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Epigenetic gene regulation by histone demethylases: emerging role in oncogenesis and inflammation

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Abstract

Histone N-terminal tails of nucleosomes are the sites of complex regulation of gene expression through post-translational modifications. Among these modifications, histone methylation had long been associated with permanent gene inactivation until the discovery of Lys-specific demethylase (LSD1), which is responsible for dynamic gene regulation. There are more than 30 members of the Lys demethylase (KDM) family, and with exception of LSD1 and LSD2, all other KDMs possess the Jumonji C (JmjC) domain exhibiting demethylase activity and require unique cofactors, for example, Fe(II) and a-ketoglutarate. These cofactors have been targeted when devising KDM inhibitors, which may yield therapeutic benefit. KDMs and their counterpart Lys methyltransferases (KMTs) regulate multiple biological processes, including oncogenesis and inflammation. KDMs' functional interactions with retinoblastoma (Rb) and E2 factor (E2F) target promoters illustrate their regulatory role in cell cycle progression and oncogenesis. Recent findings also demonstrate the control of inflammation and immune functions by KDMs, such as KDM6B that regulates the pro-inflammatory gene expression and $CD4^+$ T helper (Th) cell lineage determination. This review will highlight the mechanisms by which KDMs and KMTs regulate the target gene expression and how epigenetic mechanisms may be applied to our understanding of oral inflammation.

Keywords

epigenetics; histone lysine methylation; oncogenesis; oral inflammation

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None to disclose.

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Introduction

DNA molecules wrap around histone octamers comprised of H2A, H2B, H3, and H4 to make up nucleosomes, which are the basic units of chromatin. Chromatin remodeling plays a critical role in permissivity of gene expression by allowing access to *trans*-regulators through dynamic processes (Voss and Hager, 2014). Chromatin remodeling can occur upon methylation of the 5-position of cytosine in guanine–cytosine (GC)-rich regions called CpG islands by DNA methyltransferases (DNMTs), resulting in transcriptional repression (Hung *et al*, 1999; Martinowich *et al*, 2003). In addition, chromatin remodeling may occur with post-translational modifications (PTMs) at the N-terminus region of histone tails by means of methylation, acetylation, or ubiquitination, as well as other modifications, for example, sumoylation and phosphorylation (Shiio and Eisenman, 2003; Desjarlais and Tummino, 2016).

Histone acetylation is a common histone modification and involves the transfer of an acetyl group from acetyl coenzyme A (acetyl-CoA) to lysine and arginine residues on histone proteins by histone acetyltransferases (HATs) (Roth *et al*, 2001). This modification results in transcriptional activation and can be reversed by histone deacetylases (HDACs). Histone methylation can also occur on the lysine and arginine residues through transfer of a methyl group from *S*-adenosyl-l-methionine (SAM) by histone methyltransferases (HMTs) and be reversed by histone demethylases (HDMs) (Morera *et al*, 2016). Histone acetylation and methylation may be influenced by phosphorylation of serine and threonine residues on histone tails. Histone kinases transfer a phosphate group from adenosine triphosphate (ATP) to serine and threonine side chain hydroxyl groups (Jeong *et al*, 2013). Phosphorylation of certain serine or threonine residues, such as serine 10 on histone 3 (H3S10) or threonine 6 on histone 3 (H3T6), can result in histone acetylation or histone demethylation, respectively (Lo *et al*, 2000; Metzger *et al*, 2010). Similar to histone acetylation and methylation, histone phosphorylation can be reversed by protein phosphatases. Histone proteins can also be ubiquitinated by ubiquitin ligases, resulting in transcriptional repression or activation upon ubiquitination of H2A or H2B, respectively (Wang *et al*, 2004; Lee *et al*, 2007). Histone ubiquitination is reversible through activity of deubiquitinating enzymes, thereby contributing to the dynamicity of epigenetic gene regulation. Histone sumoylation utilizes similar enzymes as ubiquitination to covalently bind small ubiquitin-like modifier (SUMO) proteins to lysine residues (Cimarosti *et al*, 2012). This modification results in transcriptional repression and can be reversed by sentrin/SUMO-specific proteases (SENPs) (Shiio and Eisenman, 2003; Cimarosti *et al*, 2012). Among the PTMs of histone tails for epigenetic regulation, histone acetylation and methylation are the most common and well studied (Koch *et al*, 2007). Histone acetylation by HATs generally leads to gene activation (Roth *et al*, 2001), and histone tail methylation may result in gene silencing or activation, depending on the location of the amino acid substrate for methylation and the valency, that is, mono-, di-, or tri-methylation (Black *et al*, 2012).

Histone methylation has long been thought to be involved with heterochromatin formation and permanent chromosome inactivation (Peters *et al*, 2002). For instance, di- and tri-methylation of histone 3 at Lys 9 (H3K9Me2/3) causes a heritable heterochromatic state by recruiting heterochromatin protein 1 (HP1), which facilitates gene silencing through DNA

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CpG methylation (Fuks *et al*, 2003; Audergon *et al*, 2015). However, the discovery of a Lys-specific demethylase 1 (LSD1 or KDM1A) specific for H3K4 and H3K9 mono- and di-methylation raised the possibility that histone methylation is a dynamic process in transcriptional regulation (Shi *et al*, 2004). Subsequent to this initial finding, numerous studies reported the presence of histone Lys demethylases (KDMs) that commonly contain Jumonji C (JmjC) domains responsible for catalyzing the demethylation reactions. To date, more than 30 KDM family members have been reported, among which the vast majority belong to JmjC-containing KDMs, except LSD1 and LSD2, which were the original two demethylases identified (Shi and Tsukada, 2013). In general, the gene regulatory roles of KDMs are positioned highly upstream in the signaling cascade, and KDMs possess multiple target genes involved in diverse biological processes. For instance, KDM4B and KDM6B regulate osteogenic differentiation capacities of human mesenchymal stem cells (MSCs) (Ye *et al*, 2012; Xu *et al*, 2013); KDM4A regulates metastatic colonization of oral squamous cell carcinomas (OSCCs) (Ding *et al*, 2013); and KDM6B determines senescence and inflammatory programs (Agger *et al*, 2009; Barradas *et al*, 2009; De Santa *et al*, 2009). Summarized in Table 1 are the Lys histone marks with the corresponding KDMs and Lys methyltransferases (KMTs), as well as examples of known biological effects.

KDMs – basic mechanisms of enzyme functions

KDMs possess distinct functional domains and motifs that are critical to their enzyme activity, and are classified by the presence or absence of the JmjC domain. Lysine-specific demethylases (LSD) are those KDMs that lack the JmjC catalytic domain. LSD1, also known as KDM1A, instead possesses a C-terminal amine oxidase-like (AOL) domain that contains substrate binding and flavin-adenine dinucleotide (FAD)-binding domains (Chen *et al*, 2006a). The majority of KDMs, other than LSD1 and LSD2, possess the JmjC domain that confers demethylase activity and substrate specificity. This domain shares structural similarity to the cupin family of proteins and contains a cofactor coordinating region comprised of Fe(II) and α-ketoglutarate (α-KG) (Clissold and Ponting, 2001; Chen *et al*, 2006a,b). The JmjC domain also contains a zinc-finger region that brings the JmjC domain into close proximity with the C-terminal domain and plays an important role in structural stability and catalytic activity of the enzyme. The JmjC domain functions as a Fe(II) and α-KG-dependent oxygenase and confers the demethylase activity to the enzyme.

The PHD domain is commonly found in histone modifying enzymes due to its specificity to methylated lysines and also mediates substrate specificity for KDMs (Shi *et al*, 2006; Lan *et al*, 2007). Structural studies have revealed the functional interaction between PHD and JmjC domains for the recognition of methylated Lys residues, which may alter the enzyme activity. Horton *et al* (2010) showed that both PHD and JmjC domains of histone demethylase PHD Finger Protein 8 (PHF8) bind to H3K4Me3 and that such binding enhanced the rate of demethylation on its cognate substrate, H3K4Me2. In addition to these domains, KDMs contain chromodomains called ‘tudor’ motifs that consist of anti-parallel b-strands forming a tube-like structure, which is found in many proteins associated with chromatin (Maurer-Stroh *et al*, 2003; Sprangers *et al*, 2003). KDM2A contains a tandem repeat of two tudor motifs near the C-terminal region that bind to H3K4Me3 with specificity determined by side-chains from the tudor motifs (Huang *et al*, 2006). Hence, KDMs’ non-

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catalytic domains possess distinct functions that determine the substrate specificity and modulate the enzyme activity.

The first identified KDM containing the JmjC domain was FBXL11 (F-box and leucine-rich repeat protein 11), also named KDM2A, whose demethylase activity is conferred by the JmjC domain and is specific for H3K36Me2 (Tsukada *et al*, 2006). In addition to the JmjC domain, KDM2A also possesses other motifs, including the CxxC zinc-finger (ZF) domain, PHD domain, and three leucinerich repeats (Tsukada *et al*, 2006).

KDM2A directly binds to DNA via its ZF domain. An earlier study demonstrated that KDM2A is recruited to non-methylated CpG islands through the ZF domain and depletes the H3K36Me2 enrichment, causing a unique chromatin state at the CpG islands (Blackledge *et al*, 2010). KDM2A also binds unmethylated CpG islands of ribosomal DNA (rDNA) through its ZF domain during starvation, leading to reduced rDNA transcription by depletion of H3K36Me3 (Tanaka *et al*, 2014). Starvation would consequently cause reduced ribosome biogenesis and protein synthesis. Therefore, chromatin remodeling at the site of CpG islands by KDM2A is a dynamic process and responsive to environmental cues, rather than a permanent alteration.

The demethylase reaction mechanisms provide insights in design and discovery of potential inhibitors that modulate the biological functions of KDMs, which are involved in diverse human diseases. For instance, LSD1 and LSD2 can be targeted by tranylcypromine, a clinically validated inhibitor of monoamine oxidase (MAO), which structurally resembles LSDs and also requires FAD cofactor for the enzyme reaction (Binda *et al*, 2010). Likewise, the vast majority of inhibitors of JmjC-containing KDMs are the competitors of α-KG, for example, 5-carboxyl-8-hydroxyquinoline (8-HQ) compounds and GSK-J1, some of which are broad-spectrum inhibitors for all JmjC family KDMs (Hopkinson *et al*, 2013). Efforts in identifying non-competitive inhibitors of KDMs are also underway to enhance the drug specificity, such as amiodarone derivatives, which inhibit KDM5A activity by binding to the PHD domain (McAllister *et al*, 2015). Mechanism-based chemical inhibitors of KDMs are not favored as they target common KDM cofactors, such as FAD and a-KG, and thus lack target specificity. Nonetheless, KDMs represent promising therapeutic targets for a diverse array of human diseases as they regulate multiple biological processes, such as cell proliferation/differentiation, oncogenic transformation, and inflammation.

KDMs – master regulators of cell cycle progression and oncogenesis

The number of KDMs discovered so far is indicative of the diverse biological effects and target genes under regulation by the KDM family. A given KDM may elicit different biological effects based on the cell type, microenvironment, and the state of other interacting regulatory proteins. Biological effects of KDMs are further stratified by presence of non-histone target proteins that may be regulated through the demethylase activity. Importantly, individual KDMs are positioned highly upstream along the gene regulatory axis due to their mode of regulation involving chromatin state. Hence, KDMs do function as master regulators of multiple biological processes, among which regulation of retinoblastoma (Rb)-mediated cell cycle progression has been extensively studied.

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LSD1, the first KDM identified possessing flavin-dependent mono-oxidase activity (Shi *et al*, 2004), regulates cell cycle progression by demethylating Lys 442 of myosin phosphatase target subunit 1 (MYPT1), a protein phosphatase that activates the growth inhibitory function of Rb by removing Ser 807/811 phosphorylation (Cho *et al*, 2011). Consequently, transcriptional activation of E2F target genes is induced by LSD1, resulting in cell cycle progression. For example, LSD1 is associated with Rb on the Epstein–Barr virus latency gene promoter to repress the promoter activity in a cell cycle-dependent manner (Chau *et al*, 2008). Evidence also indicates the presence of methylated Lys residues on Rb associated with LSD1, possibly modulating cell cycle progression. Hence, LSD1 provides an example in which non-histone proteins, such as MYPT1 and Rb, are the substrates for its demethylase activity and augment its primary biological effect, which is to suppress target gene expression through H3K4 demethylation.

It appears that Rb plays a central role in the epigenetic regulation of cell proliferation through its interactions with multiple KDMs. In addition to LSD1, the active form of Rb recruits KDM5B, a JmjC-containing demethylase specific for H3K4Me3/2/1, to the promoter regions of E2F-responsive genes, including *Cdc25c, Cdc2, Mcm3, Mcm5*, resulting in gene silencing (Nijwening *et al*, 2011). Using an immortalized mouse cell line with the temperature-sensitive SA40 large T antigen (LTA), the authors showed rampant occurrence of senescence-like growth arrest at a non-permissive temperature, at which LTA was inactivated. However, KDM5B knockdown abrogated this senescence program, allowing for continued proliferation at the non-permissive temperature. Enrichment of H3K4Me3 on E2F-target gene promoters was markedly reduced during senescence, presumably due to demethylase activity of KDM5B. Likewise, Beshiri *et al* (2012) showed that KDM5A is highly enriched on E2F-target promoters and that H3K4 methylation was lost during growth arrest induced by terminal differentiation in embryonic stem cells. During quiescence or senescence, Rb-mediated cell cycle arrest is facilitated by the assembly of a multi-subunit complex of DP1, Rb-like 2 (RBL2/p130), E2F4, and MuvB (RBBP4, LIN9, LIN37, LIN52, and LIN54), called the DREAM complex (Osterloh *et al*, 2007). Cell cycle gene suppression by the DREAM complex is mediated in part by recruitment of KDM5A to the promoter regions of the DREAM target genes, causing H3K4 demethylation (Beshiri *et al*, 2012). These findings demonstrate the role of the dynamic functional interaction between KDMs and Rb in the epigenetic regulation of E2F target gene expression (Figure 1).

One of the distinguishing features of senescence-associated cell division arrest is induction of the *INK4A-ARF* locus, resulting in an elevation of $p16^{INK4A}$, a G1/S-specific cyclin-dependent kinase (CDK) inhibitor targeting CDK4 and CDK6 (Loughran *et al*, 1996). As a result, CDK4/6-cyclinD complex cannot phosphorylate Rb, allowing Rb (or Rb-like proteins, *e.g.*, p130) to remain complexed with E2F, thereby silencing E2F target genes. The importance of $p16^{INK4A}$ for senescence has been validated in replicative senescence (RS), oncogene-induced senescence (OIS), and stress-induced premature senescence (SIPS) (Collins and Sedivy, 2003). Molecular mechanisms governing the activation of the *INK4A-ARF* loci during senescence involve epigenetic regulation by Bmi-1, a polycomb-group (PcG) protein component of the polycomb repressive complex (PRC) 1, a multi-subunit epigenetic complex with H2A ubiquitinase activity. To initiate target gene silencing, PRC1 functions in tandem with PRC2, possessing methyltransferase activity on H3K27 through

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the *enhancer of zeste homologue* 2 (EZH2). PRC1 subunit RING finger protein 1 (RING1B) is the catalytic unit displaying E3 ubiquitin ligase activity (Li *et al*, 2006). As p16^{INK4A} elicits, its senescence-inducing effect through modulating Rb phosphorylation, altered H3K27 methylation at *INK4A-ARF* loci also regulates the Rb-mediated growth suppression.

Regulation of *INK4A-ARF* loci by PcG proteins suggested the role of KDM6A or KDM6B in regulation of senescence because these KDMs share the same histone substrate (H3K27Me3) as EZH2. This possibility was tested in OIS induced by BRAF oncogene in human diploid fibroblasts (HDFs); KDM6B was induced in cells undergoing OIS, while no changes occurred with KDM6A (Agger *et al*, 2009). Subsequent functional experiments revealed that loss of H3K27Me3 on the *INK4A* promoter by KDM6B led to upregulated p16^{INK4A} expression. A similar senescence-inducing role of KDM6B was also demonstrated in HDFs through overexpression of H-Ras oncogene (Barradas *et al*, 2009). Interestingly, a recent study showed that Rb is a direct substrate for KDM6B demethylase activity during OIS (Zhao *et al*, 2015). This study showed that demethylation of Rb at Lys 810 by KDM6B reduced Rb phosphorylation at Ser 807/811 by CDK4/6, thereby activating the Rb-mediated suppression of E2F target genes. Therefore, KDM6B utilizes its demethylase activity both on H3K27Me3 and Rb, as a non-histone substrate, to impose its effects on senescence, and joins several other KDMs, including LSD1, KDM2A, KDM5A, and KDM5B, that regulate Rb-mediated growth suppression.

Gene regulation through H3K27 methylation also extends to SIPS, which can be induced by exposure to ionizing radiation. A senescent phenotype is induced in normal human keratinocytes (NHK) exposed to ionizing radiation, concomitant with elevated KDM6B levels and a loss of global H3K27Me3 levels, as well as reduction in PcG factors, such as Bmi-1 and EZH2 (Dong *et al*, 2011). Overexpression of Bmi-1 resulted in suppressed KDM6B and p16^{INK4A} levels in the irradiated NHK, and these cells bypassed SIPS. Such radioprotective effects of Bmi-1 in NHK were not only due to suppression of *INK4A* but also increased expression of oxidase genes, for example, *Lpo, p22-phox, p47-phox, and Gp1*, which neutralize reactive oxygen species. Consequently, the cells expressing Bmi-1 demonstrate enhanced DNA repair capacities and increased survival upon irradiation.

PcG-mediated gene silencing is also linked to carcinogenesis. Using a transgenic mouse model, an earlier study found that aberrant Bmi-1 overexpression was sufficient for T-cell lymphoma development (Haupt *et al*, 1993). Aberrant overexpression of Bmi-1 and EZH2 has been reported in human cancers, including oral squamous cell carcinomas (OSCCs), and is known to promote the self-renewal capacities of cancer stem cells (Kang *et al*, 2007; Song *et al*, 2010). In addition, by suppressing the *INK4A-ARF* loci, Bmi-1 overexpression evades the senescence program and leads to cellular immortalization of human oral keratinocytes (Kim *et al*, 2010). A single heterozygous mutation in H3 genes at Lys 27 to methionine (K27M) is associated with the formation of an aggressive brain tumor known as diffuse intrinsic pediatric glioma (Chan *et al*, 2013). The glioma cancer cells harboring such a mutation demonstrated global reduction of H3K27Me3 expression but increased local enrichment of H3K27Me3 and EZH2 at the gene promoters specific to tumor suppression, including *INK4A*. A subsequent study confirmed the oncogenic potential of the H3.3K27M mutation in normal neuronal precursor cells (NPC), and the transformed NPC demonstrated

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elevated genes involved in self-renewal and proliferation, including *Lin28B and PLAG1* (Funato *et al*, 2014). As such, the aberration of epigenetic alterations by PcG proteins is strongly implicated in carcinogenesis through suppression of the senescence growth checkpoints and inducing stem characteristics.

To the contrary, KDM6B, which counteracts the PcG-mediated gene silencing, may elicit tumor suppressive effects. For instance, KDM6B expression is lost during malignant transformation of pancreatic ductal adenocarcinoma, and KDM6B knockdown led to enhanced tumorigenic ability of cells (Yamamoto *et al*, 2014). One mechanism of tumor suppression underlies induction of senescence (Lin *et al*, 2012), while another study illustrated a chromatin-independent mechanism that involves KDM6B interaction with p53 (Ene *et al*, 2012). KDM6B overexpression led to growth arrest in *INK4*-null glioblastoma stem cells by $p21^{WAF1}$ induction through a p53-dependent mechanism. Co-immunoprecipitation revealed that KDM6B binds p53 as a substrate for demethylation at Lys 372, resulting in p53 nuclear localization. Interestingly, KDM6B may utilize its same biological functions to elicit tumorigenic effects under different pathological settings. KDM6B is strongly induced by the human papilloma virus (HPV) 16 E7 oncoprotein in human foreskin keratinocytes and then leads to elevation of $p16^{INK4A}$ expression through demethylation of H3K27Me3 at the *INK4A* promoter region (McLaughlin-Drubin *et al*, 2011). In HPV+ cervical cancer cells, elevated $p16^{INK4A}$ levels were found to be required for cancer cell survival in order to neutralize the hyperactive CDK4 and CDK6, which would lead to cell death under conditions of Rb inactivation by E7 (McLaughlin-Drubin *et al*, 2013). Consequently, KDM6B inhibition by means of siRNA or a small molecule inhibitor, such as GSK-J4, led to cell death of HPV+ cancer cells.

The above studies illustrate the mechanistic involvement of KDMs in diverse cellular processes, primarily through epigenetic regulation of target gene expression by histone Lys demethylation, and secondarily through modulation of non-histone target proteins. In particular, multiple KDMs regulate cell cycle progression through functional interactions with Rb and/or E2F. These interactions elicit broad phenotypic effects in cellular senescence and carcinogenesis. Cellular senescence is also closely linked with inflammation in that senescence triggers secretion of a discrete pattern of inflammatory cytokines, such as interleukin-6 (IL-6), IL-8, and GRO-α, collectively named senescence-associated secretory phenotype (SASP) originally observed in culture models exposed to genotoxic stress (Coppe *et al*, 2008). A recent study showed that KDM6B overexpression induced senescence and SASP in glioma cell lines through its demethylase activity (Perrigue *et al*, 2015). Likewise, senescence-associated secretion of MCP-1 in human mesenchymal stem cells (MSCs) was suppressed by Bmi-1 through the loss of H2AK119 ubiquitination (Jin *et al*, 2015). Hence, there is accumulating evidence that cellular inflammatory responses are regulated epigenetically by KDMs.

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Emerging roles of KDMs in regulation of inflammation and immune functions

The first evidence supporting epigenetic regulation of inflammation was made in cultured human monocyte-derived dendritic cells (DCs) exposed to bacterial endotoxin lipopolysaccharides (LPS) (Saccani and Natoli, 2002). In this study, exposure of DCs to LPS led to rapid or delayed induction of various inflammatory cytokines, IL-8, MIP-1α, MDC, and ELC during the 72 hrs post-treatment. Among them, several genes encoding for the cytokines with delayed induction had transiently lost H3K9 methylation at the promoter regions during the 72-h period of exposure to LPS, while induction of other epigenetic marks, such as H3K4 methylation and acetylated H3 and H4, remained stable. Furthermore, altered H3K9 methylation at these promoter sites upon LPS treatment coincided with RNA polymerase II recruitment. A subsequent study showed enhanced binding of G9a, a Lys-specific methyltransferase, at the promoter region of *tumor necrosis factor alpha* (TNF-α) in human monocytes with an endotoxin tolerant phenotype, resulting in H3K9 methylation (El Gazzar *et al*, 2008). As endotoxin tolerance is linked with a loss of inducibility of cytokine expression after prior exposure to LPS, the above study indicated the mechanistic role of KTMs in such a phenomenon. In addition, G9a recruited at promoter sites forms a complex with HP1 and Dnmt3a/b, and suppresses target gene expression through hypermethylation at CpG islands. The functional significance of G9a enrichment for cytokine gene silencing was shown by knockdown of G9a and consequent reactivation of TNF-α expression in endotoxin tolerant cells. These studies suggest that epigenetic modifications triggered by H3K9 methylation played the primary role in silencing cytokine gene expression during endotoxin tolerance. The tolerant phenotype is associated with increased expression of RelB, a repressive subunit of nuclear factor-κB (NF-κB), as observed in blood mononuclear cells of sepsis patients, resulting in the suppression of inflammatory cytokines (Yoza *et al*, 2006). It was then found that RelB interacts with and recruits G9a in the tolerant phenotype, thereby causing target gene suppression via H3K9 dimethylation (Chen *et al*, 2009). These studies demonstrate that target gene regulation by histone Lys methylation is a dynamic process that modulates inflammatory responses through interactions with known inflammatory mediators.

To identify the KDMs that mediate inflammatory responses, De Santa *et al* (2009) reported screening of KDMs induced by acute LPS treatment in the Raw264.7 mouse macrophage cell line. Within 2 hrs of LPS exposure, KDM6B was strongly induced while KDM6A, a related KDM with the same substrate specificity (H3K27Me3), remained unchanged. KDM6B induction was dependent upon NF-κB, as overexpression of IκBα or deletion of IKKγ in cells abolished the inducing effect of LPS. The first intronic region of the *Kdm6b* gene was found to contain the conserved κB sites with a confirmed increase in p65 binding after LPS exposure. Chromatin immunoprecipitation (ChIP) assay combined with high throughput sequencing (ChIP-Seq) revealed enrichment of KDM6B near the target promoters at the genomic level (De Santa *et al*, 2009). ChIP assay allows for survey of DNA-protein interactions. Hence, sequencing of all DNA elements that co-precipitate with specific proteins, for example, KDM6B, would indicate potential genomic target sequences. Upon activation of macrophages with LPS and IFN-γ, KDM6B was preferentially recruited

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to the transcription start site (TSS) regions of immune regulatory genes and inflammatory mediators, and those genes involved in antimicrobial defense. Also, the vast majority (73%) of the genes with KDM6B enrichment correlated with the recruitment of RNA polymerase II (RNA Pol II) in the activated macrophages, indicative of a gene activator role of KDM6B in the inflammatory queue. As KDM6B demethylates H3K27Me3 and opposes PcG-mediated gene silencing, inflammatory signals would be diminished by PRC2 subunits possessing KMT activity, such as EZH2. In fact, Peng *et al* (2015) reported suppression of Th1-type chemokines, CXCL9 and CXCL10, in colon cancer tissues exhibiting high expression of EZH2, SUZ12, and EED, supporting the role of PcG proteins in the silencing of inflammatory genes. Apparently, the interplay between PcG and KDM6B determines the cellular responses to inflammatory and senescing signals, as well as other epigenetic modifiers, including G9a and Dnmt3a/b (Figure 2). A recent study by Kruidenier *et al* (2012) demonstrated the first small molecule inhibition of KDM6B by GSK-J4, which binds to the enzyme's active site and prevents incorporation of α-KG, a required cofactor for KDM6B demethylase activity. Cell-based assay revealed reduced expression of LPS-inducible genes, such as *TNF-α* and inflammatory cytokines, and recruitment of RNA Pol II to the *TNF-α* TSS in macrophages exposed to GSK-J4 or related compounds. Besides KDM6B, a subsequent study found the involvement of PHF2, a KDM specific for H4K20Me3, in the activation of TLR4-target inflammatory genes, *TNF and CXCL10*, upon exposure to LPS (Stender *et al*, 2012). It is not known whether KDM6B and PHF2 have redundant or complementary effects on epigenetic inflammatory signaling. Also, there may be other KDMs that control the expression of inflammatory mediators through modifying different histone marks. However, KDMs in general may be therapeutic targets of novel anti-inflammatory approaches, and further research will be necessary to elucidate the therapeutic benefits of these compounds.

A recent study demonstrated that KDM6B and KDM6A play pivotal roles in $CD4^+$ T-cell development through epigenetic regulation of S1pr1 and Klf2 expression, both of which are necessary for Th cell maturation and egress from the thymus (Manna *et al*, 2015). KDMs also modulate immune functions by determining $CD4^+$ Th cell lineage differentiation, thereby vastly affecting immune responses in multiple biological systems. Naïve $CD4^+$ T cells from the thymus can differentiate into several different Th cell lineages, including Th1, Th2, Th3, Th17, natural killer T, and regulator T helper (Treg) (reviewed in Kennedy and Celis, 2008), and would facilitate distinct immune responses based on the cytokine and gene expression profiles, as well as its interaction with other immune cells. For instance, $CD4^+$ naïve T cells differentiate into the Th1 lineage through transcription regulator T-bet expression and produce IFN-γ and TNF-α to provoke pro-inflammatory responses (Zhu *et al*, 2010). Conversely, the IL-4 and IL-2 cytokines drive the differentiation of $CD4^+$ naïve T cells to the Th2 lineage by the action of GATA3 (Zheng and Flavell, 1997). Th2 cells with discrete cytokine profiles, for example, IL-4, IL-5, and IL-13, play pivotal roles in humoral immunity through B cells against certain pathogens and allergic reactions. The Th17 lineage is induced by TGF-β and IL-6 through transcription factors RORct and pSTAT3, while the immune-suppressive Treg cell lineage is induced by TGF-β through Foxp3 (Kimura and Kishimoto, 2011). Th17 cells play critical roles in pro-inflammatory reactions in autoimmue disorders, such as rheumatoid arthritis and systemic lupus erythematosis (Singh *et al*, 2014).

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As such, perturbation of CD4+ Th cell lineage differentiation could impair the immune system with diverse health consequences.

KDM6B was found to play critical roles in lineage determination for CD4+ Th cell differentiation. Li *et al* (2014) utilized CD4-specific deletion of the *Kdm6b* gene and showed that CD4+ naïve T cells preferentially undertook the Th2 and Th17 lineages while Th1 and Treg pathways were suppressed. This finding is in keeping with the prior discussion that KDM6B in general regulates pro-inflammatory cytokine gene expression in response to LPS challenge (De Santa *et al*, 2009). *Kdm6b* ablation in CD4+ Th cells led to congruent alterations in T-cell lineage factors; T-bet expression was reduced while GATA3 was induced, shifting the lineage to Th2. Also, KDM6B appears to interact with key transcription factors for Th lineage determinants. KDM6B interacts with Smad2/3 and is recruited to the Nodal target gene promoters to activate gene expression through H3K27Me3 demethylation (Dahle *et al*, 2010). KDM6B interaction with Smad3 was also reported by Li *et al* (2014) in the context of H3K4 methylation at the *Foxp3* promoter, which drives the Treg lineage. These authors found that KDM6B also interacts with Ash2L, a KMT specific for H3K4 methylation sites that promotes gene activation, and recruits the KMT complex to the Smad3 target promoter, like *Foxp3*. *Kdm6b* ablation would therefore disrupt this Smad-Ash2l KMT complex formation at *Foxp3*, thereby suppressing Treg lineage differentiation. These findings demonstrate the critical role of KDM6B in CD4+ Th lineage determination through its histone demethylase activity and direct interactions with key transcription modulators.

KDMs – role in craniofacial and mineralized tissue formation in the oral cavity

In addition to regulating systemic developmental and diseases processes, KDMs have also been found to regulate biological processes in the oral cavity. In particular, KDMs regulate dental mesenchymal stem cell (dMSC) and bone marrow mesenchymal stem cell (BMMSC) proliferation and differentiation, which play an instrumental role in tooth and alveolar bone formation and craniofacial development. For instance, KDM2A has been found to promote ameloblast and odontoblast differentiation and suppress adipogenic and chrondogenic differentiation of stem cells from the apical papilla (SCAP), indicating its importance in tooth development (Dong *et al*, 2013; Yi *et al*, 2016). Zheng *et al* demonstrated that KDM5B and KDM6B are upregulated during tooth development (Zheng *et al*, 2014). KDM6B has been shown to transcriptionally activate bone morphogenetic protein 2 expression and promote odontogenic differentiation of dMSCs and mineralized tissue formation (Xu *et al*, 2013).

KDMs play an important role in alveolar bone formation and craniofacial development. Yang *et al* showed that KDM6A transcriptionally activates runt-related transcription factor 2 (Runx2) and Osterix, important transcription factors for osteoblast differentiation, thereby promoting mineralized tissue formation (Yang *et al*, 2015). KDM4B and KDM6B have also been shown to enhance osteogenic differentiation of BMMSCs by demethylating *H3K9me3* to activate *DLX* gene expression and demethylating *H3K27me3* to activate *HOX* expression, respectively (Ye *et al*, 2012). PHF8 regulates jaw development and neuronal survival through

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Msh homeobox 1 (MSX1), transcription factor regulating craniofacial and neuronal development, and its analog MSXB (Qi *et al*, 2010). Unlike these KDMs, which promote osteoblast and mineralized tissue formation, KDM2B has been shown to suppress the osteogenic differentiation of MSCs by demethylating H3K4 and H3K36 residues on key target gene promoters. Dysregulation KDM2B and its failure to demethylate these sites has been implicated in the development of oculo-facio-cardio-dental (OFCD) syndrome, an inherited disease that results in severe craniofacial and cardiac abnormalities (Fan *et al*, 2009).

The application of dMSCs for tissue regeneration has been an area of great interest due to ease of access and their immunomodulatory properties (Pierdomenico *et al*, 2005). KDM6B has been shown to play an important role in this phenomenon by transcriptionally activating insulin-like growth factor-binding protein 5 (IGFBP5), which is highly expressed in MSCs undergoing osteogenic differentiation (Liu *et al*, 2015). Liu *et al* demonstrated that IGFBP5 promotes the anti-inflammatory and immunomodulatory effect of dMSCs by downregulating NF-κB signaling, and that IGFBP5 expression is downregulated in periodontal tissues collected from patients with periodontitis. Thus, KDM6B is critical for the anti-inflammatory effect of IGFBP5 in periodontal tissues.

KDMs and other epigenetic modulators of oral inflammation

Chronic inflammation by means of periodontal or root canal infection allows abscess formation, causing gross tissue destruction and tooth loss. Earlier studies indicated the possible role of Th1 pro-inflammatory cytokines, including IL-1β and TNF-α, in the pathogenesis of human apical periodontitis linked to chronic inflammation (Matsuo *et al*, 1994; Formigli *et al*, 1995). Analysis of human periapical granulomas revealed significant bone resorbing activity, which could be neutralized by anti-IL-1β and anti-TNF-β antibodies (Wang and Stashenko, 1993). In rats, homologous forms to these mediators, IL-1α and TNF-α, are strongly induced during the early stages of apical periodontitis in a murine model of pulp exposure and were primarily secreted by macrophages and fibroblasts (Tani-Ishii *et al*, 1995). The functional significance of other types of Th1 and Th2 cytokines was assessed in the pulp exposure model with gene knockouts. These studies revealed that IFN-γ, IL-12, and IL-18 had little effect on apical periodontitis, while IL-6 and IL-10 showed protective effects against bone destruction in these pulp exposure models (Sasaki *et al*, 2000, 2004; Balto *et al*, 2001). The pro-inflammatory cytokine IL-17, produced principally by $CD4^+$ Th17 cells and neutrophils, is elevated in inflammatory bone lesions, including periodontitis and periapical lesions (Vernal *et al*, 2005). Ablation of the IL-17 signaling by IL-17RA knockout or IL-17 neutralizing antibody showed protective effects against apical periodontitis in the pulp exposure model, confirming its role in mediating inflammation-induced bone destruction (AlShwaimi *et al*, 2013). In another study, $CD4^+$ Treg cells expressing the master regulator Foxp3 were shown to accumulate in the periapical lesions, in parallel to an increase in the bony defect, suggesting a possible role of Treg cells in the balance of periapical inflammation (AlShwaimi *et al*, 2009). Thus, the progression of infection-related bone lesions in the oral cavity involve a complex interplay among pro-inflammatory and immunomodulatory cytokines, as well as lineage-specific differentiation of $CD4^+$ Th subsets.

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Mechanistic studies involving KMT/KDMs in oral inflammation are very limited, while studies pertaining to other epigenetic modifications, such as histone acetylation and DNA methylation, demonstrate their regulatory roles in the disease process. Xuan *et al* (2016) showed indirect evidence of KDM6B involvement in periodontal inflammation through regulation of M2 macrophage polarization, although the detailed mechanisms by which KDM6B regulates periodontal tissue responses to infection remains unknown. Downregulated expression of Lys methyltransferase EZH2 and concordant induction of KDM6B were noted in infected dental pulp, resulting in a loss of differentiation capacities of dental pulp cells exposed to inflammatory mediators, including LPS and TNF-α (Hui *et al*, 2014). Acetylation of histones is generally associated with active gene expression. All Lys sites, for example, K5, K8, K12, and K16, of H4 are acetylated in euchromatin, whereas Lys acetylation is strongly suppressed on H4 in heterochromatin (O'Neil and Turner, 1995). While H3/H4 acetylation is generally linked with gene transcription, recent studies demonstrate site-specific roles of histone acetylation or deacetylation for gene activity (Shahbazian and Grunstein, 2007). Hence, like KMT/KDMs, histone acetylation/deacetylation is considered a dynamic process to modulate gene activity. In periodontitis models, H3 acetylation was found to be induced in gingival epithelial cells through upregulation of histone acetyltransferase p300/CBP, although identification of specific gene targets mediating the periodontal inflammatory response requires further investigation (Martins *et al*, 2016). The role of DNA methylation at CpG-rich promoter regions has been more extensively studied in oral inflammatory conditions than that of histone modifications. Several studies showed reduced methylation of CpG islands on the promoter regions of inflammatory genes, such as TLR2, TLR4, and IL-6, in oral epithelial tissue with periodontitis, which presumably result from diminished expression of DNMT1 and DNMT3a (Ishida *et al*, 2012; De Camargo *et al*, 2013; Martins *et al*, 2016). Notably, DNA CpG hypermethylation is mechanistically connected with KMT activity, such as G9a, and H3K9 methylation and recruitment of HP1 and DNMT lead to CpG island hypermethylation and gene silencing (Fuks *et al*, 2003; Audergon *et al*, 2015). Likewise, EZH2, the KMT subunit of PRC2, directly modulates the DNA methylation state of the target promoter through protein interactions with DNMTs (Viré *et al*, 2006). These findings demonstrate epigenetic cross talk between histone and DNA modifications to tighten the control of target gene expression through epigenetic modulators. Further studies will be required to elucidate the details of epigenetic mechanisms underlying inflammatory lesions throughout the oral cavity and to determine whether they are similar or unique to such processes elsewhere in the body. In doing so, it may be possible to develop novel therapeutics in the management of inflammatory conditions in the oral cavity.

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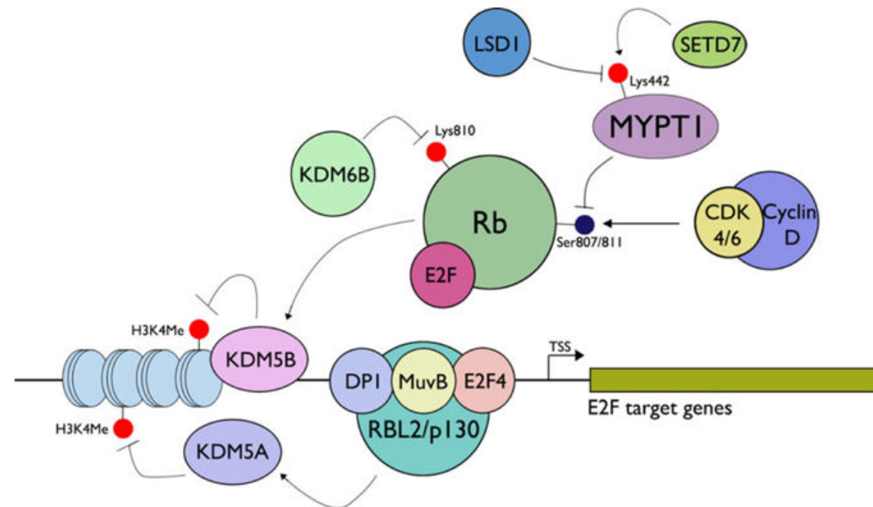

Figure 1.

Functional interaction of KDMs with Rb-mediated control of E2F target genes. SETD7 methylates Lys442 of MYPT1, a protein phosphatase targeting Rb at Ser807/811. Demethylation of MYPT1 by LSD1 downregulates MYPT1, causing increased Rb phosphorylation, thereby inactivating Rb (Cho *et al*, 2011). The active form of Rb also recruits KDM5B to E2F-responsive promoters to demethylate H3K4Me2/3, resulting in gene silencing. During senescence or quiescence, Rb-mediated cell cycle arrest is achieved through the assembly of a multi-subunit complex of DP1, Rb-like 2 (RBL2/p130), E2F4, and MuvB, called the DREAM complex (Osterloh *et al*, 2007). The DREAM complex recruits KDM5A to the promoter regions of DREAM target genes, causing H3K4 demethylation and gene silencing (Beshiri *et al*, 2012).

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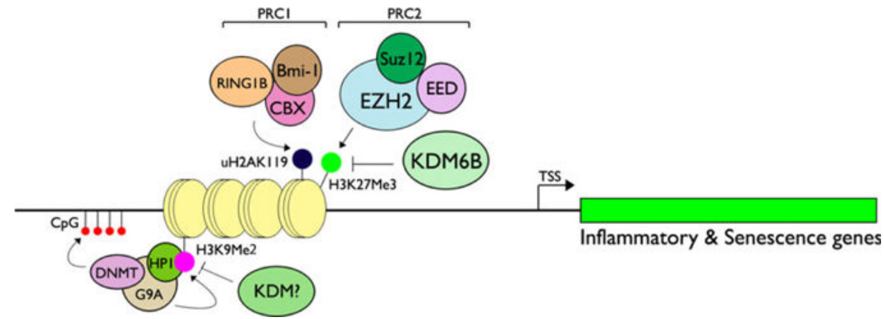

Figure 2.

KDMs and KMTs regulating inflammatory and senescence genes through epigenetic modifications. PRC2 subunit EZH2, a KMT specific for H3K27, triggers K27Me3, which is recognized by CBX subunit of PRC1, which also contains Bmi-1 and RING1B. These protein subunits of PRC1 demonstrate E3 ubiquitin ligase activity for H2AK119, causing monoubiquitination, which then inhibits Pol II elongation (Li *et al*, 2006; Zhou *et al*, 2008). During inflammatory signaling or senescence, KDM6B is induced and demethylates H3K27Me3 and suppresses PcG-mediated gene silencing. Inflammatory cytokines are also regulated through G9a, a KMT specific for H3K9Me2 that recruits HP1 and Dnmt3a/b, resulting in hypermethylation of CpG islands (Han *et al*, 2016). During inflammation, demethylation of H3K9Me2 has been found strongly associated with RNA Pol II recruitment to the pro-inflammatory cytokines (Saccani and Natoli, 2002), although the corresponding KDM is yet to be identified.

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Table 1

Summary of methylated Lys histone marks and corresponding KDMs/KMTs

Histone Marks	*KDMs*	*KMTs*	*Target Gene Effect*	*General Biological Effects*
H3K4Me2	KDM1A/LSD1 KDM1B/LSD2	SETD1A SET7	Activation	• Enriched over transcription binding regions of promoters[a]
H3K4Me3	KDM2B/FBXL10/JHDM1B KDM5A/JARID1A KDM5B/JARID1B KDM5C/JARID1C KDM5D/JARID1D	SETD1A SET7	Activation	• Enriched at the transcription start sites (TSS).[b] • Functions as the focal point for assembly of transcription preinitiation complex.[c] • Broad H3K4Me3 peak spans the enhancer regions of tumor suppressors.[d]
H3K9Me1	KDM1A/LSD1 KDM3A/JMJD1/JHDM2A KDM7A/JHDM1D KDM7B/PHF8	SUVR5 SUV39H1 SUV39H2	Repression	• Establishes constitutive heterochromatin.[e] • Regulates rRNA synthesis.[f]
H3K9Me2	KDM4A/JMJD2A/JHDM3A KDM4B/JMJD2B KDM4C/JMJD2C/JHDM3C KDM4D/JMJD2D KDM4E/JMJD2E	G9A SETDB1	Repression	• Establishes constitutive heterochromatin.[e] • Linked with endotoxin-tolerant phenotype in immune cells.[g] • Mediates gene silencing at subtelomeric region.[h] • Regulates osteogenic differentiation.[i]
H3K27Me3	KDM6A/UTX KDM6B/JMJD3 KDM6C/UTY KDM7A/JHDM1D	EZH1 EZH2	Repression	• Linked with silencing of inflammatory mediators.[j] • Enriched over senescence gene promoters.[k]
H3K36Me2	KDM2A/FBXL11/JHDM1A KDM2B/FBXL10/JHDM1B	SET2	Activation/Repression	• Determines rDNA promoter regulation.[l]
H3K36Me3	KDM4A/JMJD2A/JHDM3A KDM4B/JMJD2B KDM4C/JMJD2C/JHDM3C KDM8/JMJD5	SET2	Repression	• Associated with silencing subtelomeric transcripts.[m] • Regulates longevity through suppressing intragenic cryptic transcription.[n]
H3K56Me3	KDM4E/JMJD2E	SUV39H	Repression	• Associated with pericentromeric heterochromatin.[o]
H4K20Me1/2	KDM1A/LSD1 KDM7B/PHF8	SET8 SUV420H1		• Broadly distributed across genome.[p] • Involved in cell cycle regulation and DNA repair.[p] • Regulates chromatin structure during cell cycle progression.[p]
H4K20Me3	KDM7C/PHF2	SMYD5 SUV420H2	Repression	• Enriched at pericentric and subtelomeric heterochromatin.[q] • Regulates the expression of TLR4-target inflammatory genes.[r]

[a]Wang *et al* (2014);

[b]Lloret-Llinares *et al* (2012);

[c]Lauberth *et al* (2013);

[d]Chen *et al* (2015);

[e]Audergon *et al* (2015);

[f]Zhu *et al* (2010);

[g]El Gazzar *et al* (2008);

[h]Arnoult *et al* (2012);

[i]Deng *et al* (2015);

[j]De Santa *et al* (2009);

[k]Agger *et al* (2009);

[l]Tanaka *et al* (2015);

[m]Suzuki *et al* (2016);

[n]Sen *et al* (2015);

[o]Jack *et al* (2013);

[p]Jørgensen *et al* (2013);

[q]Shinchi *et al* (2015);

[r]Stender *et al*, 2012.

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