THE REGULATION OF THE HISTONE DEMETHYLASE KDM5B

CHARACTERIZING THE FUNCTION AND REGULATORY MECHANISMS OF THE HISTONE DEMETHYLASE KDM5B: INSIGHTS INTO THE COMPLEXITY OF EPIGENETIC REGULATION

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Abstract:

KDM5b acts as a transcriptional repressor through its ability to demethylate tri-methylated lysine (K) 4 on histone H3 (H3K4me3). Demethylation of this histone modification leads to transcriptional repression and downstream biological effects on gene expression. KDM5b is involved in the regulation of differentiation and can exert an oncogenic and a tumour suppressive role depending on cellular context, making it an attractive future target for pharmaceutical intervention. Work from our group has shown that KDM5b expression is linked to differentiation, and that recruitment of the enzyme does not always result in an alteration of H3K4me3. Additionally, work from our group, as well as others, has failed to observe H3K4me3 demethylation by KDM5b in nucleosomal preparations. We therefore hypothesized that KDM5b may exert its demethylase potential on alternative histone targets and that KDM5b requires enzymatic co-factors to demethylate nucleosomes, similar to what is observed for other histone-modifying proteins. In this thesis, we describe KDM5b as having an alternate histone target, di-methylated histone H2B lysine 43 (H2BK43me2). We show that this methyl mark is the primary target for KDM5b, and that the expression level of H2BK43me2 is directly related to the process of differentiation. We additionally present a novel co-factor for KDM5b, the co-repressor TLE4 of the Groucho/TLE family. The presence of TLE4 is required and sufficient to confer nucleosomal demethylase activity to KDM5b, a novel discovery for any of the KDM5 family members. Overall, this work has described both an additional KDM5b target, and detailed requirements for KDM5b nucleosomal demethylation, advancing our understanding of how this enzyme is regulated *in vivo*. The novel aspects of KDM5b regulation presented within this thesis provide a framework from which future studies can be designed. This work contributes to our overall understanding of epigenetic regulation and will potentially aid in the development of novel anti-cancer therapeutic strategies.

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Abbreviations

5mC	5-methylcytosine
A	Alanine
AR	Androgen Receptor
ATP	Adenosine-5'-triphosphate
Atr2	Atrophin 2
Atr2L	Atrophin 2L
Atr2S	Atrophin 2S
BAH	Bromo Adjacent Homology
BF-1	Brain Factor 1
CDKN1A	Cyclin Dependent Kinase inhibitor 1A
CFP	mCerulean Blue Fluorescent Protein
ChIP	Chromatin Immunoprecipitation
COMPASS	Complex Proteins Associated with Set1
CoREST	Corepressor of REST
CSC	Cancer Stem Cell
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic Acid
DNMT	DNA methyltransferase
DNMT1	DNA methyltransferase 1
DNMT3A	DNA methyltransferase 3A
DNMT3B	DNA methyltransferase 3B

ELM2	EGL-27 and MTA-1 homology
EMT	Epithelial to Mesenchymal Transition
ESC	Embryonic Stem Cells
ESI-LC-MS/MS	Electrospray Ionization Liquid Chromatography Mass Spectrometry
FAD	Flavin adenine dinucleotide
FBXL11	F-Box and Leucine Rich Protein 11
FLIM	Fluorescent Lifetime Imaging Microscopy
FRET	Förster Resonant Energy Transfer
Gcn5	General Control of Amino Acid synthesis proteins like-2
GFP	Green Fluorescent protein
Grg	Groucho
H1	Histone H1
H2A	Histone H2A
H2AK119 ub	Ubiquitinated lysine 119 of Histone H2A
H2B	Histone H2b
H2BK120ub	Ubiquitinated lysine 120 of Histone H2B
H2BK43	Histone H2B Lysine 43
H2BK43me2	Histone H2B Lysine 43 di-methylated
H2BK43me3	Histone H2B Lysine 43 tri-methylated
H3	Histone H3
H3K27	Histone H3 Lysine 27

H3K27me3	Histone H3 Lysine 27 tri-methylated
H3K36me	Histone H3 Lysine 36 methylated
H3K4	Histone H3 Lysine 4
H3K4me1	Histone H3 Lysine 4 mono-methylated
H3K4me2	Histone H3 Lysine 4 dimethylated
H3K4me3	Histone H3 Lysine 4- tri-methylated
H3K79me	Histone H3 Lysine 79 methylated
H3K9	Histone H3 Lysine 9
H3K9me3	Histone H3 Lysine 9 tri-methylated
H3S10	Histone 3 Serine 10
H3T6	Histone H3 Threonine 6
H4	Histone H4
H4K20me	Histone H4 Lysine 20 methylated
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
HDM	Histone Demethylase (Assay)
HEK293	Human Embryonic Kidney 293
ICM	Inner Cell Mass
ING	Inhibitor of Growth
ISWI	Imitation SWI
JmjC	Jumonji (domain)
K	Lysine

KDM1a	Lysine Demethylase 1 (A)
KDM5a	Lysine demethylase 5a
KDM5b	Lysine demethylase 5b
KDM5c	Lysine demethylase 5c
KDM5d	Lysine demethylase 5d
KLF10	Kruppel-like transcription Factor 10
KMT	Lysine Methyl Transferase
Lid	Little Imaginal Disc
me	Methyl
miR	MicroRNA
MLL	Mixed lineage Leukemia
MLL2	Mixed lineage Leukemia2
MLL3	Mixed lineage Leukemia3
MLL4	Mixed lineage Leukemia4
MRM MS	Multiple Reaction Monitoring Mass Spectrometry
MSK1	Mitogen and Stress activated protein Kinase 1
MSK2	Mitogen and Stress activater protein Kinase 2
NDM	Nucleosome Demethylase (Assay)
NEAA	Nonessential Amino Acids
NFkB	Nuclear Factor Kappa B
PARP1	Poly (ADP-Ribose) polymerase 1
Pax9	Paired Box 9

PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEV	Position Effect Variegation
PHD	Plant Homology Domain
PKCβ1	Protein Kinase C beta 1
PML	Promyelocytic
POD	PML Oncogenic Domain
PRC1	Polycomb Repressive Complex 1
PRC2	Polycomb Repressive Complex 2
PVDF	Polyvinylidene difluoride
qRT-PCR	quantitative Reverse Transcriptase PCR
R	Arginine
Rb	Retinoblastoma
RERE	Arginine Glutamic Acid Repeat Encoding protein
REST	RE-1 Silencing Factor
RNA	Ribonucleic Acid
RSK2	Ribosomal S6 Kinase 2
S	Serine
SANT	Swi3/ADA-2/NcoR/TRIIB
SDS PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SWI/SNF	Switch/Sucrose Non-fermentable
T	Threonine

TBST	Tris-Buffered Saline with Tween
TF	Transcription Factor
TF11D	Transcription Factor IID
TFA2C	Transcription Factor Activating Protein 2C
TIEG1	Transforming growth factor Beta Inducible Early Gene
TLE1	Transducin Like Enhancer of split 1
TLE4	Transductin Like Enhancer of split 4
TSA	Trichostatin-A
TSS	Transcriptional Start Site
Wt	Wild type
YFP	Yellow Fluorescent Protein
YYI	Ying Yang 1
αMEM	Minimal Essential Medium Eagle, alpha-modifications

Chapter 1 General Introduction

1.1 Transcription

Higher order organisms are made up of a wide variety of cell types that perform specific and specialized functions, however each of these cells contains the same set of approximately 50,000 genes. For specialization to be possible, tightly controlled regulation of gene expression must exist within each cell lineage. Additionally, these cells must be able to respond to both extracellular and intracellular cues, leading to appropriate alterations in the expression of specific gene products (Orphanides and Reinberg 2002). The journey from DNA to protein is a multi-step process involving regulation through various signaling pathways, co-regulator binding, and context-dependent binding of transcriptional regulators known as transcription factors (TFs) (Roeder 1996), leading to the activation of RNA polymerase II, an enzyme made up of 12 subunits that is responsible for the transcription of functional proteins (Cramer, Bushnell, and Kornberg 2001; Roeder 1996; Gnatt et al. 2001). After the recruitment of RNA polymerase II, synthesis of the RNA strand is initiated and transcription commences. Soon after initiation, RNA polymerase II pauses to allow the addition of a 5' cap structure to the nascent RNA. This structure provides protection from nucleases, and is thought to aid in the process of exporting the new transcript to the cytoplasm, where it is translated from RNA to functional protein. Upon completion of capping, RNA polymerase II moves in a 5' to 3' direction along the target DNA, resulting in the growth of the nascent RNA molecule in a process referred to as elongation. When RNA polymerase II reaches the end of the encoding DNA template, termination occurs and the process of polyadenylation adds a protective tail at the 3' end of the nascent RNA. The nascent RNA

is then exported from the nucleus to the cytoplasmic compartment where translation into a functional protein occurs (Proudfoot, Furger, and Dye 2002; Orphanides and Reinberg 2002).

1.1.1 Upstream Regulation of Transcription Factors

RNA polymerase II is unable to independently bind or recognize gene promoters and therefore requires the expression of TFs. These factors bind to regulatory elements on the DNA sequence upstream of the site at which transcription is initiated and, in a context-dependent manner, facilitate the recruitment of RNA polymerase II to gene targets (Roeder 1996). This family of proteins is vast and varied, representing 5% of the human genome (Tupler, Perini, and Green 2001). In order to maintain specificity of gene expression, several different upstream mechanisms regulate the activities of sequence-specific TFs. The regulatory sequences of most genes contain binding sites for multiple TFs, allowing expression of each gene to be regulated by multiple signaling cascades (Lefstin and Yamamoto 1998) . Further, the activity of a single TF may be affected by the presence of others, such is the case for Yin Yang 1 (YY1) which is known to act as a transcriptional repressor, activator or initiator depending on the presence or absence of additional factors (Shi, Lee, and Galvin 1997). Additionally, context dependence may occur where binding of one TF may result in turning one gene on, and turning another gene off (McKenna and O'Malley 2002). Additionally, several mechanisms exist for upstream regulation of TFs. In the simplest case, the cytoplasmic and nuclear compartments of eukaryotic cells permit a mechanism of regulation by virtue of either

sequestering of a specific TF in the cytoplasm and not allowing it access to a DNA binding site or conversely, promotion of nuclear transport to increase access to specific binding sites (Carmo-Fonseca 2002). Additionally, post-translational modifications of TFs play a role in their regulation. In order to respond quickly to environmental cues, TFs may be ubiquitinated and targeted for degradation, resulting in an accelerated mechanism for altering transcription (Salghetti et al. 2001). TFs are subject to additional post-translational modifications such as phosphorylation, acetylation and methylation, that also result in alterations to their subcellular localization, activity, or ability to recognize binding sites on DNA (Zhang and Reinberg 2001).

1.2 Chromatin Structure and DNA Packaging

The large DNA macromolecule is not present in a naked form within eukaryotic cells, but is rather packaged and compacted into a highly organized structure known as chromatin. The fundamental building block of chromatin is known as the nucleosome, an octameric protein core made up of two copies each of four histone proteins, H2A, H2B, H3 and H4, around which 146 base-pairs of DNA is wound in 1.7 super-helical turns (Kornberg and Thomas 1974; Luger et al. 1997) (luger et al, 1997). Nucleosomal units are small, positively charged, and highly conserved throughout evolution. Winding of DNA around these protein cores results in the formation of an 11nm fiber, commonly termed “beads on a string” and represents the first order of chromatin compaction. Increased compaction occurs upon the binding of an additional histone protein, H1. This leads to condensation of the 11nm fiber into a 30nm fiber, representing the second level

of chromatin compaction (Robinson and Rhodes 2006; Kornberg and Lorch 1999) (Figure 1.1)(Lodish H 2000). Though the 11nm fiber has been solved by crystal structure (Luger et al. 1997), the exact structure of the 30nm fiber remains unclear. Two distinct models have been proposed, the one-start helix/solenoid model in which nucleosomes are connected by DNA which is bent, creating a helical path (Widom and Klug 1985), and a second, the two start/zig zag model, in which nucleosomes are connected by DNA linkers which are straight, creating a zig zag pattern (Woodcock and Ghosh 2010; Williams et al. 1986). Chromatin then undergoes several levels of higher order folding and though the dynamics of this remains highly unclear, long-range interactions between nucleosomal arrays are thought to play a role in additional compaction (Li and Reinberg 2011).

1.2.1 Euchromatin Versus Heterochromatin: Patterning for Cellular Identity

The packaging of DNA into chromatin was originally thought of solely as a method to contain the large DNA macromolecule to the confines of the eukaryotic nucleus and to provide a protection mechanism for DNA during replication. However, it has become apparent that chromatin plays an important role in the regulation of gene transcription (Narlikar, Fan, and Kingston 2002; Kouzarides 2007). Transcription requires that particular subsets of genes be accessible to both sequence-specific TFs and the transcriptional machinery. The winding of DNA around nucleosomes, as well as the higher order packaging of chromatin renders this process more difficult, demonstrating that the structure of chromatin itself plays an important role in DNA

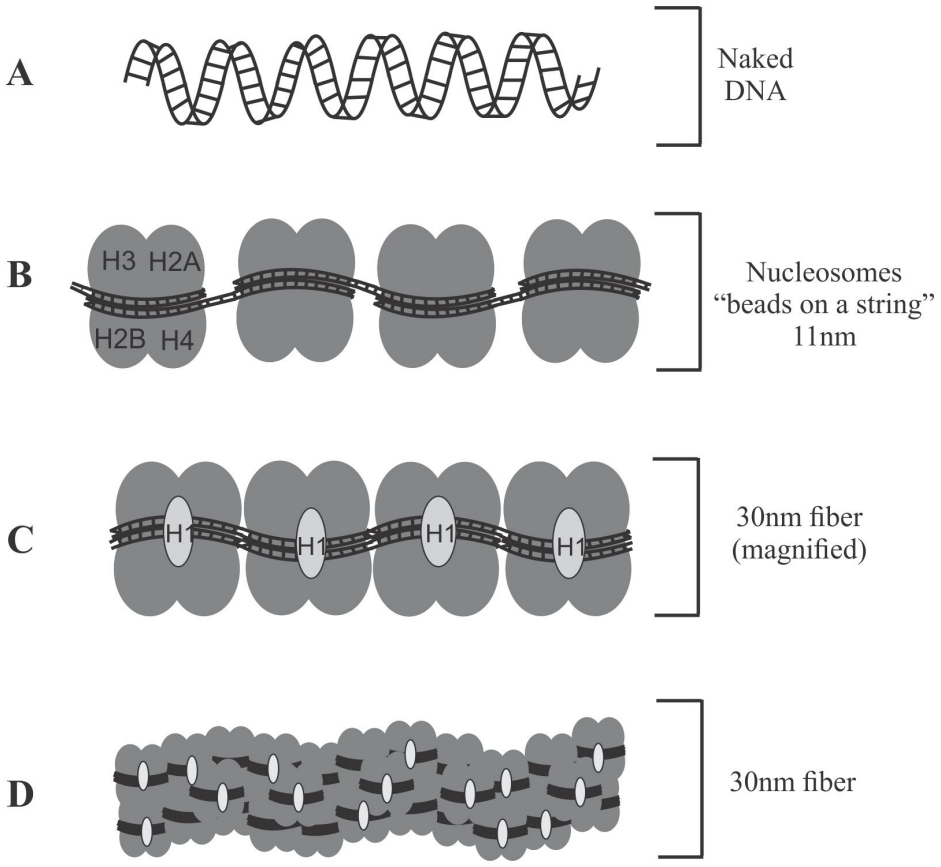


Figure 1.1 Chromatin Compaction A) The DNA macromolecule without additional proteins in the "naked" form B) Addition of Nucleosomes, octameric protein cores made up of two copies of each histone protein: H3, H4, H2B and H2A3. 146 bp of DNA is wound around each core forming an 11nm fiber commonly known as "beads on a string" C) The addition of histone H1 results in further compaction of the 11nm fiber D) Higher compaction results in the formation of a tightly packed 30nm chromatin fiber, the exact organization of which remains unknown.
Adapted from Lodish et al, 2000.

accessability. In the simplest of terms, repression is achieved by rendering DNA inaccessible, and activation is achieved by making it accessible (Urnov and Wolffe 2001; Narlikar, Fan, and Kingston 2002). Subsets of genes which are not being transcribed are packaged into highly condensed regions of chromatin referred to as "heterochromatin" whereas sections of DNA containing required genes remain in a more open conformation known as "euchromatin"(Grunstein et al. 1995). Each cell type packages DNA into a unique pattern of euchromatin and heterochromatin, a pattern which is maintained throughout the process of cellular division, contributing to the maintenance of cellular identity (Richards and Elgin 2002).

1.2.2 Co-regulators and Access to DNA

The knowledge that DNA is packaged into higher order structures denotes the importance of accessibility in transcriptional regulation. Access of a TF to its required binding site within nucleosomal DNA would depend on the location of its binding site within the chromatin fiber (Urnov and Wolffe 2001; Urnov, Wolffe, and Guschin 2001) . Activators and repressors must therefore "open" and "close" the chromatin structure respectively; a process that requires the recruitment of additional cofactors. Co-regulators are protein complexes recruited to promoters of interest by sequence specific TFs. These additional protein complexes play multitudinous roles, and are indispensable for the regulation of transcription (McKenna and O'Malley 2002). Several categories of co-regulators exist, including co-activators and co-repressors, and their individual recruitment is pertinent to the creation of promoter- or tissue-specific responses

(Hampsey and Reinberg 1999) . Many co-regulators have been found to be responsible for alterations to chromatin structure, once again connecting the importance of chromatin structure directly to transcriptional regulation. These chromatin structure-regulating protein complexes can be divided into two categories. The first represents a class of ATP-dependent chromatin remodelers and includes the SWI/SNF (Switch/Sucrose Non-Fermentable) and ISWI (Imitation SWI) families, commonly referred to as the ATP-dependent nucleosome remodeling complexes. These complexes utilize energy derived from the hydrolysis of ATP to reposition nucleosomes. The resulting conformational changes may occlude or expose particular regions of DNA on the exterior of the nucleosome core, allowing or preventing access to TFs and RNA polymerase II (Kingston and Narlikar 1999; Vignali et al. 2000). The second category of chromatin regulatory complexes is a group of co-regulators that modify the structure of histone proteins that form the nucleosomes. These co-regulators catalyze post-translational modifications of the amino terminal tail of histones, resulting in alterations to nucleosome structure (Wu and Grunstein 2000). Both types of protein complexes result in physical changes to the chromatin structure, which results in downstream transcriptional alterations important to gene regulation and cell identity. They highlight the importance of chromatin structure as a gene expression regulator. This concept was incredibly novel, as previous studies had merely highlighted the importance of chromatin in DNA packaging.

1.2.3 Epigenetics

The discovery that nuclear co-activators had intrinsic histone modifying properties that had a direct effect on the downstream transcriptional state, led to the development of a theory that the regulation of the genetic code may not only occur by DNA itself but that certain auxiliary elements may play a pertinent role as well. This realization jump-started an entirely new field of study, now commonly known as "epigenetics". Originally defined as "the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence" (Russo 1996), this definition has since been modernized several times. Epigenetics is still considered to study alterations to gene expression not resulting in alterations to the DNA structure itself, including histone modifications, and DNA methylation events.

1.3 The Histone Protein

The octameric core that makes up the protein component of the nucleosome is formed by combining two each of four histone proteins: histone H2A, H2B, H3 and H4. An additional histone, histone H1, is required for further packaging of the 11nm chromatin fiber to the more densely packed 30nm fiber (Luger et al. 1997). Each individual histone protein contains highly conserved domains; a large globular core containing three helices referred to as the histone fold, and two tails, including an extended amino terminal tail. These amino terminal tails protrude from the nucleosomal core and represent 25-30% of the total histone mass (Wolffe and Hayes 1999; Strahl and Allis 2000). Though the tails appear to be dispensable for the formation of the nucleosome subunit, evidence suggests that they are required for higher order chromatin

compaction through their role in nucleosome-nucleosome interactions (Luger et al. 1997). The protruding amino-terminal tails of histones, which extend beyond the nucleosomal core, represent a long template and a virtually infinite ability for the nucleosomal structure to be post-translationally modified. Interestingly, although histone proteins are considered to be amongst the most evolutionarily conserved proteins known, they are also among the most variable in terms of post-translational modification (Richards and Elgin 2002) due to the availability of their amino-terminal tails.

1.3.1 Post-Translational Modifications

Due to availability outside of the core nucleosome, many amino acid residues on histone tails are targets of extensive post-translational modifications. These occur on specific amino acid residues and include the acetylation of lysines, phosphorylation of serines and threonines, ubiquitination of lysines and methylation of both lysines and arginines (Turner 2000; Nightingale, O'Neill, and Turner 2006; Margueron, Trojer, and Reinberg 2005). The result of adding these molecular groups is varied and depends highly on both which amino acid was modified and the type of modification (Kouzarides 2007; Turner 2007). The addition of these various groups tends to result in one of two possible consequences. First, it may change the interaction between DNA and the histone directly, leading to an alteration of the chromatin structure as a whole. This activity is observed mostly when a post-translational modification, such as acetylation, alters the charge of an amino acid on the histone tail. The second consequence of histone modification is the alteration of non-histone protein recruitment to histone tails resulting

in downstream transcriptional effects such is the case with many methylation events (Kouzarides 2007; Strahl and Allis 2000).

1.3.2 The Histone Code

The observation that combinations of specific histone modifications were often grouped together on the same histone tail, or within the same region of chromatin, brought to birth the theory that these modifications create a type of "code", referred to as "the histone code". It was postulated that histone modifications, both type and number, could dictate a downstream transcriptional profile, and thus biological outcome (Turner 2000). Histone modifications are grouped into two categories: those resulting in active transcription (or "ON") and those resulting in transcriptional repression (or "OFF"), which characterize differences in accessible versus closed chromatin states. This code has a pertinent role in dictating DNA packaging, interactions of DNA with accessory proteins, and a strong influence on gene regulation (Strahl and Allis 2000; Turner 2000; Richards and Elgin 2002). It has recently been suggested that the terminology of the histone code be referred to as the "epigenetic code" and updated to include the importance of differentiation and development, allowing for a statement similar to the following:

> "The epigenetic code describes the way in which the potential for expression of genes in a particular cell type is specified by chromatin modifications put in place at an earlier stage of differentiation"
>
> (Turner, 2007).

It is important to consider that this more novel "epigenetic code" includes not only histone modifications but also the cooperation of additional epigenetic alterations, such as DNA methylation events (discussed below).

1.4 Epigenetic Marks

1.4.1 DNA Methylation

DNA methylation is found to occur at cytosine residues with both CG dinucleotides and CNG trinucleotides resulting in the formation of 5-methylcytosine (5mC) (Bird 2002; Jones and Takai 2001). DNA Methylation is generally considered to be repressive and tends to localize to repeated regions of DNA such as centrosomes and transposons, important for the maintenance of chromosome stability, although it is also known to occur within genes (Berger 2007). Gene promoters generally remain free of DNA methylation due to the presence of CpG islands that are methylation resistant. These resistant CpG islands remain methyl-free even when they are present at the promoter of a gene that is silenced. Some CpG islands however, become methylated during development (as seen in the case of X inactivation), leading to extremely stable

repression (Bird 2002), or they can become aberrantly hyper-methylated in cancer, resulting in extremely stable alterations to gene expression profiles(Berger 2007).

As early as 1975, it was proposed that DNA methylation might be inheritable (Holliday and Pugh 1975; Riggs 1975). A family of DNA methyltransferases (DNMT) are involved in this phenomenon. Specifically, DNMT3A and 3B are responsible for *de novo* methylation, which is required for the establishment of methylation patterns during development (Okano et al. 1999; Hsieh 1999; Okano, Xie, and Li 1998). DNMT1 prefers to methylate new CpGs that are partnered with a parental strand already possessing methylation, and is thus known as the maintenance DNA methytransferase (Bestor 1992; Pradhan et al. 1999). Since DNA methylation events can lead to alterations in gene transcription without actually changing the DNA sequence, and since these methylation marks are conserved and replaced after replication, DNA methylation is considered to be one category of epigenetic mark.

1.4.2 Histone Acetylation

One result of post-translational modifications to histone tails is an alteration of nucleosome structure leading to changes in the configuration of the basic subunit of chromatin. This is the case for histone acetylation; the first discovered and most extensively studied post-translational modification. Acetylation to lysine residues on a histone tail results in the neutralization of the basic charge of the residue, leading to a loosening of the interaction between the histone core and DNA. This structural alteration leads to an increased accessibility to certain regions of DNA, and is therefore associated

with transcriptional activation (Shogren-Knaak et al. 2006; Workman and Kingston 1998; Hebbes, Thorne, and Crane-Robinson 1988). The association of acetylated histones in locations of active transcription was noted as early as 1978 when chromatin was found to be less resistant to endonuclease A digestion after treatment with Trichostatin A, a potent, competitive Histone Deacetylase (HDAC) inhibitor (Simpson 1978). Additionally, areas of chromatin associated with high levels of acetylation were found to be more loosely packaged than those associated with areas lacking acetylation in nucleosome arrays, suggesting that acetylation of lysine resides on the histone tail resulted in alterations to higher order chromatin structure. These alterations were not directly connected to transcriptional regulation until it was found that many transcriptional co-activators, such as General control of amino acid synthesis protein 5 (GCN5) and p300/CBP, which were originally observed to have classical co-regulatory activities, including modulating the association of TFs to promoters (Guarente 1995), appeared also to have intrinsic histone acetyl transferase (HAT) activity (Zhang and Reinberg 2001; Kuo and Allis 1998). It was additionally observed that several global transcriptional repressors were associated with complexes containing HDAC activity (Kuo and Allis 1998; Kuzmichev and Reinberg 2001). As co-regulators, these HATs and HDACs do not bind to DNA directly and are generally members of large multi-subunit protein complexes that are recruited by and associate with DNA-bound activators (Utley et al. 1998). In 1998, several independent groups provided evidence that the enzymatic activities of these co-activators/repressors were required for transcriptional activation and repression (Wang, Liu, and Berger 1998; Kuo et al. 1998; Kadosh and Struhl 1998; Hassig et al. 1998). This provided the first

evidence that the histone acetylation profile of a gene was pertinent to transcriptional regulation. Acetylation occurs on Lysine (K) residues, most frequently on residues K9, K14, K18, K23 and K56 of Histone H3 as well as K5, K8, K13 and K16 of Histone H4 (Berger 2007; Strahl and Allis 2000). Understanding histone acetylation as well as the role of co-regulatory HAT and HDAC enzymes has provided the field of epigenetics with a model for studying additional novel post-translational modifications, and both HAT and HDAC enzymes have been increasingly popular targets for drug discovery (Kuo and Allis 1998; Khan and Khan 2010)

1.4.3 Histone Phosphorylation

The second result of post-translational modification to histone tails is an alteration to the binding platform for the recruitment or removal of effector proteins, a phenomenon required for transcriptional regulation. Contrary to what is observed with histone acetylation (as summarized above) where acetylation results in alterations to the nucleosome structure itself, most other post-translational modifications are categorized separately. These post-translational additions or removals result in an alteration of a particular residue to recruit other chromatin binding proteins, downstream TFs, or additional post-translational modifications, without directly affecting the interaction between nucleosomes and associated DNA. Several phosphorylations to both serine (S) and threonine (T) residues within the histone tails perform this function. Though histone phosphorylation was discovered over 30 years ago, it has only recently become the subject of intense study. Both T and S residues on most histones are the targets of

phosphorylation and modification of these residues is frequently involved in the compaction of chromatin during cell division. Phosphorylation of both T119 of H2A and H3S10 for example, play pertinent roles in this process. Phosphorylation of histone residues is extensively involved in cross-talk with other post translational modifications and downstream signaling pathways. The phosphorylation of Histone H3 serine 10 (H3S10), for example, is required for the condensation of chromatin during mitosis, resulting in transcriptional repression. Interestingly, histone phosphorylating kinases mitogen and stress-activated protein kinase 1 and 2 (MSK1/2) and ribosomal S6 kinase 2 (RSK2) tend to target this same residue, resulting in recruitment of the phospho-binding protein 14-3-3, which is thought to activate nuclear factor kappaB (NFκB)-regulated genes (Kouzarides 2007; Banerjee and Chakravarti 2011). This suggests that the post-translational modification of a single residue is required for both an opening and closing of the chromatin structure through the recruitment of differing effector proteins (berger, 2007). Phosphorylation events are implicated in a variety of cellular processes including DNA damage repair, apoptosis, transcriptional regulation and cell cycle progression, though much of our knowledge of the regulation and roles of histone phosphorylation remains preliminary (Banerjee and Chakravarti 2011).

1.4.4 Histone Ubiquitination

Ubiquitination differs from the aforementioned mechanisms because it involves the addition of large moieties to the histone tail (Berger 2007). The function of ubiquitination remains unclear but it is believed to either act to recruit supplementary

proteins to histone tails or to physically "wedge" chromatin open due to its size. H2B and H2A are both targets for mono-ubiquitination, at lysine residues 120 and 119 (H2BK120ub, H2AK119ub) respectively. Both locations represent a region on the surface of the nucleosome core, easily accessible to ubiquitination machinery (Osley 2006). Functional effects of ubiquitination appear to vary depending on the residue to which the moiety is added. Ubiquitination of H2B is associated with areas of euchromatin and is required for transcriptional elongation by RNA polymerase II (Pavri et al. 2006; Xiao et al. 2005) while H2A ubiquitination appears to be concentrated at areas of heterochromatin and is repressive to transcriptional elongation (Zhou et al. 2008). H2A ubiquitination may be required to initiate heterochromatin formation, and is involved in heritable gene silencing, and X-inactivation through its role in chromatin compaction (Smith et al. 2004; de Napoles et al. 2004).

Similar to phosphorylation, ubiquitin modifications appear to be intimately involved in cross talk with other post-translational modifications, exemplified by ubiquitination of H2B which appears to play a role in the regulation of methylation of Histone H3 at both lysine 4 and lysine 79 (H3K4 and H3K79 respectively). A reduction in H2BK120ub leads directly to a reduction of methylation at both lysine marks(Zhu et al. 2005). Ubiquitination of H2A has been found to cooperate with DNA methylation to correlate with the transcriptional silencing of Hox genes (Wright, Wang, and Kao 2011; Wu, Gong et al. 2008) and also to alter inter-histone interactions. Ubiquitination of H2A119ub1 by the Ring 1A/B components of the polycomb repressive complex 1 (PRC1) has been showed to affect the interaction of histone H2A with both linker DNA

and the linker histone H1. Deubiquitination of H2A119, in turn, results in both the phosphorylation of histone H1 and the de-association of H1 from chromatin, leading to alterations in higher order chromatin packaging (de Napoles et al. 2004). It has been suggested that the presence of an ubiquitin group may represent a required binding site for enzymes involved in histone methylation. Though, as with phosphorylation, most of the work completed on histone ubiquintation is preliminary, recent advances have supported the notion that ubiquitination represents an important post-translational modification, resulting in the modulation of chromatin structure and down-stream transcriptional effects.

1.4.5 Histone SUMOylation

Similar to histone ubiquitination, modification by small ubiquitin-like modifier SUMO involves the addition of a large moiety to the histone tail. The covalent addition of this 100 amino acid SUMO group, termed SUMOylation, occurs most frequently on the N-terminal histone tail, similar to the addition of most other post translational modifications (Nathan et al. 2006). SUMOylation is involved in the stabilization of constitutive heterochromatin in fission yeast and is generally associated with transcriptional repression, antagonizing both acetylation and ubiquitination on the same histone tail. Though details of how SUMOylation of histones affects transcription remain ambiguous (Garcia-Dominguez and Reyes 2009; Kouzarides 2007), the addition of SUMO groups directly to proteins involved in transcriptional control has proven to be an important aspect of their regulation and appears to be associated mostly with

transcriptional repression (David, Neptune, and DePinho 2002; Ling et al. 2004; Bouras et al. 2005). Further understanding how SUMOylation is involved in post-translational crosstalk may provide insights into how other post-translational marks are regulated.

1.4.6 Histone Methylation

Though histone methylation was first discovered in 1964 (Murray 1964), no information on the biological significance of this modification was collected until decades later. Recently however, much interest has been placed on the regulation of histone tail methylation. Unlike the previously mentioned modifications, methylation can occur on both lysine and arginine (R) residues on amino terminal histone tails (Sims, Nishioka, and Reinberg 2003; Shilatifard 2006). This modification is also progressive, suggesting that unlike acetylation, which is either present or absent, methylation potentially allows for an increased ability to fine-tune regulation. An arginine can become mono- or di-methylated, the latter of which can be either symmetrical or asymmetrical. Similarly, a lysine can be modified in a mono-, di- or tri-methylated form, each of which has been found to have a differing affect (Santos-Rosa et al. 2002; Cloos et al. 2008). Methylation does not alter the charge of the histone tail, suggesting that this modification does not play a direct role in DNA/ histone interactions. Rather, methylation can result in a modulation of chromatin structure, altering the accessibility to chromatin to effector proteins, or it may act as a recruitment signal for regulatory factors (Cloos et al. 2008). Thus, methylation can result in transcriptional alterations due to changes in the chromatin landscape as a whole (Bannister, Schneider, and Kouzarides 2002; Lachner et al. 2001).

Unlike histone acetylation, the mere presence of a methylation mark does not dictate transcriptional activation or repression. It is, instead, the precise location of that post-translational modification that appears to result in a specific transcriptional consequence. Histone H3, for example, contains several lysine residues that are open to methylation, with lysines 4, 36 and 79 (H3K4me, H3K36me and H3K79me respectively) being generally ascribed to transcriptional activation, and lysines 9 and 27 (H3K9me and H3K27me) being generally ascribed to transcriptional repression. Methylation of lysine 20 of H4 (H4K20me) has additionally been ascribed to sites of transcriptional repression (Sims, Nishioka, and Reinberg 2003; Kouzarides 2007).

1.4.6.1 Bivalency

Recent studies have introduced the phenomenon known as bivalency, where methyl marks considered as signs of active transcription (such as H3K4me3) and those considered as signs of repressed transcription (such as H3K27me3) co-occupy the same promoters. These promoters are considered to be "bivalent" and are generally found in lineage specific genes within pluripotent cells such as embryonic stem cells (ESC), the inner cell mass (ICM) of the mouse and pregastrulation stage zebrafish embryos (Bernstein et al. 2006; Alder et al. 2010; Dahl et al. 2010; Lindeman et al. 2010). The presence of transcriptionally repressive marks are thought to maintain lineage commitment genes in the off position while the activation marks hold the promoters in a "poised" gene state, prepared for rapid activation upon appropriate differentiation cues (Bernstein et al. 2006; Fisher and Fisher 2011). Though they are generally found within

ESC, recent work has suggested that promoters bearing bivalency can also be found within neural progenitors, hematopoietic stem and progenitor cells, and mesenchymal stem cells (Mikkelsen et al. 2007; Cui et al. 2009; Collas 2010). This phenomenon has also been observed lower on the evolutionary scale, with *C. elegans* showing H3K4me3 and H3K27me3 co-occupying promoters early in development (Wang, Fisher, and Poulin 2011). Bivalency can become resolved to univalency after a single round of differentiation takes place or genes may remain poised through several rounds of differentiation until lineage commitment occurs (Surface, Thornton, and Boyer 2010).

1.5 Methylation of Histone 3 Lysine 4 (H3K4)

Methylation at H3K4 was first identified in 1975 in trout testis (Honda, Candido, and Dixon 1975; Honda, Dixon, and Candido 1975). Since then, it has been found associated with transcriptional activation in many eukaryotic species. H3K4 can be mono-, di- or tri-methylated, with each methylation level having a distinct role and localization within the genome. Studies in yeast have demonstrated that H3K4me3 is located at the transcriptional start site (TSS) of actively transcribed genes, H3K4me2 is spread throughout active genes, mainly concentrated at the center of the transcribed region, and H3K4me1 appears to be mostly localized to the 3' region of transcribed genes (Santos-Rosa et al. 2002; Pokholok et al. 2005). Localization in vertebrates, however, appears to show co-localization of H3K4me2 and me3 in many cases, with H3K4me2 showing broad association with eukaryotic regions and H3K4me3 being specifically associated with the 5' region of actively transcribed genes (Bernstein et al. 2002; Krogan

et al. 2003; Ng et al. 2003). H3K4me3 localized to gene promoters allows for transcriptional activation by binding a subunit of transcription factor II D (TFIID), which then leads to the formation of the initiation complex (Sims, Nishioka, and Reinberg 2003; Vermeulen et al. 2007). Though both mono- and di-methylated versions of H3K4 span further into the transcribed protein and have even been found at enhancer elements (Robertson et al. 2008; Heintzman et al. 2009), H3K4me3 remains strongly localized to the transcriptional start site (Santos-Rosa et al. 2002; Cloos et al. 2008; Kooistra and Helin 2012). The physiological significance of H3K4 methylation appears to be a marker for the recruitment of effector proteins. This effector recruitment then results in either enzymatic or physiological activity at the sites of interest (Shilatifard 2008). The presence of this modification at the transcriptional start site of genes results in the recruitment of proteins containing several highly conserved chromatin binding domains, such as Tudor domains, Plant Homology Domains (PHD) and chromo domains (Berger 2007; Ruthenburg, Allis, and Wysocka 2007; Campos and Reinberg 2009). Proteins containing these domains that recognize H3K4 methylation are classically involved in further downstream chromatin modifications resulting in transcriptional activation or accessibility, including ATP-dependent chromatin remodelers, and proteins involved in histone acetylation such as Inhibitor of growth (ING) proteins (Ruthenburg, Allis, and Wysocka 2007)

1.5.1 H3K4 Methylation and Development

Genome-wide chromatin studies have suggested that the global levels of H3K4me3 decrease from the ESC stage over the course of differentiation (Ang et al. 2011) with bivalency being removed through demethylation of H3K4me3 positive promoters (Bernstein et al. 2006). H3K27me3 expression however, appears to remain present. This suggests that the presence of H3K4me3 may be required for early development, although its removal may also represent a required checkpoint for certain stages of differentiation. Selective removal of H3K4me3 seems to be required for appropriate cell fate determination to occur. Studies in *C. elegans* demonstrate that the appearance of H3K4me3 is regulated according to cell lineage and that the deposit of this tri-methylation is extremely dynamic (Wang, Fisher, and Poulin 2011) lending credence to the theory that both the presence and absence of this mark may represent significant methods of gene regulation during development. Interestingly, recent studies categorizing the role of H3K4 methylation in fully differentiated cells such as the cardiomyocyte add support to this work, suggesting that maintenance of H3K4me3 is required to maintain cellular integrity even in a non-dividing, fully committed cell type (Stein et al. 2011). This also supports the notion that although the expression of H3K4me3 may be required to be reduced at certain developmental checkpoints, re-expression of this mark occurs at later stages of development.

For cells to maintain appropriate identity and respond properly to external and internal cellular cues, a fine balance between methylation and demethylation of H3K4 must be maintained in both a lineage- and commitment-dependent manner. Slight

alterations to the expression level or localization of enzymes required to maintain this balance may result in changes in levels of H3K4me3 in either a global, or gene-specific manner which, in turn, could easily result in disease or abnormal cellular phenotypes through aberrant transcription, or lack thereof, at a subset of developmentally important genes.

1.6 Enzymes Regulating Histone Methylation

1.6.1 H3K4 Methyltransferases

The easiest method of regulating methylation at H3K4 is to exert control over the enzymes required for the placement or removal of this mark. The addition of a methyl group on the ε-Nitrogen of lysine 4 is regulated by a family of enzymes known as SET domain containing lysine methyltransferases (KMT). The first H3K4 methylase, SET1, was discovered in *Saccharomyces cerevisiae* as a member of a histone modifying complex named complex proteins associated with Set1 (COMPASS) (Miller et al. 2001). SET1 contains a classical "SET" domain common to other epigenetic regulators and originally named for a homologous domain localized within three proteins known to suppress Position Effect Variegation (PEV) in Drosopila: **S**u(var)3-9, a protein required for the methylation of H3K9, **E**nhancer of Zeste, a polycomb group protein, and the trithorax group protein **T**rithorax (Sims, Nishioka, and Reinberg 2003). SET1, now named KMT2, is not enzymatically active on its own and requires association with other members of COMPASS to mono-, di- and trimethylate H3K4 (Shilatifard 2006;

Schneider et al. 2005; Wood et al. 2007; Miller et al. 2001; Krogan et al. 2002; Roguev et al. 2001; Nagy et al. 2002).

Though yeast contains only one H3K4 KMT, mammals express 6 SET1 homologues referred to as Set1A, Set1B, Mixed Lineage Leukemia (MLL), MLL2, MLL3 and MLL4. Each of these mammalian homologues is found in a COMPASS-like multi-protein complex and has active H3K4 KMT activity (Cho et al. 2007; Hughes et al. 2004; Lee and Skalnik 2005; Wu, Wang et al. 2008) It is of interest that yeast can maintain appropriate patterning of H3K4 methylation with only one KMT, whereas mammals appear to have 6 separate enzymes that exhibit non-redundant function. Theories suggest that this added layer of regulation has appeared through evolution to deal with the complexity of mammalian development and gene expression. MLL complexes contain three additional common subunits: WDR5, RbpB5 and Ash2 which exist in a complex separate from MLL and which can associate with all MLL family members through their SET domain (Milne et al. 2002; Hughes et al. 2004; Wysocka et al. 2003). Association of this triplex of proteins with MLL is required for MLL activity on H3K4, which, similar to most other epigenetic regulators, is not enzymatically active without the presence of its complex (Dou et al. 2006).

An additional H3K4 KMT, Ash1, was first discovered in *Drosophila* and found to have KMT activity both *in vitro* and *in vivo* (Byrd and Shearn 2003; Beisel et al. 2002). The human homologue of Ash1, huAsh1, has also been found to methylate H3K4 *in vitro* and appears to colocalize with both MLL and H3K4me3 at actively transcribed genes (Gregory et al. 2007). huAsh1 contains a SET domain, required for enzymology, and is

hypothesized to play a role in the dimethylation at HOX genes during development, although its distinct role in H3K4 methylation remains unknown (Eissenberg and Shilatifard 2010).

1.6.2 Histone Demethylases

Historically, histone methylation was considered to be a mark of permanence. Without the discovery of an enzyme class capable to reverse methylation, it was thought that these marks were static, representing permanent modifications and alterations to chromatin structure. The discovery of the enzyme KDM1a (Lysine (K) DeMethylase 1, also known as LSD1, BHC110) in 2004, changed this notion. KDM1a was found to have the ability to catalyze the demethylation of histone residues by a flavin adenine dinucleotide (FAD)-dependent amine oxidase reaction. However, the enzymology of this demethylase requires a protonated methyl ε-ammonium in its substrate. This is absent in the trimethylated state of methylation, resulting in the conclusion that this enzyme was restricted to mono and dimethylated modifications and that trimethylation represented permanency (Shi et al. 2004). KDM1 was originally found to show enzymology specific to H3K4me1 and H3K4me2, though it was later discovered that it also exhibits activity on H3K9 in certain contexts (Metzger et al. 2005).

1.6.2.1 Jumonji Domain Containing Demethylases

Since the discovery of KDM1, a more novel, larger protein group named the Jumonji (JmjC) domain family of demethylases has been discovered. JmjC demethylases

catalyze the removal of methylation marks utilizing a hydroxylation reaction through their JmjC domain. Enzymatic reaction requires the use of iron (II) and alphaketoglutarate as cofactors but no longer requires a protonated methyl ε-ammonium, allowing for the demethylation of all three methylation states (Figure 1.2). In several cases, the tri-methylated version is the preferred substrate for this enzyme class (Tsukada et al. 2006; Klose, Kallin, and Zhang 2006; Christensen et al. 2007; Fodor et al. 2006; Whetstine et al. 2006). F-Box and Leu-rich repeat protein 11 (FBXL11) was the first enzyme discovered in this class, possessing demethylase activity towards both the mono- and di-methylated versions of H3K36 (Tsukada et al. 2006).

To date, JmjC enzymes of this class have been found to be active on H3K4 (Secombe and Eisenman 2007; Seward et al. 2007; Tahiliani et al. 2007; Lee et al. 2007; Klose et al. 2007; Iwase et al. 2007; Yamane et al. 2007); H3K9 (Yamane et al. 2006), H3K27 (De Santa et al. 2007; Lan et al. 2007; Agger et al. 2007), H3K36 (Fodor et al. 2006) and H4K20 (Liu, Tanasa et al. 2010). This has led to the current understanding that methylation represents an extremely flexible and dynamic modification state resulting in the active modulation of transcription.

1.7 Demethylation of H3K4

Although the addition of H3K4 methylation remains important to transcriptional regulation, the enzymes responsible for removing this mark serve an equally important role in gene regulation. The JmjC class of demethylases as a whole is an expansive protein family (the human genome encodes 30 different JmjC containing proteins, 18 of

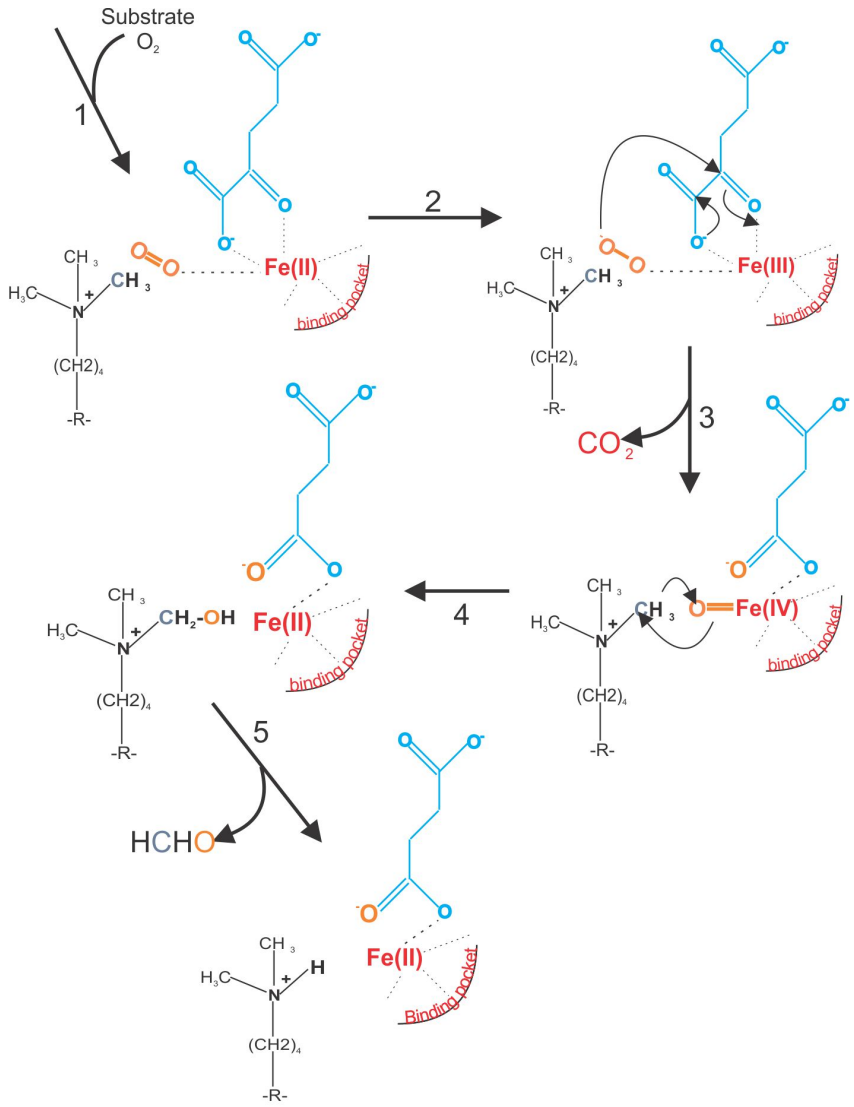


Figure 1.2 Mechanism for Demethylation mediated by JmjC demethylases 1.Substrate, molecular oxygen and alpha-ketoglutarate (blue) interact 2. An electron is transferred from Fe(II) to the molecular oxygen resulting in the formation of a superoxide radical and Fe(III) 3. The superoxide radical attacks the carbonyl group (C2) in the alpha-ketoglutarate, alpha-ketoglutarate accepts an electron from the Fe(III) resulting in the production of succinate and CO_2. 4. A highly reactive oxy-ferryl (Fe(IV)=O) is formed. This oxy-ferryl intermediate is reduced and a hydrogen atom is removed from the methylated substrate forming a carbinolamine. 5. This carbinolamine degrades into formaldehyde and a lysine minus one methyl group and the Fe(II) is regenerated.

which have been proven to show demethylase acitivity on both arginine and lysine residues (Kooistra and Helin 2012)). Phylogeny has suggested that within this family there are several clusters of proteins that appear to group together in both structure and function. The KDM5 family of demethylases, known to target all three methylation states of H3K4, represents one such cluster (Cloos et al. 2008)

1.8 KDM5 Demethylases

The KDM5 family of JmjC demethylases includes four known mammalian members: KDM5a, KDM5b, KDM5c and KDM5d (previously known as Jarid1a, Jarid1b, Jarid1c and Jarid1d respectively). These demethylases are highly conserved structurally and are characterized by the presence of five protein domains: JmjN and JmjC domains required for demethylation activity, a BRIGHT/ARID (A/T rich binding domain) which binds G/C rich DNA regions (Scibetta et al. 2007), a C5HC2-zinc finger domain and several plant homeobox (PHD) domains involved in the enzymes ability to recognize and bind methylated residues and regulate protein-protein interactions (Cloos et al. 2008) (Figure 4.4C). Deletion of any full domain of the conserved protein family results in alterations to its demethylase activity (Yamane et al. 2007) and single point mutations within the iron-binding pocket, such as the point mutation to H499 in KDM5b results in complete eradication of enzymology (Yamane et al. 2007; Xiang et al. 2007). Members of the KDM5 family of demethylases are not limited to vertebrates and have several homologues across evolution. This includes “Little Imaginal Disc” or Lid, a JmjC containing enzyme containing H3K4me3 demethylase activity in Drosophila (Eissenberg

et al. 2007), JhD2 in budding yeast (Huang, Chandrasekharan et al. 2010), KDM_5 in Saccharomyces cerevisiae and rbr-2 in *C.elegans* (Christensen et al. 2007).

This family of histone demethylases acts specifically on H3K4 methylation marks, with a preference for tri-methylated H3K4 (H3K4me3) and its members are therefore considered to be potent transcriptional repressors through their ability to remove this mark of transcriptional activation. KDM5a plays a role in Notch-mediated silencing where demethylation at specific regulator elements rather than entire promoter TSS regions is sufficient to result in gene silencing (Liefke et al. 2010). Additionally, it has recently been suggested that KDM5b may be involved in intragenic transcription and elongation of KDM5b target genes, though these results are currently under debate (Xie et al. 2011; Schmitz et al. 2011). This adds an additional layer of regulation, suggesting that the accuracy of these enzymes for transcriptional regulation is most likely extremely pertinent to sensitive biological functions within the cell, with potentially significant impact on processes including development and differentiation, and that even the smallest of perturbations could wholly or in part give rise to disease or transformation.

1.8.1 KDM5 Expression Profiles

The spatial and temporal expression of each of the four mammalian KDM5 family members differs significantly, with each appearing to play non-redundant functions throughout development. KDM5a appears to be widely expressed in all tissues but shows high expression in the hematopoietic system (Cloos et al. 2008; Christensen et al. 2007; Klose et al. 2007; Lopez-Bigas et al. 2008). KDM5c, an X-linked gene which escapes X-

linked inactivation (Wu, Ellison et al. 1994; Wu, Salido et al. 1994) appears to have more limited expression, showing neuronal expression patterns and playing a role in neuronal development (Iwase et al. 2007). KDM5b shows a completely different profile. It is widely expressed in ESC and undifferentiated progenitors (Dey et al. 2008), but limited in adult tissues: restricted to the testis and differentiating mammary gland (Lu et al. 1999; Barrett et al. 2002), and in several forms of cancer (Barrett et al. 2002; Barrett et al. 2007; Madsen et al. 2003; Xiang et al. 2007; Roesch et al. 2006; Roesch et al. 2010).

1.9 KDM5b

KDM5b appears to be a multi-functional member of the KDM5 demethylase family, with a pertinent role in development and a proposed role in oncogenesis. Originally known as Plu-1, KDM5b was first discovered as a target up-regulated in response to Her2/c-ErbB2 in breast cancer cell lines and primary breast cancers (Lu et al. 1999). Of limited expression in most adult tissues, KDM5b shows consistent up regulation in breast and prostate cancers in both human and mice, and has been shown to play a regulatory role in multiple cancer types (Lu et al. 1999; Barrett et al. 2002; Xiang et al. 2007; Yamane et al. 2007; Hayami et al. 2010; Roesch et al. 2010).

1.9.1 The Role of KDM5b in Development

In contrast to previous studies on family member KDM5a, where KDM5a does not appear necessary for embryonic development (Klose et al. 2007), KDM5b is crucial for embryonic development, as studies using KDM5b knockout mice have reported

embryonic lethality around E4.5 (Catchpole et al. 2011). This suggests that KDM5b is required in early embryonic development and that this role cannot be substituted by another KDM5 family member. Catchpole et al. report the creation of a KDM5b mouse strain containing a mutation in which the ARID domain is removed. This mutation has previously been documented to completely obliterate the demethylase activity of KDM5b (Yamane et al. 2007; Tan et al. 2003) though Catchpole et al. suggest that some residual activity is a possibility (Catchpole et al. 2011). Interestingly, these mice are both viable and fertile suggesting that the role of KDM5b in embryonic development may not hinge completely on its enzymology (Catchpole et al. 2011).

1.9.2 The Role of KDM5b in Cancer

Delineating the exact role that KDM5b exerts in cancer has become complex. KDM5b is known to be a regulator of both oncogene and tumour suppressor expression, through direct interaction with the promoters of these genes, resulting in alterations to cell properties such as cell cycle progression and invasion potential (Yamane et al. 2007; Yoshida et al. 2011). KDM5b has been associated with cell cycle control in both an accelerating (breast cancer) (Yamane et al. 2007) and decelerating (Melanoma) (Roesch et al. 2010) fashion and is directly involved in the ability of both lung and bladder cancer cells to escape apoptosis, where a reduction in KDM5b is often linked to a reduction in oncogenic potential (Hayami et al. 2010).

It has recently been suggested that KDM5b may be additionally required for the adaptation of cells to hypoxia. Solid tumors are considered to be highly hypoxic

compared to surrounding tissue, and adaptation to this state is required for tumor survival (Semenza 2003). Previous work has shown that reduced H3K4 methylation is linked to poor prognosis in cancer patients (Seligson et al. 2005), suggesting that an ability to demethylate H3K4 is important for tumor survival. Due to the requirement of dioxygenases, such as KDM5b, for molecular oxygen, it is proposed that the increased expression level of these enzymes may represent a compensatory mechanism in response to decreasing oxygen availability (Xia et al. 2009). Without this compensatory mechanism, H3K4me3 levels would be expected to increase as tumors increase in size and oxygen levels decrease, leading to the death of the hypoxic tumor cells. An increase in the expression level of demethylases such as KDM5b may provide a mechanism for the tumor to maintain low H3K4me3 levels even in situations where decreased oxygen levels are present, allowing tumour survival.

KDM5b has also recently been used as a biomarker to flag a small population of slow cycling cells within the heterogeneous population of a melanoma (Roesch et al. 2010). These "slow" cells appear to be required for tumour maintenance, giving rise to progeny which express low levels of KDM5b, and knock down of KDM5b results in an exhaustion of tumour growth. It has been suggested that the acceleration of cell cycle in these melanocytes after suppressing KDM5b expression may be due to a de-repression of E2F-target genes. KDM5b is a member of the Rb (Retinoblastoma) repression complex, required for the repression of E2F target genes during senescence (Nijwening et al. 2011; Chicas et al. 2012). Though repression of E2F targets would generally be considered a tumour-suppressive function, mutations to Rb are common in cancer progression,

allowing pro-proliferative effects to override normal suppression, which could lead to increased oncogenic potential. As noted by Chicas et al., this highlights the context-dependent role of these demethylases (Chicas et al. 2012). (Figure 1.3) These multitudinous and varied roles in different cancers suggests that though intimately involved in oncogenesis, KDM5b appears to exert its "tumorigenic potential" in different ways depending on cellular context and may respond differently depending on which upstream cellular cues become activated.

1.9.3 Regulation of KDM5b

Chromatin contains some of the most expansive and complicated complexes in eukaryotic cells. Most, if not all, transcriptional regulators require interaction with multiple co-factors both to be properly localized to targets of interest, and to perform their enzymatic role within the cell. Histone modifiers, as well as histone marks, are brought into contact with an extensive number of proteins and distinct combinations of proteins result in appropriate biological read-outs. Due to the generalized role of KDM5b as a transcriptional repressor, and the potential downstream biological effects of its activity, it is of importance to understand any and all co-regulators involved in the activity of this enzyme.

1.9.3.1 The Role of KDM5b as a Co-repressor

Previous to its enzymology as a histone demethylase, KDM5b was found to interact with the developmental regulators BF-1(FoxG1b, Brain factor-1) and Pax9 (Paired box protein-9) through yeast two-hybrid screening. Expression of KDM5b was

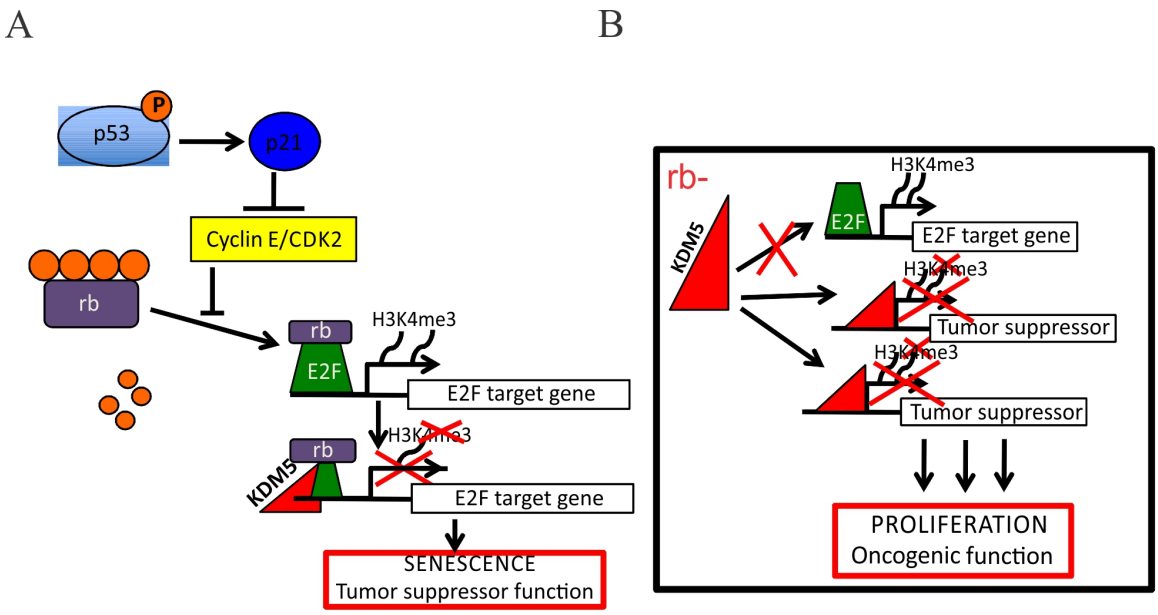


Figure 1.3 KDM5b plays a role in the rb senescence complex A)In wildtype conditions, activation of p53 leads to the activation of p21 and inhibition of Cyclin E/CDK2. This then leads to the dephosphorylation of rb allowing binding to E2F. Chromatin remodeling proteins including KDM5s which are recruited to the promoter of E2F target genes leading to the formation of heterochromatin through epigenetic modulation including the removal of H3K4me3 by KDM5. This then leads to a halt in senescence, illustrating a tumor suppressive function. B) In a rb- context, KDM5 appears to act in an oncogenic fashion. This suggests that an increased pool of KDM5is now available, but unable to bind to E2F target genes without the presence of rb. This allows a pro-proliferative function to become apparent, potentially through the down-regulation of alternate tumor suppressors.

found to overlap spatially and temporally with both BF-1 and PAX9 during development and was found to act as a transcriptional co-repressor for both TFs (Tan et al. 2003). To date, no further investigation of these transcriptional regulators on the enzymology of KDM5b has been published. Interestingly, KDM5b has since been implicated as a transcriptional co-repressor for several other TFs. For example, transforming growth factor-β inducible early gene-1/Krüppel-like transcription factor 10 (TIEG1/KLF10) was found to bind to the C-terminus of KDM5b and form complexes *in vivo*. Expression levels of KDM5b directly related to the repressive ability of TIEG1 on its downstream target, Smad7. Binding of TIEG1 was found to occur in a location that would not affect KDM5b enzymology, though the direct effect of TIEG1 on KDM5b enzymology was never directly investigated (Kim et al. 2010). Additionally, KDM5b has been implicated in the down-regulation of $p21^{CIP}$/ cyclin-dependent kinase inhibitor 1A (CDKN1A) as a co-repressor to both Myc and the transcription factor Transcription Factor Activating Protein 2C (TFAP2C). Interestingly, although mutation to the JmjC enyzmology domain of KDM5b abolishes its ability to act as a co-repressor in this context, removal of other domains appears to result in no alteration to its activity with Myc and TFAP2C. Previous studies have outlined that removal of any domain of KDM5 proteins results in a disruption to its demethylase activity, suggesting that although an intact JmjC domain appears to be required in this context, complete enzymology may not (Wong et al. 2012).

1.9.3.2 Interaction of KDM5b with Other Histone Modifiers

The cooperative effort of several chromatin-modifying proteins is often required to result in downstream biological effects. KDM1, for example, is often found to co-occupy promoters with members of the Polycomb Repressive Complex 2 (PRC2), including Suz12 and EZH2 (Tsai et al. 2010). PRC2 is a histone methylating complex required for the methylation of H3K27me3. This suggests that the demethylation of H3K4me3 and methylation of H3K27me3 are directly linked through the interaction of their regulatory complexes. Additionally, the H3K27me3 demethylase UTX has been found to interact with H3K4 methyltransferases MLL2-4, suggesting that the reverse correlation is also true (Issaeva et al. 2007).

KDM5b has also been implicated in concert with other histone-modifying complexes, much like KDM1. Direct binding of KDM5b with both class I and class II HDACS has been shown through the PHD domains of KDM5b (Barrett et al. 2007). This suggests a functional link between hypoacetylation of H3 and a demethylated state of H3K4, both resulting in transcriptional repression. Treatment with HDAC inhibitors such as Vorinostat, a class I HDAC inhibitor, results in an increase in H3K4me3 at target genes involved in differentiation and tumour suppressive functions, suggesting that HDAC inhibitors may function to reduce tumour growth both directly through inhibition of deacetylase activity, and indirectly by modulating KDM5b activity (Huang et al. 2011).

1.9.3.3 Direct Modification of KDM5b

Knowledge of post translational modifications occurring directly to KDM5b are limited, however recent work suggests that KDM5b can be PARylated by Poly(ADP-ribose) polymerase-1 (PARP-1). PARP-1, an enzyme which catalyzes the polymerization of ADP-ribose units from donor NAD+ molecules to target proteins, is generally considered to possess intrinsic enzymatic activity resulting in its own poly (ADP-Ribosyl)ation (PARylation), though additional targets of its enzymatic activity including Histone H1 and KDM5b have recently been discovered (Krishnakumar and Kraus 2010). PARP-1 acts to maintain permissive chromatin states at the promoter of PARP-1 regulated genes. This maintenance of transcription is partially mediated through the prevention of H3K4me3 demethylation through direct PARylation of KDM5b, resulting in its inhibition and exclusion from PARP-1 regulated promoters (Krishnakumar and Kraus 2010). To date, this is the only known modification of KDM5b, as well as the only data currently contributing to our knowledge of how KDM5b is recruited or blocked from access to promoters of interest.

1.9.3.4 Direct Regulation of KDM5b Activity

Though KDM5b has been suggested to have co-repressor activity in many contexts, no direct binding partners have been implicated in KDM5b enzymology. Only one binding partner affecting H3K4me3 demethylation by a KDM5 family member, KDM5d, has been identified to date. Ring6a, a ring family protein was found to directly bind to, and result in an increase in H3K4me3 demethylation potential of family member

KDM5d on a histone substrate (Lee et al. 2007). No other co-factors have been determined. Other histone modifying proteins require complex members for active enzymology and nucleosomal localization, such as the requirement of COMPASS by Set 1. Histone demethylase KDM1 shows histone demethylation activity in the absence of any cofactors, but requires association with CoRepressor of RE-1 silencing factor (Co-REST) in order to successfully demethylate a nucleosomal substrate (Lee et al. 2005). Similar to KDM1, KDM5b shows histone demethylation activity *in vitro* in the absence of co-factor recruitment. However, this activity is limited to histones, as no nucleosomal activity has ever been observed. Multiple subunits of histone modifying complexes are required to dictate both nucleosomal targeting and specificity. Histone-modifying enzymes appear to be recruited to their nucleosomal targets through multiple interactions that individually, may appear to be weak, or transient. To date, the *in vivo* mechanisms of nucleosomal localization of most modifying complexes, including that of KDM5b, remains highly elusive. This leaves a large gap in our knowledge of this transcriptional repressor.

1.9.4 Hypothesis: Understanding the regulation of the histone demethylase KDM5b may provide novel insight into both development and the process of oncogenesis

The hypothesis that garnering an increased understanding of the histone demethylase KDM5b may provide novel insight into both development and the process of oncogenesis is supported by data gathered to date on the role of H3K4 methylation in

appropriate gene patterning, and the expression profile of KDM5b. Unlike other KDM5 family members, KDM5b appears to be specifically expressed both during development and in several cancerous cell varieties, though it is of limited expression in most fully developed cell types. The role of KDM5b as a transcriptional repressor through its ability to remove the activating H3K4me3 mark at gene promoters suggests that expression of this demethylase may result in inappropriate transcriptional profiles leading to, or resulting from, the process of transformation. Increasing our knowledge of how KDM5b is regulated, including the factors involved in influencing its localization to target genes and identifying potential enzymatic targets and/or co-regulators involved in its activity, will increase our understanding of transcriptional regulation throughout development, as well as potentially provide novel therapeutic targets for the process of transformation.

1.10 Hypothesis and Study Rationale

It is clear that modulation of transcriptional regulators, including epigenetic factors such as histone demethylases, can result in downstream biological effects. Although much progress has been made towards understanding how many of these regulators function, currently, regulation of the KDM5 family of histone demethylases remains highly mysterious. KDM5b is involved in both development and transformation. and gaining knowledge on how KDM5b is regulated is pertinent to our understanding of the transcriptional regulation that occurs throughout these processes. Evidence garnered from research completed on other transcriptional regulators such as KDM1, paired with recent work from our laboratory, which shows that KDM5b localization to target genes

does not always result in alterations to H3K4me3 levels, supports the hypothesis that KDM5b may have alternate histone targets for its demethylase activity. In this thesis, we aimed to determine these alternative substrate(s) and to determine the biological pertinence of the activity of KDM5b at this location. Chapter 3 of this thesis describes a new histone target for KDM5b, di-methylated Histone H2B Lysine K43 (H2BK43me2). We characterize H2BK43me as a novel histone methylation and provide evidence that this mark represents the primary substrate for KDM5b. We additionally show that the appropriate modulation of H2BK43me2 represents a pertinent checkpoint in development. From this work and previous work on histone demethylases including KDM1(Lee et al. 2005), we further hypothesize that although KDM5b does not appear to require co-regulators to act on H2BK43me2, the addition of cofactors is required for the enzyme to demethylate H3K4me3 on a nucleosomal target. Further, a novel cofactor for KDM5b is described, the Groucho/TLE protein Transducin Like Enhancer of split 4 (TLE4). We show that TLE4 and KDM5b interact both *in vitro* and in live cells, and that TLE4 is both required and sufficient to confer nucleosomal demethylation activity to KDM5b. Additionally, we show that TLE4 represents an evolutionarily conserved cofactor for the KDM5 demethylase family, and that modifying TLE4 expression in live cells can result in a global alteration to H3K4me3 levels. This provides the first example of a KDM5 family protein acting on a novel substrate, or demonstrating demethylase activity on a nucleosomal substrate, and contributes to our understanding of the regulation of this enzyme family.

Chapter 2 Materials and Methods

Preamble

The following chapter details materials and methods utilized in all chapters of this thesis.

2.1 Tissue Culture and Transient Transfection

E14Tg Mouse embryonic stem cells, including KDM5b heterozygous (CSA022) cells were obtained from BayGenomics and maintained in ESC medium consisting of Dulbecco's modified Eagle's Medium (DMEM) lacking phenol red (Invitrogen) with 4,500 mg/liter D-Glucose and L-Glutamine (Sigma-Aldrich), 10% fetal bovine serum (FBS) (VWR), 2mM glutamine (Gibco-BRL), 0.1mM β-mercaptoethanol (Sigma-Aldrich), 1,000 U/Ml ESGRO medium (Millipore) and 2mM Sodium Pyruvate (Invitrogen). Cells were grown at 37°C in a humidified 6% $C0_2$ incubator. The cells were passaged every 48 hours and medium was changed on alternate days. HEK293 human embryonic Kidney cells were cultured in Minimal Essential Media α (αMEM) (Invitrogen) with 10% FBS (Invitrogen) at 37°C with 5% $C0_2$. Mouse Fibroblast NIH3T3 cells were grown in DMEM with 10% FBS at 37°C with 5% $C0_2$.

NIH3T3 and HEK293 cells were transfected in either six-well dishes or 3.5cm glass bottomed tissue culture dishes (Truant lab) using TurboFect (Fermentas) transfection reagent as per supplier's instructions. Cells were passaged 12-24 hours pre transfection and lysed or visualized approximately 20-24 hours post transfection.

2.2 Nucleosome Preparation for MRM-MS analysis

Cells were pelleted and resuspended in two volumes of Buffer A (10mM Tris 7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT 0.2mM PMSF) and incubated for 10 minutes on ice. Intact nuclei were the separated from cytoplasmic protein by centrifugation at 3000 rpm. Nuclei were then resuspended in one volume of Buffer C (20mM Tris 7.9, 25%

Glycerol, 0.42M NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM PMSF, 0.5mM DTT) and incubated at 4^0C for 30 minutes. Nucleoplasmic protein was then separated from chromatin by centrifugation at 12000 rpm for 30 minutes. Chromatin was then solubilized by pipette in 1M guanidine hydrochloride (pH 7.6) and incubated for 60 minutes at room temperature.

2.3 MS Analysis of H2BK43me2-3 and H3K4me2-3 Peptides

Detection and quantification of the level of H2BK43 and H3K4 methylation were accomplished by Multiple Reaction Monitoring Mass Spectrometry (MRM-MS) (Figure 2.1). For *in vivo* analysis of H2BK43 and H3K4 methylated peptides, we used nucleosomes or purified histones from ES cells. For H2BK43me2-3 analysis the nucleosomes were digested with GluC in 1M Guanidine HCl and yielded SYSIYVYKVLKQVHPD peptide which was then detected by MRM-MS by monitoring fifty four precursor-to-product ion transitions (Table 2.1) For H3K4 analysis the nucleosomes were digested with ArC in 0.1M GuHCl giving T<u>K</u>QTAR peptide which was then detected by MRM-MS by monitoring thirty one precursor –to-product-ion transitions (Table 2.2) MRM peak areas were used to compare the relative abundance of H2BK43me2 vs. H2BK43me3 and H3K4me2 vs. H3K4me3 in samples from cells at different stages of cell differentiation (day 1 to day 7).

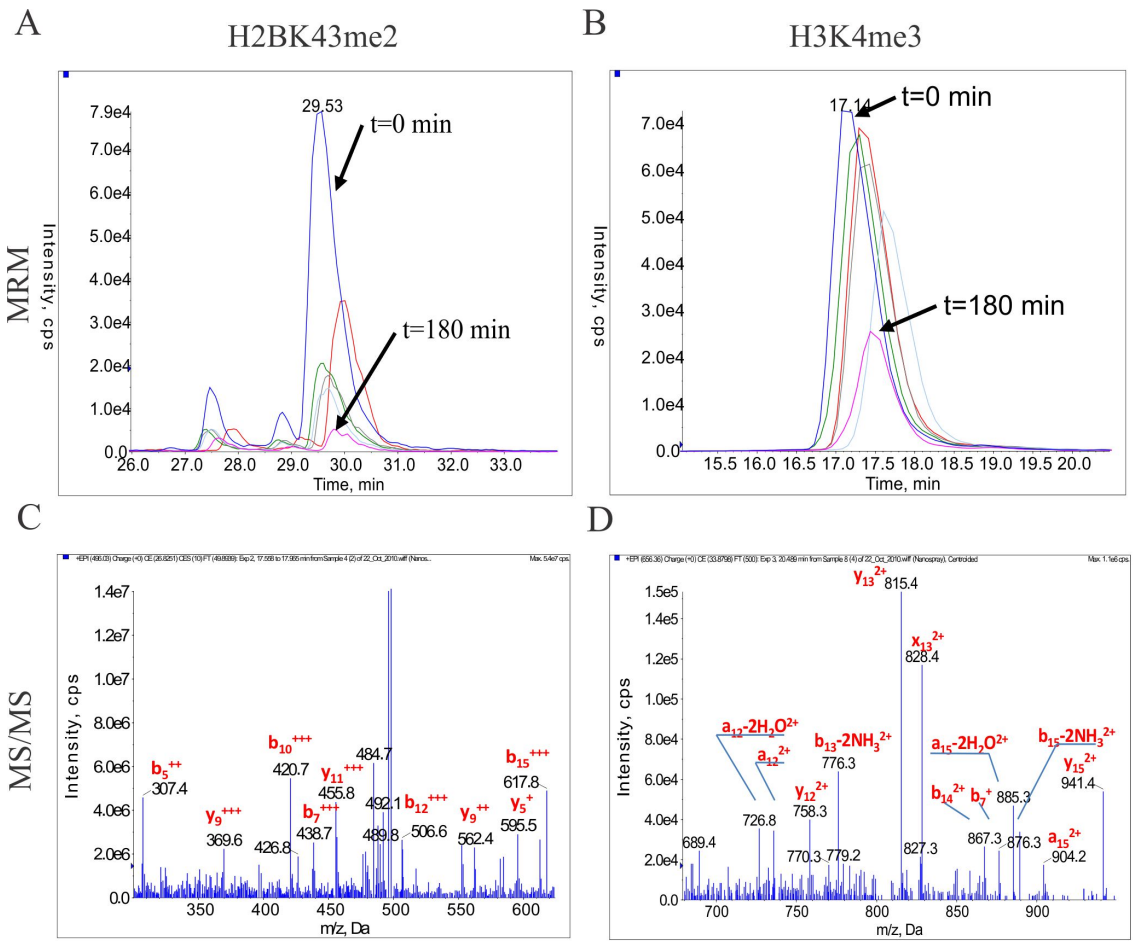


Figure 2.1 Mass spectrometric analysis of products in in vitro demethylation assays and the confirmation of H2BK43 tri- and dimethylation in mESCs by MS/MS. A) MRM peaks representing precursor-to-product ion transitions (M y_{10}-y_4) of methylated the H2BK43 peptide: Biotin-Ahx-YVY**Kme2**VLKQVHPDT. MRM peaks corresponding to samples taken from the indicated time points of the demethylation assay are shown. B) MRM peaks representing precursor-to-product ion transitions (My_8-y_2) of methylated H3K4 peptide: Biotin-AHX-ART**Kme3**QTARKS.C) A tandem MS/MS spectrum of a precursor peptide corresponding to trimethylated H2BK43 in mESCs. D) A tandem MS/MS spectrum of a precursor peptide corresponding to dimethylated H2BK43 in mESCs. A MS/MS spectrum is collected each time when a MRM peak meeting transition criteria is detected. Due to the difference in retention time, the H3K43me2 and H3K43me3 peaks can be readily separated and their identity confirmed by MS/MS data as shown.

Table 2.1 Precursor-to-product ion transitions used to monitor H2BK43 peptide SYSIYVYKVLKQVHPDH2BK43me2-3 (i*n vivo* methylation).

Molecular ion m/z	Mol. Ion Charge	Product ion m/z	Product ion	Collision Energy [V]	Dwell time time [ms]
H2BK43me2					
970.0	(+2)	1325.8	y11+	59.7	50
970.0	(+2)	1226.7	y10+	59.7	50
970.0	(+2)	1063.6	y9+	59.7	50
970.0	(+2)	1004.5	b8+	59.7	50
970.0	(+2)	1103.6	b9+	59.7	50
970.0	(+2)	1216.7	b10+	59.7	50
647.0	(+3)	935.5	y8+	27.1	50
647.0	(+3)	836.5	y7+	27.1	50
647.0	(+3)	723.4	y6+	27.1	50
647.0	(+3)	801.5	y13++	27.1	50
647.0	(+3)	744.9	y12++	27.1	50
647.0	(+3)	663.4	y11++	27.1	50
647.0	(+3)	713.4	b6++	27.1	50
647.0	(+3)	876.4	b7++	27.1	50
647.0	(+3)	1004.5	b8+	27.1	50
647.0	(+3)	672.9	b11++	27.1	50
647.0	(+3)	736.9	b12++	27.1	50
647.0	(+3)	786.4	b13++	27.1	50
H2BK43me3					
984.0	(+2)	1353.8	y11+	60.5	50
984.0	(+2)	1254.7	y10+	60.5	50
984.0	(+2)	1091.7	y9+	60.5	50
984.0	(+2)	1032.5	b8+	60.5	50
984.0	(+2)	1131.6	b9+	60.5	50
984.0	(+2)	1244.7	b10+	60.5	50
656.4	(+3)	935.5	y8+	27.4	50
656.4	(+3)	836.5	y7+	27.4	50
656.4	(+3)	723.4	y6+	27.4	50
656.4	(+3)	815.5	y13++	27.4	50
656.4	(+3)	758.9	y12++	27.4	50
656.4	(+3)	677.4	y11++	27.4	50
656.4	(+3)	713.4	b6++	27.4	50
656.4	(+3)	876.4	b7++	27.4	50
656.4	(+3)	1032.5	b8+	27.4	50
656.4	(+3)	686.9	b11++	27.4	50
656.4	(+3)	750.9	b12++	27.4	50
656.4	(+3)	800.5	b13++	27.4	50
496.0	(+4)	836.5	y7++	22.6	50
496.0	(+4)	723.4	y6++	22.6	50
496.0	(+4)	595.3	y5++	22.6	50
496.0	(+4)	684.4	y11+++	22.6	50
496.0	(+4)	634.9	y10+++	22.6	50
496.0	(+4)	553.3	y9+++	22.6	50
496.0	(+4)	577.7	y14+++	22.6	50
496.0	(+4)	548.7	y13+++	22.6	50
496.0	(+4)	511.0	y12+++	22.6	50
496.0	(+4)	614.3	b5+++	22.6	50
496.0	(+4)	713.4	b6++	22.6	50
496.0	(+4)	876.4	b7++	22.6	50
496.0	(+4)	523.8	b8++	22.6	50
496.0	(+4)	573.3	b9++	22.6	50
496.0	(+4)	629.9	b10++	22.6	50
496.0	(+4)	505.6	b12+++	22.6	50
496.0	(+4)	538.6	b13+++	22.6	50
496.0	(+4)	584.3	b14+++	22.6	50

Table 2.2 Precursor-to-product ion transitions used to monitor H3K4 peptide TKQTARme2-3 (*in vivo* methylation).

Molecular ion m/z	Mol. Ion Charge	Product ion m/z	Product ion	Collision Energy [V]	Dwell time time [ms]
H3K4me2					
732.4	(+2)	631.4	y5+	46.2	50
732.4	(+2)	475.3	y4+	46.2	50
732.4	(+2)	347.2	y3+	46.2	50
732.4	(+2)	246.2	y2+	46.2	50
732.4	(+2)	258.2	b2+	46.2	50
732.4	(+2)	386.2	b3+	46.2	50
732.4	(+3)	487.3	y4+	46.2	50
732.4	(+3)	558.3	y5+	46.2	50
366.7	(+3)	631.4	y5+	25.4	50
366.7	(+3)	475.3	y4+	25.4	50
366.7	(+3)	347.2	y3+	25.4	50
366.7	(+3)	246.2	y2+	25.4	50
366.7	(+3)	386.2	b3+	25.4	50
366.7	(+3)	487.3	y4+	25.4	50
366.7	(+3)	558.3	y5+	25.4	50
H3K4me3					
746.5	(+2)	645.4	y5+	47	50
746.5	(+2)	475.3	y4+	47	50
746.5	(+2)	347.2	y3+	47	50
746.5	(+2)	246.2	y2+	47	50
746.5	(+2)	272.2	b2+	47	50
746.5	(+2)	400.3	b3+	47	50
746.5	(+3)	501.3	b4+	47	50
746.5	(+3)	572.3	b5+	47	50
373.7	(+3)	645.4	y5+	26	50
373.7	(+3)	475.3	y4+	26	50
373.7	(+3)	347.2	y3+	26	50
373.7	(+3)	246.2	y2+	26	50
373.7	(+3)	272.2	b2+	26	50
373.7	(+3)	400.3	b3+	26	50
373.7	(+3)	501.3	b4+	26	50
373.7	(+3)	572.3	b5+	26	50

The digests were analyzed by a positive electrospray ionization-liquid chromatography mass spectrometry (ESI-LC-MS/MS) on a triple quadrupole (Q3 linear ion trap) mass spectrometer (4000 QTRAP, Applied Biosystems). A nanoAcquity UPLC system (Waters) equipped with C18 analytical column (1.7 μm, BEH130, 75 μm × 200 mm and/or 75 μm × 250 mm) was used to separate the peptides at the flow rate of 300 nL/min and operating pressure of 7000 psi (at 95/5H2O:MeCN). Eluted peptides were directly electrosprayed (Nanosource, ESI voltage +2000 V) into the QTRAP. Peptides were eluted using a 62 min gradient with solvents A (H20, 0.1% formic acid) and B (MeCN, 0.1% formic acid) 41 min from 5% B to 50% B, 6 min 90% B, 10 min 5% B. MRM transitions and *in silico* digests were obtained using Skyline™ software made available free of charge by McCoss Lab, University of Washington

2.4 Histone Demethylase Assay (HDM)

All Histone demethylase reactions including peptide, histone and nucleosome substrate assays were performed as described in Lee et al (Lee et al. 2007) using JHDM buffer (50 mM HEPES [pH 8.0], 100 mM NH_4SO_4, 1 mM a-ketoglutarate, 2 mM ascorbate, 5% glycerol, and 0.2 mM PMSF). The peptide HDM reaction was prepared on ice and a time 0 sample was removed. The samples were then incubated at 37^{o}C and samples were removed at 5, 30, 60, 90, 120 and 360 minutes. For a synthetic H2BK43 peptide (Biotin-AHX-YVY**Kme2**VLKQVHPDT) fifteen transitions were monitored (M→ y_{10}-y_4) which all co-eluted at 29.5 min (Table 2.3). For H3K4 synthetic peptide (Biotin-AHX-ART**Kme3**QTARKS) twenty transitions were monitored (M→ y_8-y_2)

Table 2.3 Precursor-to-product ion transitions used to monitor H2BK43me2 biotin-Ahx-YVYKme2VLKQVHPDT (synthetic) peptide.

Molecular ion m/z	Mol. Ion Charge	Product ion m/z	Product ion	Collision Energy [V]	Dwell time time [ms]
1004.4	(+2)	332.1	y3+	56	100
1004.4	(+2)	824.4	y7+	56	100
1004.4	(+2)	937.5	y8+	56	100
1004.4	(+2)	1036.6	y9+	56	100
669.6	(+3)	603.9	y10++	27	100
669.6	(+3)	685.4	y11++	27	100
669.6	(+3)	734.9	y12++	27	100
669.6	(+3)	332.1	y3+	27	100
669.6	(+3)	568.3	y5+	27	100
669.6	(+3)	696.3	y6+	27	100
669.6	(+3)	824.4	y7+	27	100
502.7	(+4)	696.3	y6+	22	100
502.7	(+4)	412.7	y7++	22	100
502.7	(+4)	469.3	y8++	22	100
502.7	(+4)	518.8	y9++	22	100

Table 2.4 Precursor-to-product ion transitions used to monitor H3K4me3 (synthetic) biotin-Ahx-ARTKme3QTARKS peptide

Molecular ion m/z	Mol. Ion Charge	Product ion m/z	Product ion	Collision Energy [V]	Dwell time time [ms]
764.5	(+2)	1117.68	y9+	40	100
764.5	(+2)	961.579	y8+	40	100
764.5	(+2)	860.531	y7+	40	100
764.5	(+2)	690.389	y6+	40	100
764.5	(+2)	562.331	y5+	40	100
764.5	(+2)	461.283	y4+	40	100
764.5	(+2)	390.246	y3+	40	100
764.5	(+2)	234.145	y2+	40	100
764.5	(+2)	559.344	y9++	40	100
510.0	(+3)	1117.68	y9+	21	100
510.0	(+3)	961.579	y9+	21	100
510.0	(+3)	860.531	y8+	21	100
510.0	(+3)	690.389	y7+	21	100
510.0	(+3)	562.331	y6+	21	100
510.0	(+3)	461.283	y5+	21	100
510.0	(+3)	390.246	y4+	21	100
510.0	(+3)	234.145	y3+	21	100
510.0	(+3)	559.344	y2+	21	100
510.0	(+3)	481.293	y8++	21	100
510.0	(+3)	430.769	y7++	21	100

which all co-eluted at 17.1 min (Table 2.4). The peak area being proportional to the quantity of a given peptide was used for its relative quantification in KDM5b *in vitro* demethylase assays. These samples were then subjected to MRM-MS as described above. For histone and nucleosome assays the detailed protocol follows. Western blot analysis was performed exactly as detailed in Lee et al (Lee et al. 2007)

2.4.1 Histone and Nucleosomal Demethylase Assays (HDM/NDM))

Histone Demethylase Assays and Nucleosomal Demethylase assays were completed as previously described (Lee et al. 2005). Bulk histones were purchased from Sigma, and nucleosomes were isolated as previously described (Brand et al. 2008). Nucleosomes were verified using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and agarose gel electrophoresis (Figure 2.2)

2.4.2 Quantitative HDM and NDM Assays (qHDM/qNDM)

The buffer conditions and incubations were as described above. Modifications for microplate format were made as follows; 50µl samples were incubated overnight at 37C in either 96 well plate or individual eppendorf 1.5 ml tubes in JHDM buffer plus peptide, histones (Sigma) or purified nucleosomes. Samples were then diluted to 200µl with 150µl of PBS. The samples were then crosslinked to Maleic Anhydride amine binding plates (Pierce) in duplicate and prepared following vendors protocols. Plates were then treated for standard ELISA assay by blocking in PBS, 0.1% Tween20 and 2%BSA for 1 hour at room temperature. Antibodies were then added to either methyl specific antibody or pan-

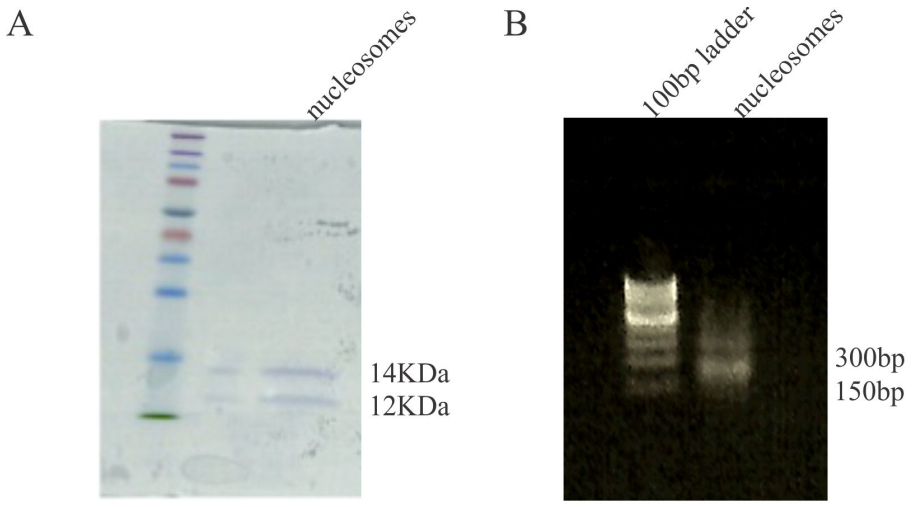


Figure 2.2 Verification of nucleosomes A) nucleosome samples analyzed by 12% polyacrylamide gel electrophoresis. Proteins at 12 and 14 KDa, corresponding to the expected size of histone proteins are present. No evidence of contaminating protein is observed. B) nucleosome samples analyzed by agarose gel electrophoresis. DNA bands of 150bp and 300bp, corresponding to mono- and di-nucleosome arrays are observed. No contaminating DNA or large multi-nucleosome arrays are present.

H3 (loading control) was then added to separate wells. Antibody to Rabbit IgG conjugated to either Alexa 488 or Alexa 647 was then used at a 1:10000 dilution. Relative fluorescence intensities were then read using a BMG Omega Fluorstar plate reader. Antibody dilutions were: H2BK43me2 (Abcam) 1:500 dilution, H3K4me3 (Active Motif) 1:2000 and pan-H3 (Millipore) both at 1:2000 dilution. For a more detailed methodology, please refer to Stalker and Wynder, 2012 (Stalker and Wynder 2012)

2.4.3 Analysis of qHDM or qNDM

Using a BMG Fluorstar Omega microplate reader, fluorescence intensity was measured. RFUs were standardized using a protein concentration standard curve (5, 2, 1, 0.1, 0.01, 0.001, 0.0001, and 0.00001 mg of either histones or nucleosomes). Additionally two blanks were used to set the non-specific background; JHDM buffer alone and KDM5b alone (no histone added). The negative control was either bulk histones or nucleosomes incubated in buffer alone. Values equal to or below the average value of the blanks were not used. These samples are then normalized to pan-H3 similar to standard practice with western blot analysis. The pan-H3 normalized number is then compared between experimental and the histone/nucleosome alone control. All numbers are expressed as fold change vs. histone/nucleosome only sample. All p values represent students t-test of experimental vs. control (nucleosomes alone or histone alone).

2.5 Immunoprecipitation and Affinity Purification

Nuclear extractions were performed as per previously published conditions (Stalker and Wynder 2012). Briefly, two pellet volumes of buffer A was added to packed cells. After incubation on ice for 5-10 minutes, cells were re-pelleted by spinning at 3000rpm for 10 minutes at 4C. The supernatent is then removed and kept as the cytoplasmic fraction. Buffer B (10x) is then added to the supernatent to a final concentration of 1x. 1 volume of buffer C was then added to the pellet fraction, incubated at 4C for 30 minutes and spun down at 12000 rpm for 30 min. The supernatent represents the nuclear fraction. All samples were maintained at -80C until time of use. Cells were cultured in 10cm tissue culture dishes as discussed above. Transfections were completed 24-48 hours before lysis. Cells were lysed using NP40 lysis buffer containing protease inhibitor (Roche) and clarified using centrifugation at 12,000rpm for 20 minutes at 4°C. Protein samples were then incubated with antibody of interest for 12-18 hours at 4°C on a nutator. Antibodies utilized for coimmunprecipitation are: GFP (1ug, Clontech, #632460), KDM5b (1ug, Bethyl, A301-813A) TLE4 (1ug, Santa Cruz, sc-9125), pCNA (Dr. Christopher Wynder), TLE1 (1ug, Bethyl, A303-545A). ProA or ProG beads (50% in NP40 lysis buffer, Calbiochem) were added to samples for 2-4 h at 4°C. Affinity purifications through 3xFLAG were completed using 3XFLAG beads (50% in NP40 lysis buffer, Sigma). Beads were then washed 3 times: once in IP buffer 350+NP40 (20mM Tris-HCL [pH7.9], 0.2mM EDTA, 10mM β-mercaptoethanol, 10% glycerol, 350mM KCL, 0.2mM PMSF, 0.1% NP40) and two times in IP buffer 350 (as above without NP40). Protein was then eluted using either 0.1M Glycine [pH1.8] and corrected for pH

using TrisHCl [pH7.9] or eluted directly using 2x SDS sample buffer (Biolabs). Affinity purification using FLAG M2 beads (Sigma) was eluted using the 3XFLAG peptide as per vendor's protocol. SDS sample buffer was then added or diluted to 1x.

2.6 Western Blot

For western blots, equal amounts of protein were loaded onto SDS-Polyacrylamide gels and electroblotted to a polyvinylidene difluoride (PVDF) membrane (PALL). Membranes were blocked with 5% (w/v) non-fat dried milk in Tris-buffered saline with tween (TBST) at room temperature followed by 12-18 hour incubation at 4°C on a nutator with primary antibody. Primary antibodies utilized for western blots are: 3xFLAG (1:5000, Sigma, F1804), GFP(1:1000, Clontech, #632460) H3K4me3 (1:1000, Active Motif, #39159), H3K4me3 (1:1000, Upstate, #07-043, discontinued), H3pan (1:1000, millipore, #07-690), H2BK43me2 (1:500, Abcam, ab4629), H3K27me3 (1:1000, Abcam, ab6002), KDM5b (1:3000, Bethyl, A301-813A) pCNA (pC8) (1:1000, Abcam, ab20237), TLE4 (1:500, Santa Cruz, sc-9125), TLE4 (1:1000, Sigma, G4044), TLE1 (1:1000, Bethyl, A303-545A). Membranes were then washed 5x in TBST at room temperature on a shaker for 10-15 minutes each wash. HRP-conjugated secondary antibody (BioRad) against either mouse or rabbit IgG was used at 1:10,000 dilution, HRP-conjugated goat anti rabbit secondary (ab97051, Abcam) was used at 1:50,000 dilution and Rabbit anti-mouse HRP conjugated secondary antibody (ab97046, Abcam) was used at 1:25000 dilution. Western blots were visualized by either Immobilon Western Chemiluminescent HRP substrate (Millipore), or ECL detection reagent (GE

Healthcare) paired with development by film, or visualization by enhanced chemiluminescence using the MicroChemi system (DNR Bio-Imaging Systems, Israel).

2.7 Verification of H2BK43me2 Antibody

Verification of recognition of H2bK43me 2 antibody was performed by dot blot analysis. Peptides of interest were diluted in ddH_20 and blotted onto a nitrocellulose membrane at different concentrations. Commercial peptides include H2B unmodified (ab13214, Abcam) and H2BK43me2 (ab23555, Abcam). All other peptides were a gift of Dr. Shawn Li (University of Western Ontario) (Table 2.5). After drying, the membrane was verified using Ponceau stain (0.1% (w/v) Ponceau S in 5% acetic acid). Membrane was washed in TBST and blotted as per western blot protocol stated above (Figure 2.3A). The H2BK43me2 antibody was further verified for binding to an H2BK43me2 peptide (Figure 2.3B) and recognition of nucleosomes (Figure 2.3C)

2.8 Neurosphere Differentiation Assay

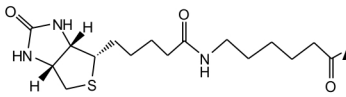
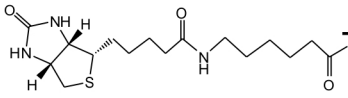
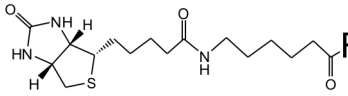
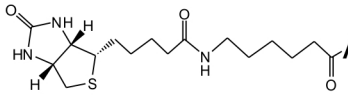
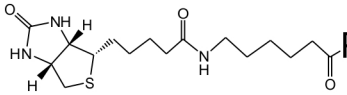
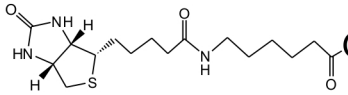
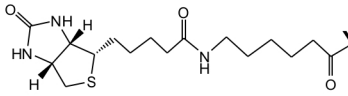
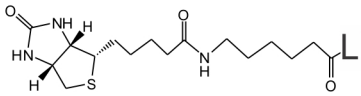
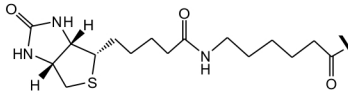
Cell lysis and nuclear extracts were performed as described above in immunoprecipitation. mESCs were grown in mESC medium (as above) to 75%-85% confluence. mESC medium was then replaced with neural differentiation medium consisting of Neurobasal (Invitrogen), 5% FBS (VWR) , 1xB-27 supplement (Gibco) and 1mM L-Glutamine (Gibco). After 48 h, the cells were gently released from the tissue culture dish and replated in a nonadherent Petri dish with a 1:5 dilution of the same mESC medium. For further differentiation, neurospheres were transferred to a poly-D-

Lysine coated tissue culture dish where they could be maintained and media was changed to neural differentiation media (5% FBS, 1x B-27, 1x sodium pyruvate, 1x Non-essential amino acids (NEAA) in 1x DMEM). For imaging, cells were fixed in 4% paraformaldehyde at 37°C for 10 minutes. The neuronal outgrowths were then counted based on images captured for mESCs and neurospheres using an Olympus DSU and CoolpicHQ camera with 10X magnification.

2.9 Chromatin Immunoprecipitation Assay (ChIP):

mESC or neurospheres were washed twice with Phosphate-buffered saline (PBS) and crosslinking was performed using 1% paraformaldehyde for 5 to 10 minutes at 37°C. The adherent mESC were then scraped, cells were washed twice with cold PBS, resuspended in 200µl of SDS-ChIP lysis buffer containing protease inhibitor (1% SDS, 10mM triton X-100,50 mM Tris-HCL[pH8.1]), and incubated on ice for 10 minutes. The total volume of each sample was then brought to 2ml with ChIP dilution buffer (0.01%SDS, 1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCL [pH8.1], 167mM NaCl), and samples were sonicated using a Diagenode Bioruptor for 12 minutes (pulses of 30 s on and 30s off). Samples were pre-cleared using 25µl of salmon sperm DNA-Protein (Sigma) and Protein A beads (50%, Calbiochem) for 1h at 4°C on a nutator unit. The salmon sperm DNA-protein A agarose beads were removed by centrifugation at 2,000rp for 2 min. at 4°C and the supernatant was collected. For the input control, 100µl of precleared ChIP sample was removed and prepared by a method similar to eluted ChIP samples (to follow). For PCR standards, 100µl total of pre-cleared ChIP sample was

Table 2.5 Amino acid sequences of peptide substrates[a] used in the KDM5b *in vitro* demethylation assay

Peptide Sequence	Histone Site
	H3K4
	H3K9
	H3K23
	H3K27
	H3K36
	H4K20
	H2BK46
	H2BK108
	H2BK43

[a]A peptide substrate was derived from the corresponding histone Lys site (identified in red) and synthesized with a biotin moiety coupled to the N-terminus of the peptide with an e-aminocaproic acid (Ahx) linker. The biotin moiety facilitated peptide isolation prior to mass spec analysis.

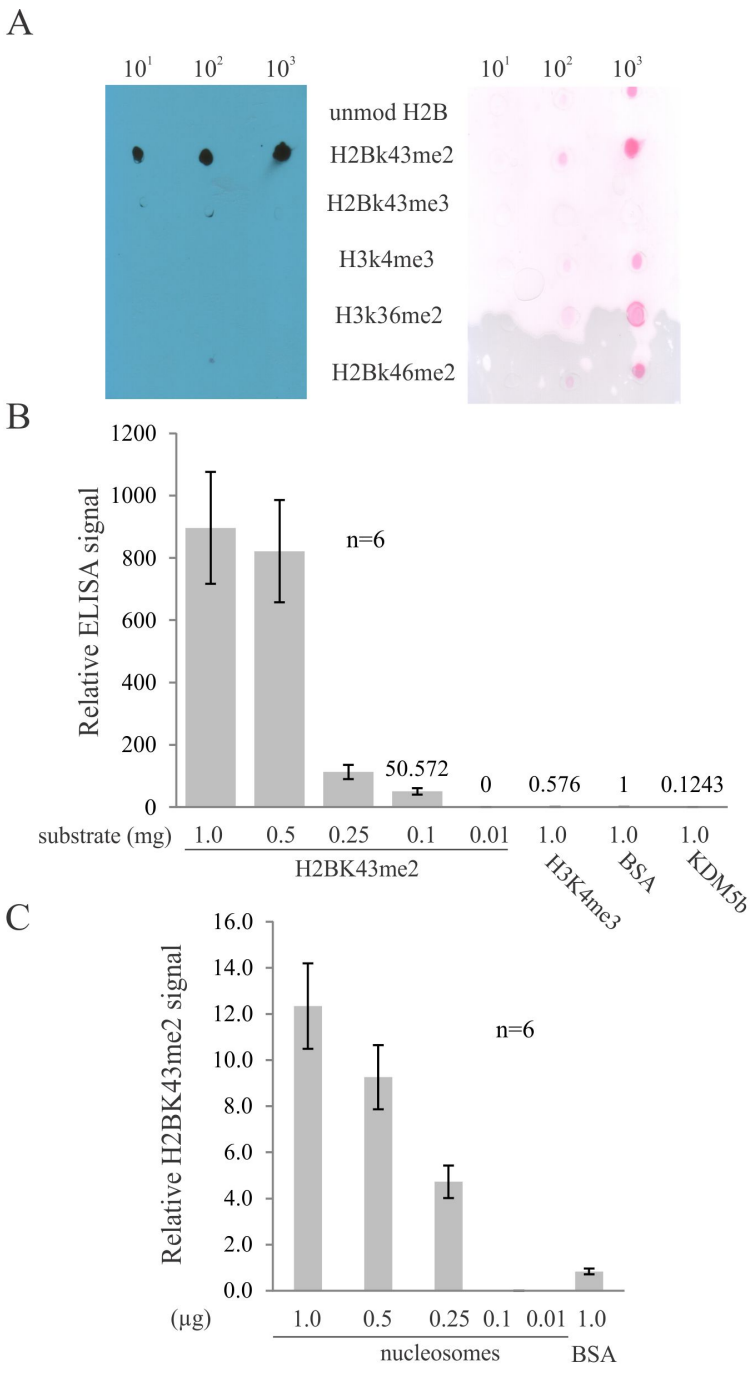


Figure 2.3 The anti-H2Bk43me2 antibody recognizes the H2BK43me2 mark specifically A)Dot blot analysis showing the specificity of the H2Bk43me2 antibody used in this thesis. Peptide concentration is shown at the top of both the blot and Ponceau membrane. Corresponding peptide identities are shown in the middle B) The antibody binds to the H2BK43me2 peptide (Biotin-Ahx-YVYKme2VLKQVHPDT) in a concentration-dependent manner in an ELISA assay. No binding is seen to H3K4me3 peptide, control BSA or KDM5b. C) The antibody detects H2BK43me2 on nucleosomes in a concentration dependent manner

combined from all samples and prepared by a method similar to eluted ChIP samples (to follow). Samples were divided equally among antibodies being analyzed and a no antibody, immunoglobulin G [IgG] control. 0.3mg Anti KDM5b (Bethyl), 3ml anti-H3K4me3 (Active Motif) and 0.5 mg anti-H2BK43me2 (abcam) were used. Samples were incubated overnight at 4°C. Antibody-chromatin complexes were precipitated with 25µl of salmon sperm DNA/Protein A beads, agitated for 2h at 4°C and centrifuged at 2,000 rpm for 2 min. at 4°C. The beads were washed five times in 1x low-salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCL[pH8.1], 150mM NaCl), 1x High-salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDA, 20mM Tris-HCL [pH8.1], 500mM NaCl), 1x in LiCl immune complex wash buffer (0.25M LiCl, 1% IGEPAL-CA630 (Sigma), 1% deoxycholic acid[sodium salt], 1mM EDTA, 10mM Tris-HCL [pH8.1]) and 2x in Tris-EDTA buffer. Chromatin was eluted from the antibody by using 100µl of ChIP elution buffer (1% SDS, 0.1M $NaHC0_3$, made fresh), for 2h at room temperature. The DNA-Chromatin complexes were de-cross linked by incubation at 65°C for 12-18h and purified using a Qiagen PCR purification kit (Qiagen). The purified samples were analyzed by quantitative polymerase chain reaction (qPCR) performed with a Chroma4 (BioRad) system and iQ SYBRgreen PCR kit (BioRad). RNA was prepared using the Qiagen RNeasy Kit (Qiagen) and reverse transcripted using qscript mastermix (Quanta). Samples were quantitated using Opticon software. All results are expressed as fold change of the normalized value vs. control. The values were normalized using the input to derive a percent of control value that was then compared between control and experimental values. Data are presented as fold difference

between control and experimental values. All values reported here were above the IgG background value. All experiments were done with biological triplicates.

2.10 Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

RNA was prepared using the Qiagen RNeasy Kit (Qiagen) and reverse-transcribed using qscript mastermix (Quanta). qPCR was performed as in the ChIP section. Values are expressed as a percentage of the control per unit of GAPDH. All experiments were done in triplicate.

2.11 Bacterial Protein Expression and Purification

KDM5b was cloned from pcDNA3.1-Plu1 (Dr. Joyce Papidimitriou) into pDEST17 using Invitrogen's gateway cloning system as per manufacturer's protocols. Recombinant protein was expressed in E.*coli BL21*, induced with IPTG and purified using Qiagen Ni^{+2} agarose as per vendor's protocol (Qiagen). TLE4 was cloned from YFP-TLE4 into pTRCHIS-C (invitrogen). Recombinant protein was expressed in E.*coli* TOP10 as per manufacturer's protocols and induced with 1mM IPTG. Recombinant proteins were dialyzed in JHDM buffer (Lee et al. 2007) overnight at 4C prior to utilization in enzymatic assays.

2.12 Generation of Constructs

2.12.1 Generation of H2B and H2BK43A Constructs and Cell Lines

3Xflag-H2B^{K43A} constructs were synthesized by IDT DNA to create pIDSMART-KAN:H2B k43A Mutant (Ref ID 43123785) and cloned into the EF1a-puromyocin vector (Wynder lab) via restriction digest with XhoI and HindIII. Stable cell lines were created by transfection into mESC and selection using Puromyocin. Verification was completed using western blot for 3xFLAG using 3xFLAG antibody (Sigma). Wild type H2B lines were created using the same protocol, using full length H2B cDNA.

2.12.2 Generation of constructs for Chapter 4

Primers to express human KDM5b were made with 5'BamH1 and 3' Xho1 (MOBIX, McMaster university) and cloning was performed using PCR product from pcDNA3.1-Plu1 (Dr. Joyce Papidimitriou), between BamH1/Xho1 sites of pEYFPC1 (BD Biosciences, Clontech). Primers to express TLE4 were made with 5'BspE1 and 3'Acc651 overhangs (MOBIX, McMaster University) and cloning was performed using PCR product from TLE4 cDNA (In pBluescript R, Open Biosystems #5296117), between BspE1 and Acc651 sites of pEYFPC1, or pemCerC1 (BD biosciences/Clontech). CMX FLAG RERE and CMX FLAG RERE 481-C were kind gifts from Dr. Chih Cheng Tsai and have been previously described (reference Wang et al, EMBO). pEYFPC1 was used as a control (BD Biosciences/Clontech)

2.13 Microscopy

All wide-field images, unless otherwise stated, were captured on a Nikon TE200 epifluorescence inverted microscope equipped with a Hamamatsu Orca ER digital camera (Hamamatsu Photonics, Japan). The objectives used were a 60x oil immersion plan apochromat NA1.4 objective or a 40x objective. All filter sets and dichroic filters were from Semrock (Rochester, NY). The filter wheel and 175W Xenon lamp with ND2 or ND4 filters were from Sutter Instruments (Novato, CA). The imaging platform controlling the scope was NIS elements 3.1. Images of TLE4 and RERE co-localization were produced by obtaining a z-Stack and performing 3-D deconvolution. Creation of image files was performed using Imarisx64 (Bitplane Scientific Sofware).

2.13.1 Fluorescent Lifetime Imaging Microscopy (FLIM)

FLIM was conducted as previously described (Munsie et al. 2011). Briefly, FLIM was conducted using a Leica TCS SP5 inverted confocal laser-scanning microscope run usings LAS advanced Fluorescence softward from Leica. Samples were excited with a two photon pulse using a tunable Chameleon Laser. mCerulean and YFP were used as FRET pairs and all imaging was completed in Hank's saline HEPES buffer pH 7.3. Photons were collected and counted using TCSPC softward from Becker and Hickl.

2.13.2 Förster Resonant Energy Transfer (FRET) analysis

FRET analysis was completed using ImageJ software and the Becker and Hickl FLIM plug in. (McMaster Biophotonics facility, www.macbiophotonics.ca) as previously described (Munsie et al. 2011)

2.14 *In vivo* Analysis of Methylation Levels

2.14.1 Primary Antibody Conjugation

Primary antibodies to H3K4me3 (#39159, Active Motif) and H3K9me3 (ab9989, Abcam) were conjugated using the APEX Alexa-Fluor 555 antibody labeling kit (Invitrogen/Molecular probes) as per supplier's instructions. 10µg of primary antibody was used in both cases.

2.14.2 Immunofluorescence

Cells were grown in glass bottomed 3.5 cm tissue culture dishes or glass-bottomed six-well dishes and transfected 24 hours before fixation. Cells were fixed using 4% paraformaldehyde solution in PBS (Sigma) for 20 minutes at room temperature. Dishes were kept covered from light unless otherwise stated. Samples were then permeabilized using ice cold methanol, on ice, at 4°C for 17 minutes. Methanol was then removed and cells were washed once and then incubated in blocking solution (2% FBS in PBS) for 1 hour at room temperature. Primary conjugated antibodies were then diluted 1:50 into antibody dilution solution (1%FBS, 0.02% Tween 20 in PBS) and incubated overnight at 4°C on a rocker. Samples were then washed 3x for 20 minutes in PBS. All dishes were visualized and stored in PBS.

2.14.3 Intensity Analysis

Intensity analysis for *in vivo* demethylation assays using primary conjugated antibodies was completed using Image J software (McMaster Biophotonics Software, www.macbiophotonics.ca). Background subtraction was completed using a rolling ball radius of 50.0 pixels for all images. The minimum and maximum displayed value for each image was set equally for each image. The region of the nucleus was selected by hand and average pixel intensity within the specified region was measured.

2.15 Analysis of TLE4 chromatin dependence

2.15.1 Hoescht addition

3T3 cells were transfected with YFP-TLE4 in 3.5cm glass bottom dishes as discussed above. At the 24 hour time point 10 µg/ml Hoescht dye was added to cell media. Cells were observed at 60x (see "Microscopy") for 1.5 hours with multichannel images captured every 10 seconds for 1.5 hours. Representative images were selected at different time points to illustrate results.

2.15.2 Actinomycin D treatment

3T3 cells were transfected with mCerulean-TLE4 in 3.5 cm glass bottom dishes as discussed above. At the 48 hour time point, 5 µg/ml Actinomycin D in serum free media was added. Representative images were captured at 0 to 18 hours. 20x widefield fluorescence microscope images were captured on an Evos digital LED inverted microscope equipped with a 20x air plan fluor NA1.2 objective (AMG, Seattle, USA).

2.16 Statistical Analysis

All statistical analysis for Chapter 3 was done using either Microsoft Excel or BMG's Omega analysis software. All p-values were derived using from student's t-test using two-sample, equal variance, two-tailed settings. Statistical analysis for Chapter 4 was completed using SigmaPlot Software 11.0 (Systat Software Inc). If data passed normality assumptions, student's t-tests were performed. If data did not pass normality assumptions, the Mann-Whitney method was utilized to analyze data.

Chapter 3 Defining a novel substrate for KDM5b

Preamble

The material presented in this chapter is a representation of the following manuscript formatted for submission to PNAS

KDM5b controls the dynamics of H2BK43 and H3K4 methylation to regulate neural differentiation of embryonic stem cells

Leanne Stalker[*,1], Marek Galka[*,2], Huadong Liu[2], Richard L. Carpenedo[3], Wendy Zhu[2], Sean Keating[1], Mark Meneghini[5], Martin L. Doughty[4], R. Truant[1], William L. Stanford[3.6], Christopher Wynder[1,2,#], and Shawn S.-C. Li[2,#]

The only changes made were for thesis formatting and continuity purposes and copy editing.

LS, CW and SL wrote the manuscript. Editing was performed by WS and RC. All experiments were performed by LS except those listed below:

MG performed mass spectrometry work and created peptides used in mass spectrometry work

CW and LS created the 3x FLAG cell lines

CW extracted 3xFLAG containing nucleosomes

WZ aided in immunofluorescence work

KDM5b controls the dynamics of H2BK43 and H3K4 methylation to regulate neural differentiation of embryonic stem cells

Leanne Stalker[*,1], Marek Galka[*,2], Huadong Liu[2], Richard L. Carpenedo[3], Wendy Zhu[2], Sean Keating[1], Mark Meneghini[5], Martin L. Doughty[4], R. Truant[1], William L. Stanford[3.6], Christopher Wynder[1,2,#], and Shawn S.-C. Li[2,#]

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3.1 Abstract

Histone lysine methylation forms part of the epigenetic program that governs the differentiation of embryonic stem cells, yet the function and mechanism of regulation for many methyllysine marks remain undefined. We report here that the methylation of Lys43 on histone H2B, a novel histone mark controlled by the lysine demethylase KDM5b, plays a critical role in the neural differentiation of mouse embryonic stem cells (mESCs). We show that dimethyl H2BK43 is a preferred substrate for KDM5b both *in vitro* and *in vivo* and that the global level of H2BK43 methylation undergoes dynamic changes during mESC differentiation in a manner that is inversely correlated to changes in KDM5b. Pluripotent cells are marked with H2BK43me3, yet this mark is lost during differentiation and replaced by H2BK43me2 in lineage-committed cells. In contrast, the global level of H3K4me3 undergoes relatively small changes in the same timeframe. H2BK43me2 functions in cooperation with H3K4me3 on the promoters of KDM5b target genes to regulate their transcription. Loss of H2BK43 dimethylation in the *Kdm5b*$^{+/-}$ mESCs or in cells stably expressing an unmethylatable H2B mutant (K43A) led to widespread changes in gene expression and a complete blockage in neural differentiation. Our work identifies H2BK43me2 as a novel substrate for KDM5b and a global mark for lineage commitment in mESCs. Moreover, the dynamic interplay between the H2BK43me2 and H3K4me3 marks control neural differentiation of mESCs by regulating the transcription of developmental genes.

3.2 Introduction

Histone Lysine methylation is a reversible, covalent modification catalyzed by lysine methyltransferases (KMTs) and erased by methyllysine demethylases (KDMs) (Klose and Zhang 2007). Lysine methylation is associated with either activation or repression of gene expression, depending on the site and degree of methylation (Esteller 2007; Paik, Paik, and Kim 2007; Barski et al. 2007). Genes marked by H3K9, H3K27, or H4K20 methylation, for example, are usually repressed whereas those associated with H3K4 and H3K36 methylation are generally activated (Barski et al. 2007). Recent studies highlight the importance of histone methylation on specific lysine residues in controlling the balance between stem cell self-renewal and differentiation in embryonic stem cells (ESCs) and during early animal development (Marks et al. 2012; Vastenhouw and Schier 2012; Rada-Iglesias et al. 2011; Rada-Iglesias and Wysocka 2011). Developmental or lineage-regulatory genes in ESCs are often associated with bivalent domains characterized by co-occupation by H3K4me2/me3 and H3K27me3 (Vastenhouw and Schier 2012; Zhao et al. 2007; Bernstein et al. 2006; Rada-Iglesias et al. 2011; Rugg-Gunn et al. 2010). H3K4me3/2 usually mark the transcriptional start sites of actively transcribed genes whereas H3K27me3 marks genes for repression (Barski et al. 2007; Schubeler et al. 2004; Azuara et al. 2006; Pan et al. 2007). Developmental genes that contain the bivalent domains are silenced in ES cells but are poised for activation during differentiation or development (Azuara et al. 2006; Bernstein et al. 2006).

Some KTMs and KDMs have also been shown to play an essential role in regulating ESC maintenance and differentiation. For instance, the H3K4me2/me3

demethylase KDM1 controls the balance between self-renewal and differentiation in human ESCs by occupying the promoters of a subset of developmental genes where it reduces H3K4 methylation(Adamo et al. 2011). KDM5b, another H3K4me3 demethylase, plays an essential role in development and neural differentiation of mouse ESCs (mESCs) by maintaining an optimal H3K4me3 level on target genes(Catchpole et al. 2011; Schmitz et al. 2011). Over-expression of KDM5b has been shown to cause a blockage of terminal differentiation in mESCs) (Dey et al. 2008).

To define the mechanism by which KDM5b regulates ESC differentiation, we used multiple reaction monitoring (MRM) mass spectrometry (Liu, Galka et al. 2010; Yocum and Chinnaiyan 2009) to define the targets of the enzyme, which led to the identification of H2BK43me2 as a novel substrate of KDM5b. This was followed by a detailed investigation into the dynamics of H2BK43me2 and H3K4me3 demethylation both *in vitro* and *in vivo*. We show that the global level of H2BK43me2 undergoes marked dynamic changes during mESC differentiation that are inversely correlated with changes in KDM5b. Disruption of the dynamic pattern of KMD5b and H2BK43me2 levels leads to aberrant gene transcription and a blockage in mESC differentiation.

3.3 Results

3.3.1 H2BK43me2 is a Novel Substrate of KDM5b

The only characterized substrate for KDM5b to date is H3K4me3. To identify novel substrates of KDM5b, we performed *in vitro* demethylation assays on peptides representing lysine sites on histones H3, H4 and H2B in both the di- and tri-methylated

states (Figure 3.1A and Table 2.5). Using the MRM-MS technique (Yocum and Chinnaiyan 2009), we quantified the change in methylation for each peptide upon incubation with the recombinant KDM5b (r.KDM5b) (Stalker and Wynder 2012) (Figure 3.1A and Table 2.5). Of the 15 peptides examined, H3K4me2 and H2BK43me2 were found to be better substrates for KDM5b-mediated demethylation than H3K4me3. Approximately 80% of the H2BK43me2 peptide was demethylated in 60 minutes compared to 20-30% for the H3K4me3 peptides (Figure 3.1A). The remarkable activity of KDM5b towards methylated H2BK43 appeared to be specific for the dimethyl state as it showed no activity on the trimethylated peptide. Thus, H2BK43me2 is a substrate of KDM5b *in vitro*.

To characterize the kinetics of demethylation of H2BK43me2 by KDM5b, we repeated the *in vitro* demethylation assay by taking samples at different time points for MRM-MS analysis to monitor the progress of the enzymatic reaction (Figure 2.1). The H3K4me3 and H2BK43me3 peptides were included as positive and negative controls, respectively. As shown in Figure 3.1B, the H2BK43me2 peptide exhibited a fast initial rate of demethylation characterized with ~50% substrate turnover in 4 min, and by 30 min, the reaction was saturated (Figure 3.1B). In contrast, only 8% of the H3K4me3 peptide substrate was demethylated in 4 min, and 20% in 30 min. No significant change in the H2BK43me3 peptide was observed within the same time frame. Thus, the H2BK43me2 mark is a better substrate than H3K4me3 for KDM5b *in vitro*.

3.3.2 KDM5b Specifically Demethylates H2BK43me2 *in vivo*

To determine whether H2BK43me2 is a specific substrate of KDM5b *in vivo,* we carried out a nucleosome demethylase assay. Specifically, nucleosomes were purified from mESCs and incubated with r.KDM5b, and the demethylation of nucleosomal H2BK43me2, H3K4me3 and H3K27me3 was assessed by Western blotting. The anti-H2BK43me2 antibody used in this study recognized both the H2BK43me2 peptide and the mark on nucleosomes in a concentration-dependent manner (Figure 2.1B-C). Consistent with results from the MS analysis, incubation of nucleosomes with 1 mg r.KDM5b resulted in a marked reduction in the H2BK43me2 level, a slight decrease in the H3K4me3 and no change in the H3K27me2 levels (Figure 3.1C). These data indicate that r.KDM5b preferentially demethylates nucleosomal H2BK43me2.

To confirm the activity of the recombinant KDM5b, endogenous KDM5b (m.KDM5b) was immunoprecipitated from mESC and used in demethylation assays on purified histones or nucleosomes. In both assays, we found that m.KDM5b was able to demethylate H2BK43me2 significantly more efficiently than H3K4me3 (Figure 3.1D & E), indicating that H2BK43me2 is a better substrate than H3K4me3 for KDM5b *in vivo*.

Because the KDM5b family, including KDM5a-d, is evolutionarily conserved, we investigated whether another mammalian member, such as KDM5d or the yeast ortholog yKDM5 might also exhibit activity towards H2BK43me2. While both r.KDM5d and yKDM5 were capable of demethylating the H3K4me3 mark, they were inactive towards the H2BK43me2 mark, suggesting that KDM5b is a specific demethylase for the latter (Figure 3.2A). Moreover, we further supported both our assay and r.KDM5b by verifying

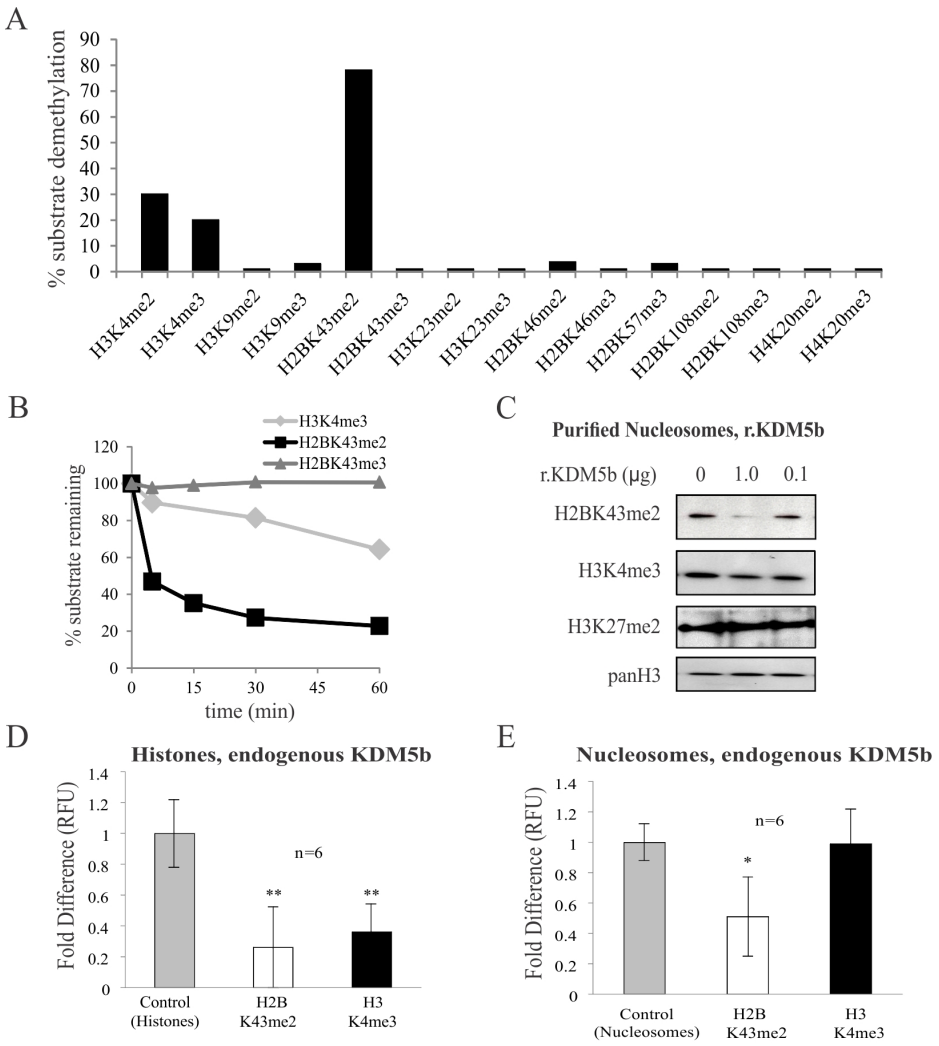

Figure 3.1 H2BK43me2 is a novel substrate for KDM5b *in vitro* and *in vivo* A) The specificity profile of KDM5b determined by *in vitro* demethylation assays on histone peptides followed by MS analysis. The amino acid sequences of the peptides are shown in Table 3.1. The known substrates, H3K4me2 and H3K4me3 were included as positive controls. The graph, showing the percentage of substrate demethylation at 60 min after the addition of r.KDM5b is representative of two independent experiments. The standard deviations were within 10% of the values shown. B) H2BK43me2,me3, or H3K4me3 was measured by MRM-MS at 0,5,15,30 and 60 minute incubation with r.KDM5b and graphed as a percentage of remaining substrate against time. C) r.KDM5b was capable of demethylating H2BK43me2 but not H3K4me3 or H3K27me3 in nucleosomes. Nucleosomes prepared from mESC were subjected to demethylation assays by r.KDM5b at indicated amounts followed by SDS-PAGE and immunoblot as indicated. pan H3 indicates total Histone H3 amount. D) Both the H2BK43me2 and H3K4me3 marks on bulk histones could be demethylated by KDM5b purified from mESC (m.KDM5b). E) H2BK43me2 but not H3K4me3 could be demethylated by KDM5b in nucleosomes purified from mESC. RFU: relative fluorescence unit, fold difference to control, all samples normalized to panH3 *, $p<0.05$,

that KDM5b could demethylate H3K43me3, as would be expected, but had no activity towards H3K9me2 on histones. Conversely, also as expected, the histone demethylase KDM4c, which is known to act on H3K9 methylation marks, was able to demethylate H3K9me2 but showed no activity towards H3K4me3 (Figure 3.2B).

3.3.3 The global level of H2BK43me2 undergoes dynamic changes during mESC differentiation

To the best of our knowledge, the function of mammalian H2BK43me2 has not been characterized to date. Because KDM5b plays an important role in stem cell differentiation (Schmitz et al. 2011; Dey et al. 2008), it is likely that the H2BK43me2 mark also plays a role in this process. To explore this possibility, we first examined whether KDM5b is regulated during neural differentiation of mESCs. The KDM5b protein level declined on day 5 of differentiation when the cells were committed to the neural lineage, and by day 7, it dropped to a level below detection by Western blotting (Figure 3.3A). The dynamic expression of KDM5b suggests that the abundance of its substrate would follow an opposite trend. To examine whether this was the case with H3K4me3 and H2BK43me2, we immunoblotted for these marks in cells collected on days 0, 5 and 7 of neural differentiation. While the H3K4me3 level remained relatively constant, a marked increase in the level of H2BK43me2 was observed on days 5 and 7 by Western blot (Figure 3.3B).

To quantify the dynamic changes of the two KDM5b substrates during mESC differentiation, we employed MRM-MS to measure the abundance of

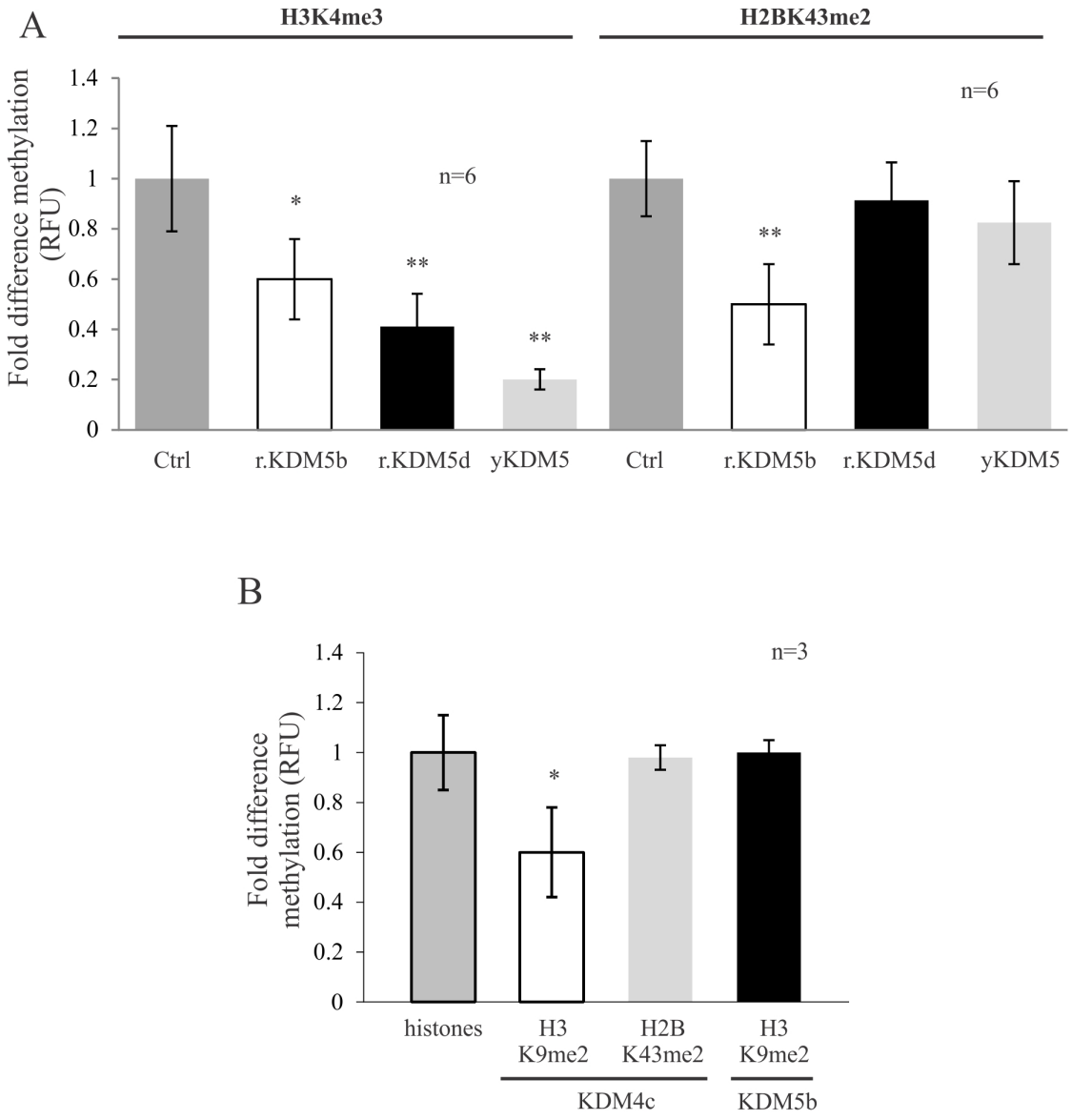

Figure 3.2 Specificity of KDM5b in comparison to other members of the KDM5 family A) Mouse KDM5b is specific for H2BK43me2. Histones were subjected to demethylation by r.KDM5b (mouse), r.KDM5d (mouse) or yKDM5 (yeast) and the corresponding amount of remaining H3K4me3 or H2BK43me2 was measured. Shown on the graph is the relative amount of a given histone mark after incubation with the enzyme relative to control (no enzyme added), set at 1. *, $p<0.05$ **,$p<0.001$. The recombinant KDM5b was expressed in bacteria, the recombinant KDM5d was expressed in baculovirus and the yKDM5 in yeast. All were purified prior to use. B) Full length r.KDM4c is active towards known substrate H3K9me2 but not H2BK43me3. Conversely, r.KDM5b shows no activity towards H3K9me2 on histones via this assay. *,$p<0.05$.

H3K4me2H3K4me3, H2BK43me2 and H2BK43me3 in cell number-matched samples taken on days 0, 3, 5, and 7 of neural differentiation. In agreement with earlier observation that H3K4me2 was a better substrate for KDM5b than H3K4me3 (Figure 3.1A), a 20-50 fold decrease in the H3K4me2 level was detected in samples taken on days 5 and 7 compared to that on day 0, while the H3K4me3 level did not decrease by day 5 and only started to decline by day 7 (Figure 3.3C). In contrast, the H2BK43me-2 and -3 marks exhibited much greater changes in the same time frame. The level of H2BK43me3 decreased gradually in the early stages of differentiation, but dropped sharply between days 5 and 7 (Figure 3.3D). By day 7, an approximately 10,000-fold decrease in the global level of H2BK43me3 was observed, relative to the level on day 0. Conversely, the H2BK43me2 mark underwent a dramatic increase between days 3 and 7, and by day 7, it reached a level approximately 10,000-fold of that on day 0 (Figure 3.3D). Therefore, the change in the global levels of the H2KB43me-2 and -3 marks spanned 8 orders of magnitude during one week of ESC differentiation, a phenomenon never before observed for any histone marks.

To ascertain that the dynamic change in H2BK43me2 was indeed caused by a decrease in the expression of KDM5b, we made use of a KDM5b gene-trap mutant ES cell line, $mESC^{KDM5b+/-}$. We showed previously that this heterozygous line expresses an unusually low level of KDM5b (Dey et al. 2008) (Figure 3.4A). Since complete knockout of *kdm5b* is embryonic lethal (Catchpole et al. 2011), the $mESC^{KDM5b+/-}$ line represents the best model available to date to test the global level changes in KDM5b substrates in a homogeneous mESC population.

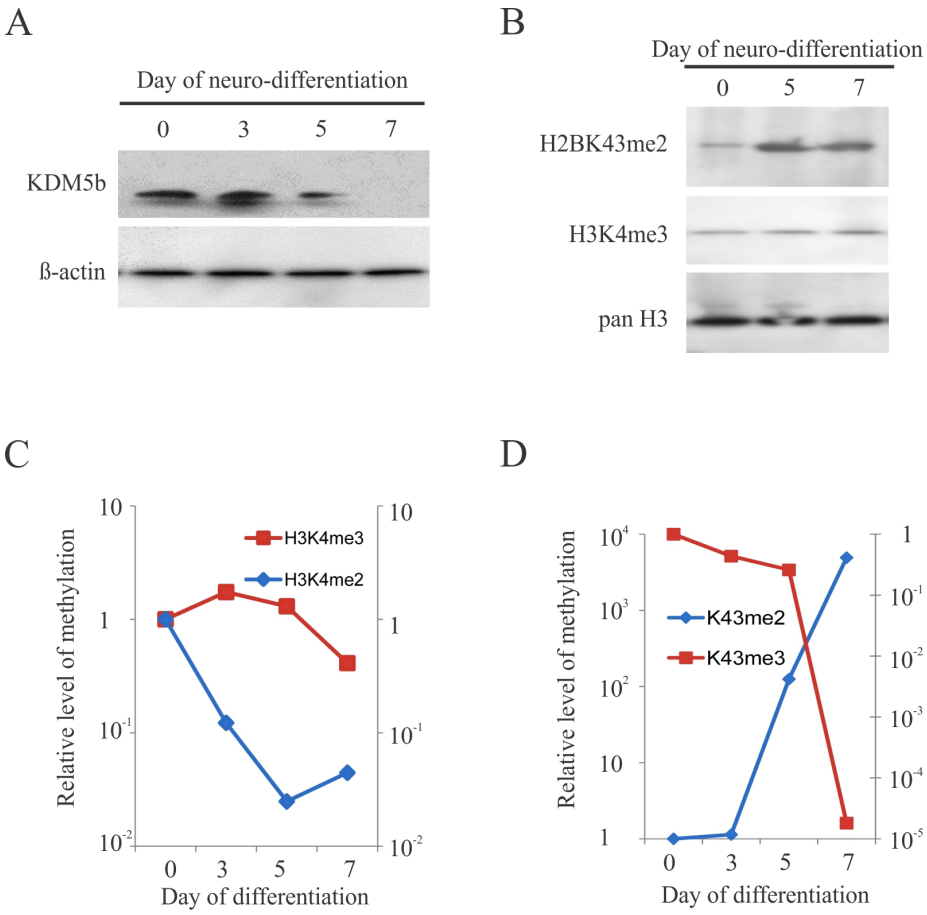

Figure 3.3 KDM5b and H2BK43me2 undergo reciprocal changes during mESC neural differentiation A) The protein level of KDM5b diminishes during neural differentiation of mESC. Cells were allowed to differentiate for 7 days and sampes were taken at days 0,3,5,and 7 respectively for western blot to determine the corresponding level of KDM5b protein expression. An anti ß-actin blow was used as a control for equal loading. B) A western blot showing the levels of H2BK43me2 and H3K4me3 during mESC neural differentiation C,D) Dynamic changes in the global levels of H3K4me2, -me3. (C) and H2BK43me-2, me-3 (D) during mESC differentiation. MRM-MS was used to measure the relative amount of the corresponding peptide. Relative level of methylation compared to time 0 shown on graph.

The abundance of H3K4me3 in mESC$^{KDM5b+/-}$ was found to be similar to that in wild-type (wt) mESC despite a marked reduction in KDM5b in the mutant line (Figure 3.4A-C). To monitor the changes in the H2BK43me2 mark during the neural differentiation of the wt mESC and mESC$^{KDM5b+/-}$ lines, we used MRM to quantify the level of the corresponding peptides in cell number-matched samples taken on days 0 and 5 of differentiation (Figure 3.4D). The dynamics of H2BK43me2 were markedly different between the two cell lines. Strikingly, the level of H2BK43me2 in the mESC$^{KDM5b+/-}$ sample before differentiation (day 0) was 10-fold over that detected for the wt mESC sample collected on day 5 of differentiation (Figure 3.4D). Moreover, the broad range of dynamics in H2BK43me2 associated with wt mESC differentiation was not observed in the mutant ESCs as the level of H2BK43me2 remained high throughout differentiation (Figure 3.4D). Consequently, neural differentiation of the mESC$^{KDM5b+/-}$ cells was completely blocked, accompanied with massive cell death after 7 days (Dey et al. 2008; Zhou et al. 2011). Collectively, these data demonstrate a reverse correlation between the global levels of KDM5b and H2BK43me2 during mESC neural differentiation and identify H2BK43me2 as a highly dynamic histone mark in this process.

3.3.4 The H2BK43me2 and H3K4me3 Marks and KDM5b are Mutually Exclusive on Gene Promoters

To characterize the role of KDM5b and its substrates in regulating gene transcription, we performed RT-PCR and ChIP assays on two previously characterized KDM5b target genes (Dey et al. 2008), namely Egr1 and TCF3. Consistent with previous

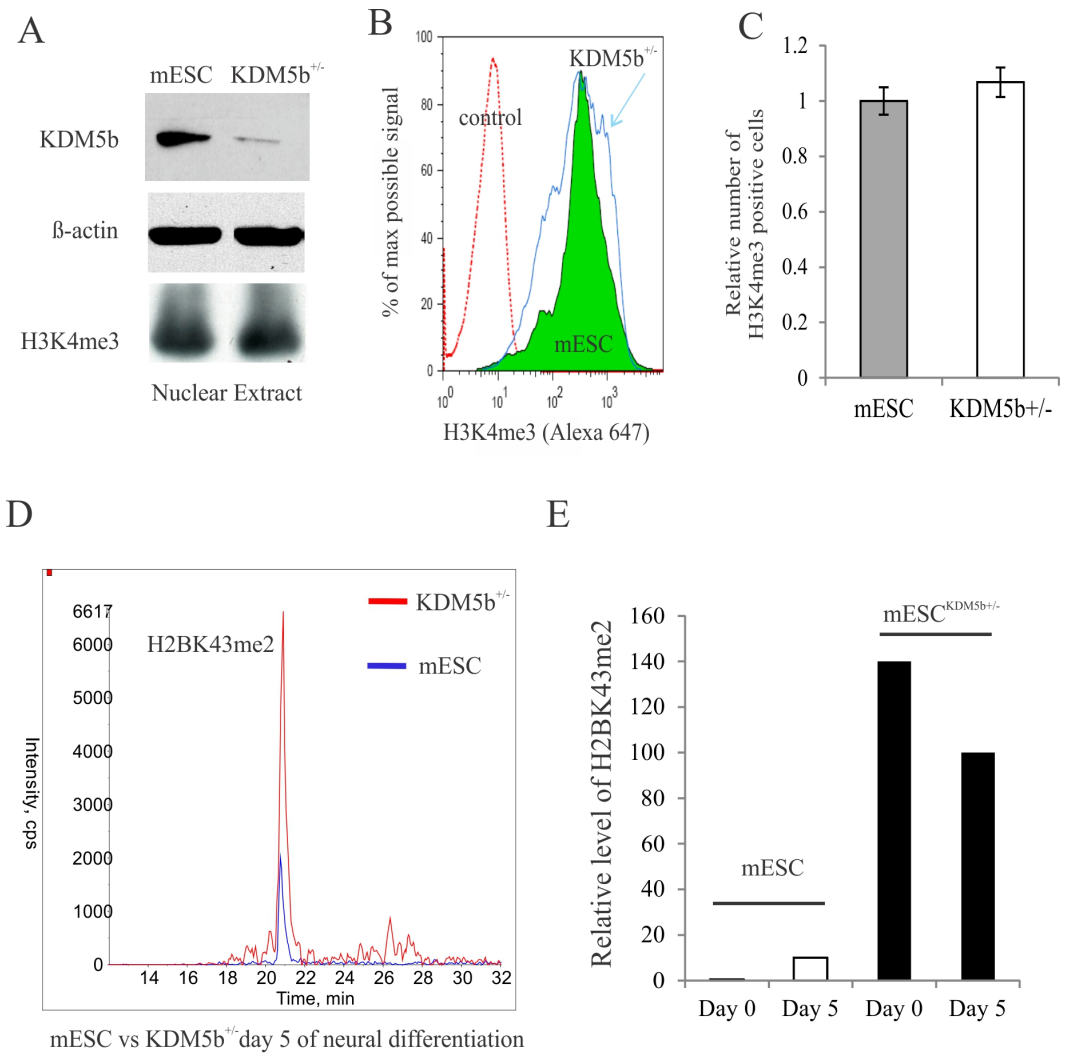

Figure 3.4 A deficiency in KDM5b leads to changes in H2BK43me2 dynamics, but not those of H3K4me3, during neural differentiation A) western blot of nuclear extracts shows that the level of KDM5b in mESC KDM5b$^{+/-}$ cells is greatly reduced compared to that in the wild-type mESC. No difference in H3k4me3, however, is observed between the wt and the mutant lines. A ß- actin blot was included as a loading control. (B,C) Flow cytometric analysis indicates that the percentage of H3K4me3-positive cels is comparable between mESC and mESC KDM5b$^{+/-}$ lines. The cells were staining with anti-H3K4me3 folowed by an Alexa-647-labeled secondary antibody before flow cytometry. The graph in C is based on the quantification of data shown in B. D) An overlay of MRM spectra for H2BK43me2 in mESC KDM5b$^{+/-}$ (red trace) and in mESC (blue trace) on day 5 of differentiation E) The mESC KDM5b$^{+/-}$ cells exhibited altered dynamicsin H2BK43me2 from that of the parent mESC on days 0 and 5 of neural differentiation as measured by MRM-MS. The graph is representative of two independent experiments with the standard deviation within 10% of the corresponding values shown, values are relative compared to mESC at day 0.

work (Dey et al. 2008), Egr1, a pro-differentiation gene negatively regulated by KDM5b, increased in transcription on day 3 of neural differentiation. The augment in Egr1 transcription correlated with an increase in H3K4me3 occupancy at its promoter (Figure 3.5A). Intriguingly, a similar trend for H2BK43me2 was observed as its association with the Egr1 promoter increased significantly on day 3 of differentiation compared to day 0. However, the difference in promoter occupancy between the day 0 and day 3 cells for the H3K4me3 mark is significantly greater than that for H2BK43me2. This might be due to the relatively low level of the latter mark on day 3 (Figure 3.3D). Importantly, the increased association of the two histone marks with the Egr1 promoter coincided with a reduction in KDM5b to the same region (Figure 3.5A). In contrast to Egr1, mTcf3, a transcriptional factor in the core regulatory circuitry of ESCs, has been shown to decrease in expression at early stages of differentiation (Cole et al. 2008). We showed that this was indeed the case as mTcf3 expression was significantly reduced on day 3 of neural differentiation compared to day 0. Contrary to what was observed for Egr1, the association of both the H3K4me3 and H2BK43me2 marks with the mTcf3 promoter was significantly reduced in day 3 cells. This was mirrored by enhanced occupancy of the mTcf3 promoter by KDM5b on day 3 of differentiation compared to day 0 (Figure 3.5B). Therefore, H3K4me3 and H2BK43me2 appear to co-mark the pro-differentiation gene Egr1 while KDM5b marks the pluripotency gene mTcf3.

To further delineate the relationship between KDM5b and the H3K4me3 and H2BK43me2 marks during mESC differentiation, we monitored the change in mTcf3 transcription and its association with H3K4me3, H2BK43me2 and KDM5b, respectively,

on samples taken on days 0, 3 and 5 of neural differentiation. The transcription of mTcf3 was repressed on day 3, but enhanced significantly on day 5 (Figure 3.5C). The association of H3K4me3, H2BK43me2 and KDM5b within different regions of the mTcf3 promoter across multiple time-points of differentiation was then examined to investigate whether the dynamic movement of epigenetic signatures correlated with the dynamic transcription pattern of mTcf3. Specifically, we probed, by ChIP, the association of KDM5b and the two histone methylation marks within four different regulatory regions of the mTcf3 gene (Figure 3.6A). On day 0, H3K4me3 and H2BK43me2 were found at the -50 to -150 and -395 to -500 loci, but absent from the transcriptional start site (TSS, +63 to -41). The majority of KDM5b, in contrast, was detected at the -395 to -500 locus. H2BK43me2, but not H3K4me3 or KDM5b, was additionally present at the upstream region from -1000 to -2000 on day 0 (Figure 3.6B). On day 3, when the transcription of mTcf3 was repressed, KDM5b was enriched at the -50 to -150 region and present at low levels at the TSS. At this time point, H2BK43me2, and to a lesser degree, H3K4me3 became enriched at the TSS as KDM5b was removed from the upstream -395 to -500 region (Figure 3.6C). Intriguingly, this pattern was reversed on day 5 when mTcf3 transcription was significantly enhanced and KDM5b was removed from the -50 to -150 region. The removal of KDM5b coincided with augmented levels of both the H3K4me3 and H2BK43me2 marks in the same region (Figure 3.6D). These data suggest that the dynamic association of H2BK43me2 and H3K4me3 with different regulatory domains of the mTcf3 gene, which was reversely correlated with

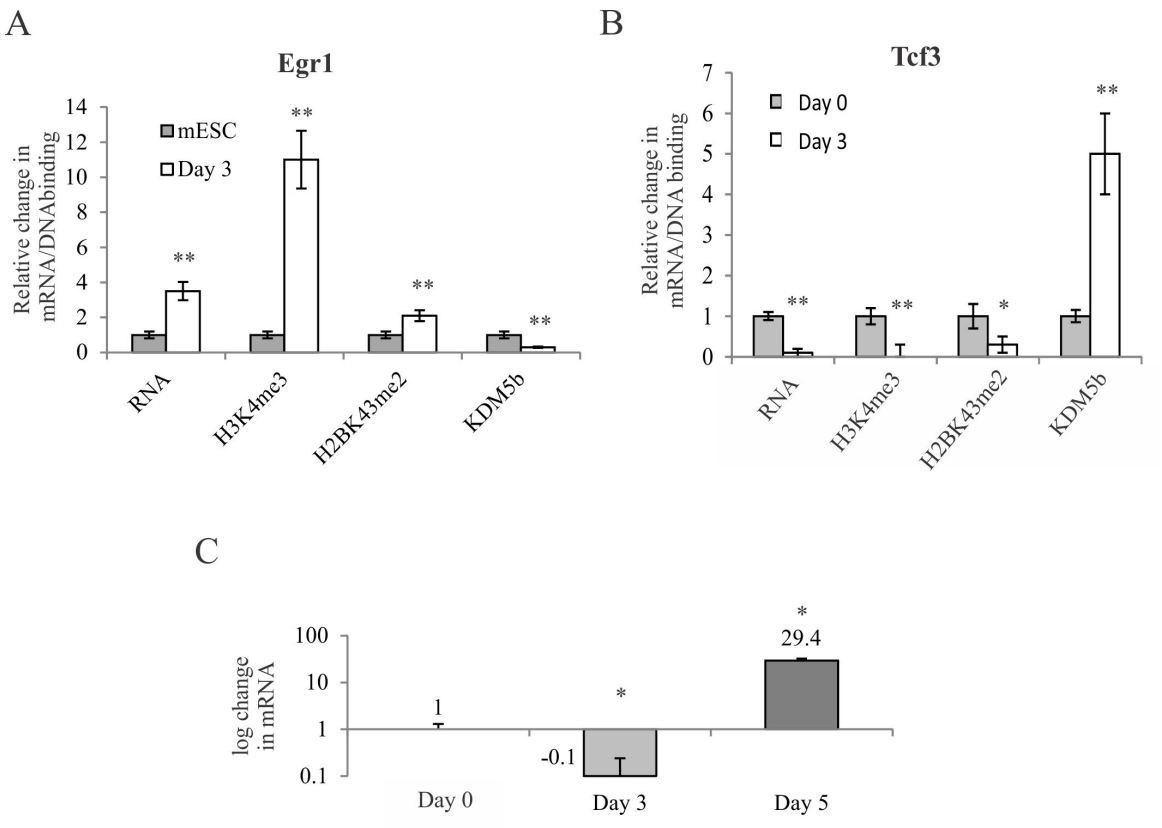

Figure 3.5 Dynamic gene transcription and association of KDM5b target genes with H2BK43me2 and H3K4me3 during mESC differentiation A,B) Expression (qRT-PCR) and association (qChIP) of the Egr1 (A) and mTcf3 (B) genes with the H3K4me3 and H2BK43me2 marks on day 0 (mESC) and 3 of neural differentiation of mESCs. C) Dynamic expression of mTcf3 during mESC differentiation as determined by qRT-PCR. All experiments represent three biological replicates. *, $p<0.05$, **, $p<0.001$

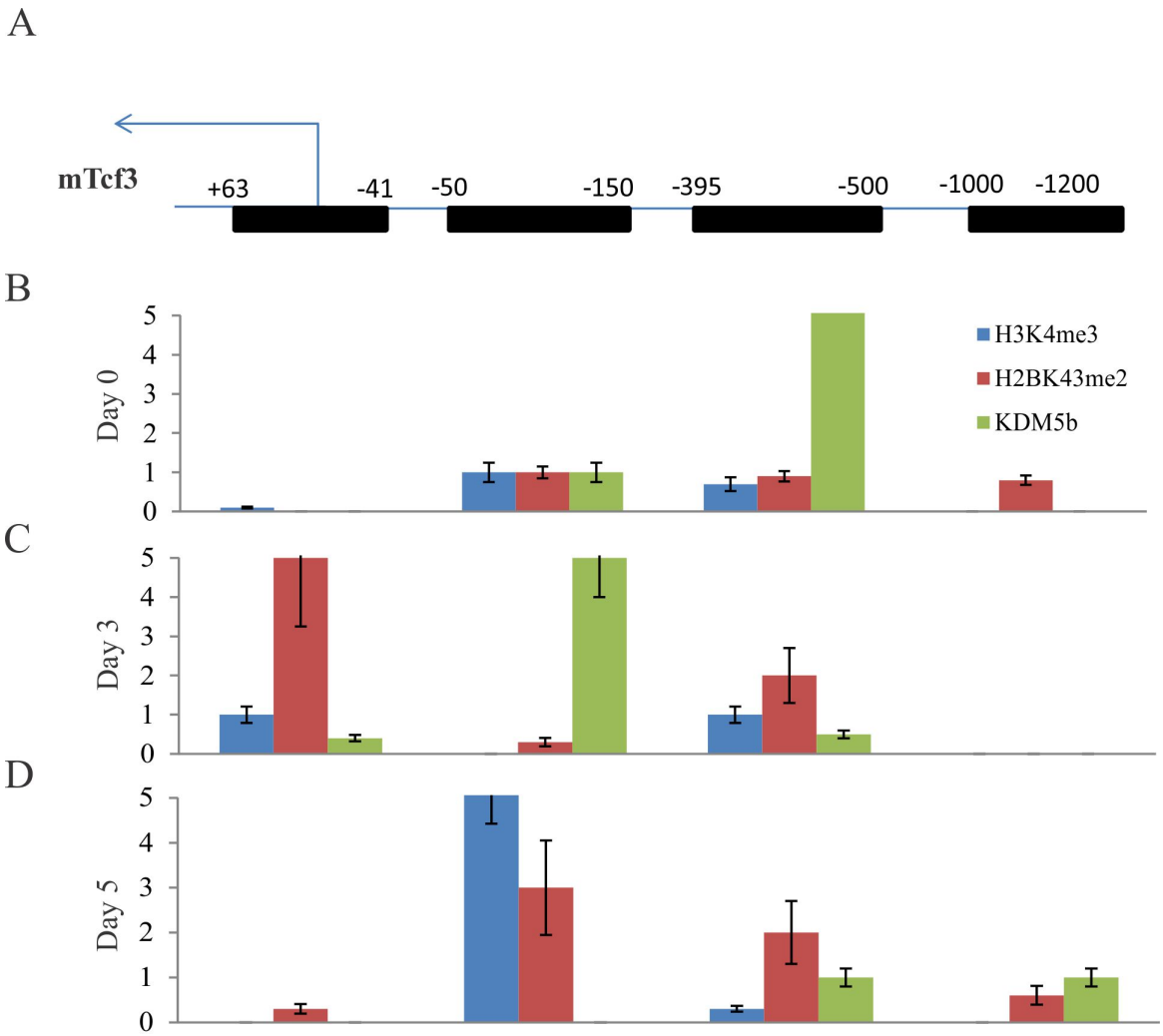

Figure 3.6 Dynamic association of KDM5b, H3K4me3 and H2bK43me2 with the mTcf3 promoter over neural differentiation Association of the mTcf3 promoter regions (identified with black bars at the top (A) with H3K4me3, H2BK43me2 and KDM5b in mESC on day 0 (B), day 3 (C) and day 5 (D) of neural differentiation. ChIP assays were carried out with antibodies against H3K4me3, H2BK43me2 and KDM5b respectively. Figure represents three biological replicates.

KDM5b occupancy regulates the dynamic transcription of this gene during mESC differentiation.

3.3.5 An H2BK43A Mutant ESC Line is Defective in Neural Differentiation

Because the dynamic change in H2BK43me2 and the resulting alteration in promoter association play an important role in regulating KDM5b target gene expression, we predicted that disrupting this mark would impede mESC differentiation. To test this hypothesis, Lysine43 was mutated to an alanine to create the mutant histone, H2BK43A. We then generated mESC clones that stably express either the wt or mutant H2B fused to a triple-FLAG (3F) tag. The resulting cell lines, mESC$^{3F\text{-}H2B}$ and the mESC$^{3F\text{-}H2BA}$, exhibited differences in KDM5b dynamics during neural differentiation; the reduction in KDM5b expression on day 5 of differentiation relative to day 0 was more pronounced for the mESC$^{3F\text{-}H2B}$ than the mESC$^{3F\text{-}H2BK43A}$ cells (Figure 3.7A). This change in KDM5b dynamics implied that a reversed change in H2BK43me2 abundance was likely. We found this to be indeed the case as both the 3F-H2BK43me2 and H2BK43me2 (endogenous) levels were increased on day 5 of differentiation for the mESC$^{3F\text{-}H2B}$, but not the mESC$^{3F\text{-}H2BK43A}$ cells. It is remarkable that even the endogenous H2BK43me2 was not detectable on day 5 in the mESC$^{3F\text{-}H2BK43A}$ cells, suggesting that the mutant histones repressed the generation of this mark on the endogenous H2B.

We have previously shown that over-expression of KDM5b leads to an increase in proliferation and a blockage in the terminal differentiation of mESCs (Dey et al. 2008). To investigate whether the altered dynamics of KDM5b seen in mESC$^{3F\text{-}H2BK43A}$ (Figure

3.7A) would cause a similar defect, we examined the expression of the pluripotency gene Sox2 and the neural marker Sox1(Kan et al. 2004). Specifically, the change in Sox2 or Sox1 expression between days 0 and 5 of differentiation in the mESC$^{3F\text{-}H2B}$ and mESC$^{3F\text{-}H2BK43A}$ cells were measured by Western blotting (Figure 3.7B). The expression of Sox2 remained generally unchanged while that of Sox1 increased with neural differentiation of the mESC$^{3F\text{-}H2B}$ cells, in agreement with earlier observations mESCs (Elkouris et al. 2011; Kan et al. 2004). The mESC$^{3F\text{-}H2BK43A}$ cells, however, showed no nuclear Sox1 on day 5, suggesting that neural differentiation was blocked for these cells.

To substantiate the above finding, we measured the levels of Sox1 and TUJ1 (β-tubulin III, neural specific) together with H2BK43me2 in mESC, mESC$^{3F\text{-}H2B}$, mESC$^{3F\text{-}H2BK43A}$ and mESC$^{KDM5b+/-}$ cells using an established method (Zhou et al. 2011). No significant difference in Sox1, TUJ1 or H2BK43me2 was seen between the mESC$^{3F\text{-}H2B}$ and the mESC cells on day 5 (Figure 3.7C). In contrast, the mESC$^{3F\text{-}H2BK43A}$ cells were characterized with significantly decreased levels of Sox1, TUJ1 and H2BK43me2 compared to the mESCs (Figure 3.7C). Intriguingly, these changes paralleled those seen in the mESC$^{KDM5b+/-}$ cells, suggesting that KDM5b controls the global H2BK43me2 level to regulate the expression of the neural genes Sox1 and TUJ1. To confirm that the phenotypic changes observed in the mESC$^{3F\text{-}H2BK43A}$ line were neural specific, we measured, by qRT-PCR, the expression levels of two additional neural genes Hes1 and PS1. AFF1, a gene that encodes a chromatin binding protein, was included as a control. The mESC$^{3F\text{-}H2BK43A}$ cells exhibited significantly reduced expression of HES1 and PS1,

but not AFF1, compared to mESC$^{3F\text{-}H2B}$, suggesting that mutation of K43 on H2B specifically affects the expression of neural genes (Figure 3.7D).

The above biochemical analysis provided a basis for the defects in neural differentiation for the mESC$^{3F\text{-}H2BK43A}$ cells. On day 5, the mESC$^{3F\text{-}H2B}$ cells formed adherent neurospheres with cells starting to migrate out of the sphere body. In contrast, the mESC$^{3F\text{-}H2BK43A}$ cells showed larger spheres that appeared to be less densely packed (Figure 3.8 A & A'). To investigate whether neural differentiation was blocked in the mutant line, we examined the ability of the corresponding neurospheres to form neurite connections as a phenotypic sign of mature neuron development. On day 10, the mESC$^{3F\text{-}H2B}$ neurospheres collapsed as the cells were spread out and connected by long threads of neurite extensions (Figure 3.8B & C). In contrast, the mESC$^{3F\text{-}H2BK43A}$ cells formed tight spheres that showed no sign of neurite outgrowth (Figure 3.8 B' & C'), consistent with a complete blockage in neural differentiation.

3.3.6 Loss of H2BK43 methylation deregulated KDM5b target gene transcription

To examine whether the loss of H2BK43 methylation would cause more wide-spread changes in gene transcription than shown above, we measured the transcriptional levels of nine additional KDM5b target genes in the mESC, mESC$^{3F\text{-}H2B}$ and mESC$^{3F\text{-}H2BK43A}$ cells, respectively (Figure 3.9A). While the level of the majority of genes remained essentially unchanged between the mESC and mESC$^{3F\text{-}H2B}$ cells, the transcription of the tumour suppressor PLAGL1 was significantly repressed and that of the cell cycle regulator Cyclin D1 was significantly enhanced in the mESC$^{3F\text{-}H2B}$ cells (Fig. 5A). In contrast, mESC$^{3F\text{-}H2BK43A}$ cells were characterized by a number of

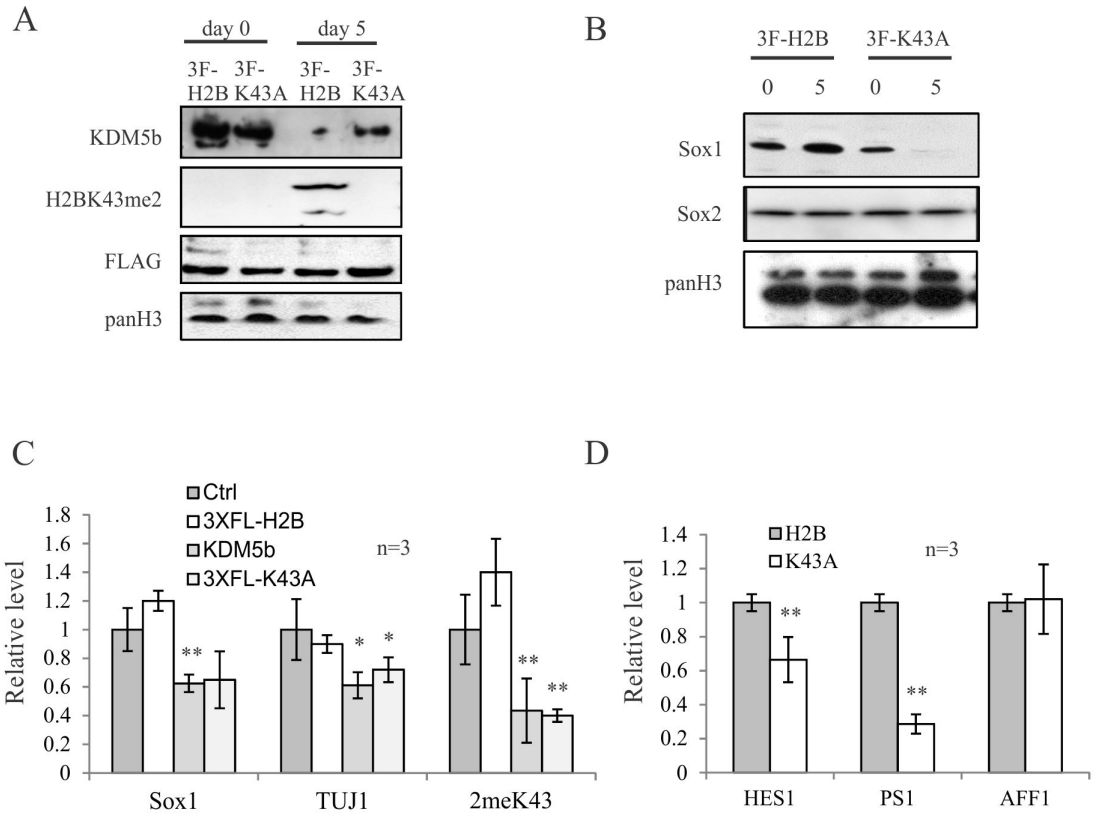

Figure 3.7 Loss of H2BK43 methylation impedes the expression of ncural differentiation markers A) mESC stably expressing either the triple FLAG-tagged wild-type (wt) histone H2B (3F-H2B) or the mutant K43A (3F-K43A) were subjected to western blot for KDM5b and H2BK43me2 on both day 0 and day 5 of neural differentation respectively B) Expression levels of Sox1 and Sox2 in 3F-H2B and 3F-K43A on day 0 and day 5 respectively. Pan H3 was used as a loading control in both A and B. C) Relative levels of RNA of Sox1, neural ß-tubulin (TUJ1) and K43me2 in mESC expressing the 3F-H2B or 3F-K43A D) Relative levels of mRNA of HES1, Ps1 and AFF in mESC expressing the 3F-H2B or 3F-K43A based on qPCR data. All data is from 3 biological replications.*,$p<0.05$, **, $p<0.001$

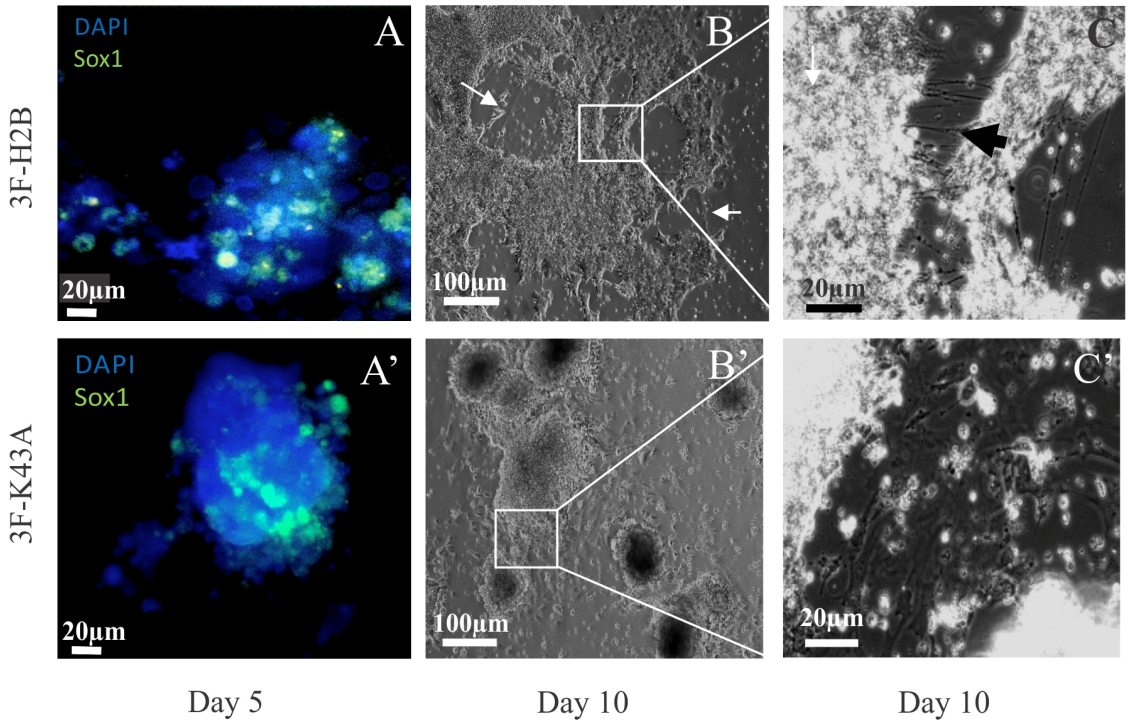

Figure 3.8 Loss of H2BK43 methylation impedes neural differentiation A/A') Anti-Sox1 immunostaining of neurospheres formed by the 3F-H2B (A) and the 3F-K43A (A') mESC on day 5 of neural differentiation B/B') Phase contrast images of the neurospheres formed by the 3F-H2B (B) and 3F-K43A (B') at day 10 of neural differentiation. White arrows in B denote areas of neurite formation not observed in B' C/C') High magnification (100x) views of the boxed regions in B/B'. Black arrow in C identifies neurites connecting two colonies of cells, missing from C'

significantly repressed genes, including Nanog, Egr1, Axin2 and CyclinD1, indicative of alterations in cell fate and cell cycle regulation (Figure 3.9A). The mTcf3 and BRCA1 genes, however, were found to be significantly over-transcribed in the mESC$^{3F\text{-}H2BK43A}$ cells.

To investigate whether the observed changes in gene transcription were caused by KDM5b, reChIP experiments were carried out to analyze the localization of KDM5b to the target genes. To this end, 3F-H2B- or 3F-H2BK43A-containing nucleosomes were purified with an anti-FLAG antibody and re-immunoprecipitated with the anti-KDM5b antibody (Figure 3.9B). Compared to 3F-H2B, the 3F-H2BK43A mutant histones recruited significantly more KDM5b at the promoters of Oct4, Nanog, Egr1 and Axin2, Cyclin D1 and p27, a group of genes that were repressed in the mESC$^{3F\text{-}H2BK43A}$ cells (Figure 3.9A & B). Conversely, we found decreased recruitment of KDM5b to the promoters of mTcf3 and BRCA1, two genes whose expression was augmented in the mutant cell line (Figure 3.9A & B). The correlation between gene transcription and KDM5b occupancy of the promoter reinforces the notion that KDM5b regulates gene transcription via H2BK43 methylation. Because H3K4me3 is also a substrate of KDM5b, we repeated the reChIP experiment using an anti-H3K4me3 antibody. A reverse correlation between H3K4me3 and KDM5b recruitment was also observed for all target genes, particularly those whose transcription levels were significantly decreased in the mutant cell line (Figure 3.9C). This suggests that similar to its role on H3K4me3, KDM5b negatively controls the abundance of the H2BK43me2 mark to regulate gene

transcription (Schmitz et al. 2011). While the above reChIP data linked both the H2BK43me2 and H3K4me3 marks with the promoters of KDM5b target genes, they did

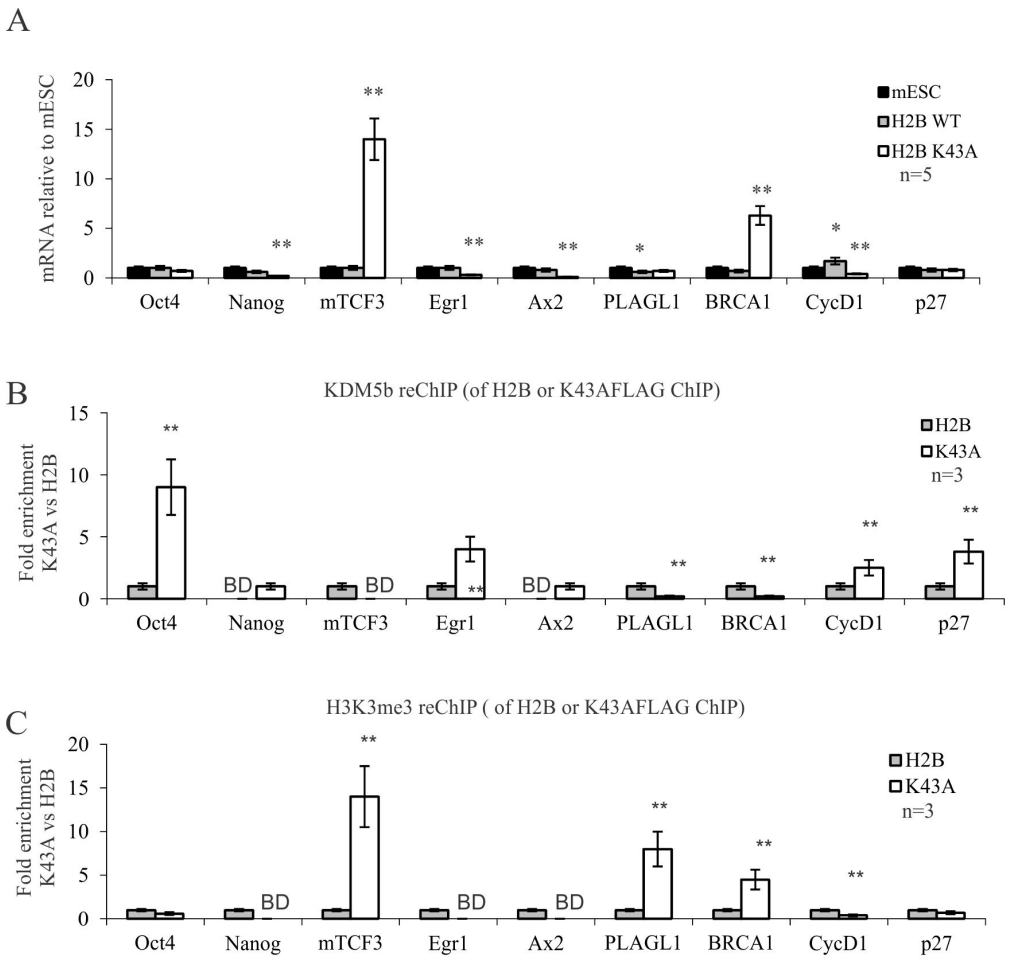

Figure 3.9 Increased KDM5b association at target genes leads to loss of H2BK43 methylation
A) Expression of KDM5b target genes in mESC and in cells stably expressing the 3F- H2B or 3F-K43A mutant, as determined by RT-PCR B) KDM5b showed distinct association with target genes in cells expressing the wt H2B from those expressing the H2BK43A mutant. A FLAG ChIP after cross linking was followed by a KDM5b re-ChIP to assess the association of target genes with KDM5b in the two cell lines. C) Target genes were differentially marked with H3K4me3 in the 3F-H2B versus 3F-K43A cell lines. A FLAG ChIP after cross linking was followed by an H3K4me3 re-ChIP to assess the association of target genes with H3K4me3 in the two cell lines. *, $p<0.05$, **, $p< 0.001$

not provide direct evidence that the two methylation events occur on the same nucleosome. To verify this, 3F-H2B and 3F-H2BK43A-containing mononucleosomes were isolated and blotted for associated KDM5b, H2BK43me2, ubiquitinated H2B (Xiao et al. 2005) and H3K4me3 (Wang et al. 2009). As expected, KDM5b was enriched in the H2BK43A-containing nucleosomes characterized with less ubiquitinated H2B and H3K4me3 (Figure 3.10A). The co-existence of the Ub-H2B and H3K4me3 marks with H2BK43me2 on the same nucleosome supports a role for the latter in transcriptional activation. This assertion is further supported by ChIP data demonstrating increased occupancy of H2BK43me2 at the promoter of mTcf3, an activated gene and conversely, decreased association with the promoter of Ax2, a repressed gene, in the mESC$^{3F\text{-}H2BK43A}$ mutant line (Figure 3.10B).

Given that a similar correlation was observed between promoter association with H3K4me3 and gene transcription, our ChIP and re-ChIP data supports a model in which H2BK43me2 and H3K4me3 together mark genes for active transcription. This model is substantiated by ChIP data for KDM5b, H2BK43me2 and H3K4me3 at the Egr1 proximal promoter using the mESC$^{3F\text{-}H2B}$, mESC$^{3F\text{-}H2BK43A}$, mESC$^{KDM5b\text{-}OXP}$, a line stably over-expressed KDM5b (Dey et al. 2008), and the mESC$^{KDM5b+/-}$ lines (Figure 3.10C). While the mESC$^{3F\text{-}H2B}$ cells showed a similar pattern of promoter association as the control mESCs, both the mESC$^{3F\text{-}H2BK43A}$ and the mESC$^{KDM5b\text{-}OXP}$ cells featured increased KDM5b and decreased H2BK43me2 and H3K4me3 occupancy at the Erg1 promoter. This is in contrast to the mESC$^{KDM5b+/-}$ cells that featured increased levels of the two histone marks but a reduced level of KDM5b. Taken together, our data indicate that

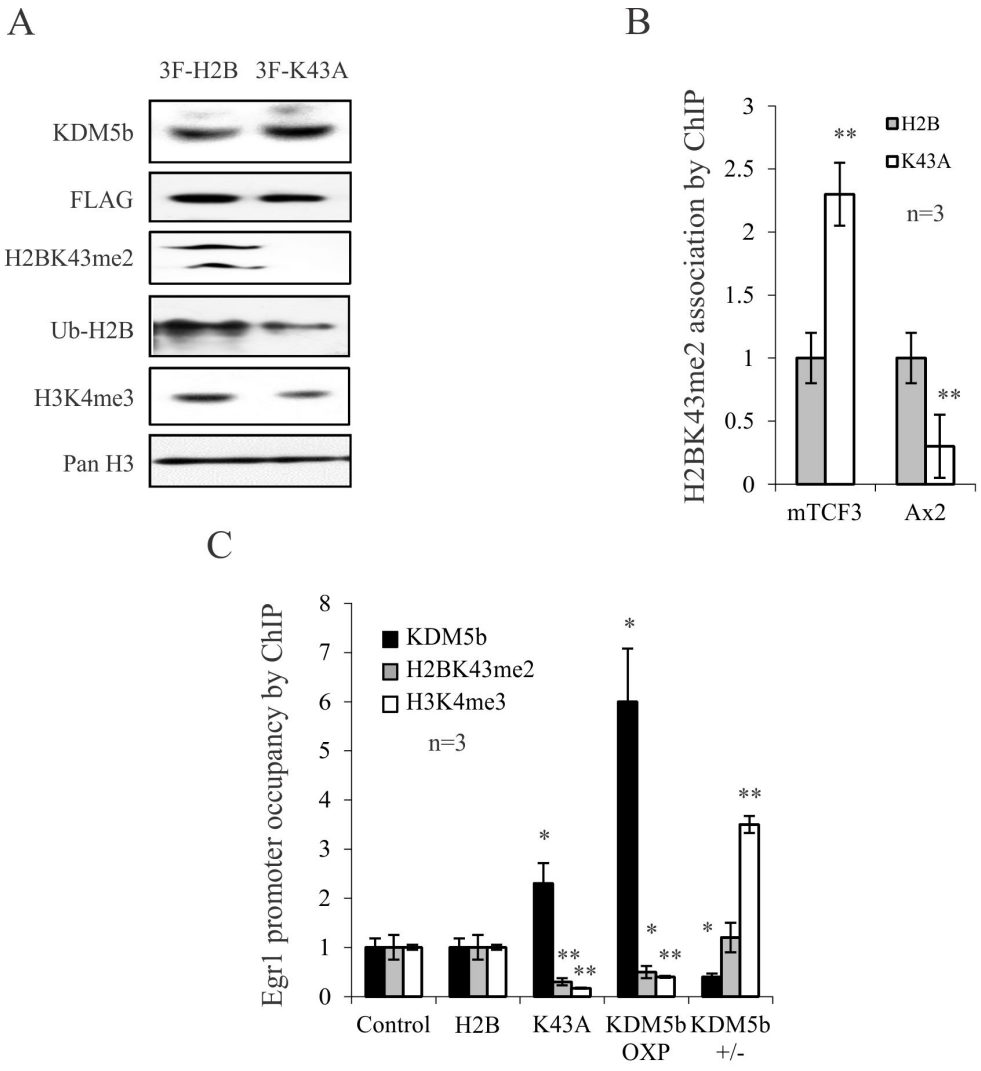

Figure 3.10 Loss of H2BK43 methylation alters nucleosome occupancy of other histone marks
A) H2B ubiquitination and H3K4me3 are diminished in H2BK43A-containing nucleosomes. Western blots were used to compare the level of KDM5b, H2BK43me2 (upper band, FLAG tagged, lower band, endogenous), and ubiquitinated H2B (Ub-H2B) and H3K4me3 in cells expressing either 3F-H2B or 3F-K43A. Anti-FLAG and pan histone H3 were used as loading controls. B) Altered H2BK43me2 occupancy (by ChIP) of the promoter regions of mTcf3 and Ax2 between cells expressing 3F-H2B or 3F-K43A. C) Comparison of the Egr1 promoter (TSS) association with KDM5b, H2BK43me2 and H3K4me3 by ChIP in control mESC, cells expressing 3F-H2B, cells expressing 3F-K43A, cells over expressing KDM5b (KDM5b OXP) and KDM5b $^{+/-}$ ESC
*p<0.05, **, p< 0.001.

KDM5b controls the association of H2BK43me2 and H3K4me3 at the promoter to regulate gene transcription.

3.4 Discussion

Under optimal conditions, ESCs may be induced to differentiate into neural stem cells and ultimately neurons. During this process, the bivalent H3K4me3 and H3K27me3 histone domains at the promoters of neural fate-specification genes are resolved by Jmjd3, a specific KDM for H3K27me3 that is required for neural commitment (Burgold et al. 2008). Herein, we showed that KDM5b, a KDM known to target the H3K4me3 mark, additionally functioned as a specific demethylase for H2BK43me2 and played an essential role in neural differentiation of mESCs by controlling the methylation status of both histone marks. MRM-MS analysis of cells undergoing neural differentiation demonstrated that KDM5b controls H2BK43me2 and H3K4me3 dynamics to affect neural commitment of mESCs. Pluripotent ESC are characterized with a high level of KDM5b and low levels of both the H2BK43me2 and H3K4me3 marks. In neural lineage-committed cells, the KDM5b level was distinctly lower and the global levels of H2BK43me2 and H3K4me3, especially the former, were markedly higher than in the ground state mESCs. Consequently, KDM5b target genes co-marked by the two modifications underwent dynamic changes in expression during the neural differentiation. Maintaining the dynamics of KDM5b and of its substrate H2BK43me2 appeared to be critical for differentiation as mESC that either over-express the enzyme or contain a

Kdm5b$^{+/-}$ allele were defective in neural differentiation as were cells stably expressing a mutant H2B histone in which K43 was replaced by an Alanine.

Our data suggests that while H3K4me3 is a general mark for transcriptional activation, H2BK43me2 is a mark specific for KDM5b target gene expression. Together, these two marks provide an important epigenetic control mechanism of the transcriptional program during ESC differentiation. It would be interesting to determine in subsequent studies whether the H2BK43me2 mark regulates gene expression on a genome-wide scale and whether KDM5b and H2BK43me2 play a role in the differentiation of pluripotent or multipotent cells into other cell types or lineages other than neurons. In this regard, it is interesting to note that KDM6b regulates the coordinated removal of the repressive mark H3K27me3 on some *Hox* gene promoters during ESC differentiation (Lan et al. 2007; Agger et al. 2007). Additionally, both KDM6b and KDM4b have been shown to play an essential role in osteogenic differentiation of human multipotent stem cells (Ye et al. 2012). Therefore, distinct combinations of KDMs and KMTs may control histone dynamics to drive different differentiation processes in pluripotent and multipotent cells.

Our work provides an example of how dynamic changes in the methylation of histone marks (i.e. H2BK43 and H3K4) regulate stem cell differentiation. Although the identification of the first histone demethylase (Shi et al. 2004) changed the long-held view that lysine methylation is a stable and irreversible PTM (Byvoet et al. 1972), it is not known whether lysine methylation is as dynamic as protein phosphorylation. McDonald and colleagues showed that, in contrast to DNA methylation that is unchanged during epithelial-to-mesenchymal transition (EMT), the global levels of H3K9me2, H3K4me3

and H3K36me3 are significantly altered in an KDM1-dependent manner (McDonald et al. 2011). The range of dynamic changes for these histone marks during EMT is, however, much smaller than what was observed here for the H2BK43 mark during mESC differentiation. The changes in the global level of tri- and dimethyl H2BK43 during neural differentiation spans orders of magnitude, a phenomenon never observed before for any histone mark. While our data indicate that KDM5b is the demethylase for H2BK43me2, we do not know what KDM or KTM is responsible for the dynamic changes seen for H2BK43me3. A direction in future studies is to identify this enzyme and elucidate how it interplays with KDM5b to control the dynamics of H2BK43me-2 and -3 to affect the decision-making process of self-renewal and versus differentiation in stem cells.

It is important to consider that the work performed within this chapter represents a mere portion of the possible experiments which could be completed in the future to complete or expand our knowledge of the interaction of KDM5b with a novel substrate. Though the utilization of recombinant techniques paired with both *in vivo* analysis, and peptide based MRM-MS provides a well supported overview of this interaction, alternate techniques may provide addtional peices of key information. Analysis of an enzymatic dead KDM5b, such as the known H499Y mutant(Yamane et al. 2007), would provide key information, supporting that the same enzymatic domains within KDM5b are required for enzymology on H2BK43me2. Creation of a H499Y stable cell line, in order to study differentiation would also aid in the seperation of two key concepts of KDM5b regulation, recruitment versus enzymology during differentiation. Utilization of a

KDM5b knockdown cell line, would also help to back up the results found using the KDM5b heterozygous line, to ensure that results are a direct result of decreased KDM5b levels and not a product of the method by which this cell line was created.

3.5 Acknowledgements

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Conflict of Interests Statement

The authors declare no conflict of interest

Chapter 4 Defining an enzymatic complex for KDM5b

Preamble

The material presented within this chapter is a representation of a potential manuscript not yet submitted.

The Groucho co-repressor TLE4 represents a novel cofactor for the histone demethylase KDM5b Leanne Stalker, Lise Munsie, Marek Galka, Shawn Shun-Cheng Li, Ray Truant, and Christopher Wynder

LS wrote the manuscript and performed all experiments and data analysis except the experiments listed below

LM aided in the collection of images for FLIM-FRET analysis
MG performed MRM-MS/MS based *in vitro* demethylase assay

4.1 Abstract

KDM5b (Lysine-specific demethylase 5b) is a potent transcriptional repressor through its ability to remove methylation residues from histone 3 lysine 4 (H3K4), a pertinent mark of active chromatin. TLE4 (Transducin like enhancer of split 4) is a transcriptional co-repressor known to bind histone H3 and result in the formation of repressive chromatin through nucleosome compaction. Here we show that TLE4 represents a novel cofactor for KDM5b. The association of TLE4 and KDM5b was found both using co-immunoprecipitation and *in vivo* using FLIM-FRET in live mammalian cells. We also show that TLE4 represents a novel enzymatic cofactor for KDM5b and that association with TLE4 confers the ability to demethylate H3K4me3 on a nucleosomal substrate *in vitro*. This ability appears to be evolutionarily conserved as TLE4 confers nucleosomal activity to both KDM5d and yKDM5 in addition to KDM5b. We also demonstrate that ectopic expression of TLE4 in mammalian cells is sufficient to result in a specific reduction in the global level of H3K4me3 by quantitative immunocytochemistry. We have therefore determined a minimal required complex for the enzymology of KDM5b on nucleosomes as well as elucidated an additional molecular mechanism by which TLE4 can act as a transcriptional repressor.

4.2 Introduction

KDM5b, or lysine (K) specific demethylase 5b is a member of the Jumonji C (JmjC) domain containing demethylase family. KDM5b, also known as JARID1b or Plu-1 exerts its demethylase activity through a hydroxylase reaction known to specifically

target tri-methylated and di-methylated Lysine 4 of Histone 3 (H3K4me3, H3K4me2 respectively) (Yamane et al. 2007; Xiang et al. 2007). As methylation of H3K4 is considered to be a mark of transcriptional activation (Berger 2007; Barski et al. 2007; Pokholok et al. 2005; Schubeler et al. 2004), KDM5b and its family members are considered to be potent transcriptional repressors.

Originally discovered as a gene up-regulated in response to c-Erb2, KDM5b shows limited expression in most adult tissues but has been found to be up-regulated in several forms of cancer (Lu et al. 1999; Barrett et al. 2002). KDM5b expression has been correlated to tumour grade, metastatic potential (Yamane et al. 2007), and invasion ability (Yoshida et al. 2011). Of particular interest, KDM5b has been found to regulate cellular proliferation in both an accelerating (Yamane et al. 2007) and decelerating (Roesch et al. 2010) fashion, and has been recently targeted as having both oncogenic and tumour suppressor potential depending on cell type (Barrett et al. 2002; Hayami et al. 2010; Lu et al. 1999; Roesch et al. 2008; Roesch et al. 2010; Xiang et al. 2007; Yamane et al. 2007). KDM5b has also been found to play a pertinent role in development through the regulation of both differentiation and proliferation depending on the level of cell fate commitment (Dey et al. 2008; Schmitz et al. 2011; Xie et al. 2011). This suggests that KDM5b localization and activity on target genes must be highly regulated, allowing specific demethylation of target genes depending on cellular context. At the current time however, very little is known about how KDM5b is localized to its gene targets, or how its enzymology is regulated *in vivo*. Recent work has suggested that the localization of

KDM5 demethylases to the promoters of target genes of interest is not sufficient to assume demethylation and that enzyme localization is often paired with the maintained presence of its enzymatic target, H3K4me3 (Lopez-Bigas et al. 2008; Schmitz et al. 2011). Additionally, KDM5 demethylases have yet to have demonstrated demethylase activity on a nucleosomal substrate *in vitro*, though the activity is well supported on a histone-based substrate. This supports a model where the localization and enzymology of these enzymes are regulated separately, potentially paired with the requirement for either cofactor recruitment, or additional chromatin landscape alterations before enzymatic activity can proceed.

The requirement for cofactors is common amongst transcriptional regulators, with these enzymes seldom working alone. KDM1 (Lysine specific demethylase 1, LSD, BHC110), for example, requires association with CoREST, the co-repressor of REST (RE-1 silencing transcription factors) in order to mediate nucleosomal demethylation of H3K4 (Shi et al. 2005; Lee et al. 2005), and association with the androgen receptor (AR) to mediate the demethylation of H3K9 (Metzger et al. 2005). Though the enzymology of KDM5 demethylase proteins was defined in 2007 (Yamane et al. 2007; Xiang et al. 2007), no enzymatic cofactors or localization landmarks have yet to be determined for this demethylase family. Ring6a represents the sole determined cofactor for any member of the KDM5 enzyme family, increasing the efficiency of KDM5d on a histone substrate. Ring6a however has not been found to allow nucleosomal demethylation (Lee et al. 2007).

Many transcriptional regulators are recruited to gene targets through interaction with DNA binding proteins and enzymatic cofactors. Most recently, the polycomb repressive complex 1 (PRC1) was found to require recruitment by core binding transcription factors (Yu, Mazor et al. 2012). One of the first co-repressor proteins required for the specific recruitment of chromatin modifying enzymes to DNA is the Groucho protein, first discovered in *Drosophila* (Jennings and Ish-Horowicz 2008). Groucho possesses both mouse (Groucho,Grg) and human (Transducin like enhancer of split, TLE) homologues which have been shown to act as co-repressors (Stifani et al. 1992; Mallo, Franco del Amo, and Gridley 1993; Grbavec et al. 1999; Fisher and Caudy 1998). Groucho/TLEs have no direct ability to bind DNA but have been shown to bind directly to histone proteins (Palaparti, Baratz, and Stifani 1997) and to exert transcriptionally repressive properties at least partially modulated through the recruitment of HDAC proteins (Sekiya and Zaret 2007; Chen et al. 1999; Chen and Courey 2000). The recruitment of TLE proteins has been found to trigger alterations to the chromatin structure, resulting in the assembly and stabilization of a repressive chromatin state through nucleosome compaction (Sekiya and Zaret 2007; Fisher and Caudy 1998; Winkler, Ponce, and Courey 2010). This compaction results in an inability of transcription factors and RNA polymerase II to access chromatin and stretches far beyond the actual binding site of TLE suggesting that the binding of TLE proteins may represent an initial step in transcriptional repression through alterations to the chromatin landscape. Despite this evidence, our

actual knowledge of the role of TLE proteins in the regulation of nucleosomal modifications remains elusive.

Recent work defines a role for Grg4/TLE4 in transcriptional repression, suggesting that Grg4/TLE4 recruitment to Pax2 DNA binding sites acts as a transcriptional switch. In the presence of Grg4, Pax 2 is able to initiate polycomb-mediated gene silencing and the methylation of H3K27 through the recruitment of PRC2. In the absence of Grg4 however, Pax2 is able to recruit the adaptor protein PTIP and associated methyl transferase complexes, including KMT2C/D resulting in the methylation of H3K4me3 resulting in transcriptional activation (Patel et al. 2012). As a possible role for KDM5b in Groucho mediated repression has been hinted at previously (Tan et al. 2003). We were led to question whether the resulting decrease in the methylation of H3K4 in the presence of TLE4 was not only due to the displacement of PTIP at Pax binding sites, but was also connected to the regulation of a histone demethylase enzyme such as KDM5b. Both KDM5b and TLE4 interact with Pax proteins (Eberhard et al. 2000; Linderson et al. 2004; Milili et al. 2002; Patel et al. 2012; Tan et al. 2003) and HDACs (Chen et al. 1999; Sekiya and Zaret 2007; Barrett et al. 2007). TLE4 expression is known to be required for neural development (Koop, MacDonald, and Lobe 1996; Yao et al. 1998; Orian et al. 2007) and has been found to show elevated expression in the testis compared to other tissues (Milili et al. 2002), a similar role and localization as KDM5b (Dey et al. 2008; Schmitz et al. 2011; Barrett et al. 2002; Madsen et al. 2003) suggesting a possible overlap in function.

In the present study, we demonstrate that TLE4 and KDM5b interact both *in vivo* and *in vitro* and that an association of KDM5b and TLE4 results in an increase in the enzymatic potential of KDM5b at H3K4me3. This association also results in the ability of KDM5b to demethylate H3K4me3 on a nucleosomal substrate *in vitro*, suggesting that TLE4 represents a specific and required cofactor for KDM5b demethylase activity, and that interaction of TLE4 with KDM5b contributes to the transcriptionally repressive role of TLE4.

4.3 Results

4.3.1 TLE4 Interacts with KDM5b and is Recruited to KDM5b Target Genes

TLE4 and KDM5b have both been directly associated in nucleosomal compaction and the resulting transcriptional repression. Additionally, both KDM5b and TLE4 have been found previously to associate with HDAC proteins and to bind preferentially to histone H3. KDM5b has previously been suggested to have a potential role in Groucho-mediated repression due to its known interaction with both Pax9 and BF-1, a known member of the Groucho repression complex. However, no direct connection between this enzyme and co-repressor has yet to be made. In order to determine if KDM5b and TLE4 interact, we performed endogenous co-immunoprecipitation experiments between KDM5b and TLE4. Although this association can be difficult to detect, we reproducibly identified endogenous KDM5b protein in the immunoprecipitates from TLE4-based pull downs in the presence of 350mM KCl (Figure 4.1A). Evidence suggests that the interaction of TLE4 with KDM5b represents a novel role for TLE4, separate from its role

with other TLEs as TLE4 and TLE1 were able to reciprocally co-immunoprecipitate each other, as would be expected through their heterodimerization domain (Gasperowicz and Otto 2005). This is not a general function of the TLE proteins since TLE1 showed no evidence of endogenous KDM5b interaction in the same precipitate.

In order to verify that this interaction is not due to the interaction of both TLE4 and KDM5b with chromatin, IPs were repeated using PCNA, another chromatin binding protein (Mello and Almouzni 2001; Strzalka and Ziemienowicz 2011) as a negative control (Figure 4.1B). Endogenous KDM5b protein was identified in immunoprecipitates from TLE4 based pull downs but not in those through PCNA. KDM5b is additionally unable to pull down PCNA supporting that the interaction of KDM5b and TLE4 is both real and specific using endogenous protein and highly stringent immunoprecipitation conditions.

Since we were interested in the role potential cofactors play in KDM5b's *in vivo* biology as a transcriptional repressor, we utilized chromatin immunoprecipitation (ChIP) to determine if TLE4 and KDM5b co-occupy KDM5b target genes of interest. Our previous work characterizes p27 as a KMD5b target involved in both its mESC and neural differentiation phenotypes (Dey et al. 2008) and TLE4 has been previously shown to bind the p27 promoter in kidney cells (Sharma et al. 2009) suggesting that it may be a shared target. Here we demonstrate that both TLE4 and KDM5b bind the p27 promoter at the same 150 basepair region in mESCs (Figure 4.1C).This supports that the interaction of TLE4 and KDM5b is likely to be localized to chromatin at the promoters of KDM5b target genes.

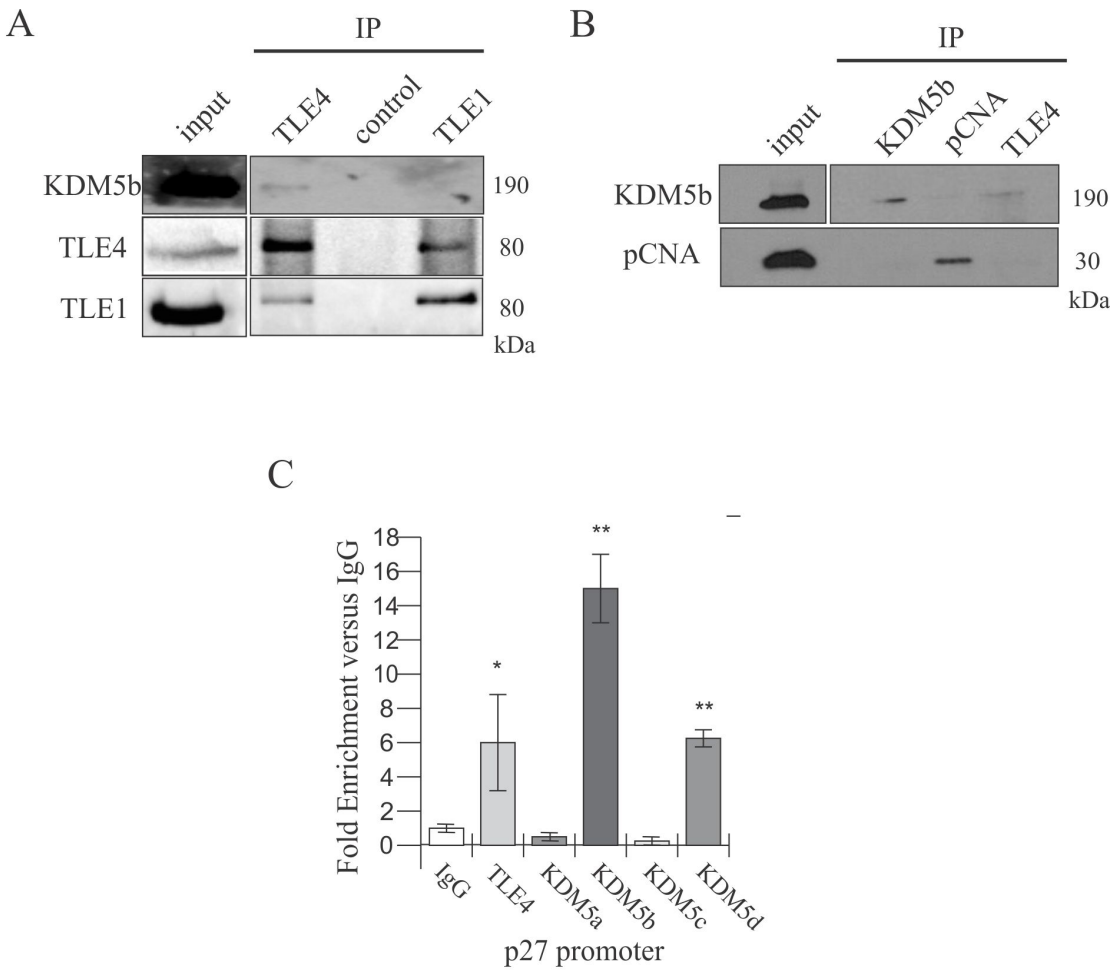

Figure 4.1 TLE4 interacts with KDM5b and is recruited to KDM5b target genes A) KDM5b immunoprecipitates with TLE4 but not with TLE1. HEK293 cell extract was incubated with antibodies against TLE4, TLE1 or with beads alone. After 350mM KCL washes, samples were subjected to western blot with antibodies against KDM5b, TLE4 and TLE1 respectively. B) KDM5b immunoprecipitates specifically with TLE4 and not with pCNA. HEK293 cell extract was incubated with antibodies against KDM5b, TLE4 or pCNA. After 350mM KCL washes, samples were subjected to western blot with antibodies against KDM5b or pCNA. C) TLE4 is recruited to the KDM5b target gene p27. ChIP assay demonstrates that KDM5b, KDM5d and TLE4 are recruited to the same 150bp region of the p27 promoter in mESC. All experiments represent biological triplicates, *, $p<0.05$, **, $p<0.001$

4.3.2 KDM5b and TLE4 Interact in Live Cells Using FLIM-FRET

We then wished to further verify the interaction of KDM5b and TLE4 in a live cell context. We therefore created fusion proteins of both KDM5b and TLE4 in which they were N-terminally tagged with eYFP (yellow fluorescent protein, venus variant) and mCerulean blue cyan fluorescent protein (CFP) respectively. Initial analysis of full-length mCerulean-TLE4 in 3T3 cells showed striking phenotype in which the fusion protein localized to distinct nuclear puncta. YFP tagged KDM5b showed an expected nuclear phenotype.

In order to measure the interaction of KDM5b and TLE4 in 3D space in live cells in a quantitative fashion we utilized Förster Resonance Energy Transfer (FRET). In this case, mCerulean blue was chosen as the donor and YFP was chosen as the acceptor in the FRET pair. mCerulean blue possesses first order exponential lifetime decay, and shows spectral overlap with YFP, with the emission spectrum of mCerulean overlapping the excitation spectrum of YFP. These properties allow for measurement of FRET using time resolved Fluorescent Lifetime Imaging (FLIM) (Rizzo et al. 2006). FLIM measures the fluorescent lifetime of the donor fluorophore in the presence of a fluorescent acceptor fluorophore. When the two fluorophores are in close proximity (less than 8nm for the CFP-YFP FRET pair) the apparent lifetime of the donor fluorophore becomes decreased. This is inversely correlated with FRET efficiency. This method is commonly used to determine the interaction between two proteins, as FRET efficiency drops off to the 6^{th} power as a function of distance (Wallrabe and Periasamy 2005). Additionally this

measurement is not affected by protein concentration or spectral bleed through, making this an extremely accurate standard of FRET measurement.

The lifetime of the donor, mCerulean-TLE4, was first measured both alone, and in the presence of YFP alone and was found to be 2.7ns (Figure 4.2A shown as green-blue in colour (b and e) and the red line on the color histogram (c and f)). This is consistent with what has been observed in the past, where non-fused mCerulean was found to have a lifetime of ~2.8ns (Munsie et al. 2011). This indicates both that fusion of TLE4 to mCerulean does not affect lifetime, and that there is no evidence of FRET between mCerulean TLE4 and YFP alone, suggesting no interaction. In the presence of YFP-KDM5b however, the lifetime of mCerulean-TLE4 was found to be significantly shorter at approximately 2.1ns (Figure 4.2A, h and i respectively, indicated as yellow orange colour and the red dashed line on the colour histogram) due to FRET. The interaction of mCerulean TLE4 and YFP-KDM5b gave an average FRET efficiency of 9% in the nuclear puncta (the highest possible FRET efficiency being ~30% for this FRET pair) versus the average of FRET efficiency of mCerulean-TLE4 interaction with the control YFP alone being 3.5% (Figure 4.2 f versus I, quantified in Figure 4.2B). These data show the TLE4 and KDM5b can directly interact within the nucleus in live cells, supporting data presented in figure 4.1, and suggesting that TLE4 may play a role in the *in vivo* biology of KDM5b. These data support that TLE4 and KDM5b interact *in vivo*.

4.3.3 TLE4 Alters the Histone Demethylase Activity of KDM5b

The association of KDM5b and TLE4 in live cells, as well as their co-occupancy of the p27 promoter within the same 150bp region suggest that TLE4 may represent a co-

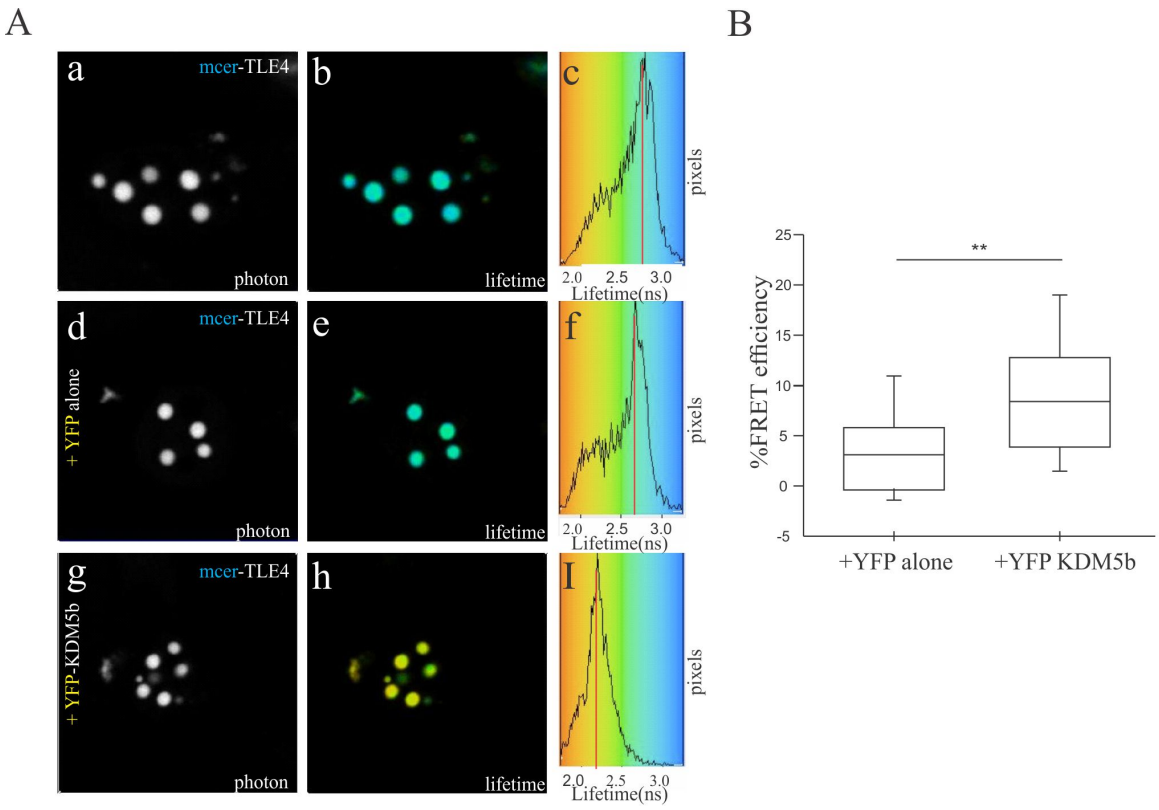

Figure 4.2 TLE4 interacts directly with KDM5b A) NIH3T3 cells were transiently transfected with mCerulean-TLE4 alone (a-c) or mCerulean-TLE4 and either EYFP alone (d-f) or EYFP-KDM5b (g-I) and FLIM analysis was performd. Fluoroescent lifetimes for mCerulean blue are presented with a continuous pseudocolour rainbow scale representing time values ranging from 1.75 to 3.25 ns. The lifetime distribution curve of the mCerulean-TLE4 is shown as a histogram on the right representing the number of pixels at each lifetime. The red vertical line marks the median lifetime distribution for the cell pictured. B) Box and whisker plot representing FRET efficiency with FRET occuring at distances <8nm for these FRET pairs. All imaging and FRET analysis were done in Hank's HEPES buffer pH 7.4. N=3, n>10 for each donor-acceptor pair. **, P<0.001 Line= mean; box= 1 standard deviation from the mean; whiskers= 2 standard deviations from the mean.

factor for KDM5b enzymology. Past work has suggested however, that many proteins that associate with the KDM5 family appear to play no direct role in demethylase activity. Pax 9 and BF-1, two KDM5b-interacting proteins identified by yeast two-hybrid screening both represent developmental transcription factors with transcriptionally repressive properties (Tan et al. 2003). Though KDM5b was found to have the ability to alter the transcriptional activity of both of these transcription factors, they have no reported effect KDM5b enzymology. More recently, a novel interactor with KDM5b, KLF10, was found to cooperate with KDM5b to enhance TGF-β signaling. KDM5b was again identified as a co-repressor of KLF10, but *in vitro* analysis of KDM5b enzymology was not completed (Kim et al. 2010).

To analyze the direct role of TLE4 in KDM5b enzymology, recombinant, full-length KDM5b was isolated from bacteria utilizing a 6xhis tag (r.KDM5b). The isolated recombinant protein was first verified to ensure that it possessed appropriate enzymology. rKDM5b was incubated in increasing concentrations (0.1ug to 5ug) with a histone substrate and analyzed by SDS-PAGE followed by western blotting using antibodies against both H3K4me3 and panH3. Isolated r.KDM5b showed enzymatic activity on H3K4me3 even at the lowest concentration (0.1μg) with pan Histone H3 used as a loading control (Figure 4.3A) suggesting that the isolated enzyme is able to perform demethylation of H3K4me3. To determine if TLE4 plays a role in KDM5b enzymology, a TLE4 complex was then immunoprecipitated from Mouse Embryonic Stem Cells (ESC) through a TLE4 antibody and was subjected to affinity purification under high salt conditions to minimize co-immunoprecipitated complexes. The affinity eluate was then

used to assess the ability of the TLE4 complex to demethylate H3K4me3 using a quantitative ELISA based read out. For detailed data analysis please see methods and materials. After an overnight incubation with a histone substrate, recombinant KDM5b showed 44% H3K4me3 remaining. The TLE4 complex showed 51% H3K4me3 remaining as compared to control (histone alone) (Figure 4.3B). These data show that TLE4 co-immunoprecipitates with an enzyme capable of demethylating H3K4me3 at a comparable rate to r.KDM5b under these conditions.

Though these data support that TLE4 interacts with an enzyme capable of demetylating H3K4me3, we wished to further analyze whether this enzyme is KDM5b. Recombinant TLE4 was therefore isolated in a similar fashion to rKDM5b, and the kinetics of KDM5b demethylation were quantified both in the presence and absence of TLE4. In order to do this, we employed Multiple Reaction Monitoring Mass Spectrometry (MRM-MS) (Yocum and Chinnaiyan 2009). MRM-MS allows us to monitor changes in a specific methyl mark over time using pre-programmed precursor to product transitions that assign to a particular peptide of interest. r.KDM5b was therefore combined with a biotin tagged peptide which corresponded to H3K4me3 both in the presence and absence of r.TLE4. Samples were drawn at both time 0 (t=0) and 60 minutes (t=60min) and the amount of H3K4me3 remaining was analyzed by MRM-MS. Combination of peptide with r.KDM5b alone resulted in a 20% decrease in the methylation of the H3K4me3 peptide substrate after 60 minutes (Figure 4.3C). Combination of peptide and r.KDM5b with r.TLE4 however resulted in a 65% decrease, a 3.25 fold enhancement of demethylation at the same time point (Figure 4.3C). Taken

together, these data suggest that TLE4 associates with a histone demethylase, specifically KDM5b, in live cells and that the association of KDM5b with TLE4 results in an increased efficiency of H3K4me3 demethylation.

4.3.4 Association of KDM5b with TLE4 allows nucleosomal demethylation

Past studies suggest that the ability to demethylate H3K4me3 on a histone substrate is not easily translated to the same capability on a nucleosomal substrate. As histones exist in nucleosomes in live cells, the ability of an enzyme to recognize, bind and act on a nucleosomal substrate is pertinent to its *in vivo* role. Most histone modifying enzymes require the recruitment of co-factors or recognition of transcription factors in order to confer nucleosomal demethylation. KDM1, for example, requires the SANT domain of coREST to recognize and act on a nucleosomal substrate (Lee et al. 2005). No such co-factor or co-repressor has yet to be identified for the KDM5 family of demethylases.

TLE4 has the ability to recognize and propagate repressive chromatin structure. This, paired with our knowledge that TLE4 increases in the efficiency of KDM5b demethylation activity on histones, led us to question if association with TLE4 would confer nucleosomal demethylation to KDM5b. Therefore, recombinant rKDM5b and rTLE4 were incubated both separately and together with a nucleosomal substrate. As expected, both rKDM5b and rTLE4 alone displayed no ability to demethylate H3K4me3 on this substrate. Interestingly however, a combination of KDM5b and TLE4 resulted in a 62% decrease of H3K4me3 (Figure 4.4A).

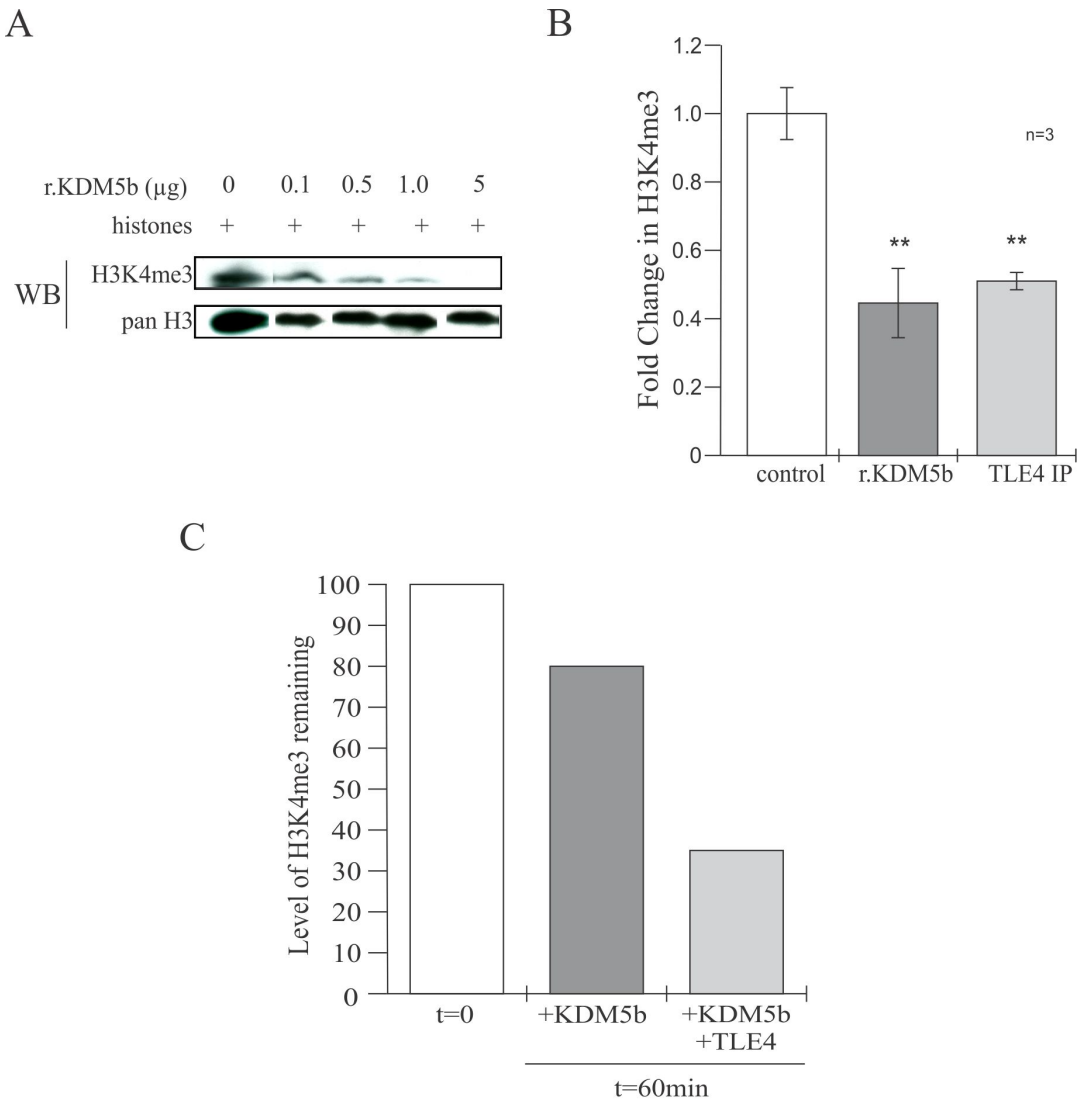

Figure 4.3 TLE4 alters the histone demethylase activity of KDM5b A) isolated recombinant KDM5b (r.KDM5b) has demethylase activity on H3K4me3. r.KDM5b was incubated in increasing quantity (0-5µg) with bulk histones (+) and incubated overnight. Samples were then subjected to SDS-PAGE and western blotted (WB) for H3K4me3. pan H3 was used as a loading control. B) complex isolated through anti-TLE4 antibody shows H3K4me3 demethylase activity. Complex isolated through anti-TLE4 antibody from mESC or r.KDM5b were incubated with bulk histones over night. Samples were then analyzed by ELISA for the corresponding level of H3K4me3 remaining. Graph shows relative amount of a given histone mark after incubating with the enzyme relative to control (no enzyme) set to 1. Graph represents a biological triplicate. C)The addition of TLE4 to KDM5b increases the efficiency of KDM5b at demethylating H3K4me3. MRM-MS was used to determine the amountof H3K4me3 remaining at 60 minutes. **p<0.001

These data suggest that TLE4 has the ability to confer nucleosomal demethylation activity to KDM5b in the absence of any other co-repressors or DNA binding proteins. This supports that the addition of TLE4 is sufficient to allow KDM5b to demethylate H3K4me3 on its *in vivo* substrate, the nucleosome.

To further analyze if TLE4 can confer nucleosomal demethylase activity to KDM5b, recombinant TLE4 (r.TLE4) was combined with 1µg r.KDM5b in increasing molar ratios. The reactions were then analyzed by SDS-PAGE followed by western blotting using antibodies against both H3K4me3 and pan Histone H3. Addition of as little as 1:0.5 molar ratio r.TLE4 resulted in an apparent increase in KDM5b demethylase activity, with increasing amounts of r.TLE4 added resulting in increased demethylation of H3K4me as compared to Pan Histone H3 (Figure 4.4B) supporting that association with TLE4 is sufficient to result in increased enzymatic potential for KDM5b

KDM5 demethylase enzymes contain a high level of sequence and structure homology (Figure 4.4C). We therefore wished to determine if TLE4 represents a co-factor that might be able to allow nucleosomal demethylation from other KDM5 family members. As previous ChIP experiments suggest that KDM5d as well as KDM5b shows occupancy of target gene promoters with TLE4, we wished to ascertain whether TLE4 represents a cofactor for KDM5d as well.

Recombinant KDM5d isolated from insect cells (r.KDM5d) was combined with rTLE4 and incubated on a nucleosomal substrate. Though the amount of methylation remaining showed high variability, the combination of r.KDM5d and r.TLE4 resulted in nucleosomal demethylation of H3K4me3 (Figure 4.4D). This supports that TLE4 has the

ability to act as a cofactor for multiple KDM5 family members and that a role in Groucho mediated repression may represent a conserved role of KDM5 demethylases.

To further analyze the ability of TLE4 to impart nucleosomal demethylase activity, we wished to determine if this role is evolutionarily conserved. The TLE/Groucho family of proteins has often been compared to the Tup1 protein found in yeast. Though there is some question as to whether Tup1 and TLE are in fact homologues and not analogues (Fisher and Caudy 1998), there are striking similarities between the two protein families. Both Tup1 and TLE contain conserved carboxyl terminal WD40 domains and have the ability to bind histones. Additionally, they have a common function, as they both act as transcriptional co-repressors. Tup1 however doesn't conform to the domain structure of the TLE/Groucho family, and does not interact directly with DNA binding partners, but rather utilizes the accessory protein CyC8 to perform this function (Fisher and Caudy 1998).

With this in mind, we isolated TAP-tagged yKDM5 from *S.cerevisiae (open biosystems)* under high salt conditions to reduce co-immunoprecipitants. We then gauged the ability of yKDM5 to demethylate H3K4me3 from a nucleosomal substrate both in the presence and absence of r.TLE4. As expected, yKDM5b showed no ability to demethylate H3K4me3 in the absence of TLE4. The addition of r.TLE4 however resulted in a 46% reduction in the amount of H3K4me3 (Figure 4.4E) remaining, suggesting that even a KDM5 obtained from an alternate species has the ability to utilize TLE4 as an enzymatic cofactor on a nucleosomal substrate, maintaining that the interaction of TLE4

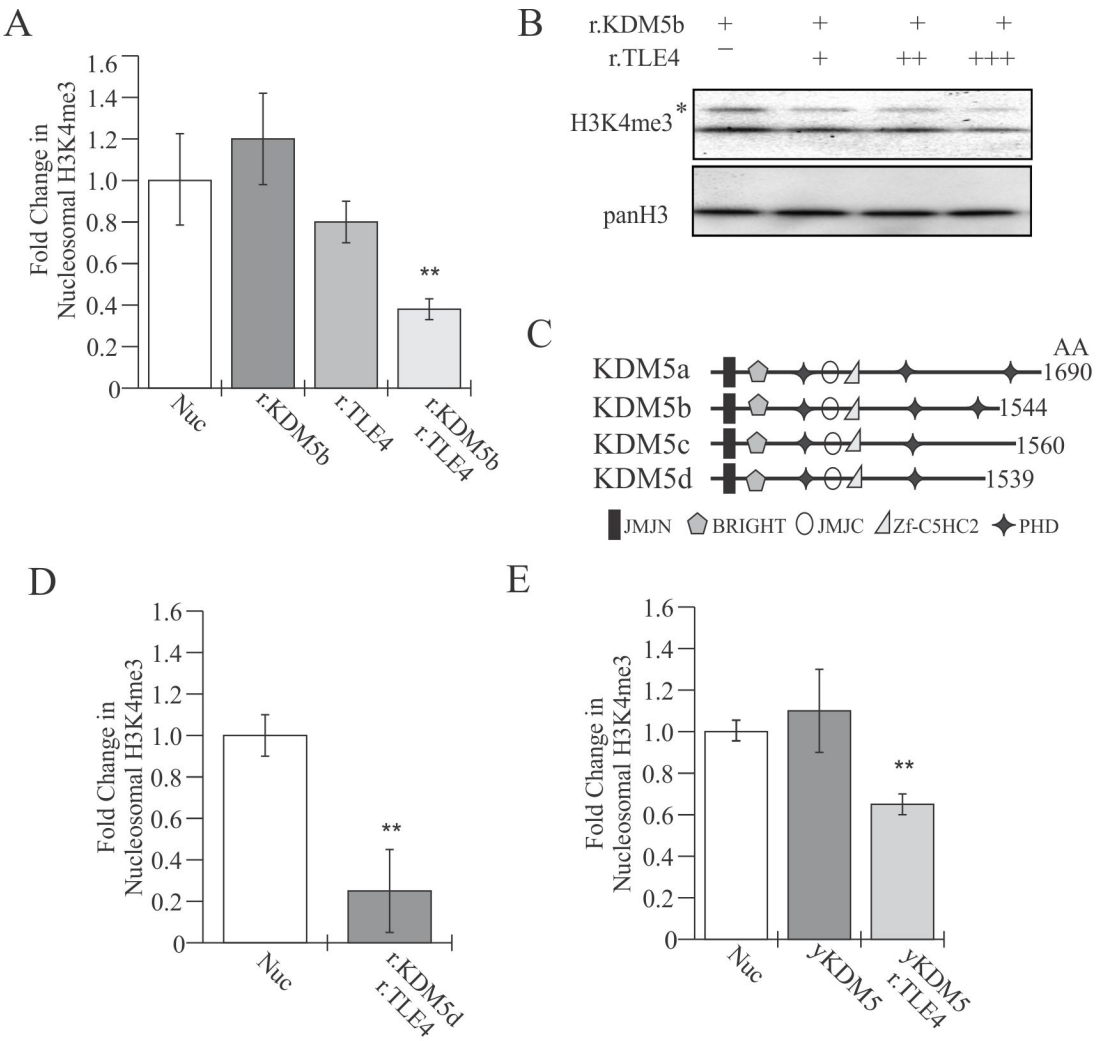

Figure 4.4 TLE4 confers nucleosomal demethylation to KDM5b A) the combination of KDM5b and TLE4 is sufficient to observe nucleosomal demethylation. r.KDM5b, r.TLE4 or a combination of both were incubated with nucleosomes overnight. The corresponding amount of remaining H3K4me3 was then measured. B) Nucleosomes were subjected to demethylation by r.KDM5b and increasing amounts of r.TLE4 overnight. The samples were then subjected to SDS-PAGE and western blotting for H3K4me3 remaining. pan H3 was used as a loading control. * corresponds to top band which represents H3K4me3. C) KDM5 family member domain structure D) TLE4 also represents a cofactor for KDM5d. rKDM5d (baculovirus) and r.TLE4 were incubated with nucleosomes overnight. The corresponding amount of remaining H3K4me3 was then measured. E) TLE4 represents a cofactor for yKDM5. yKDM5 (yeast) and r.TLE4 were incubated with nucleosomes overnight. The corresponding amount of remaining H3K4me3 was then measured. Shown on the graphs (A,D,E) is the relative amount of H3K4me3 after incubating with enzyme or enzyme and cofactor relative to control (no enzyme) set at 1. **,$p<0.001$. All experiments represent a biological triplicate.

with KDM5 family enzymes to increase KDM5 enzymology may represent a conserved function of the Tup1/Groucho/TLE protein family.

4.3.5 Effect of TLE4 on Histone Methylation *in vivo*

To further test our hypothesis that TLE4 is both required and sufficient to increase the efficiency of H3K4me3 demethylation by KDM5b, we transfected YFP tagged KDM5b and YFP tagged TLE4 separately into NIH3T3 cells. The effect of the transfection on global histone methylation was then examined using immunocytochemistry. Differing from most immunocytochemistry, where the utilization of secondary antibodies reduces the ability to examine cells side-by-side in a quantitative fashion, we instead used a primary antibody to either H3K4me3 or H3K9me3 that was directly conjugated to Alexa 555, allowing for quantitative analysis of signal. As could be expected, ectopic expression of YFP-KDM5b resulted in a distinct loss of H3K4me3 in contrast to adjacent non-transfected cells which maintained strong H3K4me3 signal (Figure 4.5A(b), quantified in Figure 4.5B), arrow heads show transfected cells). This is in contrast to the result of transfection of YFP alone, which resulted in little alteration to H3K4me3 signal (Figure 4.5A(a). Quantification of intensity taken within the nucleus only, shows a significant decrease of 66% of the H3K4me3 signal after transfection with YFP-KDM5b as compared to YFP alone (Figure 4.5B, p value<0.001).

With this in mind, we questioned whether the over-expression of YFP-TLE4 alone would result in an alteration of global H3K4me3 levels, similar to KDM5b. In theory, if TLE4 is required for KDM5b-based demethylation activity, an increase in the availability

of the co-repressor may result in an overall increase in enzymatic activity, resulting in a reduction of global levels of H3K4me3. Ectopic expression of YFP-TLE4 led to a slight decrease in H3K4me3 as compared to adjacent un-transfected cells though the difference did not appear to be as phenotypically distinct as that observed with the over-expression of KDM5b (Figure 4.5A(c)). Quantification of nuclear signal after the over-expression of YFP-TLE4 showed highly variable results, though a significant decrease of 23% of the H3K4me3 signal as compared to YFP alone is noted (Figure 4.5B, pvalue<0.001). These results are not surprising as TLE4 plays many other roles within the cell. It is therefore doubtful that ectopic expression would result in 100% of the over-expressed TLE4 being used as an enzymatic co-repressor for KDM5b. Results however still suggest that over-expression of TLE4 does result in an alteration to H3K4me3 levels, supporting a role for TLE4 in the *in vivo* demethylation of H3K4me3.

In order to ensure specificity, these experiments were repeated using H3K9me3 as a substrate. Ectopic expression of YFP-KDM5b resulted in a mild phenotypic increase in H3K9me3 signal in contrast to adjacent non-transfected cells (Figure 4.6A (b)). This result was quantified to show a 40% increase in H3K9me3 expression after transfection with YFP KDM5b (Figure 4.6B, p value<0.001) when compared to transfection with YFP alone, which resulted in no visible change to H3K9me3 levels (Figure 4.6A (a)). Though surprising that KDM5b would have such an effect on an off-target histone modification as KDM5b has no known enzymology on H3K9me3, results from H3K4me3 analysis suggest that ectopic expression of KDM5b results in a dramatic alteration to global

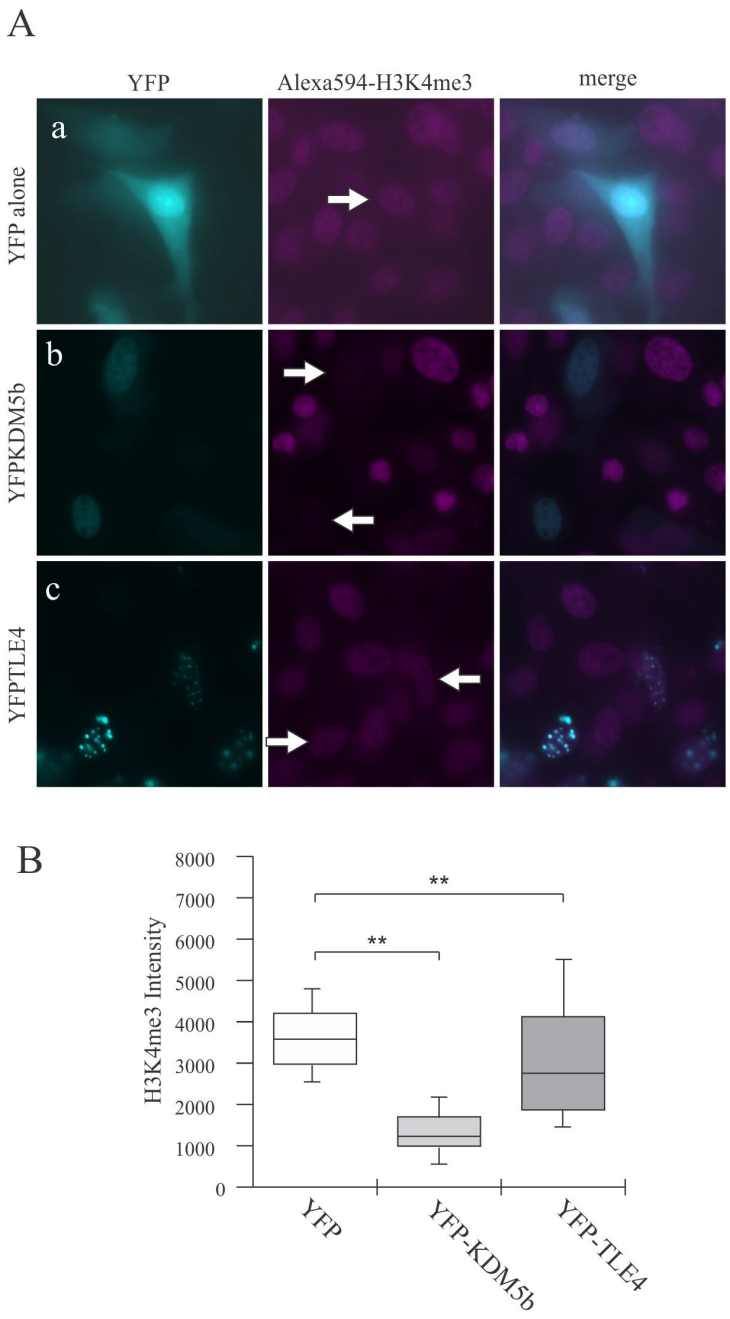

Figure 4.5 TLE4 alters global levels of H3K4me3 A) NIH3T3 cells were transiently transfected with EYFP alone (a), EYFP-KDM5b (b) or EYFP-TLE4 (c), immunoflourescence was then performed with an antibody against H3K4me3 directly conjugated to Alexa 555 and cells were imaged at 40x. White arrows represent transfected cells B) The addition of EYFP-KDM5b or EYFP-TLE4 results in a decrease in global levels of H3K4me3. Box and whisker plot representing H3K4me3 signal intensity. **, P<0.001 Line=mean; box=1 standard deviation from the mean; whiskers= 2 standard deviations from the mean. N=3, n>20 for each transfection.

chromatin signatures. As H3K9me3 is generally considered to be a histone mark of transcriptional repression, it might be expected that over expression of a transcriptional repressor would lead to an increase in its global expression. Ectopic expression of YFP-TLE4 however, led to no observable or quantifiable change in H3K9me3 levels (Figure 4.6A(c), quantified in Figure 4.6B). Taken together, these data support a model in which TLE4 plays a role as a co-repressor specific to KDM5b and the demethylation of H3K4me3 *in vivo*, and that over-expression of TLE4 alone is sufficient to observe small, yet significant changes in the global level of H3K4me3.

4.4 Discussion

Regulation of transcription is a complex and detailed process involving the cooperation of multiple enzymes, co-repressors, and modification marks to both the histone core and histone tail at the promoters of specific genes of interest. Though recent work within this field has increased our knowledge of transcription as well as the enzymes that regulate it, exact mechanisms of control over the localization and activity of these enzymes at times remains unclear. KDM5b, a member of the JmjC demethylase enzyme family, was discovered to be a histone demethylase and potent transcriptional repressor through its ability to demethylate H3K4me3 in 2007 (Yamane et al. 2007; Xiang et al. 2007), however very little is known about how this enzyme interacts with, or is recruited to promoters of interest *in vivo.* In this study, we have examined the interaction of KDM5b with TLE4, a transcriptional co-repressor of the groucho family. By identifying TLE4 as an enzymatic cofactor for KDM5b we have elucidated a minimal

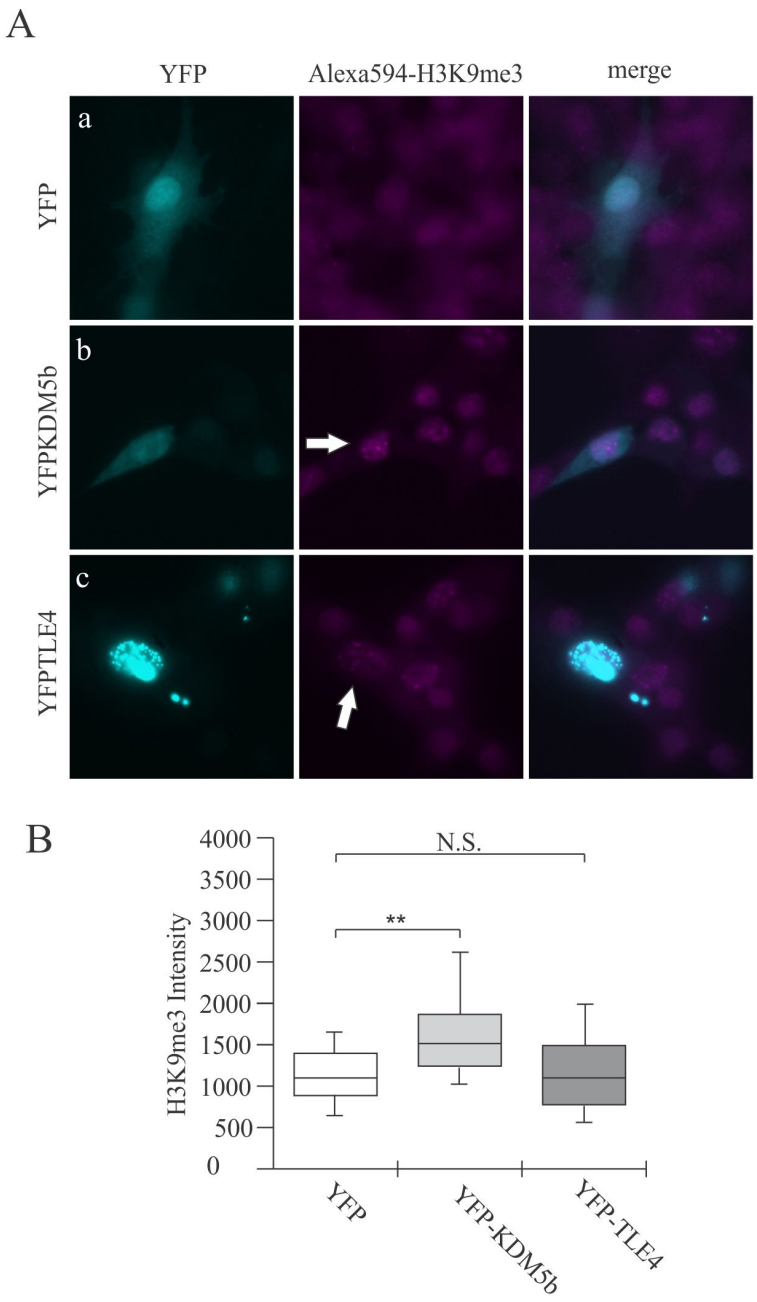

Figure 4.6 TLE4 has no effect on the global levels of H3K9me3 A) NIH3T3 cells were transiently transfected with EYFP alone (a), EYFP-KDM5b (b) or EYFP-TLE4 (c), immunoflourescence was then performed with an antibody against H3K9me3 directly conjugated to Alexa 555 and cells were imaged at 40x. White arrows represent transfected cells B) The addition of EYFP-KDM5b results in a decrease in the global levels of H3K9me3 Addition of EYFP-TLE4 results in no alteration to global levels of H3K9me3. Box and whisker plot representing H3K9me3 signal intensity. **, $P<0.001$ Line=mean; box=1 standard deviation from the mean; whiskers= 2 standard deviations from the mean. N=3, n>20 for each transfection.

required complex for the enzymology of KDM5b on its *in vivo* nucleosomal substrate. Additionally, we have identified a novel molecular mechanism by which TLE4 can contribute to transcriptional repression, increasing our knowledge of Groucho-mediated roles during epigenetic regulation.

4.4.1 The interaction of TLE4 and KDM5b is highly-context dependent

Recent work has outlined the notion that though transcriptional regulators may require precise interaction with co-repressors, that these interactions may be extraordinarily context specific. Moshkin et al. report that the enzymatic activities of both lid (dKDM5) and Rpd3 (dHDAC) may be modulated through completely separate association with differing complexes and interacting partners, including Groucho, and that stable complexes may not occur (Moshkin et al. 2009). This theory would lend credence to the reason that although the enzymatic complexes required for active nucleosomal activity for many transcriptional regulators, such as KDM1's requirement for CoREST (Lee et al. 2005), were delineated quickly, a stable enzymatic complex for any of the KDM5 family members has not yet been discovered.

TLE4 and KDM5b were shown to interact in co-immunoprecipitation experiments under physiological conditions without the requirement for ectopic expression of either the enzyme or the co-repressor. The interaction between TLE4 and KDM5b was found to be weak, yet reproducible, suggesting that it is possible that these two proteins interact only in the context of their common target genes. Our chromatin immunoprecipitation results show that they are both recruited to a small region of the promoter. Additionally,

the interaction of KDM5b and TLE4 appears to be specific as no interaction of KDM5b with TLE family member TLE1 was determined, even though TLE4 can associate with TLE1 in the same eluate.

Another possibility is that the interaction of TLE4 and KDM5b is transient, making analysis of a protein by standard methods such as SDS-PAGE difficult. In the context of this study, the interaction of TLE4 and KDM5b was additionally demonstrated utilizing FLIM FRET, a method which allows us to analyze protein interactions in real time in a live cell context. This technology is not only resistant to many of the issues usually associated with over expression, but is extremely sensitive in a time based manner, allowing even the most transient of interactions to be detected. Analysis by FLIM FRET allowed us to concentrate on specific regions within the cell, such as the nuclear puncta formed by TLE4, increasing the strength of our results by narrowing down the region of the cell analyzed in real time.

4.4.2 Association of KDM5b with TLE4 is Sufficient to Result in Nucleosomal Demethylation of H3K4me3

The interaction of TLE4 was then demonstrated to be sufficient to both increase the efficiency of KDM5b based demethylase activity on a histone substrate, and to allow for nucleosomal demethylation *in vitro.* As KDM5 proteins are highly conserved, the ability of TLE4 to confer nucleosomal demethylation properties to both KDM5d and the yeast homologue of KDM5, was then analyzed and it was determined that TLE4 represents a potentially conserved cofactor for KDM5 demethylases. TLE4 has

previously been shown to have a high binding affinity for histone H3 (Palaparti, Baratz, and Stifani 1997), and may maintain this ability in a nucleosomal context, aiding KDM5b to recognize and bind its substrate. Though TLE4 and KDM5b complexes may be recruited separately to chromatin of interest, the localization of one complex may be pertinent to the recruitment of the second. Further work will need to be completed to understand the temporal regulation of this process.

4.4.3 Recruitment of TLE4 Leads to the Formation and Stabilization of a Repressive Chromatin State

Finally, the ability of TLE4 to alter global levels of H3K4me3 was analyzed by immunocytochemistry, and it was found that the ectopic expression of TLE4 alone was sufficient to result in a reduction of H3K4me3 in a global context. Hence, TLE4 appears to act as a co-repressor of KDM5b both *in vitro* and *in vivo*. Previous work on Groucho-mediated repression has detailed a role for Groucho co-repressors in nucleosomal compaction, though the mechanism of action through which this occurred was not known. This fits in well with previously hypothesized models of Groucho mediated repression. These models have suggested a system by which after recruitment of Groucho to chromatin by specific DNA binding sites, Groucho is able to propogate a repressive chromatin state stretching far beyond its actual recruitment site by feature of its ability to tetramerize. This is similar to what is seen with Sir3/Sir4 proteins, which are required to polymerize along chromatin to exert transcriptional effects. It has been suggested that this may provide a recruitment interface for other chromatin modifying proteins and may

serve to establish or maintain silenced chromatin (Chen and Courey 2000). Though the ability of Groucho family members to interact with HDACs (Chen and Courey 2000; Chen et al. 1999; Sekiya and Zaret 2007) and contribute to transcriptional repression is well established, Groucho is well known to contain additional repressive domains that were HDAC independent. Recently, the role of TLE4 in epigenetic silencing as a recruitment "switch" for Pax 2 was completed, detailing that in the absence of TLE4, Pax2 gains the ability to recruit PTIP and associated KMT complexes leading to an increase in H3K4me3. In the presence of TLE4 however, Pax2 can initiate polycomb mediated gene silencing, resulting in an increase of H3K27me3 (Patel et al. 2012). These results, taken together with both previously modeled Groucho-mediated repression and the results shown here, provide the ground work for an eloquent system where the recruitment of TLE4, a single co-repressor, leads both to a reduction of H3K4me3 through the recruitment and association of a potent H3K4me3 demethylase, KDM5b, as well as a block in the recruitment of PTIP and KMT complexes to Pax2, preventing H3K4me3 from becoming re-methylated at these sites. At the same time, recruitment of polycomb repressive complexes would result in an increase of H3K27 methylation, leading to the formation and stabilization of a repressive chromatin state.

4.5 Conclusion

Data provided within this chapter provides evidence of an interaction between KDM5b and the groucho co-repressor TLE4. Results are provided utilizing a myriad of

techniques including pull down and *in vivo* analysis of the interaction utilizing FLIM-FRET. Though each of these techniques possess weaknesses, the combination of the two helps to provide support for the detailing of a real interaction. Alternate techniques such as bait and prey techniques like a yeast two hybrid screen, could also be performed to provide additional support, however modern techniques such as FLIM FRET are generally considered to be highly accurate at determining interactions *in vivo.* The methodology utilized for FLIM FRET is independent of concentration of the constructs, which can be further verified by utilizing differing concentrations of both the donor and acceptor and repeating the experiment, expecting to garner the same results.

Additionally, enzymology of KDM5b in the presence of TLE4 could be further analyzed by creating a recombinant verions of an enzymatic dead KDM5b mutant, ensuring that observed enzymology is that of KDM5b.

Overall however, this work, paired with previous findings, provides us with a more in depth understanding of the molecular mechanism of Groucho-mediated repression. It also provides us with the first observed ability of a KDM5 demethylase to demethylate within a nucleosomal context and provides the basis for more in depth analysis of the *in vivo* demethylation of H3K4me3 by the KDM5 family of enzymes. As KDM5b expression is of limited expression within most adult tissues, showing increased activation during oncogenesis (Barrett et al. 2002), this introduces a potentially novel role for Groucho mediated transcriptional repression in the development of several types of cancer. Further analysis, using additional Groucho family members, as well as KDM5 enzymes, will hopefully provide a more detailed understanding of this complex

interaction, and may provide pertinent insight into the development of anti-cancer molecular targets.

4.6 Acknowledgements

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Conflict of Interest Statement

The authors declare no conflict of interest.

Chapter 5 Discussion

Preamble

This body of work describes novel findings in the regulation of the histone demethylase KDM5b. We introduce two novel, and pertinent pieces of knowledge surrounding this enzyme: a novel histone mark, H2BK43me2 that can be regulated by KDM5b, and the first evidence that*, in vivo*, KDM5b is activated through association with an enzymatic complex.

The connections of this work to current theories in the transcriptional regulation field will be discussed in this chapter, arguing that these novel discoveries in KDM5b regulation could provide a model by which to increase our comprehension of transcriptional regulation. Additionally, contributing to our understanding of this enzyme will prove pertinent in our ability to delineate the exact role of KDM5b in development and disease progression.

5.1 Connections to Current Theories

5.1.1 Epigenetic Regulators as Multi-target Enzymes

5.1.1.1 Multiple Histone Targets

The recognition of enzymatic substrates is complex and often perplexing. Studies would suggest that histone methylating and demethylating enzymes are relatively non-specific, with only a few amino acids required to determine substrate selectivity. However, research on most other enzymes shows the opposite to be true, where enzymology appears to be stringently regulated at specific targets. The work presented within this thesis shows that KDM5b, a histone demethylase belonging to the KDM5 family of JmjC containing demethylases, has the ability to act on histone targets other than its classically known targets of H3K4me3 and H3K4me2. Though this represents a novel discovery for KDM5b specifically, the discovery that a histone-modifying enzyme can target multiple histone modifications is not new. Modifying enzymes capable of recognizing multiple substrates generally fit into one of two categories.; First, enzymes that are capable of recognizing multiple histone modifications all relating to the same transcritional read out and second, enzymes that may switch between activator and repressor, depending on upstream signalling. The first category is exemplified by G9a, a H3K9 methy-transferase enzyme which is known to have additional enzymatic activity on H3K27me3 (Tachibana et al. 2001) and has recently been shown to mediate the methylation of H1bK25 (Rathert et al. 2008), all marks related to transcriptional

repression. In the second category is exemplified by the histone demethylase KDM1. Although KDM1 is classically known as a transcriptional repressor through demethylation of the activating marks H3K4me2 and H3K4me1, the presence of this demethylase has also been found to result in the opposite effect through interaction with the androgen receptor (AR) (Metzger et al. 2005). Under these conditions, Protein Kinase C β1 (PKCβ1) is recruited at the receptor and mediates the phosphorylation of Histone 3 Threonine 6 (H3T6). This blocks the demethylation H3K4 by KDM1 and catalyzes the removal of H3K9 methylation instead (Metzger et al. 2010), leading to transcriptional activation.

Within this body of work, we make the first discovery that a KDM5 family member acts on a histone target other than the classically recognized targets of H3K4me3 and H3K4me2. We suggest, that although KDM5b does have the ability to demethylate H3K4me3 as previously suggested by others, H2BK43me2 represents a novel enzymatic target, additionally required to mark transcriptionally active genes. Enzymology studies, both by MRM-MS/MS and *in vitro* demethylase assay, would suggest that H2BK43me2 most likely represents the primary target for KDM5b, as enzymology appears both to be more rapid on this target, and does not require the addition of enzymatic cofactors. At first glance, it would appear as though this discovery places KDM5b within the first subset of enzymes, those capable of acting on multiple histone marks with the same transcriptional read out: gene activation. However, taking a closer look, the work presented here actually suggests a novel ideal of multi-targeting. Though G9a has the ability to recognize multiple histone modification sites leading to their methylation *in*

vitro, no evidence has been provided to suggest that the enzymology of G9a on multiple targets is required for transcriptional regulation *in vivo*. Our study however, suggests that the demethylation of both H2BK43me2 and H3K4me3 by KDM5b is required for appropriate transcriptional programming. Recent studies have demonstrated that the presence of H3K4me3 at the promoter of a gene of interest is not sufficient to assume transcriptional activation. In combination, these discoveries lead us to propose a model whereby the requirement for additional transcriptional markers, potentially H2BK43me2, may be required both for full gene activation on a promoter basis, and for appropriate differentiation to occur during development and may represent a novel form of histone cross talk.

5.1.1.2 Positional Binding and Histone Cross-Talk

A lasting question within the field of epigenetics is how histone-modifying enzymes are recruited to the appropriate promoters at the correct time. The selectivity of histone targets appears to be conferred by either binding directly with enzymatic targets, or the binding of other protein domains which positions the catalytic domain in line with particular residues. Recent studies have shown that several JmjC domain containing demethylases require the presence of non-enzymatic domains to recognize and bind off target residues in order to position catalytic domains to the residue on which enzymatic activity is directed. This is exemplified by PHF8, a JjmC domain containing demethylase with activity towards H3K9, H3K27 and H4K20. Studies show that the efficiency of PHF8 as a H3K9 demethylase is increased in the presence of H3K4me3. The PHD

domain of PHF8 binds to H3K4me3, a mark of transcriptional activation. This then positions the catalytic JmjC domain in proximity to H3K9 and results in the demethylation of this mark. This suggests that the removal of an "OFF" mark by PHF8 depends on the binding of the same enzyme to an "ON" mark (Horton et al. 2010). This theory of multiple binding points is supported by KDM5C, a family member of KDM5b. KDM5C, a H3K4 demethylase can both recognize and bind H3K9me3 through its PHD domain (Iwase et al. 2007; Li et al. 2008). Recognition then leads to the demethylation of H3K4me3, suggesting that removal of an "ON" mark by KDM5C depends on the binding of the same enzyme to an "OFF" mark, similar, yet opposite to what is observed with PHF8.

It has been suggested that binding multiple substrates in concert could represent a method of both enhancing an enzyme's activity, and regulating enyzme specificity. Work presented here however points to a theory of histone cross talk, rather than downstream binding of a non-enzymatic domain. This theory of histone crosstalk is common within the field, lending support to the histone code hypothesis. It appears that histone crosstalk, or the requirement for multiple histone modifications to occur either simultaneously, or in sequence, is often required for appropriate epigenetic signaling. In addition to aiding in the binding and positioning of modifying enzymes, it is very often the case that the presence or removal of completely separate histone modifications may be required in order for enzymology to occur. These models of cross talk can occur within the same histone tail, as is the case with histone 3. On histone 3, acetylation of both H3K9 and H3K14 must be removed prior to the methylation of H3K9 being able to occur (Rea et al.

2000; Zhang and Reinberg 2001), potentially due to steric hindrance. Cross-talk may also occur on separate histones, such as the methylation of H3K27me3 being a required to localize the ubiquination of H2AK119 (Simon and Kingston 2009). Using the work presented within this thesis, we propose that within differentiating cells, the presence of H2BK43me2 may act as a type of signaling mechanism to recruit KDM5b to required promoters to allow differentiation to proceed. This is similar to the recruiting mechanisms mentioned above as it provides a theory in which a post-translational modification on one histone is required to recruit a histone-modifying enzyme to another histone of interest. However, this model is different in that recruitment is followed by demethylation of both the original recruitment signal and an additional substrate, H3K4me3, by the same enzyme, resulting in downstream transcriptional activation. Methylation of H2BK43 may represent a developmentally specific recruiter for KDM5b, allowing this demethylase to act on H3K4me3 at specific promoters at specific times. Abolishing the ability of H2BK43me2 to be methylated leads to a block in terminal differentiation. This is similar to the downstream result of over-expressing KDM5b within ESC. Interestingly, the over-expression of KDM5b does not appreciably alter global levels of H3K4me3. Lack of global alteration to H3K4me3, suggests that within the context of ESC the requirement for appropriate H2BK43 methylation is intimately tied to the ability of cells to differentiate, at least towards the neural lineage. Conversely, the global level of H3K4 methylation, which may be rescued by other demethylases targeting this mark, does not appear to be required as a checkpoint for differentiation in the context studied within this thesis.

5.1.2 KDM5b Plays a Role in Groucho-Mediated Repression

5.1.2.1 Historical Context

Though the direct connection between KDM5b and TLE4 is a novel finding presented within this thesis, the biological roles of these two proteins overlap in several contexts.

The TLE family of proteins, including the mouse Groucho family and the Tup1 protein found within yeast, represent transcriptional co-repressors, part of a family of proteins known to play a role in transcriptional repression without the ability to directly bind to the DNA molecule itself (Chen and Courey 2000). This distinct lack of inherent enzymology, paired with work indicating binding to histones, with a preference and specificity for histone H3, provides a logical framework on which to assume that the TLE family is involved in epigenetic remodeling, leading to transcriptional repression.

KDM5b itself has been shown to recognize and bind H3 *in vitro*, with a specificity for H3K4me3, however this ability to target appears to be lost *in vivo* within the context of the nucleosome. This is not surprising, given that the majority of transcriptional regulators require the presence of an enzymatic complex be active. However, a complex or cofactor required to enhance KDM5b enzymology, has, until now, remained elusive.

Findings presented here argue that the co-localization of both KDM5b and TLE4 in a distinct and precise location on the promoter of a gene of interest is required for enzymatic activity of KDM5b to proceed, and thus transcriptional repression to occur. The theory of a connection between KDM5b and Groucho-mediated repression is not altogether novel, with the origin of the theory being presented in 2003 when it was found

that KDM5b enhanced the repressive ability of the developmental transcription factors Pax9 and BF-1 (Tan et al. 2003). Since Pax9 and BF-1 are unrelated, it was surprising, at the time, that KDM5b could act as a transcriptional co-repressor for both. A striking similarity between the two however, is that both BF-1 and PAX proteins are known to interact with members of the Groucho family. It is within this work that the first suggestion of a role for KDM5b in Groucho-mediated repression was made.

5.1.2.2 A Requirement for Multiple Complexes

Why then, if the interaction between KDM5b and the Groucho family is plausible, and has been suggested previously, did the relationship between KDM5b and TLE4 take this long to uncover? The most important aspect of the answer to this question is the method utilized within the field to determine and discover transcriptional complexes. It is very often assumed that epigenetic regulators exist within large and sometimes varied multi-protein complexes. As was outlined in chapter 1 of this thesis, proteins such as MLL and KDM1, though they possess enzymology *in vitro*, require the presence of distinct cofactors in order to possess enzymology in the context of the nucleosome (Lee et al. 2005). Given the extraordinary complexity of chromatin folding (see chapter 1), the requirement for cofactors involved in the recognition and localization to the targets of the enzyme is not surprising

. In order to act appropriately on a substrate, an enzyme must be able to both find, and come in to contact with this substrate in an *in vivo* context. The field of transcriptional regulation has long assumed therefore, that enzymes responsible for transcriptional

remodeling must exist within their multi-protein complexes in all contexts *in vivo,* as they are required to perform their function. This theory however has recently been debated, with the suggestion that the requirement of enzymes to be localized within distinct and non-changing complexes is antiquated. Moshkin et al. for instance, instead present a model in which a single transcriptional regulator, dKDM5 (LID), is present within several different complexes. These complexes may then, in turn, interact with additional complexes of co-repressors that are localized completely separately to a gene of interest. This complex web of interaction leads to potentially several downstream epigenetic modifications; the cross talk of which leads to transcriptional repression or activation (Moshkin et al. 2009). Other groups have also suggested similar theories, in which the recruitment of histone modifying enzymes to their genomic targets is mediated through multiple required interactions. Individually, such as is noted by KDM5b and TLE4, these interactions may be weak or extremely transient, however taken in the full context, they together result in stable binding or enzymology between the modifying enzyme and target sequence (Ruthenburg, Allis, and Wysocka 2007).

The classical way of thinking within the field, where enzymatic complexes remain intact throughout the cell rather than in a context dependent fashion, would explain why the interaction between KDM5b and TLE4 has been long awaited. Though KDM5b and TLE are localized to the same distinct area on the promoter of genes of interest, the direct relationship between the two in the context of a pull down, though consistent, is considered weak. Attempts to utilize classic techniques such as Mass Spectrometry to prove a connection between the two has been unsuccessful (see chapter 6), suggesting

that the interaction is transient, or extremely regulated. Utilization of more novel methodologies, such as FLIM-FRET, which allows us to study the interaction of proteins in 3D space within live cells, paired with ChIP, has aided to support the conclusions presented in this thesis, that TLE4 and KDM5b do interact, and that this interaction is more than likely represented by a small subset of each protein located specifically at promoters of interest.

The requirement of additional cofactors in separately recruited complexes provides a further explanation for the observation that KDM5 enzymes have often been found to be localized to promoters which have a maintained presence of H3K4me3 (Schmitz et al. 2011). The mere presence of other epigenetic regulators, such as the H3K27me3 demethylase UTX, at a promoter of interest results in distinct removal of the targeted modification. UTX and H3K27me3 are rarely observed at the same promoter (Islam et al. 2011). This suggests that coupling of an enzymatic reaction to enzymatic recruitment may occur in some cases, such as that of UTX, but remain separate in others. Even the recruitment of closely related histone modifying enzymes appears to be regulated differently. It appears that KDM5b can be recruited to certain KDM5a target genes only in the absence of KDM5a expression. Under wildtype conditions, in the presence of KDM5a, KDM5b is completely absent from these locations. This is of interest since it suggests that under normal circumstances; KDM5b is physically blocked from these promoters, either by the presence of KDM5a itself, or potentially by KDM5a binding partners(Islam et al. 2011). This further supports a model in which recruitment to genes of interest is not simply mediated by the presence of the target itself, but instead

by a far more complex cascade of cellular signaling and protein recruitment. Additional experiments which evaluate whether the localization of TLE4 is also altered in the absence of KDM5b would be of interest. Further more, the utilization of enyzmatic dead mutants of KDM5b may aid to seperate the concepts of enzymology and localization, helping to determine if active enzymology is required for KDM5b's localization to target genes.

5.1.2.3 Requirement for Additional Complex Members

Downstream transcriptional changes often occur after a complex series of alterations to a promoter of interest (Moshkin et al. 2009), resulting from the removal and addition of multitudinous post-translational modifications at different locations within a single promoter. TLEs and KDM5b have both been shown to interact with HDACs, and hypoacetylation of promoters of interest is also considered to be required for transcriptional silencing. However, most *in vitro* studies concentrate on a specific alteration at a specific target (i.e. removal of H3K4me3) and therefore do not illustrate the full requirements for *in vivo* gene silencing. Though the combination of TLE4 and KDM5b appears to be sufficient for the demethylation of H3K4me3 in a nucleosomal context *in vitro,* it is unlikely that these two proteins represent a complex unto themselves. The reality is more likely that a complex interaction between multiple proteins is required for downstream transcriptional changes to occur.

5.2 KDM5b as a Pharmaceutical Target: what do we know?

5.2.1 Epigenetic Regulators and Pharmaceutical Intervention

Interest in the development of pharmaceutical intervention targeted to histone modifying proteins has been gaining momentum. Alterations to epigenetic organization have been found to be involved in both the development and pathogenesis of several types of cancer. HDAC inhibitors have, until recently, been the main focus of the epigenetic pharmaceutical industry. The HDAC inhibitors Vorinostat (suberoylanimide hydroxamic acid, SAHA) and Romidepsin (depsipeptide, FK-228) have recently garnered FDA approval for the treatment of refractory cutaneous T-cell lymphoma and are under clinical trial for the treatment of additional cancer types (Mai 2010). Additional HDAC inhibitors such as Entinostat, and the anticonvulsant valproic acid are also being currently studied in clinical trials (Song, Han, and Bang 2011). However, though over-expression, mutation and alteration to many histone demethylase enzymes has been seen in several types of cancer, little has been done to target demethylase enzymes as possible treatment options. Recent studies have detailed several KDM1 inhibitors with the hope that these compounds may be useful in the treatment of several types of cancer(Huang, Greene et al. 2007). Inhibitors against JmjC domain-containing enzymes have not been identified, though compounds targeting the cofactors essential to their enzymology have proven to be promising. These include zinc ejecting compounds (Sekirnik et al. 2009), iron chelators (King et al. 2010), and analogues to α-ketoglutarate (Chen et al. 2007), which have been moderately successful for the JMJD2 (KDM4) sub-class. Novel assays are

being developed to screen and identify novel candidates against these targets (Yu, Fisch et al. 2012; Rose et al. 2010)

Many aspects of KDM5 demethylases, including their apparent roles in oncogenesis and development, make them potentially lucrative targets for pharmaceutical intervention. Specifically, KDM5b represents a tempting target due to its low expression level in most adult human tissues and over-expression or re-expression in many cancer cell types (Barrett et al. 2002; Xiang et al. 2007). Immunotherapy approaches against KDM5b have been investigated recently with results suggesting that KDM5b may represent a tumor associated antigen (TAA) for breast cancer (Coleman et al. 2010), although inhibitors to this subfamily have yet to be investigated.

The results presented in this thesis draw attention to the requirement for a full understanding of how these enzymes function and are regulated *in vivo* in order to competently design and target small molecule inhibitors.

5.2.2 Novel Model of KDM5b Mediated Transcriptional Repression

The discovery that KDM5b represents a multi-targeting enzyme is a novel discovery for the KDM5 family of demethylases. This notion opens the doors for furthering the understanding that most transcriptional regulators act in a far more complex way than directly on one histone substrate, resulting in a direct downstream transcriptional readout.

5.2.2.1 The Potential for non-Histone Modification

This complexity of regulation is again multiplied through the more recent discovery that many histone-modifying proteins are not sequestered to histones and can lead to the modification of non-histone substrates. In many cases, the modification of these proteins leads to distinct downstream effects. Recent studies have found a role for histone modifiers in both the acetylation and methylation of several important cellular regulators, such as p53. p53 is an important tumour suppressor, known to be a DNA binding protein related to transcriptional activation and is found to be mutated in more than 50% of all human cancers (Vogelstein, Lane, and Levine 2000). p53 represents the first non-histone substrate to be subject to acetylation and is additionally known to be both SUMOylated and ubiquitinated. Acetylation of p53 occurs mostly at the C-terminus, with lysines 320, 373, and 382 representing the most commonly modified targets. Lysines 370, 372 and 382 have also been reported to show acetylation (Bode and Dong 2004). More recently, methylation of p53 has been added to the list of post-translational modifications affecting this tumour suppressor, occurring at K370, K372 and K382 (Orphanides and Reinberg 2002; Chuikov et al. 2004; Shi et al. 2007; Huang et al. 2006) and mediated by HMTs including G9a (Huang, Dorsey et al. 2010). The presence of acetylation and methylation marks on p53 appear to function in an extraordinarily similar way to those present on histone targets, with acetylation leading to the induction of p53 as a transcription factor, and the result of methylation depending on both the particular residue that is modified and the level of methylation present (ie mono, di, tri-methylation) (West et al. 2010; Orphanides and Reinberg 2002). KDM1 is a demethylase that targets

p53 (Huang, Sengupta et al. 2007), binding to p53 and leading to the demethylation of K370. This demethylation results in both the repression of p53-mediated transcriptional activation and an inhibition p53 involvement in apoptosis, similar to the role of KDM1 as a transcriptional repressor when mediating the removal of K4 methylation from a histone substrate(Huang, Sengupta et al. 2007). Interestingly, no additional demethylases have yet to be discovered for p53, leaving the possibility that a JmjC domain enzyme, such as KDM5b, may target this non-histone substrate. This raises the question of the exact role of KDM5b in cancer. Though KDM5b has been considered play both oncogenic and tumour suppressive role, its exact involvement in the process of transformation has yet to be uncovered. Work proposed in this thesis suggests that KDM5b acts on additional histone targets, and presents the possibility that KDM5b may target non-histone proteins as well. This is similar to what is observed with KDM1. The role of KDM1 in cancer progression is unknown due this demethylase being involved in several aspects of cellular biology. KDM1 is involved in the demethylation of multiple histone substrates (leading both to transcriptional repression and activation), has a role in the regulation of p53 (a known tumour suppressor), and has additionally been found to regulate DNA methylation (another contributive force in transformation). In order to target inhibitors efficiently again KDM5b, it is pertinent for researchers to understand the breadth of reaction capability possible for this enzyme. Without knowing all the downstream targets of the enzyme of interest, there is no ability to have an accurate read out of the functionality of an inhibitor.

5.2.2.2 Utilization of H2BK43me2 as an Epigenetic marker for Cancer

Presented here, we identify a novel histone mark, the methylation of Histone H2B at lysine 43. Previously, this modification was not characterized within mammals, and our work has just presented a starting place for the study of its exact role in mammalian development and gene expression. We present a theory in which the levels of H2BK43me2 methylation dictate the ability of the cell to respond appropriately to cellular cues in order to differentiate properly down the neural lineage. It is unknown at this time whether the level of H2BK43 methylation is pertinent to the differentiation of alternate lineages, however this would be an interesting question to address in future studies. The expression level of H2BK43me2 was altered dramatically over the process of differentiation, with the expression of this histone mark being 10,000-fold higher on day 7 of neural differentiation than on day 0 (Chapter 3). Blocking the methylation of H2BK43, or over-expression of KDM5b which reduces overall methylation levels, prevented cells from responding to differentiation cues and led to an increase in proliferative capacity. These alterations in cellular dynamics are similar to those observed in cancer, where tumour cells garner the ability to increase proliferation and do not respond properly to cellular cues regulating cell survival. Parallels are often drawn between embryonic phenotypes and tumour cells, including the expression level of KDM5b. Though KDM5b is often considered to be "over-expressed" in several cancers (Barrett et al. 2002; Xiang et al. 2007; Hayami et al. 2010), an alternate view-point is that this embryonic demethylase has simply become "re-expressed" at the wrong time. If this is indeed the case, the expression levels of H2BK43me2, which are directly regulated by

KDM5b, may provide an epigenetic marker for tumour cells, with a dramatic decrease in H2BK43 dimethylation representing a de-differentiation event that could lead to inappropriate cellular control. There remains the possibility that the role of KDM5b in the regulation of H2BK43me2 is developmentally specific, and that this histone mark plays no role in the regulation of cancer cells, however the possibility of a novel epigenetic biomarker, and a possible explanation for the role in KDM5b re expression in many cancer cells types, is well worth future investigation.

5.2.3 TLE4 as an Additional Cancer Target

Data presented here supports the role of TLE4 as an enzymatic cofactor for KDM5b, with the presence of TLE4 conferring nucleosomal demethylase activity to this enzyme. It remains unknown however if TLE4 plays any role in the oncogenic or tumour suppressive functions of KDM5b. We suggest the possibility of three potential hypotheses. First, that the interaction of TLE4 with KDM5b is involved in the role of KDM5b as an oncogene or tumour suppressor depending on context. Second, that TLE4 plays no role in KDM5bs oncogenic or tumour suppressive functions, and may merely represent a co-repressor during normal cellular functions. This idea is driven by our hypothesis that KDM5b is located within several different and often changing complexes. The third hypothesis implicates TLE4 and KDM5b in the development of some cancers, but that this role is disconnected from their interaction with each other.

Similar to KDM5b, TLE4 has been implicated as both a potential oncogene and as a tumour suppressor, through differing actions in different cancer types. The loss of both

TLE1 and TLE4 has been connected to a decrease in cell death and apoptosis and an increase in the rate of cellular proliferation in a Kasumi-1 myeloid cell line (Acute myeloid Leukemia, AML) (Dayyani et al. 2008). After over-expression of either TLE4 or TLE1, or a combination of the two, there was a marked increase in apoptosis and cell death, supporting that in AML, these Groucho co-repressors appear to act as tumour suppressors.

Contradictory to this, TLE4 itself has been connected to the proliferative capacity of human colon cancer cells through its regulation by the micro RNA miR-93 (Yu et al. 2011). miR-93 was found to be down-regulated in SW1116 cancer stem cells (Sw1116csc) when compared to SW1116 colon cancer cells. Cancer stem cells are thought to represent the tumourogenic component of cancer cells, possessing many of the qualities normally assigned to normal stem cells: heightened proliferative capacity and differentiation potential (Yu et al. 2011), therefore identification of differentially regulated products within these cells often provides useful therapeutic targets. Using target prediction tools, target mRNAs of miR-93 were determined and found to include both TLE4, and HDAC8, a relatively novel HDAC family member (Van den Wyngaert et al. 2000; Hu et al. 2000). Forced over-expression of miR-93 within SW1116csc leads to a decrease in cellular proliferation as well as a reduction in both the mRNA and protein levels of both TLE4 and HDAC8, found to have heightened expression in SW1116csc when compared to SW1116. The heightened expression of both TLE4 and HDAC8 within SW1116csc suggests that these proteins may play a pertinent role in tumour maintenance. TLE4 and HDAC8 share other similarities, including a connection to the

developmental transcription factor Otx2. Knock down of HDAC8 results in lethality soon after birth, resulting from hemorrhaging due to inappropriate development of the mouse skull. This is similar to what is observed with over-expression of Otx2, suggesting that HDAC8 may either directly regulate this transcription factor, or play a role in regulating an Otx2 regulator. Interestingly, TLE4 is a co-repressor for Otx2, competing with the co-activator Meis2 to regulate expression levels of this transcription factor throughout development (Heimbucher et al. 2007; Agoston and Schulte 2009). This suggests a potential connection between TLE4 and HDAC8, pertinent to both development and cancer progression. A connection through HDACs was one of the first lines drawn between TLEs and KDM5b, both of which are known to interact with HDAC1 family members(Chen et al. 1999; Barrett et al. 2007). Though no direct connection between HDAC8 and KDM5b has been made to date, it would be interesting to investigate whether HDAC8 may represent an additional factor co-recruited to both TLE4 and KDM5b targets, and if the connection of these three transcriptional regulators exerts an effect in the process of transformation.

5.2.3.1 TLE4 may Play a Role in Cancer that Differs from its Mechanism with KDM5b

Historically, the role of TLEs in cancer progression has been more concentrated on the homologue TLE1, the expression of which has been found to exert both survival and anti-apoptotic cues within several cancer cell types including breast (Brunquell et al. 2012), brain (Cuevas et al. 2005), lung (Allen et al. 2006) and synovial sarcoma (Terry et

al. 2007). TLE1 levels appear to be selectively increased in invasive breast tumours when compared to their non-invasive counterparts (Brunquell et al. 2012), similar to what is observed for KDM5b (Yoshida et al. 2011), and TLE1 expression appears to result in both anchorage-independent growth (Sonderegger and Vogt 2003) and resistance to anoikis (cell death caused by extracellular matrix detachment) (Brunquell et al. 2012). Additionally, the over-expression of TLE1 in primary neural cultures was found to reduce differentiation, resulting in an increased number of neural progenitors that exhibit increased levels of proliferation(Buscarlet et al. 2009) also similar to what is observed during the over-expression of KDM5b in a stem cell context (Dey et al. 2008). These points of analysis for TLE1 raise an interesting possibility. We have provided evidence that supports the role of TLE4 in KDM5b enzymology as specific for this TLE family member, and that TLE1 exhibits no interaction with the histone demethylase under the conditions tested. It is therefore possible that TLE4 may play a role in oncogenesis that are similar to what has been observed with TLE1, but this involvement may differ from that of its interaction with KDM5b. The over-expression of TLE1 results in anchorage-independent growth, which raises an interesting parallel to an observation from our studies. Creation of a cell line in MCF7 cells which stably over-expressed TLE4 resulted in the detachment of MCF7 cells from a normal adherent state into what appeared to be a mammosphere-like phenotype prior to cell death. This suggests that the over-expression of TLE4 may also result in a disruption of anchorage dependent growth. Paired with the similar phenotypes between TLE1 and KDM5b over-expression with no evidence that there is an enzymology involvement of TLE1 with KDM5b, this observation suggests that

the oncongenic role of the TLE family members may be completely separate from that observed with KDM5b.

Due to TLE4 interacting with a large array of transcription factors and cellular signaling networks involved in cancer progression, including regulation of the Wnt (Huelsken and Behrens 2002; Brantjes et al. 2001), Notch (Hasson and Paroush 2006) and Nf-KappaB (Tetsuka et al. 2000) pathways, it is likely that TLE4 function differs depending both on cell type and timing of expression throughout development. This, paired with the knowledge surrounding the complexity of the role of KDM5b in oncogenesis (see chapter 1), suggests that evaluation of their connection in cancer progression is not a task to be taken lightly. This being said, continuing efforts to understand both the connection between, and the role of these proteins in the process of transformation may shed light on previously non-investigated options for novel weapons against many types of cancer.

5.3 Conclusions

The work presented within this thesis exemplifies the need to fully characterize all the substrates (both histone and non-histone) of epigenetic enzymes to better understand their individual roles in the writing and maintenance of the epigenome. It is additionally important to understand the cofactors involved in regulating the enzyme of interest *in vivo*. This is especially important when considering the use of therapeutic small molecule inhibitors in the treatment of disease. KDM5b is considered to be an ideal target for

chemical intervention due to its low expression level in non-cancerous human tissues and its enzymatic specificity. Our work emphasizes the importance of further study of this enzyme prior to small molecule intervention, contributes to furthering our understanding of any downstream effects that may occur as a result of small molecule therapy and provides a framework on which to continue these studies in the future.

Chapter 6 Future Directions and Conclusion

Preamble

This chapter presents some unpublished results from preliminary experiments done in follow up to the discoveries made in chapters 3 and 4 of this thesis. Not all experiments completed within this chapter represent a biological triplicate.

Mass spectrometry work was done in collaboration with Dr. Marek Galka and the University of Western Ontario Proteomics Facility (University of Western Ontario).

6.1 Future Directions and Conclusion

The body of work described within this thesis has led to discoveries into the enzymology and regulation of the histone demethylase KDM5b. We first defined a new enzymatic target of KDM5b, H2BK43me2, which may represent a novel epigenetic biomarker, or additional read-out for the modulation of KDM5b activity (chapter 3). We then defined a novel interaction partner of KDM5b, TLE4, the presence of which is required and sufficient to confer H3K4me3 nucleosomal demethylase activity to KDM5b, a phenomenon never before seen in the literature (chapter 4). This work can now be taken forward and utilized to define other possible enzymatic interacting partners for KDM5b, and will hopefully be useful in the eventual development of pharmaceutical inhibitors or modulators of KDM5b activity that may represent novel weapons in the fight against cancer.

6.2 Identification of TLE4 Spot Phenotype

In chapter 4 of this thesis we described that the *in vivo* interaction of KDM5b and TLE4 occurs within nuclear puncta present within the nucleus of cells (see Figure 4.2) TLE4 protein appears to be sequestered to distinct locations within the nuclear compartment, in which, interaction with KDM5b occurs. We hypothesized that this distinct nuclear localization might represent a chromatin dependent phenomenon and the regulation of TLE4 localization may result in an alteration to its interaction with KDM5b. We therefore sought to determine if the nuclear phenotype of TLE4 could be disrupted by the alteration of chromatin structure.

6.2.1 Hoescht Dye Does Not Eliminate TLE4 Phenotype

To analyze the possibility that the TLE4 nuclear puncta phenotype was dependent on chromatin structure, NIH3T3 cells were transfected for 24hours with YFP-TLE4 and cells were verified as displaying the expected spot phenotype. At the 24 hour time point, 10ug/ml Hoescht dye was added to the cell media and cells were observed at 60x with multichannel images captured every 10 seconds. Hoescht dye is a bis-benzimide which exhibits low levels of fluorescence on its own, but is known to bind the minor grooves of DNA, resulting in a strong fluorescent signal and an ability to visualize DNA within cells (Latt et al. 1975). The addition of Hoescht dye and subsequent intercalation of DNA results in alterations to chromatin structure and can, at times, be used to delineate if cellular localizations are dependent on chromatin arrangement. Additionally, the entrance of Hoescht dye into live cells can be easily monitored by florescence microscopy. Addition of Hoescht dye however, resulted in no alteration to TLE4 spot phenotype by visual inspection as even after 75 minutes of observation, nuclear puncta of TLE4 remained within observed cells (Figure 6.1). This experiment was repeated, and in no case was the spot phenotype of TLE4 eliminated. It is important to draw attention to the observation that this exact number and location of TLE4 puncta was not analyzed, and therefore may represent an interesting avenue of future investigation.

6.2.2 Actinomycin D treatment Does Not Eliminate TLE4 phenotype.

The addition of Hoescht dye resulted in no alteration to TLE4 spot phenotype, however we wished to pursue an additional DNA disruption methodology to verify this

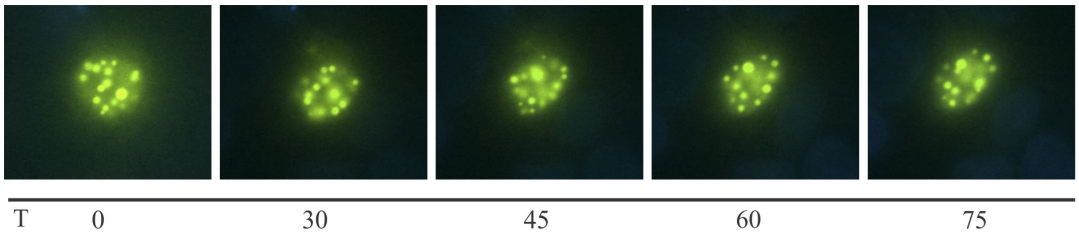


Figure 6.1 Hoescht Dye intercalation does not disrupt TLE4 puncta morphology
10ug/ml Hoescht dye was added to 3T3 cells transfected with YFP-TLE4 for 24 hours. Cells were observed for 90 minutes at 60x with multi-channel images captured every 10 seconds. Above images represent selected frames from a live cell movie. Hoescht dye is able to enter cells and intercalate DNA however TLE4 puncta phenotype does not appear to be altered by visual inspection.
Yellow: YFP-TLE4 Blue: Hoescht. T: Time in minutes

result. Actinomycin D is a cyclic-peptide containing antibiotic that is known as a general transcriptional inhibitor. Inhibition of transcription occurs through the binding of the peptide to DNA leading to an inhibition of RNA synthesis(Sobell 1985). Actinomycin D intercalates into DNA, leading to a disruption of RNA polymerase binding and alterations to chromatin structure(Casse et al. 1999). To determine if treatment with Actinomycin D would result in an alteration to TLE4 phenotype, NIH3T3 cells were transfected with mCerulean-TLE4 for 48 hours and verification of spot phenotype was performed by visual inspection at 20x. 5ug/ml actinomycin D was then added in serum free media and representative images were captured over an 18hour time period. Though significant cell death was observed within the treated dishes, presumably due to transcriptional inhibition or serum free conditions, TLE4 spot phenotype was still observed in cells remaining viable at the 18hour time point (Figure 6.2). This observation supports that disruption of chromatin structure through DNA intercalation does not result in an alteration to TLE4 localization within the nucleus.

6.2.3 Identification of TLE4 Spot Phenotype: Conclusions and Future Directions

Experimental evidence supports that the addition of the DNA intercalators, Hoescht or Actinomycin D does not result in an elimination of the TLE4 spot phenotype. This observation leads to two possible conclusions. The first possible conclusion is that TLE4 localization to distinct puncta is not chromatin structure dependent. This would explain why disruption of chromatin structure by either Hoescht dye or actinomycin D treatment did not affect localization. The second possibility is that the TLE4 containing

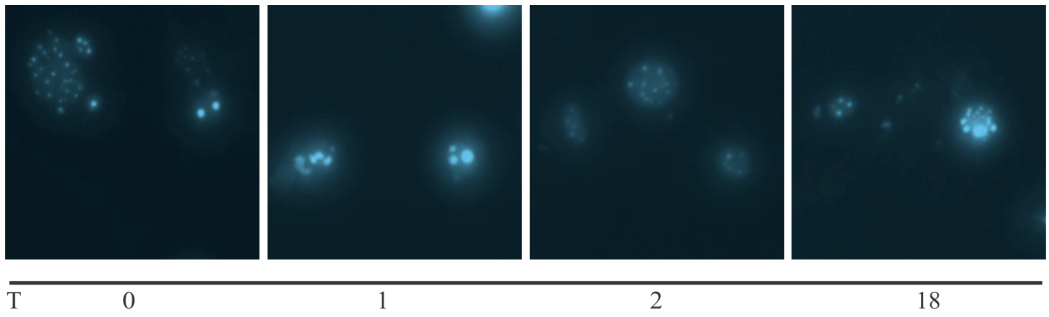


Figure 6.2 Treatment with actinomycin D does not eliminate TLE4 puncta
3T3 cells treated with 5ug/ml actinomycin D after 48 hour transfection with mCerulean-TLE4. Representative images were captured with a 20x lens on an EVOS LED benchtop microscope. At 18 hours after treatment, TLE4 puncta remain present within the cell nuclei.
Blue: mCerulean-TLE4 T: time in hours

puncta may represent areas of chromatin that are highly compacted into heterochromatin. DNA in these areas could be protected from intercalation from a high density of recruited proteins at these locations and a tightly wound chromatin structure. Data presented within this thesis suggest that TLE4 is intimately involved in transcriptional repression through its involvement with HDACs and KDM5b. The chromatin structure in areas of transcriptional repression usually shows compaction of the nucleosomes, potentially reducing the accessibility of DNA to intercalation.

Work performed within this thesis suggests that the localization of TLE4 and KDM5b to these nuclear puncta may be pertinent to their interaction, and it is therefore of interest to identify both the role of these nuclear puncta, and other possible proteins,localized within these distinct loci, in order to further our understanding of the KDM5b-TLE4 interaction and its downstream role in transcriptional repression.

6.3 The Scaffolding Protein RERE Represents an Additional Complex Member

In chapter 4 of this thesis we describe the interaction between KDM5b and the Groucho protein TLE4. We describe the interaction between these two proteins in both an *in vitro* immunoprecipitation model, and using FLIM-FRET to analyze the interaction in live cells. Though immunoprecipitation experiments reproducibly showed the interaction between KDM5b and TLE4, the interaction appeared to be weak in both a whole cell extract and nuclear fractionation based pull down. This is suggestive of a model whereby KDM5b and TLE4 may interact directly at the promoter of a gene of interest, but are not maintained in a complex throughout the cell. Though the association of TLE4 with

KDM5b is both required and sufficient to confer nucleosomal demethylation capability to KDM5b, most histone modifying enzymes require larger, more dynamic complexes to complete full enzymology *in vivo.* This led us to hypothesize that KDM5b and TLE4 may interact with additional cofactors to either increase the efficiency of their roles at the promoter, or aid to recruit KDM5b and TLE4 to the appropriate promoters at the right time.

To investigate this possibility, MS/MS analysis was completed on complexes immunoprecipitated from MCF7 cells through both KDM5b and TLE4. An IgG based pull down sample and a randomly sliced gel section were utilized as negative controls, and any peptide hits present in the negative controls were automatically discarded. As expected due to the requirements for KDM5b/TLE4 interaction, the enzyme and cofactor did not show up with each other as major hits on the MS/MS analysis, they both precipitated with a common protein known as Arginine-Glutamic acid repeat encoding protein, RERE. (University of Western Ontario Proteomics facility)

6.3.1 RERE

RERE, or Atrophin 2 (Atr2) is a member of the atrophin family of co-repressors that also contains the protein Atrophin-1(Shen et al. 2007), the polygluatmine expanded version of which results in neurodegenerative dentatorubral-pallidoluysian atrophy(Koide et al. 1994). Two different protein products are expressed from *Atn2 (Rere)* locus. The first is a full length Atrophin2 (Atr2L, RERE) which contains an Atrophin homology domain at its carboxy terminus including the arginine-glutamic acid repeat segments, and

an animo-terminal Metastasis Associated Protein-2 (MTA-2) homology domain. The second protein product, Atrophin 2 short (Atr2S) is produced from an internal promoter, and contains only the C-terminal atrophin homology domain(Shen et al. 2007). Atr2L is required for embryogenesis and alterations to its expression level results in severe developmental defects ending in lethality at E9.5(Zoltewicz et al. 2004).

6.3.1.1 RERE and Histone Modifying Proteins

The amino-terminus of RERE is of interest to our work, due to its homology to MTA-2. The MTA-2 homology domain is so named due to the amino terminus of RERE encoding the same series of domains in the same order as the MTA-2 protein, including a BAH (bromo adjacent homology) domain, an ELM2 (EGL-27 and MTA-1 homology) domain and a SANT (Swi3/ADA2/N-coR/TFIIIB) domain. MTA-2 is a scaffolding member of the NuRD chromatin-remodeling complex, which is known to associate with histone demethylases such as KDM1. Recent work has also implicated KDM5b as having an association with NuRD(Li et al. 2011). SANT domains are well known as domains that enable transcriptional regulators to carry out their functions in a context-dependent manner, for instance, the SANT domain of CoREST is involved in the histone demethylation of KDM1(Shi et al. 2005). The ELM2 and SANT domains of RERE specifically, have recently been found to be involved in mediating the interaction of RERE with HDAC1/2, and the SANT domain of RERE has been found to mediate binding of RERE to the histone methyltransferase G9a (Wang et al. 2006; Wang et al. 2008). The removal of acetylation marks at Histone H3 is a prerequisite for the

methylation of H3K9. As RERE can interact with both HDACs and G9a, this scaffold may act to coordinate the actions of several histone modifiers by localizing them to a specific promoter of interest. In support of this theory, treatment of a RERE complex with Trichostatin A (TSA) a potent HDAC inhibitor, led to an impaired ability of the complex to mediate H3K9 methylation (Wang et al. 2008). This is suggestive of RERE playing a role as a chromatin modifying protein scaffold, acting to recruit specific chromatin-modifiers in concert at specific locations.

6.3.2 RERE Recruits both KDM5b and TLE4

We hypothesize that the role of RERE as a chromatin scaffold is pertinent to the co-recruitment of KDM5b and TLE4 for transcriptional repression. RERE has previously been associated with several chromatin modifying proteins (Wang et al. 2008), and in *Drosophila*, a suggestion that Atro (d-atrophin) and Groucho may be recruited to the same transcription factors has been made (Wehn and Campbell 2006), however no direct connection between RERE at KDM5b or TLE4 has been previously detailed.

6.3.2.1 RERE Interacts with Both KDM5b and TLE4

We wanted to ascertain whether RERE interacts either with KDM5b, TLE4, or both. In order to do so, FLAG-tagged RERE was obtained (Dr. Tsai, UMDNJ) and co-transfected into HEK293 cells with YFP-KDM5b, YFP-TLE4 or YFP alone. Transfection for YFP was verified both visually and by western blot for YFP in the input samples. Pulldowns were completed through 3xFLAG followed by SDS-PAGE and western

blotting for YFP. Flag tagged RERE exhibited no ability to interact with the YFP alone control, however binding to both YFP-KDM5b and YFP-TLE4 was reproducibly observed. (Figure 6.3) This supports that full length RERE interacts with both YFP-KDM5b and YFP-TLE4 in this context.

6.3.2.2 RERE Localizes to TLE4 Puncta

The interaction of KDM5b and TLE4 observed in chapter 4 of this thesis is supported by FLIM-FRET within the nuclear puncta formed by YFP-TLE4. Previous studies of RERE within several cell types have shown a sub-cellular localization of full length RERE to distinct nuclear speckles, which phenotypically resemble the nuclear puncta formed by TLE4 (Yanagisawa et al. 2000; Waerner et al. 2001). This localization is dependent on the carboxy terminus, and has been suggested to represent the recruitment of RERE to promyelocytic leukemia (PML) oncongenic domains (PODs) (Waerner et al. 2001). Both the acetyltransfease p300, and HDAC1 have also been shown to be enriched in PODs (Salomoni and Pandolfi 2002), and RERE has been shown to interact with both acetyltransferases (via its carboxy terminus), resulting in transcriptional activation, and HDACS (via its amino terminus), resulting in transcriptional repression (Shen et al. 2007). It is therefore a possibility that RERE acts to modulate chromatin structure and downstream transcription by assembling distinct transcriptional regulatory complexes at PODs, or punctate locals within the cell nucleus.

Analysis of the interaction between TLE4 and RERE was unable to be completed by FLIM-FRET within the time constraints of this project due to the low expression

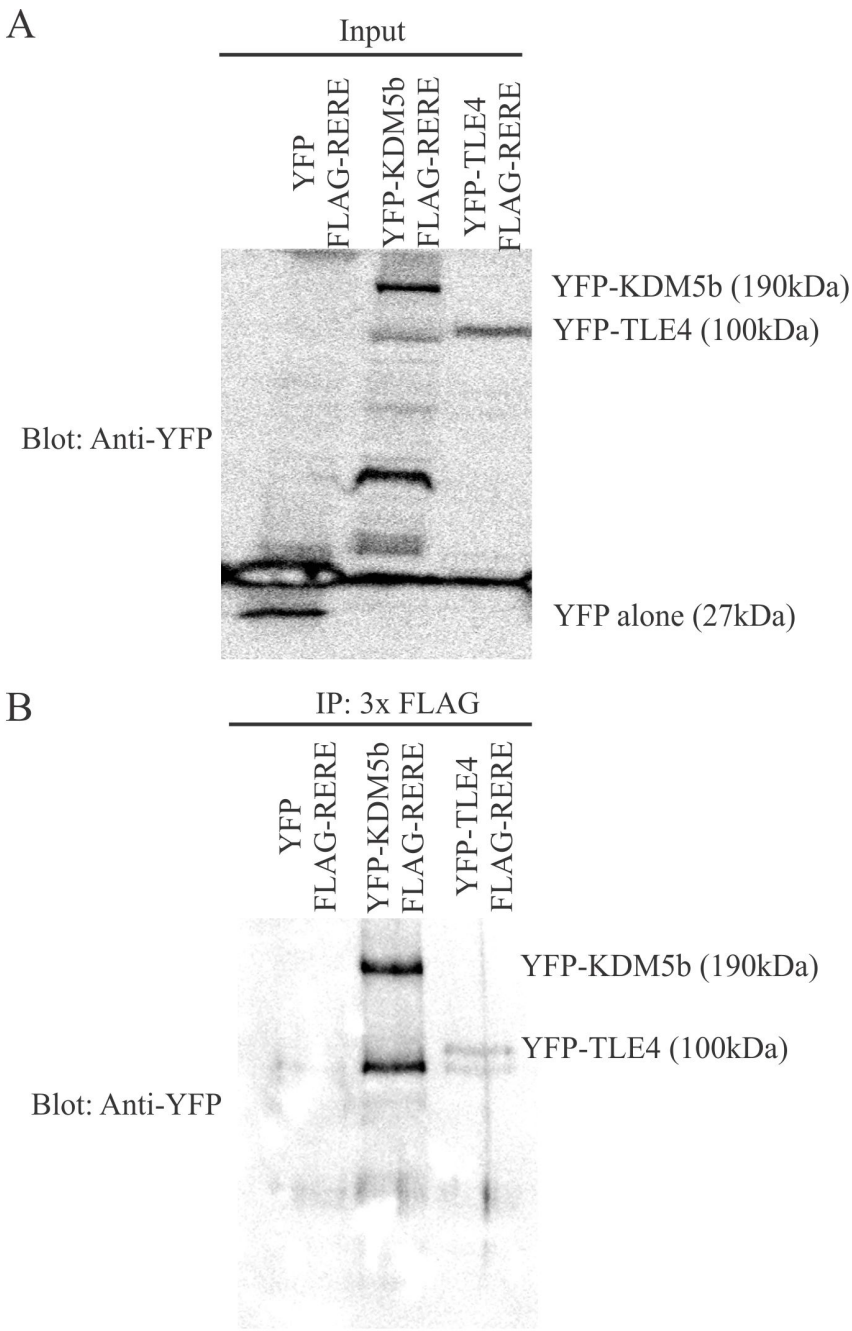


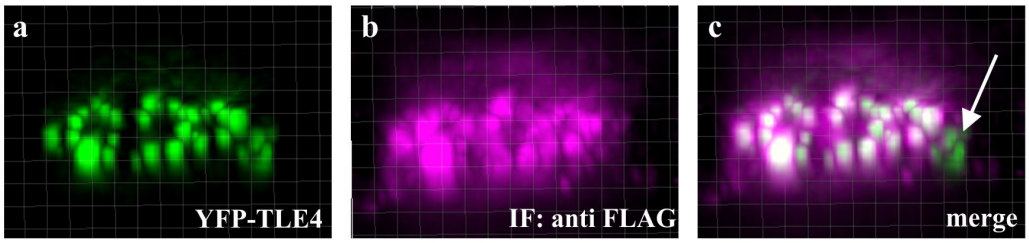
Figure 6.3 RERE interacts with both YFP-KDM5b and YFP-TLE4
Co-immunoprecipitation in HEK293 cells co-transfected with FLAG-RERE and YFP alone, YFP-KDM5b or YFP-TLE4 respectively. Pull downs were then completed through 3xFLAG and western blotting was completed for YFP. A) input samples run on 12% gel show the presence of all three YFP constructs within isolated samples. B) Pull down through 3xFLAG run on a 10% gel shows the presence of YFP-KDM5b and YFP-TLE4 but no pull down of YFP alone.

levels of fluorescently tagged RERE. However, co-transfection of FLAG-RERE and YFP-TLE4 paired with immunofluorescence against FLAG and deconvolution microscopy supports a strong co-localization between TLE4 and RERE within nuclear puncta (Figure 6.4A). Through utilization of IMARISx64 and isosurfacing, the colocalization of YFP-TLE4 (green) and FLAG-RERE (purple) becomes even more apparent, supporting a common role for these proteins within these nuclear puncta (Figure 6.4B).

6.3.3 Analysis of Mutant RERE Lacking Known Chromatin Binding Domains

In order to further analyze the interaction of RERE with both KDM5b and TLE4, we garnered a FLAG tagged version of RERE that is lacking amino acids 1-481 (CMX-RERE-481-C, Dr. Tsai). This mutant lacks the MTA-2 homology domain, including the domains known to mediate the interaction of RERE with several chromatin-modifying proteins. Previous analysis of this mutant shows that it is no longer capable of binding to G9a due to the absence of the SANT domain (Wang et al. 2008). Immunoprecipitation experiments through 3xFLAG were therefore repeated as in section 6.2.1, and interestingly RERE-481-C did appear to pull down both YFP-KDM5b and YFP-TLE4 but shows no affinity for YFP alone (Figure 6.5). This suggests that the domains present in the amino-terminal regions of RERE, including the ELM2 and SANT domains, are not required for the binding of KDM5b or TLE4.

A



B

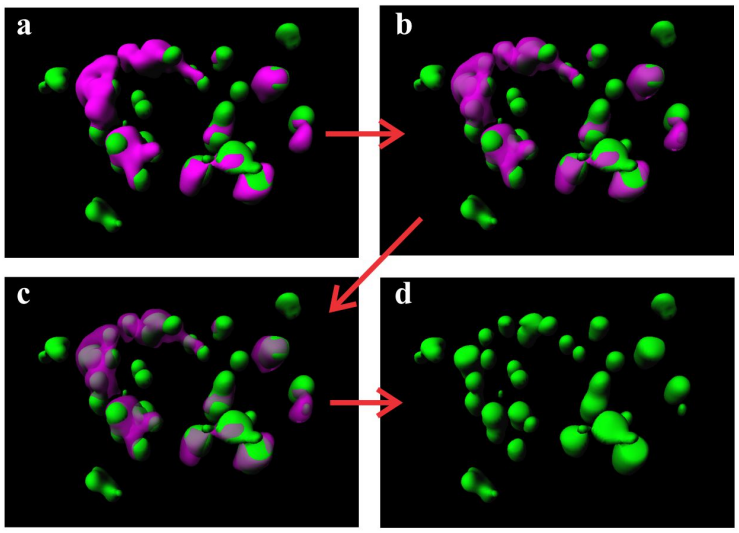


Figure 6.4: Full length RERE localizes to TLE4 puncta
A) Immunofluorescence in NIH3T3 cells. Cells were transfected with YFP-TLE4 (green) and FLAG-RERE for 24hours. immunoflouresence against FLAG was then completed using an Alexa 594 (purple) secondary antibody. a) YFP-TLE4 after 24 hour transfection b) FLAG-RERE after 24 hour transfection c) merge. Merged overlapping is pseudo-coloured in white. Arrow denotes where TLE4 signal shows no overlap with RERE. B) Isosurfacing of the same cell shown in (A). (a-d) progressive removal of FLAG-RERE staining demonstrates colocalization to nuclear puncta.

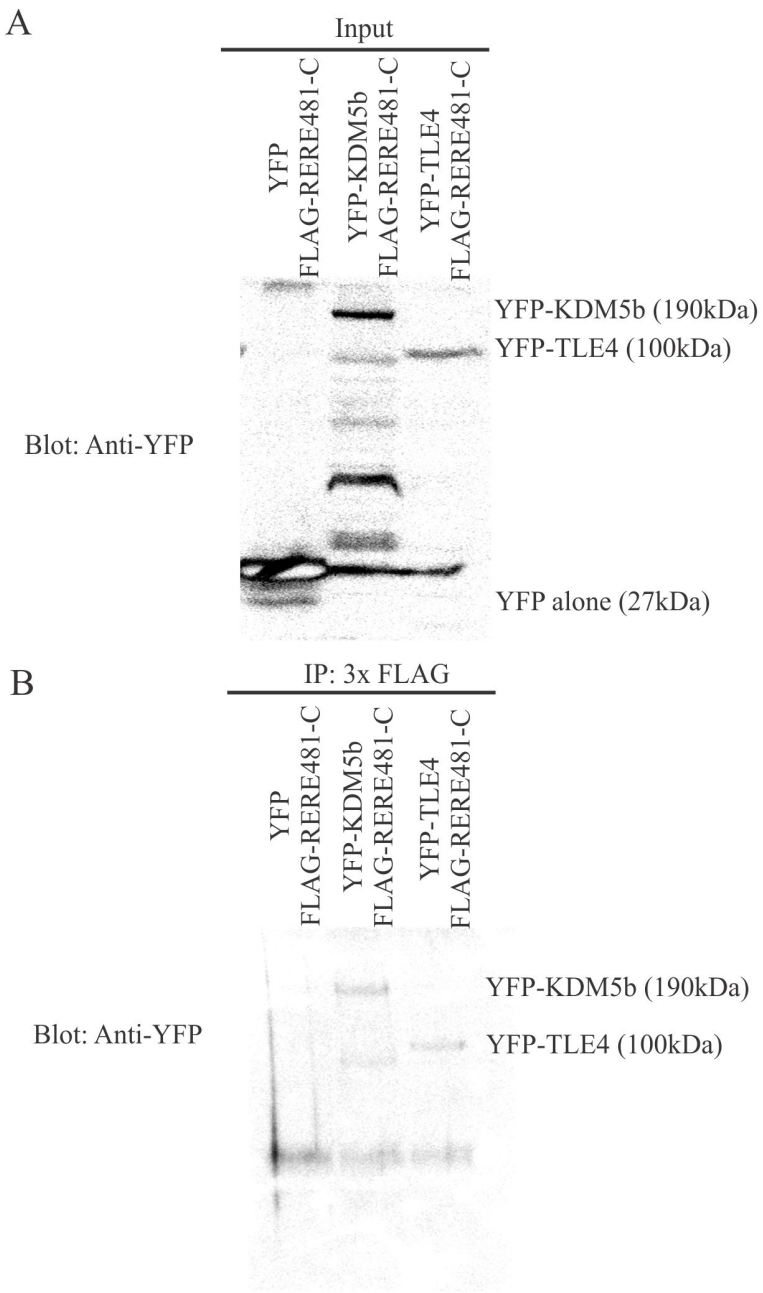
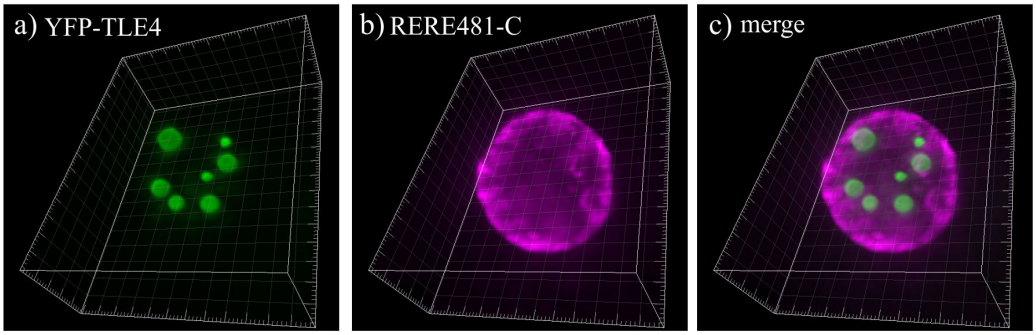


Figure 6.5 RERE481-C mutant interacts with both YFP-KDM5b and YFP-TLE4
Co-immunoprecipitation in HEK293 cells cotransfected with FLAG-RERE481-C mutant and YFP alone, YFPKDM5b or YFP-TLE4 respectively. Pull downs were then completed through 3xFLAG and western blotting was completed for YFP. A) input samples run on 12% gel show the presence of all three YFP constructs within isolated samples. B) Pull down through 3xFLAG run on a 10% gel shows the presence of YFP-KDM5b and YFP-TLE4 but no pull down of YFP alone.

In order to follow up on this, we wished to observe the localization of RERE-481-C within cells utilizing immunofluorescence. Previous studies have demonstrated that the nuclear speckling phenotype of RERE requires the carboxy-terminus and the RE repeat region, but that the amino-terminus is dispensable for this phenotype(Shen et al. 2007; Yanagisawa et al. 2000). Other groups however have proposed that the SANT domain, present within the amino terminus of RERE, is required for nuclear speckling(Wang et al. 2006). As the interaction of TLE4 with RERE appears to occur within distinct nuclear puncta, we questioned whether removal of the amino-terminus would alter the co-localization of RERE with TLE4. RERE-481-C was therefore co-transfected with YFP-TLE4 followed by immunofluorescence and deconvolution microscopy as completed in section 6.2.2. RERE-481-C was found to localize to the nucleus of transfected NIH3T3, but the distinct speckling pattern observed with full length RERE, as well as distinct colocalization with TLE4, was absent (Figure 6.6)

This suggests that though KDM5b and TLE4 both appear to be pulled down by mutant RERE, the distinct co-localization of RERE and TLE4 is absent by immunofluorescence. Previous work has suggested that RERE can hetero-dimerize with Atrophin 1, which is similar in structure to short atrophin 2, or the mutant RERE utilized here (Yanagisawa et al. 2000). This suggests that results observed using co-immunoprecipitation may represent the portion of KDM5b and TLE4 pulled down through dimerized full length RERE, and explains why colocalization with the specific FLAG-tagged mutant RERE is not observed. It is interesting to note however, that this

A



B

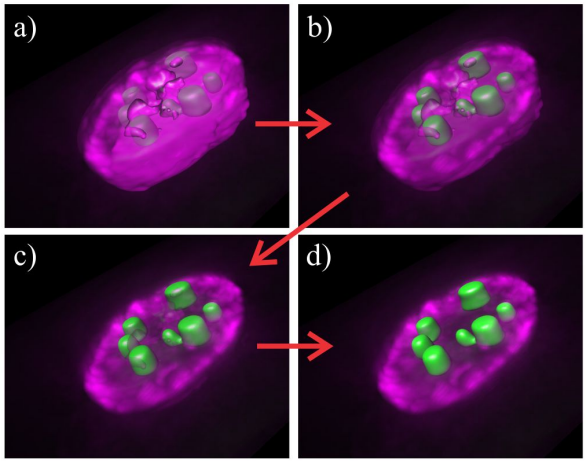


Figure 6.6 Mutant RERE481-C does not localize to TLE puncta
A) Immunofluorescence in NIH3T3 cells. Cells were transfected with YFP-TLE4 (green) and FLAG-RERE481-C for 24 hours. Immunoflouresence against FLAG was then completed using an Alexa 594 (purple) secondary antibody. a) YFP-TLE4 after 24 hour transfection
b) FLAG-RERE481-C after 24 hour transfection c) merge. Merged overlapping is pseudo-coloured in white. B) Isosurfacing of the same cell shown in (A). (a-d) progressive removal of FLAG-RERE481-C staining demonstrates no colocalization to nuclear puncta.

dimerization phenomenon does not appear to alter the interaction of RERE with G9a, as in previous publications, RERE mutants lacking the SANT and ELM2 domains do not co-precipitate with G9a.

6.3.4 The Atrophin Protein RERE Represents an Additional KDM5b Complex Member: Conclusions and Future Directions

Taken together these data suggest that RERE plays a role in the scaffolding of KDM5b and TLE4, and that this interaction is most likely taking place with the nuclear puncta (or speckling) formed by both TLE4 and RERE within live cells. It remains unknown however if the presence of RERE is required for the enzymology of KDM5b. Studies completed *in vitro* within chapter 4 of this thesis suggest that the presence of TLE4 alone is both required and sufficient to confer nucleosomal demethylation ability to KDM5b. Paired with this, over-expression of TLE4 alone appears to result in a decreased global level of H3K4me3. However, this does not eliminate the possibility that RERE may be required in an *in vivo* context. We propose the possibility that RERE may act as a chromatin modifying scaffold, recruiting subsets of chromatin-modifying proteins to specific sub-nuclear localizations within the nucleus, resulting in downstream transcriptional changes. Future experiments should concentrate on the *in vivo* analysis of the TLE4-KDM5b interaction in both the presence and absence of RERE to determine if RERE is required for their interaction (utilizing FLIM-FRET for instance) and/or downstream enzymology. Additional work, utilizing knock down of RERE could evaluate whether the presence of RERE is required to recruit KDM5b or cofactors such as TLE4 to

target genes of interest.. Though the exact role of RERE has yet to be delineated in this context, this protein provides a potentially interesting novel interaction partner for both KDM5b and TLE4, and may represent an additional future drug target.

6.4 Conclusion

This body of work describes several new features of the histone demethylase KDM5b. Specifically, we have found that KDM5b represents a histone demethylase that is not specific to H3K4me2 and me3. We find that H2BK43me2 represents a novel target of KDM5b emzymology, and indicate that this novel histone modification represents the primary target of this enzyme. We find that the methylation status of H2bK43 is directly related to the regulation of differentiation, and suggest that this novel histone modification may represent an epigenetic biomarker for cancer.

We also characterize a novel interacting partner for KDM5b, demonstrating that the groucho co-repressor TLE4 interacts with KDM5b both in co-immunoprecipitation experiments, and within live cells using FLIM-FRET. We went on to characterize an enzymatic complex required for the demethylation of H3K4me3 by KDM5b in a nucleosomal context, demonstrating that the presence of TLE4 is both required and sufficient for KDM5b to recognize and demethylate H3K4me3 in nucleosomes. We find that TLE4 represents an evolutionarily conserved cofactor for KDM5, and that TLE4 can confer nucleosomal demethylation ability to both yeast KDM5 and KDM5d in addition to its role with KDM5b. Furthermore, we find that the expression level of TLE4 is linked to level of H3K4me3 expressed globally within cells.

We then went on to further characterize additional interaction partners for KDM5b, suggesting that the arginine repeat protein RERE may represent a scaffold required for the recruitment of both TLE4 and KDM5b to promoters of interest.

The literature strongly suggests that the histone demethylase KDM5b plays a role in the regulation of gene transcription during oncogenesis, and that this enzyme may represent a potential therapeutic target. It is our hope that novel discoveries in KDM5b regulation will increase our understanding of how KDM5b may be involved in transformation. We hope that the novel aspects of KDM5b regulation presented within this thesis may provide the framework on which future studies of KDM5b can be completed, and may lead to novel cancer therapeutic strategies, or biomarkers which can ameliorate cancer treatment options in the future.

6.5 Acknowledgements

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