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# Chapter 4. Histone demethylase KDM3 (JMJD1) in transcriptional regulation and cancer progression

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## Abstract

Methylation of histone H3 lysine 9 (H3K9) is a repressive histone mark and associated with inhibition of gene expression. KDM3 is a subfamily of the JmjC histone demethylases. It specifically removes the mono- or di-methyl marks from H3K9 and thus contributes to activation of gene expression. KDM3 subfamily includes three members: KDM3A, KDM3B and KDM3C. As KDM3A (also known as JMJD1A or JHDM2A) is the best studied, this chapter will mainly focus on the role of KDM3A-mediated gene regulation in the biology of normal and cancer cells. Knockout mouse studies have revealed that KDM3A plays a role in the physiological processes such as spermatogenesis, metabolism and sex determination. KDM3A is upregulated in several types of cancers and has been shown to promote cancer development, progression and metastasis. KDM3A can enhance the expression or activity of transcription factors through its histone demethylase activity, thereby altering the transcriptional program and promoting cancer cell proliferation and survival. We conclude that KDM3A may serve as a promising target for anti-cancer therapies.

### Keywords

histone demethylase; KDM3A; KDM3B; KDM3C; JMJD1A; JMJD1B; JMJD1C; cancer; epigenetics; transcriptional regulation

## 1. Introduction

The fundamental unit of chromatin is the nucleosome, which is comprised of a segment of DNA that wraps around the octamer of core histones, which consists of two copies each of H2A, H2B, H3 and H4. The specific amino acid residues in the histone tails are subject to various types of posttranslational modifications such as phosphorylation, ubiquitination, acetylation and methylation. These modifications alter chromatin conformation and recruit additional epigenetic regulators and transcription factors, thereby regulating transcriptional gene expression in response to specific signals during various biological processes. Methylation of specific lysine residue(s) in the histone tail can activate or repress gene

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expression. For example, methylation of histone-3-lysine-4 (H3K4) is an active histone mark associated with the transcriptional gene activation, whereas methylation of histone-3-lysine-9 (H3K9) is a repressive histone mark associated with the inhibition of gene expression [1]. Methylation of H3K9 can recruit heterochromatin protein 1 (HP1) and corepressor complexes for heterochromatin formation and silencing of gene expression.

The steady state of histone methylation is determined by the balance between addition of methyl groups by histone methyl transferases (HMTs) and removal of methyl groups by histone lysine demethylases (KDMs). Based on the enzymatic mechanism, the KDMs can be categorized into two main families [2]. The first family includes two members, KDM1A (also known as lysine specific demethylase 1, LSD1) and KDM1B (LSD2). They are the flavin adenine dinucleotide (FAD)-dependent amine oxidases, and can remove mono- and dimethyl histone marks. The second family of KDMs is characterized by presence of the Jumonji C (JmjC)-domain, which is the catalytic domain for histone demethylation. They are the Fe(II) and α-ketoglutarate (KG)-dependent dioxygenases, and can remove mono-, di- and tri-methyl marks from specific histone lysines. Over 30 members of the JmjC family histone demethylases have been identified, and they can be categorized into seven subfamilies based on the sequence or structure homologies [3]. KDM3A (also known as JMJD1A or JHDM2A) belongs to the KDM3 subfamily, which includes two other members, KDM3B (also known as JMJD1B or JHDM2B) and KDM3C (as known as JMJD1C or JHDM2C). The genes encoding KDM3A, B and C are located on human chromosomes 2p11.2, 5q31.2 and 10q21.3, respectively. The KDM3 subfamily is evolutionally conserved, with orthologs of KDM3A, B, and C found among all vertebrates. The KDM3A, B and C proteins share around 50% sequence identity, and have a C2HC4 zinc finger and a C-terminal JmjC domain (Figure 4.1). They can function as coactivators for specific transcription factors, remove the repressive mono- or di-methyl marks from H3K9 (H3K9me1/2), and facilitate the binding of transcription factors to their cognate DNA sequence (Figure 4.2) [4-6]. However, KDM3C only showed *in vitro* activity against H3K9me1 peptide [7]. KDM3B was recently shown to also demethylate H4R3me2s and H4R3me1 [8]. Among the three KDM3 members, KDM3A is most extensively studied. Hereby, we will mainly review the function of KDM3A in transcriptional gene regulation, normal physiology and cancer biology.

## 2 Discovery of KDM3A as an H3K9me1/2 demethylase

The Zhang group was the first to characterize the function of KDM3A as an H3K9me1/2 demethylase [4]. In a demethylation assay using methylated histone as substrates, they detected potential H3K9 demethylase activity in one fraction of HeLa cell nuclear extracts. This demethylase activity was dependent on cofactors Fe(II) and α-KG, a requirement of the JmjC family of demethylases. Further fractionation coupled with mass spectrometry analysis identified KDM3A as a candidate for the demethylase activity. Purified KDM3A protein possessed the histone demethylase activity, but not the KDM3A mutant protein with truncation or a point mutation in its JmjC domain. In the demethylation assay using methylated lysine sites in histone H3 (K4, K9, K27, K36, K79) and H4 (K20), KDM3A only demethylated the methylated H3K9, demonstrating that KDM3A is a H3K9-specific demethylase. Lysine methylation can exist in three states as mono-, di-, and trimethylation. Using the methylated H3K9 peptides as substrates, KDM3A could demethylate both mono-

and dimethyl-K9, but no the trimethyl-K9 peptide, demonstrating that KDM3A selectively demethylates mono- and dimethylated H3K9 (H3K9me1/2) [4].

## 3. KDM3 in the demethylation of non-histone proteins

KDM3A can demethylate the substrate protein other than H3K9me1/2. For example, KDM3A can demethylate tumor suppressor p53 in breast cancer cells [9]. Knockdown of KDM3A in MDA-MB-231 or Hs578T cells enhanced apoptosis in response to chemotherapeutic drugs such as cisplatin or paclitaxel. Knockdown of KDM3A in these breast cancer cells increased the expression of pro-apoptotic genes such as PUMA, NOXA or BAX. Methylation of p53 at K372 was reported to enhance p53 activity [10]. KDM3A was co-precipitated with p53 and knockdown of KDM3A increased the level of p53-K372me1 in breast cancer cells or in *in vitro* reaction, which suggests that KDM3A can demethylate p53 at K372. KDM3A knockdown increased the level of p53 or p53-K372me1 on PUMA and NOXA promoters. Taken together, these results suggest that knockdown of KDM3A increases p53-K372me1 levels and p53 activity for the expression of some pro-apoptotic genes.

KDM3C can also demethylate the substrate protein other than H3K9me1/2. One such example is Mediator of DNA damage checkpoint protein 1 (MDC1), which functions as a regulator of cell cycle checkpoints and recruits repair proteins to the site of DNA double-strand breaks. KDM3C was found to demethylate MDC1 at lysine 45, which, in turn, promoted MDC1-RNF8 interaction, RNF8-induced ubiquitination of MDC1, and recruitment of DNA repair protein RAP80–BRCA1 to polyubiquitinated MDC1 [11].

## 4. Physiological Functions of KDM3A

Five *Kdm3a*-deficient mouse lines have been established and reported thus far (Table 4.1). These mouse models offer the opportunity to evaluate the physiological role of KDM3A. The phenotypes of *Kdm3a*-deficient mice include defects in spermatogenesis, fat metabolism, sex determination and stem cell activity. We will summarize the main phenotypes of *Kdm3a*-deficent mice and the possible mechanisms that underlie these mutant phenotypes.

### 4.1 Spermatogenesis

KDM3A was initially identified as a transcript that was highly expressed in the male germ cells [12]. The levels of KDM3A mRNA and protein were increased during the process of spermatogenesis in mice [13]. Four out of five *Kdm3a*-deficient mouse lines (Table 4.1) showed phenotypes of spermatogenesis defect, smaller testes and male infertility [13-16]. The spermatids from the *Kdm3a*-mutant mice showed defects in the chromatin condensation [13,16], an important event required for spermatogenesis. In the male germ cells, transcription factor Crem and its coactivator Act control expression of multiple genes including Tnp1, Tnp2, Prm1 and Prm2, which are required for the chromatin condensation [17,18]. In one study, KDM3A was shown to promote the expression of Tnp1 and Prm1 by binding to and removing the H3K9 methylation marks at their promoters [13]. In another study, KDM3A was shown to increase the expression of Tnp1, Tnp2, Prm1 and Prm2 by

promoting Crem recruitment and Act expression [15]. These studies suggest that the reduced expression of genes required for chromatin condensation may underlie the spermatogenesis defect observed in *Kdm3a*-deficient mice. Similarly, the *Kdm3c*-knockout mice showed spermatogenesis defects as well as male infertility [19,20], which further supported the key role for the KDM3 family histone demethylases in spermatogenesis.

### 4.2 Metabolism

Two *Kdm3a*-deficient mouse lines showed phenotypes of obesity and metabolism defects [14,21]. The *Kdm3a*-deficienct mice showed accumulation of large fat droplets in the adipose tissue, muscle and liver, as well as increased serum lipid content [14] and metabolic syndromes that include hypertriglyceridemia, hypercholesterolemia, hyperinsulinemia and hyperleptinemia [21]. The gene expression profile studies on the wild-type and *Kdm3a*-deficient skeletal muscles [14] revealed downregulation of genes associated with the PPAR signaling pathway, the master regulator of adipogenesis and obesity. Reduced expression of Ppara was found in the *Kdm3a*-deficient myocytes, concomitant with an increased level of H3K9me2 at the PPAR responsive element of the Ppara gene [14], which indicated that KDM3A increased the expression of Ppara by H3K9 demethylation on the Ppara gene.

Ucp1 is a protein important for cold-induced heat generation in brown fat tissues. In response to β-adrenergic stimulation, KDM3A was found to increase the expression of Ucp1 in brown adipose cells by binding to and demethylating H3K9 at the enhancer of the Ucp1 gene, facilitating the recruitment of several transcription factors and co-activators [14]. In another study, β-adrenergic stimulation was found to induce the phosphorylation of KDM3A at S265 by protein kinase A (PKA), and this promoted the interaction of KDM3A with SWI/SNF nucleosome remodeling complex and DNA-bound PPARγ. This phosphorylation is important to the KDM3A-dependent expression of β1-adrenergic receptor gene (Adrb1) and metabolic regulators including Ucp1 in brown adipose cells [22]. These studies suggest that downregulation of metabolic regulators such as Ppara and Ucp1 may underlie the obesity and metabolism defect observed in the *kdm3a*-deficient mice.

### 4.3 Sex determination

One *Kdm3a*-deficient mouse model, which was established from C57BL/6 (B6) x CBA F1 embryonic stem cells [23], was reported to have the male-to-female sex reversal phenotype [24]. Some of the *Kdm3a*-deficient XY mice were sex-reversed, either partially with one testis and one ovary or completely with two ovaries [24]. The *Syr* gene is located on Y chromosome and encodes a transcription factor required for male development [25]. Expression of Syr was decreased in the *Kdm3a*-deficient gonadal somatic cells, concomitant with the increased level of H3K9me2 within the *Syr* locus. Forced expression of *Sry* transgene in the *Kdm3a*-deficient mice rescued the defect of testis cord development in the XY gonads [25]. GLP/G9a methyltransferase complex was found to induce H3K9 methylation at the Sry locus and counteract the role of KDM3A in the sex determination [26]. A heterozygous GLP mutation or a GLP/G9a inhibitor restored Sry expression and rescued the sex-reversal phenotype in the *Kdm3a*-deficient mice [26]. These studies demonstrate role of KDM3A in the expression of Syr and male development of mice.

### 4.4 Stem cell pluripotency

Embryonic stem cells (ESCs) are pluripotent and are able to self-renew indefinitely. KDM3A was reported to regulate the self-renewal of ESCs [21]. Knockdown of KDM3A in ESCs resulted in the cell differentiation, accompanied by reduced expression of pluripotency-associated genes such as Tcl1, Tcfcp2/1, and Zfp57. ChIP-PCR analysis revealed an increased level of H3K9me2 on the Tcl1 promoter upon KDM3A knockdown. Forced restoration of Tcl1 expression in KDM3A-knockdown ES cells reversed the differentiation phenotype and maintained ES cell morphology. This study revealed that KDM3A promotes the expression of Tcl1 by H3K9 demethylation on the Tcl1 promoter and that Tcl1 is an important downstream effector of KDM3A for ESC self-renewal. Double knockout of *Kdm3a/Kdm3b* caused the elevation of H3K9me2 marks and perturbed gene expression in ESC, resulting in rapid ESC death and embryonic lethality [27]. This study further demonstrates a critical role for KDM3A/KDM3B-meditated H3K9 demethylation in ESC maintenance and early embryogenesis.

Somatic cells can be reprogrammed to pluripotency through fusion with ESCs, and reactivation of transcription factor Oct4 is a hallmark for effective reprograming. Ectopic overexpression of KDM3A in adult neural stem cells (NSCs) resulted in the global H3K9 demethylation and increased Oct4 reactivation upon ESC fusion, whereas overexpression of a catalytic-inactive KDM3A had no effect [28]. This study revealed that KDM3A and its H3K9 demethylase activity could promote the reprogramming efficacy of NSCs upon fusion with ESCs. Somatic cells can be reprogrammed to pluripotency through the ectopic expression of Oct4, Sox2, Klf4, and c-Myc (OSKM), which results in the generation of induced pluripotent stem cells (iPSCs). The porcine iPSCs induced by OSKM and KDM3A were shown to express higher levels of pluripotency-associated genes, compared with those induced by OSKM alone [29]. Thus, these studies reveal a role for KDM3A in the reprograming of somatic cells to pluripotency.

## 5 Roles of KDM3A in gene regulation and cancer

KDM3A was upregulated in various types of cancers, such as neuroblastoma [30], sarcoma [31], prostate cancer [32], breast cancer [9], hepatocellular carcinoma [33], renal cell carcinoma [34], colon cancer [35], cervical cancer [36], gastric cancer [37] and non-small cell lung cancer [38]. KDM3A knockdown studies in various cancer cells largely point to a tumor-promoting role of KDM3A. KDM3A has been found to promote the activity and/or expression of several key transcription factors and related transcriptional programs through its demethylase activity (Figure 4.3). Here, we will highlight some key transcription factors that are regulated by KDM3A and role of KDM3A-dependent gene regulation in cancer biology.

### 5.1 KDM3A is a coactivator for the androgen receptor and estrogen receptor

The KDM3 protein contains a LXXLL sequence (Figure 4.1C), which is a signature motif that mediates the binding of transcriptional coactivators to nuclear receptors [39]. Androgen receptor (AR) belongs to the steroid nuclear receptor superfamily. It plays a key role in the progression of prostate cancer and is the primary therapeutic target for metastatic prostate cancer. Knockdown of KMD3A was found to decrease ligand-induced expression of AR

target genes such as PSA, NKX3.1 and TMPRSS22 in LNCaP cells [4]. KDM3A was found to interact with AR and binds to the enhancers of PSA and NKX3.1 in a ligand-dependent manner, concomitant with a reduction of H3K9me2 levels on those enhancers. In contrast, knockdown of KDM3A reduced the recruitment of KDM3A to the enhancers of PSA and NKX3.1 and diminished the ligand-induced reduction of H3K9me2 on these enhancers [4]. Hypoxia induced the upregulation of KDM3A expression in LNCaP cells, which is associated with the elevation of PSA expression, as well as an increased recruitment of KDM3A to and H3K9 demethylation of the PSA enhancer [40]. These studies showed that KDM3A promoted ligand- or hypoxia-induced expression of example AR targets in LNCaP cells through H3K9 demethylation. CWR22Rv1 cells are androgen-independent prostate cancer cells [41]. AR was implicated to be one of the top transcription factors regulated by KDM3A in CWR22Rv1 cells, based on the analysis of differentially expressed genes following KDM3A knockdown [42]. KDM3A was shown to promote H3K9 demethylation, AR chromatin recruitment and expression of select AR target genes in CWR22Rv1 cells [42,43]. AR-V7 is a constitutively-active truncated form of AR and associated with the resistance of prostate cancer to the AR-targeted therapy [44]. KDM3A was found to promote the recruitment of a splicing factor HNRNPF to a cryptic exon3b and enhance the alternative splicing of AR-V7 [45]. These studies revealed two mechanisms for KDM3A in the regulation of AR transcriptional activity by increasing the AR chromatin recruitment and AR-V7 generation. The future development of KDM3A inhibitors may offer an opportunity to inhibit AR activity and thus serve as a potential therapy for advanced prostate cancer.

KDM3A was also found to promote transcriptional activity of estrogen receptor (ER) in breast cancer cells [46]. The profiling array analysis showed that 42% of estrogen-stimulated or repressed genes were down- or up-regulated at least 1.5-fold in the KDM3A-knockdown MCF-7 breast cancer cells, which suggests that KDM3A positively regulates a significant portion of the ER transcriptional program. Re-expression of wild-type, but not the catalytically-inactive KDM3A, significantly rescued the downregulation of ER target genes such as pS2, GREB1 and CCND1 in the KDM3A-knockdown MCF-7 cells, which indicates that the demethylase activity of KDM3A is required for the expression of these ER target genes. Knockdown of KDM3A in MCF-7 cells increased the H3K9me2 and decreased the recruitment of ER to the pS2 and GREB1 genes. Knockdown of KDM3A in MCF-7 or T47D cells resulted in G1 cell cycle arrest and inhibition of cell proliferation. Hormone therapy with the estrogen-receptor modulator tamoxifen is effective for the treatment of ER-positive breast cancer, but resistance to the tamoxifen invariably occurs. Knockdown of KDM3A inhibited the proliferation of MMU2 cells, which are a tamoxifen-resistant line derived from MCF-7 cells. These results suggest that targeting KDM3A may inhibit the activity of ER and thus contribute to the improved endocrine therapy for breast cancer.

Overexpression of the HER2 receptor tyrosine kinase is known to promote resistance of breast cancer to tamoxifen therapy [47]. ACK1 is a non-receptor kinase that can be activated by various receptor tyrosine kinases including HER2 [48]. One study found that heregulin-mediated ACK1 activation promoted ER activity in the presence of tamoxifen through tyrosine phosphorylation of KDM3A [49]. HOXA1 is a potent oncogene that can transform human mammary epithelial cells upon overexpression [50]. ACK1 inhibitors blocked the heregulin-induced upregulation of HOXA expression, concomitant with the accumulation of

H3K9me2 and loss of ER binding at the intron one of HOXA gene. This study suggests that ACK1 induces tyrosine phosphorylation of KDM3A to enhance ER transcriptional activity and contributes to tamoxifen resistance. Interestingly, ACK1 was also found to induce tyrosine phosphorylation of AR and promote the AR transcriptional activity under androgen deprivation conditions [51]. It will be interesting to test whether ACK1 also induces tyrosine phosphorylation of KDM3A in prostate cancer and whether this contributes to the transcriptional activity of AR.

### 5.2 KDM3A acts as a coactivator for other transcription factors

In addition to AR and ER, KDM3A was reported to function as coactivators for other transcription factors. JAK-STAT3 signaling is known to play an oncogenic role in various malignancies. KDM3A can contribute to the activation of JAK-STAT3 signaling, by serving as a coactivator for STAT3 to promote STAT3-mediated gene expression [52]. TEAD1 is a transcription factor mediating the expression of hippo target genes in the Hippo signaling pathway. KDM3A can interact with and function as a coactivator for TEAD1 in colorectal cancer cells [53]. KDM3A can also interact with and function as a coactivator for β-catenin to promote the Wnt signaling in colorectal cancer cells [54,55].

### 5.3 KDM3A regulates c-Myc expression

c-Myc is a master regulator in cell proliferation and transformation [56]. Overexpression of c-Myc is an important factor in development of various cancers [56]. Recent studies revealed that KDM3A regulated the expression of c-Myc to drive the progression of prostate, cervical, colorectal and mammary tumors. Knockdown of KDM3A was found to reduce the level of c-Myc in prostate cancer [42]. In AR-positive CWR22Rv1 or LNCaP cells, KDM3A promoted H3K9 demethylation of and recruitment of AR to the c-Myc gene enhancer.

In AR-negative PC3 and DU145 cells, KDM3A also increased the levels of c-Myc protein through its interaction with E3 ubiquitin ligase HUWE1 and inhibition of HUWE1-induced degradation of c-Myc. This stabilizing effect of KMD3A on the c-Myc protein did not require its histone demethylase activity. Of note, there are other examples for the catalytic-independent function of KDM3A. For instance, KDM3A was found to interact with and stabilize GL1 through the inhibition of its proteasomal degradation, thereby regulating the Hedgehog signaling pathway [57].

Knockdown of KDM3A blocked the proliferation of prostate cancer cells *in vitro* and orthotopic tumor formation in nude mice, whereas forced re-expression of c-Myc in these cells partly reversed the growth defects caused by KDM3A knockdown [42]. Thus, c-Myc is a key downstream effector of KDM3A in the growth and tumorigenesis of prostate cancer cells. Staining of KDM3A and c-Myc in a tissue microarray (TMA) of prostate cancer showed that high level of c-Myc was associated with high level of KDM3A, which implicates KDM3A in the upregulation of c-Myc in a subset of human prostate cancer [42].

Similar findings were reported in cervical and colorectal cancer cells. Knockdown of KDM3A in HeLa cells led to decreased expression of c-Myc and increased levels of H3K9me2 on the c-Myc gene promoter. In addition, knockdown of KMD3A reduced the growth, migration and invasion of Hela cells *in vitro*, whereas restoration of c-Myc

expression in KDM3A-knockdown cells partly rescued these defects [36]. Staining of cervical cancer specimens showed a positive correlation between KDM3A and c-Myc expression, whereby high levels of KDM3A and c-Myc were also strongly associated with poor patient survival [36]. Similarly, KDM3A was overexpressed in colorectal cancer specimens, and positively correlated with the expression of Wnt/β-catenin target genes including c-Myc [54]. KDM3A was found to promote the expression of c-Myc via β-catenin in colorectal cancer cells [54].

KDM3A was found to promote the transformation of breast cells [58]. In a previously established transformation model, transduction of human primary mammary cells with Large T antigen, TERT, and RAS (V12) can generate cell lines representing different stages of transformation. KDM3A was found to gradually increase during the progression of transformation, concomitant with a decrease of H3K9me2 levels. KDM3A increased the expression of cancer-related genes such as c-Myc and Pax3 through H3K9 demethylation in transformed cells. Knockdown of KDM3A, c-Myc or Pax3 reduced the proliferation of transformed cells. Thus, this study suggests that KDM3A promotes the tumorigenesis of breast cancer through the upregulation of c-Myc and Pax3 oncogene.

Together, these studies indicate that KDM3A can promote c-Myc expression and cancer progression in several types of cancers.

### 5.4 KDM3A regulates expression of other transcription factors

KLF2 is a transcription factor of the Krüppel zinc-finger family, and IRF4 is a member the interferon regulatory family of transcription factors. KDM3A was shown to promote the expression of KLF2 and IRF4 by demethylating H3K9me1/2 at the core promoter of these two genes. KDM3A-mediated expression of KLF2 and IRF4 played a key role in the survival and bone marrow homing multiple myeloma (MM) cells [59]. YAP1 is a coactivator for the TEAD family of transcription factors in the Hippo signaling pathway. KDM3A promoted the expression of YAP1 via H3K9me1/2 demethylation on the YAP1 promoter, and enhanced the TEAD1-mediated expression of hippo targets in colorectal cancer cells [53]. KDM3A was also found to promote the expression of β -catenin by H3K9me2 demethylation of β-catenin gene promoter in colorectal cancer cells [54].

### 5.5 KDM3A in hypoxia response

Hypoxia is a common feature in solid tumors. HIF-1α is the master transcription factor that controls adaptive gene expression under hypoxia. The level of KDM3A is upregulated under hypoxia in various normal and cancer cells [35,60-63]. KDM3A has been demonstrated to be an HIF target gene [63-66]. KDM3A levels were downregulated upon knockdown of HIF-1α, and upregulated upon HIF activation [63-66]. ChIP-qPCR revealed that HIF-1α could bind to the Hypoxia-Response Element (HRE) sequences located at the KDM3A promoter [64,65]. Hypoxia or HIF-1α overexpression could increase luciferase reporter activity of a KDM3A promoter construct, but not the KDM3A promoter with HRE sequence mutations [64,66].

The upregulation of KDM3A by HIF contributes to the expression of a subset of hypoxia-induced genes [60-63]. However, it remains unclear which transcription factor(s) is regulated by KDM3A under hypoxia. In one study, KDM3A was shown to interact with HIF-1α and

facilitate HIF-1α-dependent expression of GLUT3 in endothelial cells to enhance glucose uptake under hypoxia [60]. Mechanistically, KDM3A was recruited by HIF-1α to demethylate H3K9me2 at the proximal promoter and enhancers of GLUT3 gene, thereby increasing the expression of GLUT3 [60]. In another study, KDM3A was shown to mediate hypoxia/HIF-induced expression of Mmp12 to promote trophoblast cell invasion [61]. The regulatory sequence of Mmp12 showed KDM3A accumulation and H3K9 demethylation, but did not contain the HRE motifs [61]. The results suggest that KDM3A may regulate a transcription factor other than HIF to promote the Mmp12 expression in trophoblast cells under hypoxia.

Targeting the VEGF/VEGFR pathways to inhibit angiogenesis serves as an anti-cancer treatment, but such cancer cells can become resistant and refractory to such antiangiogenic therapy [67,68]. KDM3A was implicated to confer resistance of xenograft tumor cells to antiangiogenic treatment [62]. KDM3A, VEGF-A and FGF18 were upregulated in cancer cells (HeLa, HepG2, A431, and T98G) under hypoxia and nutrition deprivation condition. KDM3A mRNA expression was also upregulated in tumor tissues at the preangiogenic switch in HSML cells and B16 cells and at the refractory phase of anti-VEGF treatment. siRNA knockdown of KDM3A in HeLa and A673 cells had a minor effect on cell proliferation *in vitro*. In contrast, KDM3A inhibition significantly suppressed tumor growth upon injection of HeLa or A673 cells into immuno-deficient mice. Expression of angiogenic factors including FGF2, HGF, and Ang2 was significantly decreased in those KDM3A-knockdown tumor tissues, which also showed reduced CD31-postive vessel formation and CD11b-positive macrophage infiltration. These results suggest that KDM3A enhances the expression of some angiogenic factors, and promotes tumor angiogenesis and inflammatory cell infiltration. Importantly, KDM3A inhibition significantly enhanced the tumor-suppressive effects of the anti-VEGF antibody (bevacizumab) or antiangiogenic VEGFR inhibitor (sunitinib) in the xeonograft model. This study suggests that targeting KDM3A may enhance the efficiency of antiangiogenic therapy.

KDM3A was found to be involved in the development of neuroendocrine prostate cancer (NEPC) [32]. NEPC is a rare type of prostate cancer, but its incidence is increased after treatment of prostate cancer with the new generation of AR pathway inhibitors [69]. Siah2 is an E3 ubiquitin ligase and plays a key role in the availability and activity of HIF-1α by ubiquitination and degradation of several HIF inhibitory proteins [70,71]. Crossing Siah2 knockout mice with the TRAMP model reduced the formation of NEPC [32]. Siah2 was found to promote the expression of a subset of HIF-1α target genes, among which are KDM3A, HES6 and SOX9. Forced expression of KDM3A, HES6 and Sox9 in Siah2-inhibited prostate tumor cells partly recued the defect of TRAMP-C cells in the formation of xenograft prostate tumors in nude mice and the defect of CWR22Rv1 cells in the expression of NE marker under hypoxia [32]. Future work will be needed to determine the precise mechanisms of KDM3A in the neuroendocrine phenotype of prostate cancer.

KDM3A was upregulated in the hypoxic cells within colorectal cancer (CRC) liver metastases, and was identified to be an independent prognostic factor for CRC [35]. Knockdown of KDM3A inhibited the growth of subcutaneous xenograft tumors formed by the CRC cell line HCT116 [35,63] and DLD1 [35]. Similarly, KDM3A was upregulated hepatocellular carcinoma samples in relative to normal liver tissues, and high expression of

KDM3A in hepatocellular carcinoma was also associated with a higher rate of recurrence [33]. Hypoxia induced upregulation of KDM3A in hepatocellular carcinoma (HCC) cell line HepG2 and Hep3B, and knockdown of KDM3A in those HCC cell lines reduced their growth under hypoxia. Knockdown of KDM3A in HepG2 cells reduced the growth of xenograft tumors upon the subcutaneous injection into nude mice [72].

Together, these studies reveal that KMD3A is upregulated under hypoxia and contribute to the hypoxia-associated aggressive phenotypes of various cancers.

### 5.6 KDM3A in anoikis

Anoikis is a specialized form of apoptosis induced when epithelial cells detach from the extracellular matrix (ECM) [73]. It plays an important role in preventing metastasis by eliminating the tumor cells that lose the proper ECM cues. In a genome-wide shRNA screen, KDM3A was identified as one of top candidates that could promote the survival of MCF10A cells, a non-tumorigenic mammary epithelial cell line, in the absence of ECM attachment [74]. Following detachment from ECM, increased levels of KDM3A were detected in MCF10 cells, but not other breast cancer cells that resist anoikis. Upregulation of KDM3A upon detachment could partly be due to the loss of integrin/FAK and EGFR/MEK signaling. KDM3A was found to promote expression of pro-apoptotic genes BNIP3 and BNIP3L by H3K9me1/2 demethylation of their promoters. Knockdown of KDM3A in non-metastatic mouse breast cancer cell lines 67NR or 4T07 promoted their lung metastasis. This study suggests that KDM3A can induce anoikis in non-transformed mammary epithelial cells or early-stage breast cancer cells to inhibit their metastatic potential.

## 6 Roles of KDM3B and KDM3C in cancer

Acute promyelocytic leukemia (APL) can be effectively treated with differentiation therapy using all-trans-retinoic acid (ATRA), which induces terminal differentiation followed by apoptosis. KDM3B was downregulated during the differentiation of human promyelocytic leukemia cell line HL-60, and this was accompanied by the enrichment of H3K9me2 and recruitment of several corepressors to the KDM3B promoter [5]. Knockdown of KDM3B in HL-60 cells enhanced differentiation, whereas overexpression of KDM3B had opposite effect. KDM3B was found to promote the expression of leukemogenic oncogene lmo2 by H3K9me1/2 demethylation of the Imo2 promoter. KDM3B and Imo2 were upregulated in blood cells from acute lymphoblastic leukemia (ALL)-type leukemia patients. This study suggests that KDM3B promotes leukemogenesis via activation of lmo2 through its H3K9 demethylase activity.

KDM3C has been shown to promote leukemogenesis as a co-activator for transcription factor RUNX1-RUNX1T1 [7] and HOXA9 [75]. RUNX1–RUNX1T1 is a transcription factor generated by the t(8;21) translocation in acute myeloid leukemia (AML), and it can increase self-renewal and inhibit the myeloid differentiation. KDM3C was found to interact with and function as a coactivator for RUNX1–RUNX1T1. KDM3C was recruited by RUNX1–RUNX1T1 to its target genes and regulated their expression by H3K9me2 demethylation. Conditional knockout of *Kdm3c* inhibited the proliferation of hematopoietic stem cells (HSCs) driven by RUNX1–RUNX1T1. Knockdown of KDM3C inhibited the proliferation

of multiple AML cell lines. This study suggests that KDM3C is required for maintenance of multiple types of leukemia driven by different mutations.

KDM3C was also demonstrated to promote leukemogenesis driven by translocation of the mixed lineage leukemia (MLL) gene [76]. The most common translocation of MLL gene in acute myeloid leukemia (AML) is t(9;11) which encodes the oncogenic MLL-AF9 fusion protein. MLL-AF9 leukemia has been shown to follow a leukemia stem cell (LSC) model in which LSCs are enriched in a subset population of leukemia cells [76]. Transformation by MLL-AF9 induced aberrant gene expression, which includes the canonical MLL-AF9 target genes homeobox A9 (HOXA9) and Meis homeobox 1 (MEIS1) that are important to the LSC self-renewal. The shRNA screening on the mouse LSCs identified KDM3C as one of the top MLL-AF9 target genes that are essential for MLL-AF9 leukemia [75,77]. Conditional knockout of *Kdm3c* in the mouse LSCs led to a decreased self-renewal of LSC. KDM3C was found to interact with HOXA9, and may serve as a co-activator to modulate a HOXA9-dependent gene expression [75]. This study suggests that KDM3C may drive the MLL-AF9 leukemia by promoting the activity and transcriptional program of HOXA9 through its H3K9 demethylase activity.

KDM3C was found to promote the expression of YAP1 in esophageal cancer (EC) cells [78]. Staining of KDM3C in 100 paired esophageal cancer tissues showed that KDM3C was highly upregulated in the cancer tissues compared to the adjacent normal tissues. Knockdown of KDM3C reduced the expression of YAP1 and inhibited the growth of two esophageal cancer cell lines, Eca109 and EC18, whereas the forced re-expression of YAP1 in the KDM3C-knockdown cells rescued the cell growth *in vitro*. KDM3C was shown to bind to and remove the H3K9me2 mark on the YAP1 promoter. This study indicates that KDM3C promotes the growth of esophageal cancer cells through upregulation of YAP1 expression.

## 7 Regulation of KDM3A by posttranslational modifications

KDM3A can be regulated by a variety of posttranslational modifcaitons such as phosphorylation, ubiquitination and acetylation. Phosphorylation of tyrosine resiudes located in the JmjC domain of KDM3A can enhance its H3K9 demethylase activity. Tyrosine 1114 of KDM3A can be phosphorylated by the non-receptor tyrosine kinase ACK1, and this leads to increased H3K9 demethyation and co-activation of some ER target genes in breast cancer cells [49]. Tyrosine 1101 of KDM3A can be phosphorylated by JAK2, and this increases the H3K9 demethyation and co-activation of STAT3 target genes in Hela cells after IL-6 treatment [52]. Lysine 918 of KDM3A can be modified by ubiquitin ligase HUWE1 through a K27/K29-linked noncanonical ubiquitination, and this promotes the co-activation of c-Myc for the expression of DNA repair factors in prostate cancer cells [79]. Ubiqutin ligase STUB1 can induce the canonical ubiquitination and degradation of KDM3A through the ubiquitin-proteasome pathway, and thus the low expression of STUB1 may contribute to the upregulation of KDM3A protein in advanced prostate cancer [80]. Lysine 421 of KDM3A can be acetylated by acetyltransferase p300, and this leads to recruitment of the bromodomain family member BRD4 to block KDM3A–STUB1 interaction and enhance the KMD3A protein stability [80]. Importantly, inhibitors of ACK1, JAK2 or BRD4 can be used

to inhibit the KDM3A activity in cancer cells [49,52,80]. The posttranslational modifcations of KMD3A may provide new targets to antagonize KDM3A activity for poteintal cancer therapy.

## 8 Regulation of KDM3A by microRNAs

MicroRNAs (miRNAs) are small non-coding RNAs that silence target gene expression through inhibition of mRNA translation or by mRNA decay. KDM3A was targeted by several tumor-suppressive miRNAs such as miR-627 [81], miR-22 [82] and let-7c [38]. In addition, KDM3C was targetd by miR-590-3p to inhibit mitochondrial dysfunction and oxidative stress [83].

KDM3A was shown to be a target of miR-627 in colon cancer cells [81]. Overexpression of miR-627 reduced the level of KDM3A and inhibited the activity of KDM3A 3′UTR reporter construct, but not the reporter construct with mutation on the potential miR-627 binding site. Calcitriol is an active form of vitamin D, which has been shown to inhibit proliferation and induce apoptosis in colon cancer cells [84]. Calcitriol treatment of colon cancer cell line HCT-116 led to upregulation of miR-627 and downregulation of KDM3A, whereas inhibition of miR-627 blocked calcitriol-induced downregulation of KDM3A. Correspondingly, calcitriol treatment inhibited the xenograft tumor formation by HCT-116 cells, and such inhibition on cell growth was blocked when miR-627 was inhibited. Moreover, knockdown of KDM3A or overexpression of miR-627 in HCT-116 cells inhibited the cell growth. This study suggests that calcitriol inhibits growth of colon cancer cells through downregulation of KDM3A via upregulation of miR-627.

KDM3A was shown to be an miR-22 target in Ewing Sarcoma cells [31]. Overexpression of miR-22 reduced the KDM3A level in the Ewing Sarcoma cell line A673, and inhibited the activity of KDM3A 3′UTR reporter construct, but not the reporter construct with mutation on the potential miR-22 binding site. Ewing Sarcoma pathogenesis is driven by oncoproteins that arise from the fusion between the EWS gene and one of Ets transcription factor genes, among which EWS/Fli1 is the most common fusion oncoprotein and underlies 80–90% of Ewing Sarcoma cases [82]. One of the miRNAs repressed by EWS/Fli1 in Ewing Sarcoma was miR-22 [85], which inhibited the growth of Ewing Sarcoma cell lines upon the ectopic overexpression [31]. As KDM3A is a miR-22 target, the repression of miR-22 by EWS/Fli1 may increase the level of KDM3A in Ewing Sarcoma cells. Consistently, published gene profiling data showed a positive correlation between KDM3A and EWS/Fli1 in Ewing Sarcoma. Knockdown of KDM3A in three different Ewing Sarcoma cell lines inhibited cell growth *in vitro*. Knockdown of KDM3A in A673 cells reduced the growth of xenograft tumors in nude mice. This study suggests that the fusion oncoprotein EWS/Fli1 promotes the growth of Ewing Sarcoma cells by upregulating KDM3A through the repression of miR-22.

A mutual regulation between KDM3A and miRNA let-7c was identified in non-small lung cancer cells (NSCLC) [38]. KDM3A was found to repress the expression of miRNA let-7c, as indicated by the increased let-7c level following KDM3A knockdown. On the other hand, KDM3A was shown to be a target of let-7c. Overexpression of let-7c in H1299 and

A549 cells reduced the level of KDM3A, and repressed the activity of KDM3A 3′UTR reporter construct, whereas the repression was partially alleviated after mutation of the potential let-7c-binding site in the reporter construct. Thus, this study reveals a feedback loop between KDM3A and let-7c, through which KDM3A maintains its high expression through repression of the miRNA let-7c in NSCLC.

## 9 Conclusion and Future Perspective

The KDM3 family of histone demethylases, particularly KDM3A, has been demonstrated to regulate transcription factors and transcriptional program through its H3K9me1/2 demethylase activity, and thus promotes cancer development and progression. Mechanistically, KDM3A can regulate gene expression by functioning as coactivators for transcription factors and/or upregulating the expression of transcription factors (Figure 4.3). Several issues on KDM3A-mediated gene regulation are anticipated to be resolved in future research. First is the role of KDM3 in the tumor initiation and progression. The current evidence for the tumor-promoting role of KDM3 is mainly based on the siRNA knockdown or conditional knockout in cells. Future studies to cross KDM3 knockout mice with relevant mouse transgenic tumor models are preferable and will allow better evaluation of the role of KDM3 in transcriptional gene regulation and cancer biology. The second issue is to identify the key transcriptional factor(s) and transcriptional program(s) that underlie KDM3-driven cancer progression. KDM3 may potentially regulate multiple transcription factors in cell-type, cancer-stage or context-specific manners. This issue will be better addressed with the additional studies that use the genome-wide analyses (e.g. ChIP-seq, RNA-seq, etc) on KDM3-inhbited cancer cell lines or models. Third, evidence suggests that KDM3A can demethylate the non-histone substrate or alter the function of its interactors independent of its catalytic activity, and further studies are needed to explore these directions. Finally, it is crucial to develop selective KDM3 inhibitors. Although selective inhibitors have been identified to target several JmjC subfamily members [86], selective inhibitors that target the KDM3 subfamily are currently unavailable. Further modifications of known JmjC KDM inhibitors or *in silico* screen may help identify selective KDM3 inhibitors. The structures of KDM3 have not yet been solved, and future determination of KDM3 structures is expected to facilitate the identification of selective inhibitory compounds or peptides targeting KDM3.

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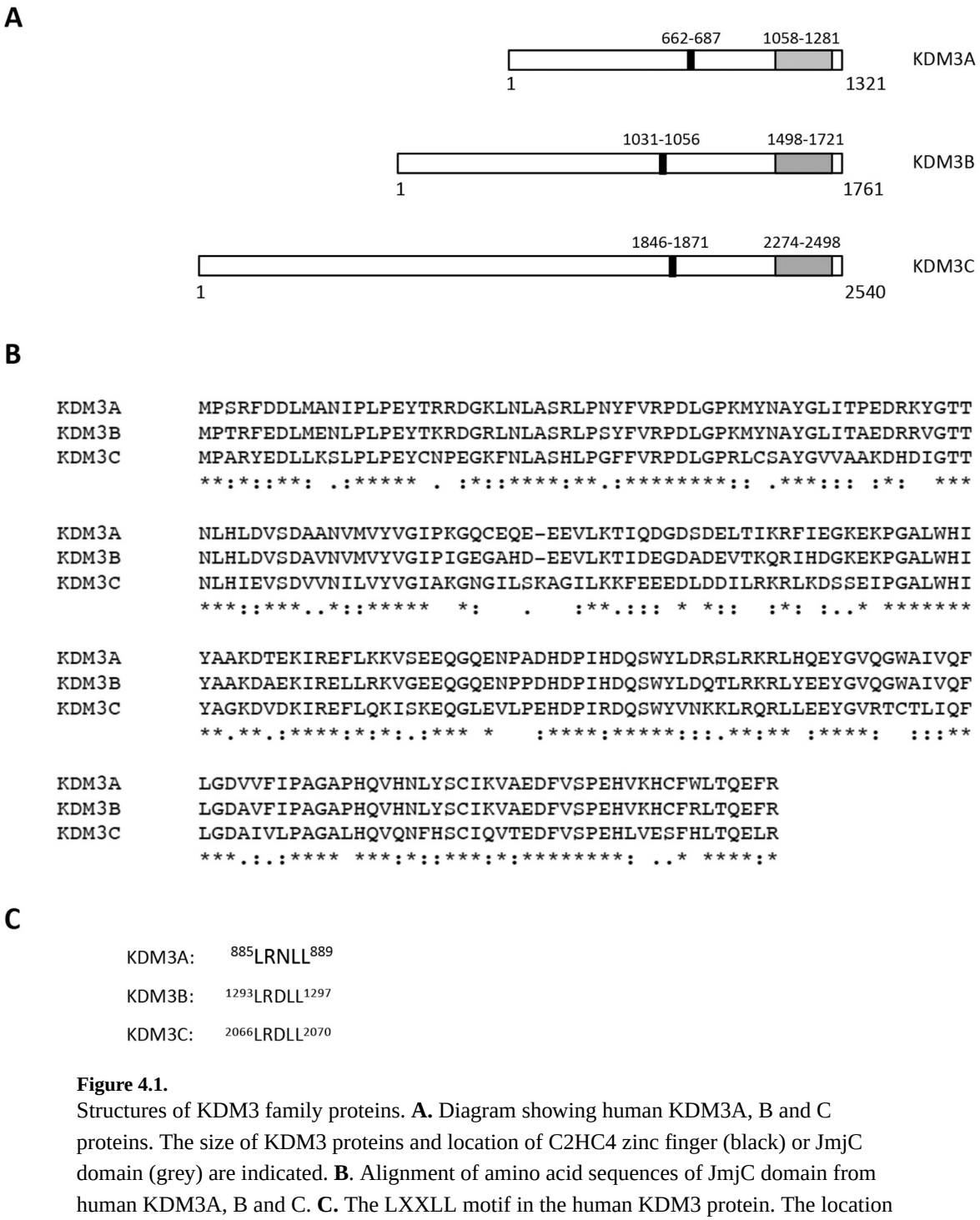


**Figure 4.1.**

Structures of KDM3 family proteins. **A.** Diagram showing human KDM3A, B and C proteins. The size of KDM3 proteins and location of C2HC4 zinc finger (black) or JmjC domain (grey) are indicated. **B**. Alignment of amino acid sequences of JmjC domain from human KDM3A, B and C. **C.** The LXXLL motif in the human KDM3 protein. The location of KDM3 LXXLL motif is indicated.

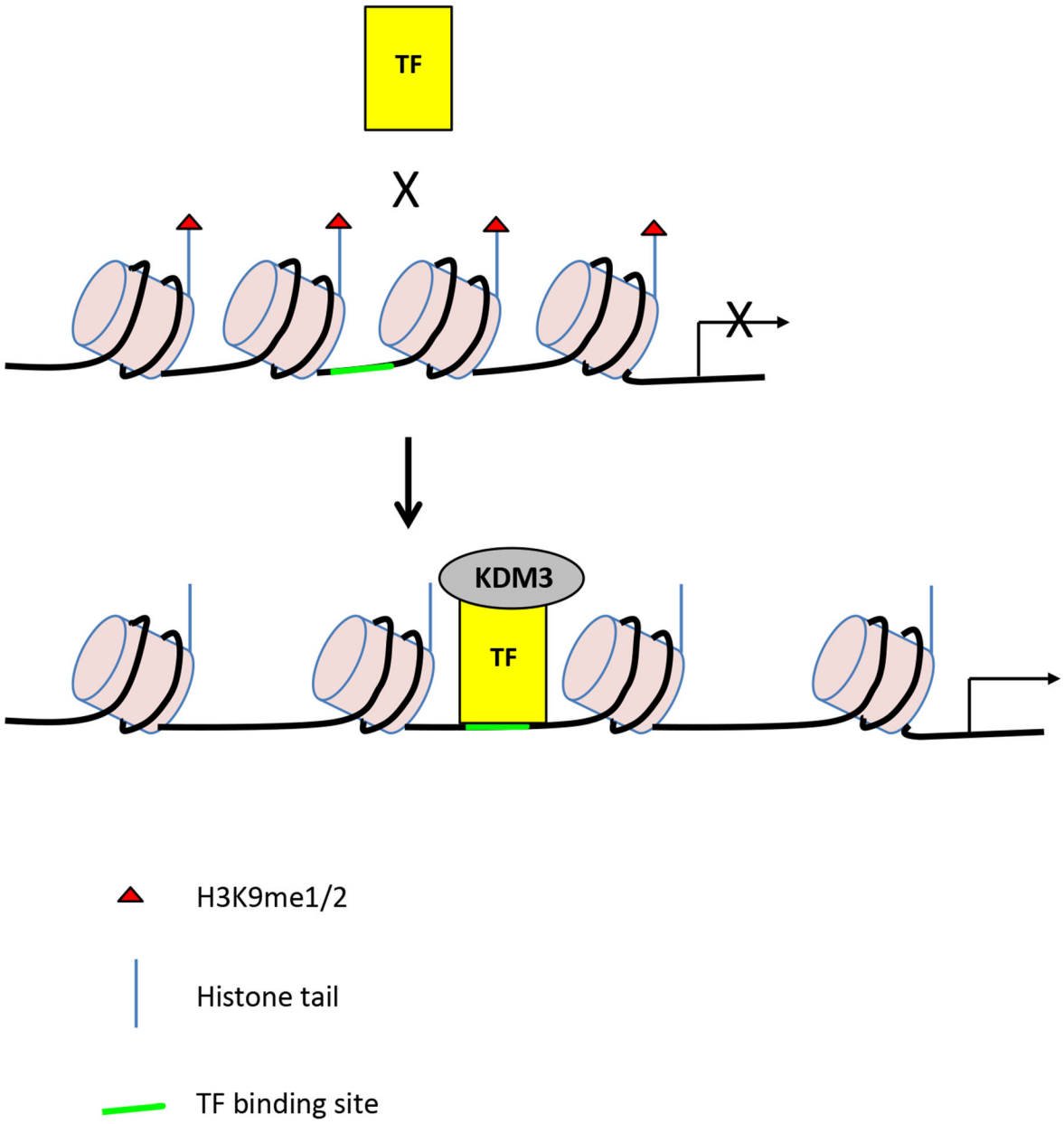


**Figure 4.2.**

Model of KDM3 in the regulation of gene expression. H3K9 methylation silences gene expression by compacting chromatin and blocking the access of transcription factor (TF) to its cognate DNA-binding site. KDM3A functions as coactivators of TF, removes the H3K9me1/2 marks, relaxes chromatin, and allows the access of TF to its cognate DNA-binding site, leading to activation of gene expression.

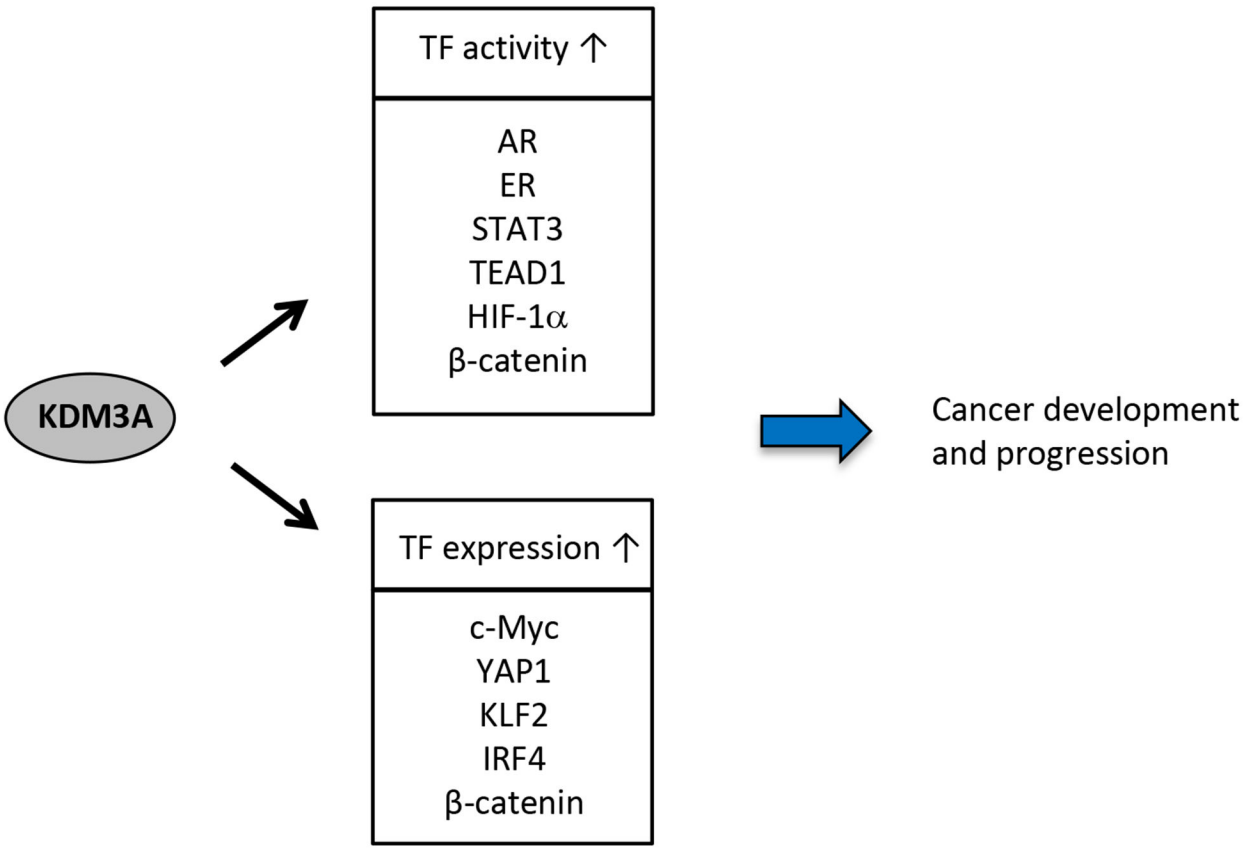


**Figure 4.3.**

Mechanisms of KDM3A in the transcriptional gene regulation in cancer cells. KDM3A functions as coactivators for transcription factor (TF) and/or promotes the expression of TF, resulting in the transcriptional gene alteration to drive cancer development and progression.

**Table 4.1.**

Summary of *Kdm3a*-decificient mouse models. Five lines of *Kdm3a*-deficient mice have been established. Lists are methods used to generate the *Kdm3a*-deficient mice and main phenotypes observed.

| Knockout Approach | Main Phenotype | Reference |
|---|---|---|
| Deletion of the catalytic JmjC domain by an insertion in intron 10 (or intron 7 of splicing variant 2) of the *Kdm3a* gene. | Spermatogenesis defect, smaller testes, fewer sperm numbers and male infertility. | Okada et al., 2007 |
| Deletion of *Kdm3a* exons 24-27 encoding the catalytic JmjC domain using the Cre-lox system. | Spermatogenesis defect, obesity and metabolism. | Tateishi et al., 2009 |
| Deletion of *Kdm3a* exons 20–25 encoding the JmjC domain by an insertion-type vector. | Obesity, metabolic syndrome, sex reversal. | Inagaki et al, 2009 |
| Deletion of *Kdm3a* exons 17-25 encoding the JmjC domain. | Spermatogenesis defect, smaller testes, fewer sperm numbers and male infertility. | Liu et al., 2010 |
| Deletion of KDM3A full-length protein by an insertion within the *Kdm3a* intron 5-6. | Spermatogenesis defect, smaller testes and male infertility. | Kasioulis et al., 2014 |