The role of α-ketoglutarate–dependent proteins in pluripotency acquisition and maintenance

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Khoa A. Tran[‡§1], **Caleb M. Dillingham**[‡¶], **and Rupa Sridharan**[‡‖2]

From the [‡]*Wisconsin Institute for Discovery,* [‖]*Department of Cell and Regenerative Biology,* [§]*Molecular and Cellular Pharmacology Program, and* [¶]*Cellular and Molecular Pathology Program, University of Wisconsin-Madison, Madison, Wisconsin 53715*

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α-Ketoglutarate is an important metabolic intermediate that acts as a cofactor for several chromatin-modifying enzymes, including histone demethylases and the Tet family of enzymes that are involved in DNA demethylation. In this review, we focus on the function and genomic localization of these α-ketoglutarate–dependent enzymes in the maintenance of pluripotency during cellular reprogramming to induced pluripotent stem cells and in disruption of pluripotency during *in vitro* differentiation. The enzymatic function of many of these α-ketoglutarate–dependent proteins is required for pluripotency acquisition and maintenance. A better understanding of their specific function will be essential in furthering our knowledge of pluripotency.

Pluripotent stem cells have the ability to self-renew indefinitely and give rise to all the cell types of a multicellular organism. Pluripotency appears transiently during early embryonic development and can be captured with the correct culture conditions to obtain embryonic stem cells (ESCs).[3] Pluripotent stem cells can also be derived from somatic cells either by the transfer of a somatic cell nucleus to an oocyte (SCNT) (1, 2) or through reprogramming, which is the overexpression of a small set of factors, usually Oct4, Sox2, Klf4, and c-Myc (OSKM) to generate induced pluripotent stem cells (iPSCs) (3–6) (Fig. 1). *In vitro* pluripotency is thought to exist in a continuum that is profoundly affected by growth conditions (7) For example, when ESCs are grown in the presence of signaling inhibitors to mitogen-activated protein kinase and glycogen synthase kinase (2i), their transcriptional profile better resembles the *in vivo* equivalent from the blastocyst stage of early embryonic development than that of ESCs grown in serum. Both the maintenance of pluripotency and its acquisition from somatic cells are affected by culture conditions. α-Ketoglutarate (α-KG)–dependent enzymes are important regulators of chromatin structure and are particularly sensitive to levels of intracellular metabolites as well as external components of the growth medium. Pluripotent cells can be grown in serum replacement medium, which contain vitamin C (Vc), that can affect the rate of catalysis of α-KG enzymes (Fig. 2) (8, 9). In the 2i conditions mentioned above, mouse ESCs (mESCs) utilize both glucose and glutamine in the medium to maintain high levels of α-KG to alter chromatin modifications (10, 11).

The plasticity of pluripotent stem cells is associated with a hyperdynamic chromatin structure where histones and heterochromatin-associated proteins have a higher mobility than in somatic cells (12). Pluripotent stem cells also have a reduced amount of heterochromatin, usually associated with gene repression, which is enriched for histone modifications such as histone H3 lysine 9 methylation (H3K9me2 and H3K9me3) (13–15). Another chromatin feature that is more prevalent in pluripotent cells are the "bivalent" domains. These are regulatory regions that contain both an activating histone modification, histone H3 lysine 4 methylation (H3K4me3), and a repressive modification, histone H3 lysine 27 methylation (H3K27me3), that is mediated by the polycomb repressive complex 2 (PRC2) (1, 16). Several lineage specification genes are held in such a bivalent state and are resolved into either expression by retaining the H3K4me3 mark or repression by retaining the H3K27me3 mark (1, 2). Thus, the bivalent state can be considered poised for gene transcription. The H3K27me3 mark is usually found in opposition to H3K36me, a mark for active transcription elongation in gene bodies (3–5). In addition to histone modifications, DNA can also be methylated. In general, DNA methylation correlates with gene repression when present at regulatory regions. A pathway to active DNA demethylation

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[2] To whom correspondence should be addressed: Dept. of Cell and Regenerative Biology, University of Wisconsin-Madison, Madison, WI 53715. Tel.: 608-316-4422; E-mail: rsridharan2@wisc.edu.

[3] The abbreviations used are: ESC, embryonic stem cell; SCNT, somatic cell nuclear transfer; OSKM, Oct4, Sox2, Klf4, and c-Myc; iPSC, induced pluripotent stem cell; 2i, inhibitors to mitogen-activated protein kinase and glycogen synthase kinase; α-KG, α-ketoglutarate; Vc, vitamin C; mESC, mouse ESC; PRC, polycomb repressive complex; 5mC, 5-methylcytosine; 5hmC, 5-hydroxymethylcytosine; Jmjd, Jumonji domain; TSS, transcription start site; H3K9me, histone H3 lysine 9 methylation; KD, knockdown; KO, knockout; DKO, double KO; TKO, triple KO; hESC, human ESC; EpiSC, epiblast stem cell; MEF, mouse embryonic fibroblast; ARF, ADP-ribosylation factor; ZF, zinc finger; MET, mesenchymal-to-epithelial transition; FBS, fetal bovine serum; HIF, hypoxia-inducible factor; miR, microRNA; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; 5fC, 5-formylcytosine; 5caC, 5-carboxylcytosine; Tet, ten-eleven-translocation; 5hmU, 5-hydroxymethyluracil; TSKM, Tet1, Sox2, Klf, and c-Myc; Ogt, *O*-linked GlcNAc-transferase; N6-mA, N^6-methyladenine; PHD, prolyl-hydroxylase domain enzyme; FIH, factor inhibiting HIF.

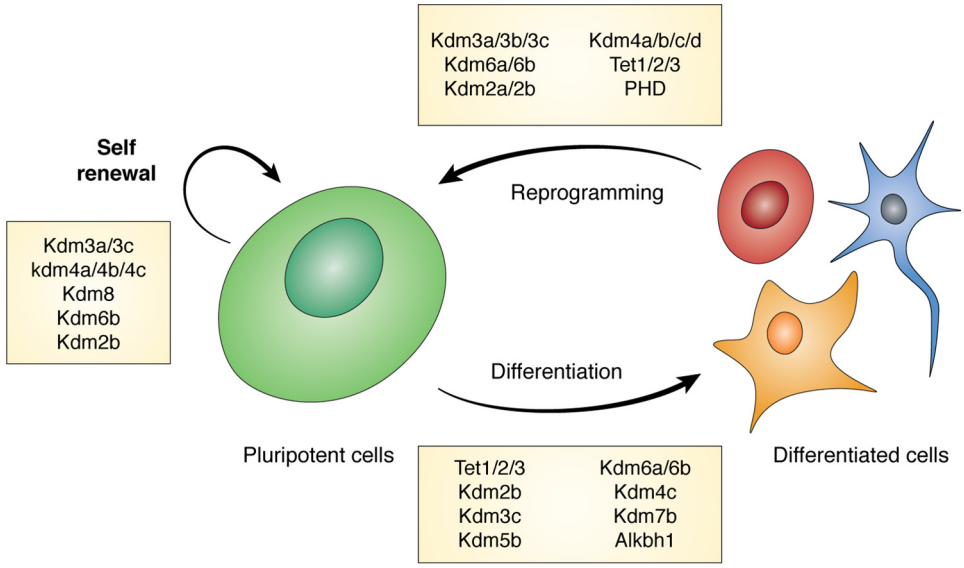

Figure 1. Pluripotent stem cells can self-renew indefinitely and differentiate into a multitude of cells. Pluripotent cells can be isolated both from the inner cell mass of the blastocyst and the reprogramming of differentiated cells. In the *boxes* are the α-ketoglutarate–dependent proteins that are known to modulate each process.

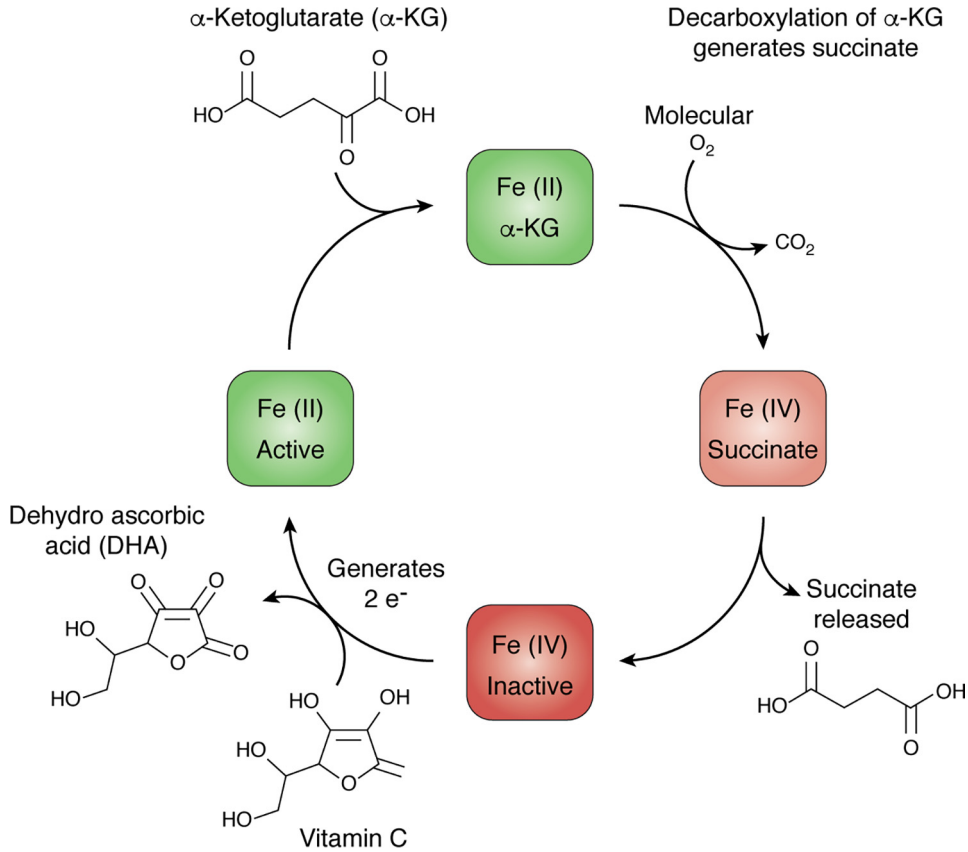

Figure 2. Fe(II) recycling of α-KG–dependent dioxygenases. The catalytic activity of α-KG dioxygenases utilizes oxygen for the decarboxylation of α-KG and oxidation of Fe(II) to Fe(IV), rendering the enzyme inactive. Vitamin C can be used to regenerate iron back to the Fe(II) state, thus restoring catalytic activity.

can be provided by the conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) (4–6). The levels of 5hmC are higher in ESCs than most differentiated cells. α-KG–dependent enzymes include the jumonji domain–containing histone demethylases, Tet proteins that modify DNA methylation, and RNA-modifying enzymes of the Alkbh family, thus modulating the delicate state of pluripotency itself.

In this review, we focus on the changes to genomic localization of the cognate histone or DNA modifications resulting from perturbation of levels of α-KG–dependent enzymes as well as the subsequent changes in transcriptional output. We

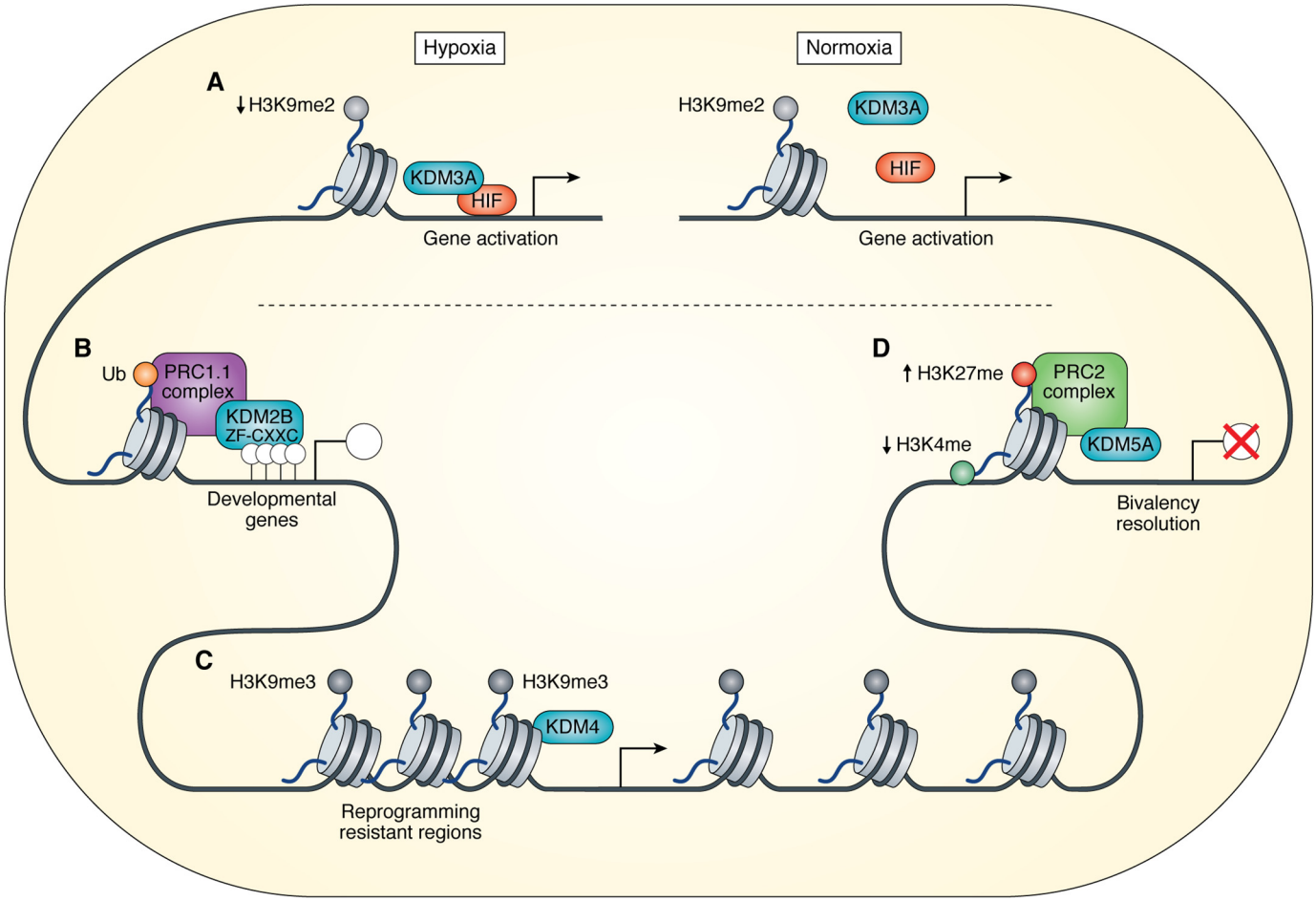

Figure 3. *A*, HIF1 recruits Kdm3a under hypoxic conditions, regulating gene expression by the removal of H3K9me2. *B*, Kdm2b binds unmethylated CpG islands via a ZF-*CXXC* domain. Kdm2b is part of the PRC1.1 complex that mediates gene repression through the ubiquitination (*Ub*) of histone H2A. *C*, the Kdm4 family of proteins reduce the levels of H3K9me3 at reprogramming-resistant regions, opening the condensed heterochromatin and allowing transcription. *D*, Kdm5a interacts with the PRC2 complex, removing H3K4me at bivalent genes, leading to gene repression.

organize the review based on the phenotypes obtained during 1) the maintenance of the pluripotent state, 2) the acquisition of pluripotency from somatic cells, and 3) its disruption during *in vitro* differentiation.

Jumonji domain (Jmjd)-containing histone demethylases

Kdm5 family: Demethylases of histone H3K4

Kdm5a, Kdm5b, and Kdm5c share specificity for demethylating H3K4me2/3. Because H3K4me3 is enriched at the transcription start sites (TSSs) of poised or active genes, it is expected that a loss of these enzymes would result in increased gene expression.

Kdm5a was found to be an interacting partner of the PRC2 complex that contains the H3K27 methyltransferase Ezh2 (17). Thus, there is an association between the methyltransferase and demethylase of histone marks that are correlated with opposing effects on gene expression. When bivalent domains are resolved during differentiation of pluripotent cells, the regions that become silenced and retain H3K27me3 have the potential to simultaneously be demethylated at H3K4me3. In mESCs, Kdm5a preferentially binds the TSS of genes that are activated during differentiation (8, 9). Kdm5a-depleted mESCs are pluripotent but display increased expression of specific PRC2 target genes (17). Kdm5a depletion does not impact PRC2 binding or the levels of H3K27me3; however, knockdown (KD) of PRC2 component Suz12 leads to a reduction of Kdm5a binding at shared promoters and a subsequent increase in H3K4me3 (17) (Fig. 3). Thus, although Kdm5a is recruited to shared locations by PRC2, it does not appear to affect the function of PRC2 (10, 11).

There have been conflicting reports on the role of Kdm5b in pluripotency. Although overexpression of Kdm5b in mESCs led to increased proliferation (1, 16), there are contradictory reports on the effects of Kdm5b depletion as well as its genomic localization. The KD of Kdm5b compromised self-renewal of ESCs and led to spontaneous differentiation (18). In this study, Kdm5b was found localized intragenically and seemed to be recruited by MRG15, a protein that binds to transcription elongation–associated H3K36me. Therefore, a role for Kdm5b activity in suppressing cryptic transcription initiation from within the gene body was proposed (18).

In contrast to these results, genomic deletion of Kdm5b in ESCs was compatible with self-renewal and pluripotency, but differentiation toward ectoderm was compromised (19). Kdm5b was found enriched at both the TSS and intragenic

regions that did not coincide with H3K36me. The depletion of Kdm5b increased H3K4me3 at the TSS but did not cause many gene expression changes. Kdm5b-localized promoters had lower levels of H3K4me3, suggesting that it functioned in maintaining levels rather than eliminating this mark (19). Corroborating this idea, Kdm5b KD ESCs were refractory to embryoid body differentiation due to continued pluripotency gene expression as well as maintenance of H3K4me3 at bivalent genes (20). During the acquisition of pluripotency by SCNT, the KD of Kdm5b by siRNA led to an increase of cloned embryos arresting in the four-cell stage of the blastocyst, suggesting an essential role for appropriate localization of H3K4me3 (21).

Another study found that Kdm5b was localized to promoters, intragenic regions, and enhancers. Kdm5b KD led to an increase of H3K4me3 at the gene body, a decrease at promoters, and spreading at enhancers (20). Thus, Kdm5b may function in restricting distribution of H3K4me3. Kdm5b KD also increased the expression of alternatively spliced exons in ESCs, suggesting a role for intragenic localization (22).

Kdm5c binds to both proximal promoter regions and distal intergenic enhancer regions in mESCs. Upon KD of Kdm5c, pluripotency is maintained, and global H3K4me3 levels remained unchanged. However, at loci that are greatly enriched for Kdm5c binding, H3K4me1 levels were gained at the expense of H3K4me3 levels. This effect was magnified at regions bound by the transcription factor c-Myc, which is a direct interacting partner of Kdm5c (23).

Thus, the Kdm5 family is involved in fine-tuning both the degree and localization of H3K4me3. The loss of each individual enzyme seems compatible with pluripotency, suggesting redundancy in function. Site specificity may be conferred by interacting factors other than c-Myc and PRC2. In this regard, it is interesting that in nonpluripotent cells, Kdm5a interacts with the Sin3 histone deacetylase complex specifically at sites that are bound by the transcription factor E2F4 (24). The role of such an interaction in pluripotent cells has not been determined.

Kdm6 family: Demethylases of histone H3K27 methylation

Kdm6a (UTX) and Kdm6b (JMJD3) can demethylate H3K27me2/me3, whereas the mouse Kdm6a paralog, Kdm6c (UTY), is located on the Y chromosome and lacks this activity (25). Similar to the reciprocal recruitment observed in the case of the Kdm5b–PRC2 complex, Kdm6a, the H3K27 demethylase, is part of the mixed-lineage leukemia H3K4 methyltransferase complex (26).

Both mouse and human Kdm6a, as well as mKdm6b, are dispensable for maintenance of self-renewal as knockout (KO) ESCs display normal morphology, cell proliferation rates, and high levels of pluripotency gene expression (27–34). Male Kdm6b KO mESCs up-regulate lineage commitment genes upon differentiation and contribute toward adult chimeras (27). Unlike the Kdm5 family, there are nonredundant functions of Kdm6a and Kdm6b in pluripotency. Overexpression of the Kdm6b catalytic domain (but not that of Kdm6a) leads to spontaneous differentiation in human ESCs (hESCs) and is accompanied by depleted global levels of H3K27me3 (18, 35). This is surprising given that the catalytic Jumonji domains are the most conserved regions between members of the same subfamily (36). Remarkably, ectopic Kdm6b expression in conjunction with lineage-defining transcription factors promotes hESC differentiation into multiple lineages (18, 35). Because directed differentiation of ESCs into specific lineages remains a goal of regenerative therapy, the transient overexpression of Kdm6b could be a useful tool for accelerating differentiation into desirable cell types.

Box 1: Aspects of Pluripotency, Reprogramming, and Differentiation

A. Pluripotency:
- Self-renewal in ESCs can be assessed by intact morphology of colonies which are dome-shaped, stain positive for alkaline phosphatase (AP), and express the pluripotency related genes Oct4, Sox2 and Nanog.
- Injection of pluripotent cells into blastocysts contributes to the developing embryo and generates chimeric animals.
- Epiblast-derived stem cells (EpiSC) can differentiate into all three germ layer – endoderm, ectoderm, and mesoderm, but unlike naïve ESCs, cannot form chimeras.

B. Reprogramming:
- Reprogramming can occur through overexpression of candidate factors in somatic cells to make iPSCs and occurs in multiple stages. When starting from mouse embryonic fibroblasts, early events include downregulation of somatic cell expression, mesenchymal to epithelial transition (MET), change to an ESC-like morphology; while a late event is the activation of pluripotency related genes such as Nanog and Oct4.
- Alternatively, reprogramming can occur by transplanting of differentiated somatic nuclei into enucleated oocytes referred to as somatic cell nuclear transfer (SCNT).

C. Differentiation:
- In vitro differentiation can be mediated by withdrawal of LIF in the absence of a mouse embryonic fibroblast (MEF) feeder layer in a monolayer, or in droplets that cause the formation of embryoid bodies (EB) that contain cells from all three germ layers. Retinoic acid (RA) skews differentiation towards ectoderm lineage.

Kdm6a and Kdm6b also have opposing roles in generating iPSCs with well-defined mechanisms. Kdm6a promotes the reprogramming of both epiblast stem cells (EpiSCs) to naïve pluripotency (Box 1) and somatic cells to iPSCs in a catalysis-dependent manner (19, 27). In the absence of Kdm6a, several important pluripotency genes (*e.g.* Sall4) are not activated due to retention of the repressive H3K27me3 that silences their expression in somatic cells such as mouse embryonic fibroblasts (MEFs). Kdm6a is likely to be targeted to these loci by direct interaction with the Oct4, Sox2, and Klf4 reprogramming factors (19, 27). In contrast to Kdm6a, Kdm6b depletion improves reprogramming of MEFs to iPSCs by two independent activities. First, its depletion decreases Ink4/ARF transcription, removing a senescence block and leading to increased reprogramming (20, 37). Second, it promotes the TRIM26 ubiquitin ligase–mediated degradation of a particular scaffold protein, Phf20. Phf20 is required to assemble a transcription activation complex at the Oct4 locus during reprogramming (20, 37).

In contrast to their opposing roles in gaining pluripotency, Kdm6a and Kdm6b enhance *in vitro* differentiation from ESCs (32, 38, 39). Hox clusters are large bivalent domains that are important for patterning the body plan during development. Surprisingly, the deletion of either Kdm6a alone (22, 40) or combined with Kdm6b (23, 31) in mESCs is accompanied by a loss of H3K27me3 rather than a gain as would be expected when deleting a demethylase. This suggests that the down-regulation of PRC2 expression may contribute to passive dilution of the H3K27me3 mark during differentiation.

Both the Kdm6a and Kdm6b KOs decrease the levels of the mesoderm-specifying gene Brachyury during differentiation.

Interestingly, the introduction of a catalytically inactive mutant of Kdm6a restores Brachyury expression, suggesting functions beyond H3K27 demethylation (25, 28, 29, 34). Thus, during differentiation, the function of Kdm6a and Kdm6b may be linked to both H3K27 demethylation and the structural role they provide as part of protein complexes.

Kdm2 family: Demethylases of histone H3K36 methylation

H3K36me1/2, a histone modification associated with active transcription elongation, can be demethylated by Kdm2a (Jhdm1a) and Kdm2b (Jhdm1b). In mESCs, both Kdm2a and Kdm2b are preferentially recruited to non-DNA–methylated CpG islands (27–34, 41, 42). The depletion of Kdm2b, but not Kdm2a, leads to spontaneous differentiation of mESCs, indicating a nonredundant role for Kdm2b in maintaining pluripotency (42).

Both Kdm2a and Kdm2b share a ZF-C*XX*C domain that targets the enzyme to unmethylated CpG islands throughout the genome; however, they interact with different partners to mediate gene regulation (41, 43). Kdm2b is part of the noncanonical PRC1 complex, PRC1.1, which has E3 ubiquitin ligase activity that mediates monoubiquitination of histone H2A, leading to gene repression (Fig. 3). In mESCs, Kdm2b recruits PRC1.1 to targets that are not specifically depleted for H3K36me enrichment (44). Furthermore, spontaneous differentiation of mESC upon Kdm2b depletion can be rescued by the catalytic mutant but not the C*XX*C-deleted Kdm2b mutant.

Interestingly, the reverse phenomenon where a PRC1 component, Phf19, recruits H3K36me demethylase NO66 to polycomb-related regions also occurs in mESCs. However, the deletion of NO66 does not have effects on pluripotency or differentiation (27, 45).

The role of Kdm2a and Kdm2b has also been assessed in several reprogramming systems. The overexpression of Kdm2b enhances OSKM reprogramming by promoting the mesenchymal-to-epithelial transition (MET) (46), an early event in the reprogramming of MEFs in serum (FBS)-containing medium (Box 1). This enhancement occurs in a catalysis-dependent manner by demethylating H3K36me at epithelial genes such as E-cadherin (46). The addition of Vc in general can improve reprogramming efficiency, which is further enhanced by the overexpression of either Kdm2a or Kdm2b. However, unlike the FBS condition, under Vc conditions, Kdm2b seems to function in the suppression of the Ink4/Arf senescence block (47). In fact, in the presence of Kdm2b and Vc, Oct4 alone (as compared with the entire set of OSKM) is sufficient to generate iPSCs through the activation of the ESC cell cycle–specific microRNA miR-302 cluster (47). Signaling through BMP4 counteracts this Oct4/Vc/Kdm2b-mediated reprogramming in a PRC1-dependent manner because under these conditions the levels of H2A ubiquitination genome-wide are significantly reduced (48). Taken together, depending on the conditions of reprogramming, the exact targets that are causal for increased reprogramming efficiency may differ, but the activity of Kdm2b is important for this process.

The Kdm2 family has been less characterized during *in vitro* differentiation, although Kdm2b-depleted ESCs cannot fully silence pluripotency genes. This may lead to the impaired activation of lineage-specific genes (42).

Kdm3: Demethylases of H3K9me1/me2

Kdm3a, Kdm3b, and Kdm3c share specificity for demethylating the repressive histone modifications H3K9me1/2 but play different roles during reprogramming and differentiation. The depletion of any of the three Kdm3 family members seems to compromise self-renewal of ESCs (49). Kdm3a depletion down-regulates pluripotency gene Tcl1 expression, which may be causal for the self-renewal phenotype (50). When ESCs are exposed to Vc, germline-associated genes of the blastocyst are up-regulated in a Kdm3a- and Kdm3b-dependent manner (51).

Given the responsiveness of the Jmjd enzymes to the antioxidant Vc, it is not surprising that they may also act as oxygen sensors. Under conditions of low oxygen tension, *i.e.* hypoxia, proteins called hypoxia-inducible factors (HIFs) are stabilized. Kdm3a has been shown to impact gene expression in an interaction with HIF1 in both cell culture and *in vivo* systems (52) (Fig. 3). The environmental milieu of early development is hypoxic and instructive for placental development. Under such hypoxic conditions, HIF1 is able to recruit Kdm3a to demethylate the promoter of MMP12, a gene important for placental development, spiral artery remodeling, and trophoblast migration. This leads to activation of gene expression specifically under hypoxic conditions (53).

Depletion of Kdm3c in ESCs leads to disruption of self-renewal and a decrease in the expression of microRNAs of the miR-200 family and the miR-290/295 cluster (49). This miR dysregulation leads to an increase in ERK/MAPK signaling and drives cells toward epithelial-to-mesenchymal transition that can be partially rescued by ectopic miR expression (49). Although the catalytic activity of Kdm3c has been disputed (54), there is a global increase of H3K9me1/2/3 upon Kdm3c KD in mESCs.

Overexpression of Kdm3a improved the efficiency of Oct4 reactivation in ESC fusion-induced reprogramming of neural stem cells (55). This improvement depended on the catalytic activity of Kdm3a as the overexpressing cells exhibited a widespread loss of H3K9me2 (55). Interestingly, only Kdm3b is essential in the Vc-mediated conversion of partially reprogrammed intermediates (pre-iPSCs) to iPSCs (56, 57). Kdm3b was also recently shown to have activity in demethylating H4R3 arginine methylation in hematopoietic stem cells (58). It needs to be determined whether these modifications on H3 and H4 are coordinately regulated. Kdm3c KD in OSKM-mediated reprogramming of MEFs leads to a decrease in obtaining *bona fide* iPSC colonies (49, 59). These effects could be mediated through the regulation of the Oct4 locus because Kdm3c is enriched at its distal enhancer, reducing H3K9me2 and promoting FACT (facilitates chromatin transcription) chaperone recruitment (59). Thus, although all three Kdm3 enzymes are required for reprogramming, they seem to contribute to the process in distinct mechanisms.

Contrary to the phenotype in mESCs, when Kdm3c is knocked down in hESCs, Oct4 and Nanog levels as well as pluripotency are maintained. However, neuronal differentiation is

much more rapid due to the reduced expression of ESC-specific miR-302 in these cells (60).

Kdm4 family: Demethylases of histone H3K9me2/me3 and H3K36me3 methylation

The Kdm4 family consists of four members, Kdm4a–d, which have demethylase activity toward H3K9me2/3 with Kdm4a–c also having specificity to H3K36me3. This substrate specificity is interesting as H3K9me2/3 are repressive modifications, whereas H3K36me3 is associated with actively transcribed genes.

Conflicting studies complicate the roles of the Kdm4 family in pluripotency. KD of both Kdm4b and Kdm4c in mESCs led to morphological changes that could be partially rescued by the catalytic mutant proteins, suggesting additional roles beyond H3K9 demethylation (61). Kdm4c was thought to function through demethylating the promoter of the pluripotency gene Nanog (50). However, this was not replicated in another study that instead found a connection between Kdm4b and Nanog target genes (61). Although Kdm4b and Kdm4c share some locations in ESCs, the unique targets of each protein show differential association: Kdm4c is localized with bivalent and PRC2-occupied promoters in ESCs, whereas Kdm4b is associated with H3K4me and activating marks (61).

The phenotypic results of Kdm4 depletion are disputed because individual-KO ESCs of Kdm4a, Kdm4b, and Kdm4c remain pluripotent (62–64). Only the combined deletion of Kdm4a and Kdm4c (DKO) impaired proliferation of mESCs in a catalytic activity–dependent manner (62). Nonetheless, all three proteins are localized to H3K4me3-enriched regions. At highly enriched Kdm4a/4c sites, there is an increase in H3K9me3 levels upon Kdm4a/4c deletion. Furthermore, the DKO cells display a propensity to express endoderm markers when grown in serum but not 2i conditions (62). This sensitivity to culture conditions was further borne out when the localization of Kdm4c switched from largely TSS-enriched in 2i to more distally located in serum-grown ESCs (64). This switch may be related to the post-transcriptional regulation of Kdm4c because the mitogen-activated protein kinase inhibitor in 2i medium prevents the phosphorylation and degradation of Kdm4c (65).

Although the roles of the Kdm4 proteins in the maintenance of pluripotency remain conflicting, the overexpression of several Kdm4 family members promotes acquisition of pluripotency in several systems. In mice, injection of catalytically competent Kdm4d mRNA during SCNT increases efficiency and improves the developmental potential of SCNT embryos (66). Excitingly, the inclusion of the primate Kdm4d resulted in the recent cloning by SCNT of monkeys that survived to term (67). The function of Kdm4d seems to be to decrease H3K9me3 levels at so-called "reprogramming-resistant regions" that are repressed in SCNT at the two-cell stage, leading to their expression (21, 66) (Fig. 3). Kdm4a performs a similar function when ectopically expressed during human SCNT (68). Kdm4b can also improve the developmental potential of SCNT-derived embryos (69, 70). Furthermore, depletion of Kdm4b or Kdm4c reduces Vc-mediated conversion of pre-iPSCs to iPSCs, whereas overexpression of Kdm4b promotes MEF reprogramming to iPSCs (57).

Deletion of Kdm4c resulted in the misregulation of lineage-specific genes upon differentiation (64). Interestingly, at distal regions, Kdm4c colocalizes with the H3K9me2 methyltransferase G9a. However, these sites lack H3K9me2, suggesting constant turnover of the modification at such loci (64).

Kdm7 family

The Kdm7 family consists of three family members, Kdm7a (KIAA1718), Kdm7b (PHF8), and Kdm7c (PHF2), that can all demethylate H3K9me2; however, Kdm7b has additional specificity to H4K20me1, and Kdm7c has additional specificity to H3K27me1/2. Kdm7a promotes neural differentiation from mESCs by demethylating H3K27me2 at the FGF4 locus (71). Deletion of Kdm7b in mESCs does not compromise maintenance of pluripotency, but upon differentiation, Kdm7b KO mESCs showed an increase in expression of mesodermal and cardiac lineage genes by controlling apoptosis (72).

Kdm8

The demethylase activity of Kdm8 toward H3K36me2 is disputed. Depletion of Kdm8 in hESCs compromises pluripotency and up-regulates lineage specification markers. KD of Kdm8 in hESCs leads to a higher percentage of cells in G_1 phase due to elevated levels of the cell cycle gene CDKN1A, but it is unclear whether this is due to direct regulation of H3K36me2 levels. The changes in cell cycle precede down-regulation of pluripotency gene expression upon Kdm8 depletion, suggesting that the disruption of the cell cycle may lead to pluripotency defects. This corroborates the idea that certain phases of the cell cycle are more conducive to exit from pluripotency (73). Additional regulation of pluripotency by Kdm8 comes from direct binding and regulation of the pluripotency-related miR-302 cluster (74).

Taken together, several common themes emerge from the studies on the α-KG–dependent histone demethylases in pluripotency thus far. 1) They can have overlapping functions that may rely on interacting proteins. 2) They can have functions that do not rely on catalytic activity. 3) They can localize to the same genomic locations as the mark that they modify. Technically confounding results are obtained between KD and KO studies. With the advent of CRISPR-Cas9 technology it may be preferable to perform KOs to obtain clearer phenotypic results.

Tet: DNA 5-methylcytosine modifiers

As mentioned above, DNA can be methylated on the 5th position of cytosine, known as 5mC, which is usually associated with gene repression when found at regulatory regions. Its removal can occur by the iterative oxidation by the ten-eleven-translocation family of enzymes (Tet1, Tet2, and Tet3) from 5mC to 5hmC to 5-formylcytosine (5fC) to 5-carboxylcytosine (5caC). 5fC and 5caC can be excised by thymine-DNA glycosylase to generate cytosine. 5mC is also passively diluted during DNA replication (75). Tet1 and Tet2 can also oxidize thymine to 5-hydroxymethyluracil (5hmU), although the functional role of this modification is unknown (76).

Tet1 and Tet2 are highly expressed in ESCs (75). Global levels of 5hmC are maintained at higher levels in pluripotent cells as compared with most somatic cells. This suggests that the 5hmC modification may function as an epigenetic mark in addition to being an intermediate for demethylation (75). Genome-wide 5hmC profiling in mESCs revealed localization at both active and repressed genes. Increased gene expression was associated with low 5hmC at the promoter and high enrichment at gene bodies (77–81). At distal enhancers, 5hmC, 5fc, and 5caC are enriched at regions bound by pluripotency factors (77, 78, 80, 82).

Initial KD of Tet1 alone or both Tet1 and Tet2 in mESCs decreased the expression of pluripotency genes (77, 83). However, both the Tet1 KO or Tet1 and Tet2 DKO mESCs remain pluripotent despite a global loss of 5hmC (84, 85). Both mouse and human triple-Tet KO ESCs are able to self-renew but have impaired differentiation potential (86, 87). Further functional diversity is also provided by a full-length pluripotency Tet1 isoform and a shorter isoform that lacks the C*XX*C DNA-binding domain that is up-regulated during differentiation (88).

Tet proteins and 5hmC are enriched at bivalent domains (77, 78, 80, 81). Upon deletion of Tet proteins, an increase of 5mC at bivalent domains is observed (84, 85, 87). Interestingly, depletion of Tet1 in mESCs decreased PRC2 binding at bivalent regions (80). Furthermore, the Tet DKO resulted in the loss of H3K27me3 in a large subset of bivalent CpG islands, whereas H3K4me3 remained unchanged (89). These observations may explain the differentiation defects observed in the Tet KO ESCs. Deletion of Tet2 in mESCs led to hypermethylation at a few enhancers, a subset of which also lost H3K27 acetylation, leading to reduced expression of associated genes (90). Tet1 depletion led to a reduction of 5hmC at both the TSS and gene body, whereas Tet2 KD decreased 5hmC within the gene body but increased levels at the TSS. Furthermore, 5hmC enrichment is reduced at exon boundaries in high- and low-expressed genes upon Tet2 and Tet1 depletion, respectively. However, there is no specific pattern of 5hmC that correlates with gene expression changes upon Tet1 or Tet2 depletion, suggesting a complex relationship with transcription (91).

In addition to protein-coding gene expression, the Tet enzymes also regulate telomere stability, although the telomere length in Tet TKO is variable (92). Tet1 is specifically enriched at young L1 transposable elements and regulates their silencing in a 5hmC-independent manner through the recruitment of Sin3a that is independent of catalytic function (93).

The Tet proteins also seem to regulate transition to cell types that resemble other developmental stages *in vitro*. Tet TKO mESCs show increased expression of Zscan4, which is a marker for the totipotent two-cell stage of development. ESCs in culture go through a two cell–like state at low frequency (94) that is enhanced in the Tet TKO (92). The addition of Vc increases global levels of 5hmC in mESCs and up-regulates a subset of germline-related genes through Tet-mediated 5hmC conversion at these genes (95). Supplementing retinol in 2i-containing medium can also modulate 5hmC through up-regulation of Tet2 and promote the reprogramming of EpiSCs to naïve iPSCs. Moreover, low retinol concentrations and Vc can synergistically promote EpiSC reprogramming (96) (Box 1).

Tet function has a supportive role in reprogramming of somatic cells to iPSCs. Ectopic expression of Tet1 promotes reprogramming that depends on catalytic function. Tet1 is localized to both the promoter and enhancer region of Oct4, leading to elevated 5hmC and decreased 5mC levels, resulting in endogenous Oct4 activation. Interestingly, Tet1 can replace Oct4 in the OSKM mixture to generate TSKM-derived pluripotent iPSCs (97). Global analysis of TSKM reprogramming showed a gradual increase of 5hmC during reprogramming, whereas 5mC levels transiently increase followed by a reduction in the late stage of reprogramming (97). Furthermore, depletion of Tet1 also reduces human OSKM reprogramming efficiency (98). Tet2 can promote reprogramming by binding to regulatory regions of pluripotency genes Nanog and Esrrb to promote 5hmC accumulation (99). Tet TKO MEFs could not be reprogrammed as they fail to undergo MET but can be rescued by ectopic expression of the catalytic domain of any Tet protein. Failed activation of MET in TKO is due to lack of DNA demethylation of the miR-200 family that down-regulate mesenchymal genes (100). Surprisingly, overexpression of Tet1 (but not Tet2) in conjunction with Vc inhibited OSK reprogramming by prohibiting MET (101). Tet proteins also promote reprogramming of pre-iPSCs, which have undergone MET. Tet1 and Tet2 can interact with Nanog, and the overexpression of both Nanog and Tet1 synergistically promotes pre-iPSC to iPSC conversion. This is mediated at least in part by elevating 5hmC levels at the Esrrb locus (102). Pre-iPSCs can be robustly converted into iPSCs in the presence of Vc and 2i (56), and this is diminished by combined depletion of Tet1 and Tet2 (56).

Tet enzymes can interact with a multitude of different proteins that alter their localization and function. Tet proteins in conjunction with pluripotency-related protein Prdm14 can promote active demethylation at germline genes (103). Idax can modulate Tet2 stability during differentiation by interacting with Tet2 and triggers its degradation through caspase activation (104). Interestingly, certain proteins can bind to 5hmC and regulate Tet function. Mbd3 is reported to bind 5hmC, and deletion of Mbd3 disrupts localization of Tet proteins (105, 106). In addition, pluripotency-related Sall4 binds 5hmC and promotes 5hmC oxidation and active demethylation (107). Tet regulation can also occur at the expression level as several enzymes and microRNA have been shown to regulate Tet expression and influence pluripotency and differentiation (108–110). Non-5hmC–related functions of the Tets can be mediated by their association with *O*-linked GlcNAc-transferase (Ogt) and the repressor Sin3a complex in mESCs (79, 93, 111–113). Ogt alters the post-translational modification of the Tet proteins, which could impact the function of these enzymes. Although inhibition of Ogt does not alter global 5hmC levels, the interaction of Ogt and Tet proteins has a significant role in targeting these proteins to the genome. In mESCs, Ogt can recruit Tet1 to regulate developmental genes. The depletion of Ogt diminishes Tet1 recruitment and decreases 5hmC levels at targeted genes. By contrast, Tet2, recruits Ogt to the genome and mediates gene regulation through *O*-GlcNAc on histone H2B, which is independent of the catalytic activity of Tet2. Depletion of Tet2 abolishes Ogt recruitment to these specific sites and leads to gene repression.

Box 2: Future Directions
1. What are the redundant roles of Kdm family members? For example, the roles of the Kdm5 family are conflicting and the generation of single, double, and triple knockouts may clarify the functions in pluripotency.
2. Why are only some features of pluripotency dependent on the catalytic activity of the enzymes?
3. How do the protein complexes associated with the α-kg dependent enzymes influence the functions of these proteins?
4. Are both catalytic activities of enzymes that have dual specificity such as Kdm4 and Kdm3b required for their role in pluripotency?

Furthermore, in nonpluripotent cells, the recruitment of Ogt by Tet2 can promote binding of SET1/COMPASS (Complex of Proteins Associated with Set1) to promote H3K4me3 and gene activation. Whether similar mechanisms are utilized in mESCs requires further investigation.

Alkbh1: DNA N^6-methyladenine modifier

Beyond cytosine methylation, adenine methylation on DNA N^6-methyladenine (N6-mA) was recently identified in mESCs (114). Alkbh1 has been shown to demethylate N6-mA in mESCs (114). Alkbh1 KO ESCs maintain pluripotent traits, exhibiting unchanged (114) or higher (115) Nanog and Oct4 expression. However, Alkbh1 KO ESCs show defects in their ability to differentiate, especially to the neuroectoderm lineage (115, 116). This could be due to the inability of the Alkbh1 KO ESCs to remove N6-mA from pluripotent genes during differentiation because higher levels of N6-mA correlate with gene silencing (114).

Prolyl-hydroxylase domain enzyme (PHD) and factor inhibiting HIF (FIH): Regulators of hypoxia-inducible factor

The HIF proteins are regulated by the α-KG–dependent dioxygenases PHD1–3 and FIH-1. HIF protein stability has both positive and negative effects on pluripotency (117–119). PHDs hydroxylate proline residues within the oxygen-dependent domains, resulting in degradation of the HIF proteins. In contrast, FIH-1 hydroxylates asparagine residues within the C-terminal transactivation domain of HIF, which prohibits the interaction with its coactivator, p300. Under hypoxic conditions, PHD and FIH-1 function is blocked, allowing HIF nuclear translocation to regulate hypoxia-related genes. Chemical inhibition of PHD or the α-KG analog dimethyloxalylglycine can regulate Oct4 expression (120), and PHD inhibitors can increase OSKM-mediated human reprogramming (121, 122). Taken together, the myriad functions of α-KG–dependent enzymes in pluripotency are only beginning to be uncovered and will be interesting to investigate in the future (Box 2).

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