al. demonstrated that KDM6A was important for mesoderm differentiation of ESCs. They found that KDM6A KO EBs could not differentiate into mesoderm lineage, as the cells retained the expression of *Nanog* and *Oct4* during the later stage of differentiation and could not induce the key mesoderm markers *Brachyury* (or *T*) and *Wnt3*. KDM6A was found to be enriched at the promoter of *Brachyury* and to regulate *Brachyury* expression through the Wnt/ β -catenin signaling pathway independently of its demethylase activity. Ectopic UTY expression in KDM6A KO cells could rescue Wnt/ β -catenin signaling-induced *Brachyury* expression, suggesting a compensatory function of UTY (7). Morales Torres et al. also confirmed the role of KDM6A, but not its enzymatic activity, in the induction of mesoderm markers

during both retinoic acid (RA)-induced monolayer differentiation and spontaneous EB differentiation of ESCs. They further showed that KDM6A KO impaired the induction of key ectoderm markers, such as *Sox1*, *Pax6*, *Msi1*, and *Otx2*, under both RA-induced and EB differentiation conditions (21). Mechanistically, KDM6A is recruited to the promoters of the marker genes of ectoderm and mesoderm to activate the differentiation programs (21). A recent study with human ESCs showed that KDM6A interacts with 53BP1 to regulate a subset of genes during ESC differentiation into human neural progenitor cells (hNPCs). KDM6A depletion in hNPCs downregulates many neurogenic genes, including *SOX4*, *FOXG1*, and *SIX3*, at day 22 of monolayer differentiation (28).

Early embryonic development. KDM6A is widely expressed in embryos and is critical for early embryonic development of mice. Homozygous KDM6A KO female embryos (*Kdm6a*^{-/-}) show more severe defects than KDM6A KO males carrying an intact *UTY* allele (*Kdm6a*^{-/γ}). Female homozygous KDM6A KO (*Kdm6a*^{-/-}) embryos die around embryonic day 12.5 (E12.5) to E13.5 (7, 29, 30). These embryos show severe midgestational embryonic defects around E9.5 to E10.5, including small, abnormal, truncated posterior and cardiovascular and neural tube closure defects, while male KDM6A KO (*Kdm6a*^{-/γ}) and female heterozygous KO (*Kdm6a*^{+/-}) embryos appear normal at this stage (7, 16, 29, 30).

The loss of one *Kdm6a* allele in male mice causes less severe embryonic defects than the deletion of both *Kdm6a* alleles in females. Several studies found that the majority of KDM6A KO males (*Kdm6a*^{-/γ}) showed perinatal lethality and only about 20 to 25% could survive through adulthood (7, 16, 29). The surviving males were significantly smaller and less viable than wild-type (WT) males (16, 29). Additionally, male embryos lacking both KDM6A and *UTY* alleles phenocopy homozygous KDM6A KO females (*Kdm6a*^{-/-}), indicating that *UTY* can partially compensate for the loss of KDM6A during embryonic development (16). Interestingly, female heterozygous KDM6A KO (*Kdm6a*^{+/-}) mice can survive through adulthood and appear normal, whereas KDM6A KO males (*Kdm6a*^{-/γ}) die around birth, suggesting that one copy of KDM6A would suffice to maintain normal development and that *UTY* cannot compensate for the loss of KDM6A in postnatal development (7, 16).

Tissue development. Beside its role in early embryonic development, KDM6A also plays a tissue-specific role in cardiac, mammary, and immune development (Table 2).

(i) Cardiac development. KDM6A, but not its enzymatic activity, is important for embryonic development of cardiac tissues. Female homozygous KDM6A KO embryos show a lack of cardiac looping and defective chamber formation. *UTY* compensates for KDM6A loss in cardiac development of male embryos (8, 16, 30). The requirements for KDM6A in cardiac development appear to be stage dependent. KDM6A KO embryos show a normal induction of cardiac TFs *GATA4*, *Nkx2.5*, *SRF*, and *Tbx5* in the myocardial lineage at E8.5. However, female homozygous KDM6A KO E9 embryos show lower overall levels of *Nkx2.5* and *Tbx5* than WT embryos (8). Consistent with the observations *in vivo*, a majority of cardiac lineage-committed embryoid bodies derived from KDM6A KO ESCs failed to develop spontaneous cardiac-like contractions in culture and could not induce expression of many cardiomyocyte genes, such as *ANF*, *α-CA*, *MLC2v*, and *Myh6* (8). Mechanistically, KDM6A is recruited to enhancers that contain response elements for key cardiac TFs *GATA4*, *Nkx2.5*, *SRF*, and *Tbx5* and acts as a coactivator for these TFs. KDM6A also promotes the interaction between *Tbx5* and *BRG1* to facilitate the recruitment of *BRG1* to enhancers of cardiac genes (8).

(ii) Mammary development. KDM6A controls the development of mammary luminal cell lineage and the expression of luminal cell-specific genes independent of its enzymatic activity. It was shown that the loss of KDM6A in mouse mammary luminal epithelium leads to a disorganized layout of luminal and basal cells, a decrease in luminal cell populations, an increase in basal cell populations, and misplaced alveoli from the main ducts during mammary development. As a consequence, these mice are unable to nourish offspring despite retaining milk production (31). Interestingly, a significant portion of luminal cells show basal cell-like characteristics in KDM6A KO

TABLE 2 Summary of mouse models used to study the functions of KDM6A in the development of different tissues

Tissue	Mouse model	Reference	Observed phenotype
Heart	Kdm6a ^{fl} ox; Ella-Cre	8	Defective cardiac looping; chamber formation around E8.5–E10.5
	Kdm6a ^{fl} ox; Sox2-Cre	16	
	Kdm6a ^{fl} ox; Vasa-Cre		
	Kdm6a ^{fl} ox; Prm1-Cre	7	
	Kdm6a ^{fl} ox; Pcgk1-Cre	30	
Mammary tissue	Kdm6a ^{fl} ox; MMTV-Cre	31	Aberrant luminal cell layout and misplaced alveoli in adult female mice. Unable to nourish offspring.
	Kdm6a enzyme-dead KI (H1146A/E1148A)		No effect on mammary tissues
	Kdm6a ^{fl} ox; Prm1-Cre	7	KO females display anemia at E10.5.
	Kdm6a ^{fl} ox; Cre-ERT2	30	KO females display myelodysplasia and splenic erythropoiesis.
Hematopoiesis and T cells	Kdm6a ^{fl} ox; CD4-Cre	32	Increased CD4-SP thymocytes and decreased CD4 ⁺ T cells in the spleen
		34	Reduced iNKT cells in the liver
	Kdm6a ^{fl} ox; Lck-Cre	33	Reduced CD4 ⁺ and CD8 ⁺ T cells and Tfh cells
	Kdm6a ^{fl} ox; Vav-Cre	35	Reduced iNKT cells in the liver, thymus, and spleen
Neural tissues	Kdm6a ^{fl} ox; nestin-Cre	37	Anxiety-like behaviors, impaired cognitive ability, aberrant hippocampal synaptic transmission and plasticity
		38	Decreased NSC differentiation
Retina	Kdm6a ^{fl} ox; Dkk3-Cre	39	Reduced PKC α -positive bipolar cells in retinas at P10
Muscle	Kdm6a ^{fl} ox; Pax7-CreER Kdm6a enzyme-dead KI (H1146A/E1148A)	22	Impaired muscle regeneration upon injury

mice. The expression of genes encoding luminal fate-determining TFs, such as STAT3, SOX9, and GATA3, is reduced, whereas basal signature genes, such as *Krt5*, *Krt14*, and *Acta2*, are induced upon KDM6A deletion in luminal cells (31). Mechanistically, more than half of genes enriched in luminal cells are bound by KDM6A at their promoters and enhancers. KDM6A colocalizes with mammary lineage-determining TFs, including NFIB and STAT5, on enhancers of luminal cell-specific genes, such as *Sox9* and *Krt8*, to regulate their expression, suggesting that KDM6A is a transcription coactivator important for maintaining the luminal lineage identity during mammary development (31). The mammary development defects were not observed in enzymatically dead KDM6A KI mice and the global H3K27me3 was unchanged in KDM6A KO mammary luminal cells, indicating that the function of KDM6A in mammary epithelial development is independent of its demethylase activity (31).

(iii) Immune development. Early work has shown that female homozygous KDM6A KO mouse embryos experience anemia around E10.5, suggesting that KDM6A is important for hematopoiesis (7). Another study utilized a tamoxifen-inducible KDM6A KO mouse model to demonstrate that depletion of KDM6A in female mice causes weight loss, anemic bones, myelodysplasia, and splenic erythropoiesis. In contrast, KDM6A depletion appears to have little effect on male mice, suggesting that UTY can compensate for the role of KDM6A in mouse hematopoiesis. Primary HPSCs isolated from KDM6A-deficient female mice also show a defect in cell migration (30). More recent studies have focused on the critical role of KDM6A in the development of T cells, including CD4 T cells and natural killer T (NKT) cells. Using a CD4-Cre-mediated KDM6A conditional knockout mouse model, Manna et al. revealed that the loss of KDM6A results in an increased amount of CD4 singly positive (SP) thymocytes in the thymus and a reduced amount of CD4⁺ T cells in the spleen while having no effect on cell proliferation, suggesting that KDM6A is important for the late-stage development of CD4⁺ T cells (32). KDM6A KO downregulates the expression of thymic egress gene *S1pr1* in CD4 SP thymocytes, leading to the accumulation of CD4 SP thymocytes and CD4⁺ lymphopenia. These defects caused by KDM6A KO could not be rescued by UTY, suggesting a requirement for KDM6A demethylase activity (32). Another group used a T cell-specific *Lck* promoter-driven Cre model to delete KDM6A in mice. The resulting

KDM6A KO mice showed moderate decreases in CD4⁺ and CD8⁺ T cells in the spleens. They also showed that KDM6A could support the development of CD4⁺ T follicular helper (Tfh) cells and that KDM6A was required for clearance of chronic viral infection by regulating the expression of Tfh-related genes, such as *Il6ra* (33).

KDM6A was also found to be important for invariant NKT (iNKT) cell development in mice. Using a similar CD4-Cre-mediated conditional knockout model, Northrup et al. showed that KDM6A loss leads to a significant reduction of iNKT cells in the liver, in addition to impaired CD4 T cell development (34). Specifically, KDM6A loss in thymocytes blocks the cells from undergoing thymic iNKT maturation. UTY could not support iNKT development in male KDM6A KO mice, indicating that the function of KDM6A is dependent on its demethylase activity (34). Using a Vav-Cre-mediated conditional knockout model, Beyaz et al. demonstrated that KDM6A was required for iNKT cell development in the liver, thymus, and spleen. They further showed that KDM6A was required for the expression of lineage-specific developmental genes, such as *Cxcr3*, *Il2rb*, *S100a6*, and *Tbx21*, in iNKT cells (35). Mechanistically, KDM6A is recruited to the promoters of many iNKT-specific genes and associates with iNKT lineage-determining TFs JunB and PLZF to regulate the expression of their target genes. The loss of KDM6A could hinder the superenhancer accessibility on iNKT cell identity genes, such as *Il2rb* and *Tbx21*, correlating with an increase of H3K27me3 signals on these superenhancers. Enzymatically inactive KDM6A fails to rescue the expression of these cell identity genes in KDM6A KO iNKT cells, suggesting that KDM6A has a demethylase-dependent function in the development of iNKT cells (35). In addition to T cell development, KDM6A has also been implicated in development of B cells (36).

(iv) Development of other tissues. KDM6A also plays a role in the development of other tissues. Two studies used nestin-Cre to conditionally knock out KDM6A in neural stem cells (NSCs) in mice. In one study, KDM6A KO mice show anxiety-like behaviors and impaired spatial learning and memory. The hippocampal regions of these mice displayed aberrant synaptic formation, transmission, and plasticity and impaired dendritic development, suggesting that KDM6A is important for neural development and cognitive behaviors (37). In another study, the loss of KDM6A increased NSC proliferation and downregulated a subset of genes related to NSC differentiation (38). A recent study also suggests that KDM6A is important for the differentiation of a subset of retinal cells. In particular, retina-specific knockout of KDM6A using *Dkk3-Cre* mice leads to a reduction in protein kinase C α (PKC α)-positive rod “on” bipolar cells in postnatal day 10 mouse retinas while having no effect on retinal progenitor cell proliferation (39). Using a CRISPR-Cas9 KO system in C2C12 myoblasts to screen a group of candidate lysine demethylases, a group reported that KDM6A was required for osteoblast differentiation in culture (40). However, the *in vivo* relevance of this study needs to be verified through animal models.

Regeneration. KDM6A plays a significant role in satellite cell (SC)-mediated muscle fiber regeneration. Using an SC-specific KDM6A KO mouse model, Faralli et al. (22) found that SC-specific KDM6A KO male and female mice experience impaired muscle regeneration after cardiotoxin-induced injury (Table 2). These mice showed a reduction in myofiber density, an increase in necrotic tissues and inflammatory cell infiltration, and a delayed recovery time. Interestingly, female mice with homozygous whole-body enzyme-dead KDM6A KI also showed impaired muscle regeneration after cardiotoxin treatment, whereas heterozygous SC-specific KDM6A KO females showed normal muscle regeneration (Table 2). These results indicate that KDM6A demethylase activity is important for SC-mediated muscle regeneration (22).

Mechanistically, KDM6A mediates the transition between proliferating and differentiating muscle progenitors during regeneration. KDM6A was shown to be dispensable for maintaining SC proliferation and the expression of SC identity genes, including *MyoD*, *Myf5*, and *Pax7*. However, KDM6A and its enzymatic activity are required to initiate differentiation of muscle progenitor cells, as the key TF MYOG could not be induced in either KDM6A KO or KI cells. During differentiation, KDM6A loss reduces the

expression of muscle regulatory gene *Myog* and increases H3K27me3 marks on myotube-specific enhancers. Additionally, differentiating myoblasts isolated from homozygous KDM6A KI mice show an enrichment of H3K27me3 signals at the promoters of myogenic genes, such as *Myog*, *Tnnc2*, and *Ckm*, suggesting that KDM6A regulates the terminal differentiation stage of muscle progenitor cells through a demethylase-dependent function (22).

KDM6A MUTATIONS IN DEVELOPMENTAL DISEASES AND CANCER

Kabuki syndrome. Kabuki syndrome (KS) is a rare human congenital craniofacial disorder manifesting as abnormal facial features, skeletal deformities, and heart and cognitive defects. Sixty percent to 89% of KS cases are caused by KMT2D (MLL4) mutations, whereas a smaller number of patients (6 to 14%) carry KDM6A mutations (41–44). There are no distinct phenotypic differences between KMT2D and KDM6A mutant KS patients (42). However, some studies reported that KMT2D KS patients appear to have more prominent facial abnormalities, while KDM6A KS patients show more growth defects (41, 45).

A recent study found that KDM6A was critical for craniofacial development in mice and for neural crest (NC) cell survival after the cell migration stage in embryos. NC-specific loss of KDM6A causes human-like Kabuki syndrome in mice (19). That study showed that about 40% of NC-specific KDM6A KO female ($Kdm6a^{-/-}$) pups died before weaning, while the male KO ($Kdm6a^{-/Y}$) mice could survive at least until weaning age. All NC-specific male KDM6A KO ($Kdm6a^{-/Y}$) and surviving female KO ($Kdm6a^{-/-}$) mice showed growth retardation and aberrant craniofacial features, such as frontonasal hypoplasia, increased facial angle, and reduced palpebral fissures, similar to the human KS phenotype. NC-specific KO ($Kdm6a^{-/Y}$) males showed milder defects than females ($Kdm6a^{-/-}$), suggesting that KDM6A loss could be partially compensated by UTY. Mechanistically, KDM6A regulates expression of craniofacial NC genes. In particular, KDM6A KO causes a downregulation of the NC stem cell signaling pathways, including PCP/Wnt/ β -catenin and Notch (19).

Interestingly, whole-body homozygous enzyme-dead KI female mice have normal body weight and facial development, suggesting that craniofacial development does not require KDM6A demethylase activity. However, females with one KI and one KO allele show human-like KS features similar to those in NC-specific KO males. These findings suggest that at least one WT KDM6A allele or two enzyme-dead KDM6A alleles are required for normal cranial NC development in mice (19).

KDM6A mutations are frequently present in cancers. An early study reported that KDM6A mutations are present in many tumor types, including multiple myeloma, esophageal squamous cell carcinoma, renal cell carcinoma, glioblastoma, and breast and colorectal cancers. Among 58 multiple-myeloma patient samples tested, about 10% had KDM6A mutations (46). A more comprehensive study of 4,742 tumor samples from 21 cancer types demonstrated that KDM6A is highly mutated across multiple cancer types, especially in bladder cancer (47). Urothelial bladder carcinoma is the most common type of bladder cancer and one of the most frequent cancers in men in developed regions of the world. Ler et al. used Sanger sequencing to analyze 176 urothelial bladder carcinoma patient samples and reported that KDM6A mutations were found in 45% of nonmuscle invasive urothelial bladder carcinoma, 28% of muscle-invasive tumors, and 28% of tumors of unknown stages. Further combining these findings with additional published data, they showed that KDM6A mutations were present in 29% of urothelial bladder carcinoma samples. The types of KDM6A mutations are widespread across the entire coding region of the gene and include frameshifting (insertion/deletion), nonsense, splice site, and missense mutations (48).

KDM6A mutations are also frequent in pancreatic cancer, one of the most common and lethal cancers in the world. According to public databases of pancreatic cancer genomes, the *KDM6A* gene is mutated in about 10.7% to 21.6% of pancreatic cancer patient samples (24). Mutations of KDM6A are also present in other cancers, including

TABLE 3 Summary of mouse models used to study the tumor-suppressive role of KDM6A in different cancer types

Cancer type	Mouse model	Reference	Observed phenotype
Bladder	Kdm6a ^{fllox} ; Upk2-Cre	53	Increased tumor burden and reduced survival rate
		54	Increased cancer development and CD44-positive stem cell proliferation
Lung	Kdm6a ^{fllox} ; Kras ^{G12D} ; Ad-Cre	55	Increased cancer progression and tumor burden. Shortened life span.
Pancreas	Kdm6a ^{fllox} ; Kras ^{G12D} ; Pdx1-Cre	24	More aggressive tumor development. Shortened life span.
	Kdm6a ^{fllox} ; Kras ^{G12D} ; Ptf1a-Cre		
	Kdm6a ^{fllox} ; Kras ^{G12D} ; Pdx1-Cre	20	
Blood	Kdm6a ^{fllox} ; Mx1-Cre	11	Higher chance to develop AML in female KO mice. Increased HSPC self-renewal and decreased HSPC differentiation.
	Kdm6a ^{fllox} ; CD19-Cre	36	Accelerated E μ -Myc-induced B cell lymphomagenesis
	Kdm6a ^{fllox} ; CreER	57	Accelerated chronic myelomonocytic leukemia and shortened life span

chronic myelomonocytic leukemia, metastatic castration-resistant prostate cancer, subgroups of medulloblastoma, and acute myeloid leukemia (AML) (46, 49–52).

KDM6A is a tumor suppressor. An early study showed that reintroduction of WT KDM6A into KDM6A-deficient esophageal squamous carcinoma cells results in slower cell growth, suggesting a role for KDM6A as a tumor suppressor (46). Later studies have reported a role for KDM6A as a tumor suppressor in different cancer types (Table 3). In one study, the loss of KDM6A in a bladder carcinoma cell line increased the cell proliferation rate by about 25%. Analysis of urothelial bladder carcinoma samples revealed that KDM6A loss activates EZH2-dependent transcriptional repression. EZH2 is a histone methyltransferase that, in contrast to KDM6A, adds a methyl group to H3K27 to downregulate gene transcription. Further analysis of KDM6A-deficient urothelial bladder carcinoma cells showed that H3K27me3 signals are enriched at the promoter regions of EZH2 target genes, including *PIP5K1B* and *GHR*. This enrichment could be reversed by ectopic KDM6A. These findings suggest that KDM6A functions as a tumor suppressor in urothelial bladder carcinomas by antagonizing EZH2-mediated transcriptional repression (48). Kaneko and Li showed that urothelium-specific KDM6A KO increases bladder cancer risk in female mice. These mice have a significantly lower survival rate upon bladder cancer induction than do female WT mice. The loss of KDM6A also reduces the expression of the tumor suppressor genes *Cdkn1a* and *Perp*. Ectopic expression of WT and enzyme-dead KDM6A could suppress cell proliferation and colony formation of a KDM6A-null bladder cancer cell line, suggesting that the tumor suppression function of KDM6A is demethylase independent (53). Another study also used a urothelium-specific KDM6A KO mouse model and reported that KDM6A-deficient mice can develop bladder cancer and have an increased number of CD44-positive bladder stem cells. The loss of KDM6A in these mice also upregulates proinflammatory cytokines such as *Cxcl1*, *Ccl2*, and *Il6* (54).

Another study has identified KDM6A as a tumor suppressor in a mouse model of metastatic lung cancer. Using the oncogenic Kras^{G12D}-induced lung cancer mouse model, Wu et al. found that deletion of KDM6A promotes lung cancer progression and increases tumor burden (55). KDM6A loss also shortens the life span of Kras^{G12D} mice from 30 weeks to 17 weeks. Tumors extracted from KDM6A KO Kras^{G12D} adult mice are significantly larger and have a higher cell proliferation rate than those from control mice. KDM6A loss promotes primary lung cancer cell growth, which could be rescued by ectopic expression of KDM6A. Notably, KDM6A KO in Kras^{G12D} lung tumors upregulates the expression of H3K27 methyltransferase EZH2 at both the mRNA and protein levels. KDM6A loss also downregulates the expression of tumor suppressors and cell cycle inhibitors CDKN2A and CDKN2B, which may explain the increase in cell proliferation in KDM6A KO tumors (55).

Three studies have identified KDM6A as a tumor suppressor in pancreatic cancer. By CRISPR-Cas9-mediated deletion of KDM6A in two human pancreatic ductal adenocarcinoma cell lines, Watanabe et al. showed that the loss of KDM6A promotes cell

proliferation, colony formation, and migration, whereas overexpression of KDM6A could suppress these traits. They further revealed that KDM6A depletion decreases the expression of tumor suppressor genes, including *CDKN1A*. Interestingly, H3K27ac is downregulated at the enhancers of *CDKN1A* in KDM6A KO cells (56). Using a *Kras*^{G12D}-induced cancer mouse model, Andricovich et al. showed that pancreas-specific KO of KDM6A causes squamous-like, metastatic pancreatic cancer in females. These mice show aggressive tumor development and shortened survival time. Interestingly, the induction of squamous-like, metastatic pancreatic cancer in male mice requires the loss of both KDM6A and the homologous UTY (24). Mechanistically, KDM6A loss deregulates MLL4 genomic binding and activates the superenhancers (SEs) controlling the expression of oncogenes *Myc*, *ΔNp63*, and *Runx3*. Ectopic expression of the WT, enzyme-dead KDM6A, or UTY in human KDM6A KO pancreatic tumor cell lines downregulates *Myc* and *ΔNp63* expression and inhibits cell proliferation, indicating that the tumor suppressor role of KDM6A in pancreatic cancer is demethylase activity independent (24). A recent study confirmed the role of KDM6A as a tumor suppressor in pancreatic cancer using the *Kras*^{G12D} mouse model. Mechanistically, KDM6A is recruited by HNF1A to regulate the enhancer landscape of acinar cells and activate the epithelial gene expression program that indirectly suppresses oncogenic pathways. HNF1A is a homeodomain transcription factor that had been thought to be a pancreatic tumor suppressor. It was also shown that HNF1A loss partially phenocopies KDM6A KO in *Kras*^{G12D}-induced pancreatic cancer (20).

KDM6A also plays a role in suppressing myeloid leukemogenesis independently of its demethylase activity. Using a hematopoietic stem and progenitor cell (HSPC)-specific KDM6A deletion mouse model, Gozdecka et al. showed that female homozygous KDM6A KO (*Kdm6a*^{-/-}) mice have a high chance (63%) to develop AML (11). These mice show an increase in HSPC self-renewal and myeloid expansion and a decrease in differentiation. In contrast, female heterozygous KO mice (*Kdm6a*^{+/-}) and male KDM6A KO mice (*Kdm6a*^{-Y}) do not manifest AML. Ectopic expression of KDM6A, enzyme-dead KDM6A, or UTY in a KDM6A/UTY-deficient AML cell line could suppress cell proliferation, suggesting that the function of KDM6A in AML suppression does not require its demethylase activity. KDM6A loss in HSPCs leads to a change in SWI/SNF-dependent chromatin accessibility, indirectly facilitating the oncogenic ETS transcriptional program while simultaneously impairing the tumor-suppressive GATA program (11). In addition to AML, KDM6A has also been identified as a tumor suppressor in other types of blood cancer, including B cell lymphoma, chronic myelomonocytic leukemia, multiple myeloma, and T cell acute lymphoblastic leukemia (T-ALL) (36, 57–60).

Together, these studies demonstrate that KDM6A functions as a tumor suppressor independently of its demethylase activity in a variety of cancer types by regulating different transcriptional programs, especially those involved in oncogenesis and tumor suppression.

Pro-oncogenic role of KDM6A. Even though many studies have reported the role of KDM6A as a tumor suppressor, some studies have provided evidence to suggest that KDM6A could play a pro-oncogenic role. Despite KDM6A being previously described as a tumor suppressor in T-ALL (59, 60), a 2016 study reported that KDM6A functions as a pro-oncogenic coactivator in a subgroup of T-ALL that expresses the oncogenic TF TAL1. KDM6A physically associates with TAL1 and is recruited by TAL1 to its target genes. Depletion of KDM6A in several TAL1-positive cell lines leads to an increase in apoptosis, whereas overexpression of KDM6A promotes cell growth (61). Another study suggests that KDM6A plays a role in promoting estrogen receptor (ER)-positive breast cancer cell proliferation and migration (26). The loss of KDM6A in a human breast cancer cell line, MCF-7, causes a significant decrease in estrogen-induced cell proliferation. Depletion of KDM6A also suppresses, whereas overexpression of KDM6A enhances, cell migration. Mechanistically, KDM6A colocalizes with ER α on a subset of ER α target genes and regulates their expression in MCF-7 cells. These genes include *GREB1*, *TFF1*, *MYB*, and *CXCR4*, which are known to be involved in breast cancer development.

KDM6A also recruits the H3K27 acetyltransferase CBP to ER α target genes to regulate H3K27ac signals (26). Interestingly, while KDM6A controls the estrogen-induced expression of the oncogene *CXCR4*, ectopic expression of enzyme-dead KDM6A could not rescue *CXCR4* expression in KDM6A KO MCF-7 cells, suggesting that KDM6A plays a demethylase-dependent role in facilitating the ER α -mediated transcriptional program to promote breast cancer cell migration (26). Whether KDM6A facilitates or impedes ER-positive breast cancer progression *in vivo* remains to be determined.

FUTURE DIRECTIONS

Many studies in the past decade have demonstrated that KDM6A functions in normal development and tumor suppression are largely independent of its enzymatic activity. For example, mouse embryonic midgestational development and development of certain tissues do not require a catalytically active KDM6A (7, 8, 16, 31). As a tumor suppressor in pancreatic cancer and AML, KDM6A also plays a demethylase activity-independent role (11, 24). It remains unclear how KDM6A regulates these processes without its enzymatic function. One hypothesis is that KDM6A regulates gene expression and enhancer activation through its interaction with MLL3/4, since MLL3/4 is known to be critical for enhancer activation and KDM6A protein stability depends on MLL3/4 (Fig. 3) (18, 25). Supporting this hypothesis, it has recently been shown that G137V and D336G mutations in the TPR domains of KDM6A impair its physical interaction with MLL3/4 and destabilize the KDM6A protein. G137V mutation also impairs the ability of KDM6A to suppress colony formation in a soft-agar assay, suggesting that KDM6A functions as a tumor suppressor through its interaction with MLL3/4 (62). The mechanism behind KDM6A genomic targeting also requires further investigation. It has been hinted that certain TFs could interact with KDM6A, which, in turn, recruits MLL3/4 to activate enhancers (20, 24).

While many findings have shown the enzymatic activity-independent role of KDM6A in normal development, several studies have shown that KDM6A demethylase activity is important in certain cellular contexts, such as T cell development and muscle regeneration (22, 35). Future work is needed to validate the tissue-specific role of KDM6A demethylase activity *in vivo* by utilizing enzyme-dead KDM6A KI mice. Unlike mammals, *Drosophila* has only one KDM6 ortholog, dUtx. It was reported that dUtx demethylase activity was important for embryonic and larval development, as enzyme-dead dUtx *Drosophila* flies die during the larval stage. These observations in *Drosophila* suggest that there could be some redundancy between the enzymatic activities of KDM6A and other KDM6 family members, in particular KDM6B, in mammals (63). One approach to study this redundancy is to generate mice harboring both demethylase-inactive KDM6A and KDM6B. It also remains unclear whether KDM6A enzymatic activity can target nonhistone substrates other than the known target H3K27me2/3.

The rapid progression and development of new tools and technology have vastly accelerated the studies of protein structure and function, which can be utilized to facilitate a more in-depth understanding of the KDM6A protein. Glycogen synthase kinase J4 (GSK-J4) is a potent small-molecule inhibitor for KDM6A. It can effectively inhibit KDM6A enzymatic activity, with a half-maximum inhibitory concentration (IC₅₀) of 6.6 μ M. However, this inhibitor is not specific to KDM6A, as it can also potentially inhibit KDM6B (IC₅₀ = 8.6 μ M) and other demethylases (64). The development of a highly specific small-molecule inhibitor of KDM6A would facilitate in-depth studies to distinguish the functions of KDM6A protein and its enzymatic activity. Advanced live-cell imaging has been one of the most rapidly developing technologies in the past decade and could be employed as a powerful tool to determine KDM6A cellular localization and its dynamic interactions with TFs, MLL3/4, or other chromatin regulators during enhancer activation, gene expression, and cell differentiation. Recently, cryo-electron microscopy (cryo-EM) has been used to study the structure of the MLL3 catalytic module consisting of the catalytic SET domain of MLL3 and associated regulatory factors ASH2L, RBBP5, WDR5, and DPY30 (65). However, the catalytic C-terminal SET domain of MLL3 does not associate with KDM6A. Future studies using cryo-EM are

needed to determine how KDM6A interacts with full-length MLL3/4 in the intact holocomplex.

As KDM6A is a known tumor suppressor in many cancer types, many attempts have been made to develop potential therapeutics to counteract cancer growth in KDM6A-mutated tumors. For example, KDM6A-deficient urothelial bladder carcinoma cells were found to be more sensitive to EZH2 inhibitors, such as GSK343 and GSK126. These inhibitors could effectively suppress *in vivo* onset and growth of KDM6A-deficient tumors in mice (48). Consistent with the study, another EZH2 inhibitor, JQEZ5, suppresses the growth of KDM6A-deficient lung tumors in a *Kras*^{G12D} mouse model (55). In pancreatic cancer, KDM6A-deficient cell lines are sensitive to the bromodomain and extraterminal domain inhibitors (24). These findings suggest promising strategies for developing treatments of KDM6A-mutated cancers, which should be further explored. Finally, mutation of KDM6A is one of the main causes of Kabuki syndrome (KS). Since this rare genetic disorder currently has no cure, targeted gene therapy can be a potential treatment to correct the KDM6A mutations in KS patients.

KDM6A has also been proposed to be an oxygen sensor (66). It was shown that KDM6A protein and the isolated KDM6A enzymatic domain display low oxygen affinities *in vitro*. Hypoxia appears to moderately increase cellular H3K27me3 levels in a KDM6A-dependent manner in several cell types. Using myogenesis as a model system, the authors showed that either hypoxia treatment or KDM6A depletion blocks cell differentiation. These results suggest that hypoxia inhibits KDM6A-mediated removal of H3K27me3 in cells (66). However, it is unclear whether hypoxia directly inhibits KDM6A enzymatic activity. Future work is needed to establish the functional relevance of KDM6A as an oxygen sensor *in vivo*.

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