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# Epigenomic Regulation by Labile Iron

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## Abstract

Iron is an essential micronutrient metal for cellular functions but can generate highly reactive oxygen species resulting in oxidative damage. For these reasons its uptake and metabolism is highly regulated. A small but dynamic fraction of ferrous iron inside the cell, termed intracellular labile iron, is redox-reactive and ready to participate multiples reactions of intracellular enzymes. Due to its nature its determination and precise quantification has been a roadblock. However, recent progress in the development of intracellular labile iron probes are allowing the reevaluation of our current understanding and unmasking new functions. The role of intracellular labile iron in regulating the epigenome was recently discovered. This chapter examine how intracellular labile iron can modulate histone and DNA demethylation and how its pool can mediate a signaling pathway from cAMP serving as a sensor of the metabolic needs of the cells.

## Graphical Abstract

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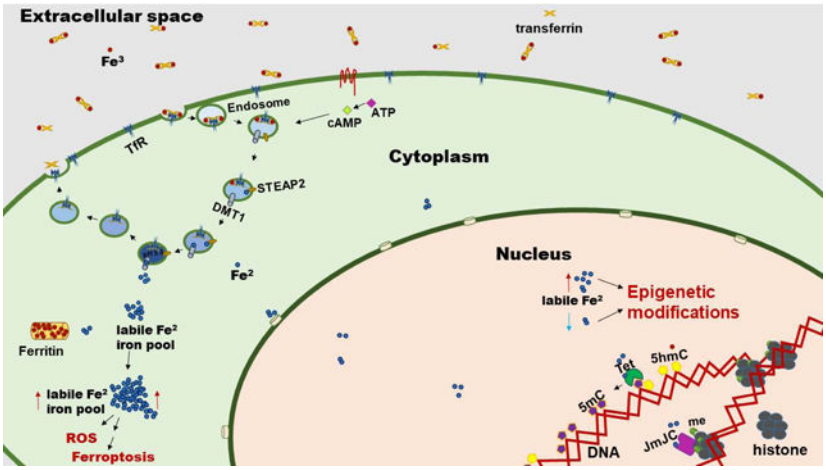


**Keywords**

Iron; intracellular labile Fe(II); reactive oxygen species; DNA methylation; histone methylation; TET methylcytosine dioxygenases; JmjC domain-containing demethylases; G-protein coupled receptor; cAMP; RapGEF2

## 1. Introduction

Iron is an essential metal needed by cells to properly function. The reduced form of iron, Fe(II), is a cofactor in numerous enzymatic reactions in the cell and is therefore required for many vital physiological functions. However, Fe(II) is highly reactive and must therefore be tightly controlled in order to reduce the risk of generating reactive oxygen species (ROS) and eliciting profound cellular damage. Therefore, several cellular pathways have evolved to tightly regulate iron uptake, sequestering, and metabolism to safely utilize iron for vital physiological processes. Furthermore, cells have evolved mechanisms to sense and utilize labile Fe(II) via epigenetic pathways that allow them to adapt and fine tune their responses to the dynamic nature of their environment. Recent insights and technological developments have shed light on the dynamic nature of labile Fe(II) and its novel role in epigenetic regulation of both DNA and histone methylation. In this chapter, we will briefly describe how cells generate a pool of intracellular Fe(II) and how this pool changes due to metabolic needs and environmental pressures. We will focus on the novel role of the labile Fe(II) pool in modulating transcription via epigenomic events, such as DNA and histone methylation, and how these events may serve as therapeutic targets for various diseases including cancer.

## 2. Overview of iron uptake and metabolism

Iron is one of the most abundant metals in cells. It is an important cofactor and a vital nutrient required for proper cellular function. It is tightly regulated, the demand can be localized inside the cell, and its excess can generate free radicals that cause cellular damage. For these reasons, a delicate balance of iron uptake, storage, utilization, and export must be struck for the maintenance of cellular functions.

Dietary iron is first absorbed in the duodenum by enterocytes[1]. Most of the unbound iron in the intestinal lumen is oxidized and present as ferric iron, Fe(III). Prior to absorption, Fe(III) is reduced to ferrous iron, Fe(II), by the duodenal cytochrome B (Dcytb) enzyme presented in the brush border of the cells[1]. Ferrous iron is able to enter the duodenal cells through the divalent metal transporter 1 (DMT1)[1–3]. Iron can also enter the duodenal cells when bound to hemeproteins[1] and is transported inside cells by the heme carrier protein 1 (HCP1)[4]. Iron within the enterocyte can go into the bloodstream by two sequential steps: 1) ferrous iron crosses the basolateral membrane via the iron exporter ferroportin; 2) ferrous iron is oxidized to ferric iron by hephaestin ferroxidase[5]. Once in circulation, iron is largely bound to transferrin[5,6]. Diferric transferrin can bind to the membrane-bound transferrin receptors (TFR1 and TFR2) on target cells and is subsequently internalized via clathrin-mediated endocytosis[1]. It is likely that most iron enters the cells using this mechanism, since transferrin is only 30% saturated under physiological conditions[1,7]. The acidification of the internalized endosomes will result in the release of the ferric iron from transferrin[7]. Subsequently, the endosomal ferrireductase STEAP3 converts ferric into ferrous iron[7]. DMT1 can then transport the ferrous iron into the cytosol[7]. Once in the cytoplasm, ferrous iron can be transported to other organelles or stored within ferritin via Fe(II) oxidation by the intrinsic ferroxidase activity of ferritin. The transferrin receptor and transferrin without iron (apo-transferrin) are sorted to recycle endosomes back to the cell surface where apo-transferrin dissociates[6].

Iron bound to transferrin is not the only mechanism that cells use to internalize iron. Mutant mice lacking transferrin receptors die in utero from severe anemia and have defects in the development of erythrocytes, muscle cells and neurons[6,8,9]. However, other types of cells are still able to internalize iron without major dysfunction in the knockout animals, suggesting that there must be other mechanisms for iron internalization. An additional mechanism for iron internalization is through metal transporters. The ferrous iron not bound to transferrin can enter cells using transporters like DMT1, natural resistance-associated macrophage protein 1 (NRAMP1), or ZRT/IRT-like proteins (ZIPs)[7,10]. Heme-bound iron can provide a source of iron for certain cell populations. Macrophages can obtain iron from heme after phagocytosis[10]. Moreover, other cell types could obtain heme-iron through various effectors such as using the heme importer Heme-Responsive Gene 1 (HGR1), the heme-carrier protein 1 (HCP1), the feline leukemia virus group C cellular receptor 1 (FLVCR1) , the heme-hemopexin complex via the LDL receptor related protein (LRP1), or the haptoglobin hemoglobin complex via the CD163 receptor[6,10,11]. Lipocalin 2 bound to catechol-ferric complexes can also enter the cell via the solute carrier protein SLC22A17[12]. In addition, ferritin released to the serum and bound to iron can enter cells via TFR1 in humans, via the T cell immunoglobulin and mucin domain containing 2 receptor or the scavenger receptor class A member 5 in mice[13,14–16].

Cytoplasmic iron can be transported to different organelles like mitochondria, stored with ferritin, exported out of cells by ferroportin 1 (FPN1), or can be part of the labile iron pool and used in different metabolic processes. Within the cytoplasm, iron is transported by the iron chaperone poly C binding protein (PCBP) family. The PCBP family of proteins delivers iron to specific locations like ferritin for storage and can also deliver iron to enzymes which require iron as a cofactor for enzymatic activity, such as ribonucleotide reductase,

deoxyhypusine hydroxylase, and iron-dependent and 2-oxoglutarate (2OG, also known as α-ketoglutarate)-dependent dioxygenase family proteins, like prolyl hydroxylase 2 (PHD2) and the asparaginyl hydroxylase factor inhibiting HIF-1α (FIH)[17,18]. Ultimately, a large portion of internalized iron is localized to the mitochondria where it is incorporated into heme and iron-sulfur proteins that are part of the electron transfer chain[17,19].

## 3. Detection and characterization of labile iron

Labile iron is hard to define because of its dynamic nature and difficulty to detect. The terminology was initially used to describe the iron release from choleglobin and hemoglobin after an acid treatment[20,21]. Later it was discovered with experimental transfusion of radioactive iron that a portion of it remained in the blood as an intermediate stage before it is incorporated into hemoglobin[22]. This iron was first termed the labile iron pool and later the extracellular labile iron pool.

A pool of free intracellular iron was also suspected after initial cellular experiments with iron chelators[23] and was later named by Allan Jacobs as the intracellular labile iron pool[24]. While chelatable iron suggested that there was a pool of free intracellular iron, a direct quantification method for free intracellular iron was missing. The chelator itself can affect the metabolism of iron by inducing intracellular shuffling of iron and by sequestering iron bound with low affinity to other proteins or lipids[25,26]. Initial experiments to determine the intracellular iron pool were done with chelators having a high affinity to iron (transferrin and deferoxamine), or with a low affinity and specificity (EDTA, phenanthroline, and DTPA). Most of these chelators have higher affinity to Fe(III) or are bound to Fe(III) because the Fe(II) is oxidized once they are formed in a complex[27,28]. For example, radiolabeled iron was supplied to cells and after cellular fractionation, iron not bound to ferritin, or bound to other molecules, were identified[25,29,30]. Another method was to chelate iron and determine the relative amount of free iron using electron paramagnetic resonance[31,32]. Additional studies with chelators also determined the functional role of “intracellular labile iron”[28]. These methods shed light on intracellular iron dynamics and definitively indicate the existence of several intracellular iron pools. However, these methods lack specificity and sensitivity, since chelators change the iron balance and composition within the cell and fractionation techniques damage cellular compartments that result in leakage of its milieu. Moreover, probable redistribution of iron binding molecules, changes in the relative amount of Fe(II) and Fe(III), and changes in endocytosis dynamics that influence the rate of iron uptake from the extracellular space affect these interpretations.

For these reasons, fluorescence cellular probes were recently developed with the goal of specifically measuring iron without affecting cellular function or viability. The first set of probes contained phenanthroline moiety, or calcein fluorescence molecules, with EDTA-like moiety[28,33–39]. These probes are very convenient because they can be used to measure the abundance of iron in living cells and determine its localization. However, most of them are unable to distinguish clearly between Fe(II) or Fe(III) and other metals like Cu or Mg. Additionally, they exhibit unequal cellular distribution, are based on “turn off” mechanisms, and are unable to function in very low pH environments, such as lysosomes, due to their pH-dependent sensitivity[40]. Nevertheless these fluorescence probes successfully steered research

into new directions and next-generation probes were subsequently developed that can target specific cellular compartments like lysosomes and mitochondria or utilize a “turn on” mechanism[41–45].

Only recently were fluorescence-based probes developed that are specific to either Fe(II) or Fe(III)[42,46–50]. This means that we have to be very cautious in interpreting the extensive previous literature that claims to detect “label intracellular iron” based on techniques that result in a disruption of cells or probes that are not specific to iron or its ionic states. For example, calcein-AM, one of the most widely used probes for iron, was initially believed to be a good sensor for Fe(II) but was later found to be a sensor for Fe(III) and not especially specific to iron[40,51]. Interpretations about the increased level of labile iron due to ROS in cancer cells, based on calcein or PhenGreen experiments, should therefore be carefully reevaluated or replicated using next-generation probes.

One of the biggest challenges was to develop probes that could sense labile Fe(II) without chelation and sequestration of iron. To overcome this obstacle, activity-based probes were developed to be able to emit fluorescence by reacting with Fe(II). These types of probes are very sensitive to nM concentrations of intracellular labile Fe(II)[46,52–58]. For example, an Fe(II)-dependent probe was designed to be able to modulate the fluorescence resonance energy transfer (FRET) between two dyes linked by an Fe(II)-responsive trigger . These types of probes allowed a more accurate quantification, localization, and detection of labile ferrous iron in living cells[59]. This activity-based approach was used in synthetic biology to design artificial molecules to control gene activation, signal transduction, and cytoskeletal remodeling in response to Fe(II) and as a specific delivery method of medication to cells with high concentration of Fe(II)[60,61]. Additionally, efforts to develop intracellular labile iron probes that could be used in living organism are under way[62–65].

The intracellular labile iron pool is very dynamic in its size and availability, which affects downstream functions of cells that are iron-dependent. A complete picture of its role inside the cell is still lacking due to difficulties in detection and quantification. However, it is clear that it performs essential functions in modulating the kinetics of iron-dependent enzymes, such as Fe(II) and 2OG-dependent dioxygenases, and iron-sulfur center proteins[66].

## 4. Labile iron as a determinant of epigenomic control.

### Labile iron as a modulator of DNA and histone demethylation

The epigenome comprises all the modifications to the chromatin that regulates the expression of the genome without altering the DNA sequence. This control of gene expression needs to be fine-tuned to the environment so that cells can appropriately, and sometimes relatively rapidly under certain situations, respond to environmental challenges. Intracellular iron plays a central role as one of the many mechanisms that inform cells of environmental changes. The function of many epigenetic effectors which regulate the epigenome requires, or is influenced by, intracellular iron levels. Changes in the environment or in the metabolism of the cell could therefore result in fluctuations of the iron pool, and consequently in the epigenome. Therefore, labile iron can be a determining factor that controls the epigenome.

Ten-eleven translocation (TET) enzymes and JmjC domain-containing demethylases are two important classes of epigenetic regulators which require Fe(II). TET enzymes (TET1/2/3) catalyze the cascade oxidation of 5-methylcytosine (5mC, the classical mark of silenced transcription) into 5-hydroxymethylcytosine (5hmC) and furthermore into transient intermediates which are excised by DNA base excision repair and are ultimately replaced by unmodified cytosine, thus completing the active DNA demethylation. Besides being a DNA demethylation intermediate, 5hmC is also a stable epigenetic mark which itself can influence transcription. Conversely, JmjC domain-containing demethylases (~20 members) are the chief enzymes which demethylate histone lysine residues and subsequently regulate chromatin accessibility and transcription. Both TETs and JmjC domain-containing demethylases belong to the Fe(II) and 2-oxoglutarate-dependent dioxygenase superfamily, a large class of enzymes which require Fe(II) for proper enzymatic activity. This suggests that changes in the intracellular labile Fe(II) pool can influence the activity of these enzymes and subsequently mediate downstream epigenetic changes.

Experiments with iron chelators were the first indication that iron is involved in the epigenetic landscape. For example, the transcription of a gene termed serpina3g, which encodes serine protease inhibitor A3G to be involved in the differentiation of memory T cells, was found to be downregulated by exposure to iron chelator deferoxamine (DFO) and by nickel exposure[67,68]. Chromatin immunoprecipitation (ChIP) assays following the treatment demonstrated a change in the methylation pattern of the serpina3g promoter which affects Serpina3g transcription. DFO chelates iron while nickel displaces iron from the active site of enzymes, like the Fe(II) and 2OG-dependent dioxygenases, thus resulting in reduced activity of these enzymes[68,69]. The interpretation of these experiments were hard to make at the time since TETs and JmjC domain-containing demethylases were discovered later[70,71]. A similar effect of iron chelators in changing histone methylation patterns was observed later in human breast cancer cell lines treated with DFO[72]. Moreover, *in vivo* depletion of ferrous iron with ferrous chelator thiosemicarbazone-24 reduces 5hmC expression and blastocyst formation during embryo development[73]. This effect in 5hmC expression and blastocyst formation can be explained by a lower TET enzyme activity, as the opposite is seen with increased TET activity with vitamin C[74–76]. A reduction of intracellular labile iron could be responsible for the alteration of DNA and histone hippocampal methylation in neonatal iron deficiency[77–79]. Vitamin C is considered a cofactor for Fe(II) and 2OG-dependent dioxygenases, such as collagen hydroxylases, TETs and JmjC domain-containing demethylases, by converting catalytically inactive Fe(III) to catalytically active Fe(III)[80]. Thus, the bioavailability of vitamin C, via Fe(II), regulates the epigenome. Moreover, quinones, a large class of cyclic organic compounds, were also found to increase the labile iron pool and consequently increase global levels of genomic 5hmC[81]. Quinone-induced 5hmC elevation was found to be mediated by TET enzymes and can be blocked by iron chelator treatment[81]. These findings gave the first indications that cellular determinants which regulate the labile iron pool result in epigenetic consequences.

The correlation of 5hmC and intracellular labile Fe(II) levels suggests that both events might be directly connected. Labile iron could act as a propagating signal that regulates the Fe(II)-dependent oxidase activity of TETs, subsequently affecting the epigenetic landscape. This previously unrecognized function for iron is persuasive since an essential characteristic of

cellular iron metabolism is its entry into the cytosolic Fe(II) labile pool from cellular iron uptake or from ferritin that is able to store large quantities of Fe(III) and release it as Fe(II). Even though this process is clearly involved in maintaining iron homeostasis and the biosynthesis of Fe(II) cofactors, it appears to have been appropriated for cellular signaling with enzymes that employ labile Fe(II), like TETs and JmjC domain-containing histone demethylases.

A mouse model of hemochromatosis that resulted in an increase of labile iron in the brain was found to result in a reduced global brain DNA methylation[82]. This reduction was correlated with a reduction of DNA methyltransferase activity by iron *in vitro*[82]. It won't be a surprise to find out that in this model TET activity could be increased, which would contribute to the global DNA demethylation[83–85].

## cAMP signaling and labile iron

Growing evidence suggests that the labile iron pool may be part of a global signaling pathway for epigenomic regulation of DNA and histone methylation. Recent work found that changes in intracellular cAMP, the second messenger for numerous signaling pathways, increases global 5hmC levels in primary Schwann cells and other cell types[86]. This increase correlates with cAMP-induced elevation of the intracellular labile Fe(II) pool and was abolished by treatment with iron chelators. cAMP was found to propagate this effect through RapGEF2 which causes enhanced acidification of endosomes that resulted in increased Fe(II) release to the labile Fe(II) pool[86]. As a result of the changes in global 5hmC levels, the transcriptional profile of these cells subsequently changed. The effect of cAMP could also be mimicked by treatment with adenylate cyclase activators, phosphodiesterase inhibitors, and by GPCR ligands which increased intracellular cAMP[86], suggesting that changes in cAMP signaling alter the labile Fe(II) pool which subsequently regulates DNA demethylation. These changes in cAMP and iron could be modulated by a non-canonical GPCR pathway connecting the dynamic changes in the cellular environment with an epigenetic response.

Like TET enzymes, JmjC domain-containing histone demethylases which modulate histone methylation, also require Fe(II) for proper enzymatic activity. Therefore, cellular determinants which alter labile Fe(II) are also expected to influence histone methylation dynamics. Recent work found that cAMP signaling and subsequent Fe(II) elevation resulted in increased demethylation of H3K4me3, a histone mark of actively transcribed genes[87]. Interestingly, while prolonged stimulation maintained decreased H3K4me3, brief stimulation resulted in only a transient increase of Fe(II) and decreased H3K4me3, both of which returned to baseline levels shortly after removal of the stimulus[87]. This suggests that the labile Fe(II) pool is sensitive to fast changes in cAMP signaling and thus allows for cells to dynamically respond to environmental stimuli through rapid alterations of histone methylation. cAMP-induced Fe(II) elevation was found to be mediated by RapGEF2 which causes an increase in endosome acidification by vacuolar $H^+$-ATPase assembly, thus resulting in endosomal iron release and increased cytosolic iron[86,87]. Removal of iron from the media or treatment with iron chelators abolished cAMP-induced demethylation of H3K4me3[87]. Moreover, inhibition of KDM5 demethylase, which antagonizes H3K4me3,

also abolished cAMP-induced demethylation[87]. Overall, these data highlight a growing body of evidence showing that labile Fe(II) is part of a broad signaling pathway which regulates DNA and histone methylation in response to environmental stimuli, thus allowing cells to rapidly alter transcription and dynamically respond to extracellular challenges.

## 5. Labile iron and disease pathogenesis.

It is clear that the intracellular ferrous iron pool isn't static, but is instead dynamically needed for the activity of downstream enzymes and reactions, including those that could affect the epigenetic program of cells. While deficiency diminishes these vital functions, its excess could result in the generation of damaging ROS or cell death via ferroptosis. Therefore, it should be no surprise that the amount of labile iron has been implicated in several cellular pathological states. It also implies that therapeutic approaches could be improved or conceived by modulating the levels of labile ferrous iron. For example, the levels of labile iron has been implicated in numerous diseases beyond the scope of this chapter, including aging[88–92] , the immune response[93,94], Parkinsons disease[95–97], age related macular degeneration[98–100], retinitis pigmentosa[101], kidney disease[102], neurodegeneration[103–105], malaria[106,107], viral infections[108,109], and inflammation[110]. How labile iron flux results in epigenetic changes to drive pathogenesis of these diseases or can be a therapeutic target has yet to be thoroughly explored.

The best studied role of labile iron in a pathology is cancer. For example, downregulation of ferritin by H-Ras results in a rise of the labile iron pool that leads to cell growth stimulation[111,112]. Increase of the labile iron pool is probably needed for the high metabolic requirements of cancer cells. This correlation of decreased ferritin levels and increased labile iron was also observed in cancer cells like adenocarcinoma and breast cancer[113,114]

In adenocarcinoma the increased labile iron pool also increases ROS and contributes to the epithelial mesenchymal transition (EMT) observed in this cancer between the early stage and the later most invasive stage[113]. The EMT and changes in cell growth and proliferation implicate a change in the transcription program which may be driven by epigenetic signatures of these cells. It will be important to elucidate the role of increased iron in cancer pathogenesis and the contribution of iron-induced epigenetic changes in disease progression. Also, it will be important to interrogate these questions using the newly-developed iron probes, since some measurements of labile iron were previously done using probes like calcein that detect Fe(III) rather than Fe(II). If the ferrous iron pool is involved in the epigenetic signature of cancer cells, then any approach to change the pool could result in changes to the epigenome and may be a therapeutic target for cancer treatment. Interestingly, iron chelation with deferoxamine in colorectal cancer results in significant inhibition of cell growth and an increase in global histone methylation[115]. Aberrant histone methylation has been observed in numerous cancers and has long been a therapeutic target in pre-clinical studies. The labile iron pool may therefore be targeted to modulate aberrant histone methylation, restore transcriptional programs, and treat cancer progression. Another potential therapeutic approach is to use the increase labile iron to trigger ferroptosis which has been used in glioma, renal cell carcinoma, and in neuroblastoma[116,117]. Future work

should continue exploring the therapeutic value of labile iron modulation in cancer treatment and in the numerous diseases where disrupted iron metabolism has been observed.

## 6. Concluding Remarks

Intracellular labile Fe(II) is like a “fire” that cells require and must maintain to sustain a plethora of intracellular enzymatic reactions. But this fire in cells can result in the production of damaging reactive oxygen species (ROS). Intracellular labile Fe(II) can burn the cells via a Fenton reaction that results in the generation of hydroxyl radicals which damage the cell. Yet enzymes such as the Fe(II) and 2OG-dependent dioxygenase superfamily require Fe(II) as do many critical cellular functions. These contrasting pressures require cells to sustain a delicate balance: Cells must tightly regulate the uptake and storage of iron to maintain a pool of intracellular labile iron large enough to perform vital cellular functions while minimizing the subsequent damage resulting from ROS. This pool of iron must shrink or swell depending on the cell’s metabolic need and its dynamic environmental pressures. For these reasons, it is probable that cells developed these mechanisms for sensing intracellular iron fluctuations and utilizing labile Fe(II) to rapidly respond to environmental stimuli. Epigenetic enzymes which utilize Fe(II) can rapidly alter transcription in response to cellular stress or environmental challenges. Within this group of enzymes, JmjC domain-containing histone demethylases and TET methylcytosine dioxygenases, which depend on Fe(II), are essential in modulating the balance of methylation-demethylation of histones and DNA, respectively. The methylation changes in the DNA or histones can result in profound alterations in gene expression and cellular identity. Additionally, these enzymes are highly sensitive to changes in the intracellular labile Fe(II) pool which can be modulated by extracellular signaling from GPCRs. Therefore, by regulating the intracellular labile Fe(II) pool, GPCRs via cAMP can change the methylation status of both DNA and histones and subsequently alter transcription in response to environmental stimuli. Signaling oscillations and rapid changes in the extracellular environment results in dynamic changes to the intracellular labile Fe(II) pool, a vital “fire” that keeps the epigenetic pot set at the right temperature for proper cellular function. Dysregulation in the intracellular labile Fe(II) pool is present in several diseases and might be an important contributing factor that alter the epigenetic landscape of those diseases.

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## Highlights

- Labile Fe(II) is redox-reactive, dynamic and required for enzymatic reactions.
- Labile Fe(II), as an essential cofactor, is involved in the demethylation of DNA and histones.
- Diseases that alter the intracellular labile Fe(II) pool can affect the epigenome.
- cAMP signaling promotes DNA and histone demethylation by increasing intracellular labile Fe(II) pool.