ROLE OF ARG DEMETHYLATION IN REGULATING P53 PATHWAY

A Thesis in
Cell and Developmental Biology

by

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Abstract

Post-translational histone modifications control transcription by regulating higher order chromatin structures and access to the underlying DNA. Previous studies have shown that histone Arg methylation and Lys acetylation coordinate to regulate the expression of p53-target genes. However, whether Arg demethylation also plays a role in regulating p53 pathway is largely unknown. PAD4 catalyzed histone deimination and demethylimination could turn Arg and monomethyl-Arg into citrulline, respectively. In my thesis, I have studied the function of PAD4 in the formation of a decondensed chromatin structure called neutrophil extracellular traps (NETs) and analyzed the interaction between PAD4 and HDAC2 by GST-pull down and re-CHIP experiments. Besides histone demethylimination by PAD4, Jumonji domain-containing 6 (JMJD6) was reported to be an Arg demethylase. To investigate the function of JMJD6 and its homolog JMJD4, I have established Flag-JMJD6 and Falg-JMJD4 expressing cell lines, purified the JMJD4 and JMJD6 fusion proteins and tested their demethylation function both in vivo and in vitro. Further, I depleted JMJD4 and JMJD6 by shRNA or siRNA in U2OS cells and tested the changes of p53-target gene expression. Through my thesis study, I found that the formation of NETs is associated with PAD4 activation and global histone citrullination. I found that PAD4 and HDAC2 coordinate with each other and act as p53 corepressors. My study also showed that the JMJD4 and JMJD6 both have weak but detectable demethylation activity and bind to p53 to regulate the expression of p53-target genes. Additionally, I analyzed the newly synthesized derivatives of PAD4 inhibitor Cl-amindine and found several candidates with improved PAD4 inhibition activity.
Table of Contents

Chapter One. Introduction ................................................................. 1

Chromatin structure and post-translational histone modifications............1
Histone Arg methylation and its reversal mechanisms:
demethylimmination and demethylation...........................................2
Peptidylarginine deiminase 4 – PAD4.................................................5
Does JmjC domain containing protein 6 mediates Arg demethylation?....6
Role of p53 in regulating the cell cycle and apoptosis.............................8

Chapter Two. Materials and methods ..................................................11

Chapter Three. Results ........................................................................24

Release of isocyanic acid (HNCO) is a novel diagnostic marker
for mass spectrometric identification of citrullination..........................24
Chromatin decondensation is associated with PAD4 activation
and histone citrullination..................................................................................25
PAD4 and HDAC2 interact and simultaneously bind to the
p21 promoter.....................................................................................................27
Comparison of PAD4 Inhibitors’ efficiency by citrullination activity........29
The putative demethylation activity of JMJD4 and JMJD6 .......................32
Testing the demethylation activity of JMJD4 and JMJD6 .........................35
Interaction of JMJD4 with Hsp70 p53 JMJD6...........................................40

Chapter Four. Discussion .....................................................................44

Releasing of isocyanic acid (HNCO) could be used to detect
the exact site of citrullination.................................................................44
Functional connection between demethylation and deacetylation of histones ................................................................. 44
PAD4 inhibitors are served as potential anticancer drugs .............. 45
JMJD6 and JMJD4 have potential Arg demethylation function ......... 46
A potential function of JMJD4 and JMJD6 in the p53 pathway .......... 58
Depletion of JMJD4 or JMJD6 affects the expression of a subset of p53 target genes .......................................................... 49

Reference .......................................................................................... 51
Chapter One Introduction

1. Chromatin structure and post-translational histone modifications.

In eukaryotic nucleus, the genetic material DNA is packed around histones to form nucleosomes, which are the structural units of chromatin. Without this package, the DNA will be quite long---about 1.8 meters in human cells, making it hard to fit into eukaryotic nucleus. However, wound on the histones, DNA will be condensed to 90 millimeters chromatin which during mitosis will be further condensed and form 120 micrometers of chromosomes (Redon et al., 2002). To form nucleosomes, 146 bp DNA interacts with a core histone octamer including two of each four histone proteins: H2A, H2B, H3 and H4. The interaction between DNA and histones not only condenses the length of DNA but also regulates nuclear events such as gene regulation, DNA damage and repair (Jenuwein and Allis, 2001; Kouzarides, 2002; Li et al., 2007; Shilatifard, 2006). This structural organization inhibits the access of many DNA and histone-binding proteins to their binding sites on chromatin (Narlikar et al., 2002; Workman and Kingston, 1998).

Recent studies have found that post-translational histone modifications play pivotal roles in the regulation of chromatin templated nuclear events. Each histone contains an N-terminal tail extruding from the octamer. The N-terminal tail maintains chromatin structural stability and interacts with neighboring nucleosome (Rhodes, 1997). The N-terminal tail could be posttranslationally modified in many ways, such as methylation, acetylation, phosphorylation, ubiquitination and citrullination. These post-translational histone modifications work in either a synergistic or antagonistic way to
activate or repress gene transcription, known also as “histone code” (Jenuwein and Allis, 2001).

Histone modifications are proposed to play a role in epigenetic regulation of gene expression. Epigenetics was defined as "a change of phenotype that is inheritable but does not involve DNA mutation" by Dr. Gottschling (Gottschling, 2004). Since histone modifications do not change genetic composition but lead to differences in cellular phenotypes, histones can serve as epigenetic information carriers. Histone modifications can change the charged properties of histones to affect the chromatin structure. For example, acetylation of Lys and citrullination of Arg neutralize basic charges. Also, the histone modifications can be recognized by specialized protein domains such as chromodomains, bromodomains, tudor domains and PHD fingers to regulate downstream events after histone modifications (Huang, 2006; Li et al., 2007; Wysocka, 2006).

Certain histone modifications inhibit or activate the addition and/or removal of other modifications via various histone cross-talk (Fischle et al., 2003). For example, a study shows that in yeast, methylation of histone H3 at lysine 4 (Lys 4) is dependent on a ubiquitin-conjugating enzyme Rad6 (Ubc2) through ubiquitination of H2B at Lys 123, indicating that ubiquitination of H2B (Lys 123) is a prerequisite for H3 (Lys 4) methylation (Sun and Allis, 2002).


Histone methylation occurs at two kinds of residues: Lys and Arg. Histone Arg methylation is found on residues 2, 8, 17 and 26 of histone H3, and residue 3 of histone H4 (Schurter et al., 2001; Wang et al., 2001).
Histone Arg methylation can produce the monomethyl-Arg, symmetrical dimethyl-Arg (addition of one methyl group to each nitrogen of the guanidinium group) or asymmetrical dimethyl-Arg (addition of both methyl groups to one nitrogen of the guanidinium group) (Figure 1). The enzyme catalyzed histone Arg methylation are protein Arg methyltransferases (PRMTs), which transfer methyl groups from S-adenosyl-L-methionine (SAM) to epsilon nitrogen atoms of guanidinium side chains of Arg (Krause et al., 2007; Tang et al., 1998). So far, nine PRMTs classified to two groups have been identified in the human genome. Type I PRMTs including PRMTs1, 2, 3, 4, 6 and 8 produce asymmetrical dimethyl-Arg, while type II PRMTs including PRMTs 5, 7 and 9 produce asymmetrical dimethyl-Arg.

Previous research shows that global turnover of histone methyl groups occurs at a similar rate to histone turnover, indicating that the histone methylation is a static modification (Byvoet et al., 1972; Duerre and Lee, 1974). On the other hand, since PRMTs catalyze histone Arg methylation is involved in the dynamic regulation of gene expression (An et al., 2004), there should be certain mechanisms to reverse histone Arg methylation. Histone replacement and histone tail cleavage have been considered as alternative ways to remove methylated histone (Bannister and Kouzarides, 2005; Bannister et al., 2002). However, both events generate new unmodified nucleosome by disassembly of the nucleosome and replacement of the histone, leading to a loss of other important histone modifications on the same histone. So, it is very likely there are other efficient ways to remove the methyl groups.

Peptidylarginine deiminases 4 (PAD4) was known to convert both Arg
Figure 1: Pathways of histone Arg modifications. Both type I and II protein Arg methyltransferases (PRMTs) can modify Arg to generate monomethyl-Arg (MMA). Type I PRMTs can further produce asymmetrical dimethyl-Arg (ADMA), while type II PRMTs produce symmetrical dimethyl-Arg (SDMA). PAD4 can convert both Arg and MMA to citrulline (Cit). How Cit in histone turns into Arg is currently unclear. Recently, JMJD6 was found to demethylate both ADMA and SDMA to generate MMA (Chang, 2007), while had little affect towards MMA. Therefore, both PAD4 and JMJD6 mediate the demethylation of histone Arg with methylation state preference.
and monomethyl-Arg to citrulline in processes called citrullination and demethylimation, respectively (Figure 1). The functions of histone demethylimation and citrullination are not only decreasing the amount of methyl-Arg on histones but also preventing methylation by PRMT to regulate chromatin functions (Li et al., 2008).

Recently, Chang et al. showed that Jumonji domain-containing 6 (JMJD6) demethylates histone H3 at Arg 2 (H3R2) and histone H4 at Arg 3 (H4R3). While the result is still in debate, JMJD6 might be the first demethylation enzyme found to reverse Arg methylation by a direct demethylation reaction (Figure 1).


Peptidylarginine deiminases 4 (PAD4) belongs to a family of enzymes termed peptidylarginine deiminases (PADs) which could hydrolyze Arg guanidinium side chains and yield peptidylcitrulline and ammonium. Five human PAD proteins named PAD1-4 and PAD6 have been identified. PAD4 is distinctive from other PAD family members: first, while other PAD proteins are known to produce citrulline from unmodified Arg residues, PAD4 was the first to be found to modify both unmodified Arg residues and monomethyl-Arg residues. Further, it remains unclear whether PAD4 also functions on dimethyl-Arg residues (Wang et al., 2004). Second, PAD4 is the only PAD protein that localizes in the cell nucleus (Nakashima et al., 2002).

PAD4 is a Ca$^{2+}$-dependent enzyme with an N-terminal immunoglobulin like domain (Ig like 1), a middle immunoglobulin like domain (Ig like 2), and a C-terminal catalytic domain. Five Ca$^{2+}$ ions can bind to PAD4 and induce conformational change in the enzyme, affecting its catalytic activity (Arita et
In our previous studies, we found that PAD4 represses the expression of estrogen receptor target genes in the breast cancer MCF-7 cells and also the p53 target genes such as p21, GADD45 and PUMA in several cancer cell lines (Wang et al., 2004; Li et al., 2008; Li et al., 2010). Further, PAD4 inhibitor Cl-amidine could inhibit the growth of the MCF-7 cells, suggesting PAD4 is potential drug target for cancer treatment.

4. Does JmjC domain containing protein 6 mediates Arg demethylation?.

JMJD6 belongs to a family called the jumonji C (JmjC)-domain containing proteins which catalyze a wide range of iron- and α-ketoglutarate-dependent oxidative reactions. The jumonji C domain was first found by a Japanese group in mouse (Takeuchi et al., 1995). Many JmjC-domain-containing proteins are demonstrated to be histone lysine demethylase and each function at specific histone sites: JHDM1 demethylates H3 monomethyl- and dimethyl-Lys 36 (Chen et al., 2006; Klose et al., 2007; Tsukada et al., 2006; Tu et al., 2007; Whetstine et al., 2006), JHDM2 demethylates H3 monomethyl- and dimethyl-Lys 9 (Chen et al., 2006; Whetstine et al., 2006; Yamane et al., 2007), and JHDM3/JMJD2 proteins are tri-methyl Lys demethylases (Cloos et al., 2006; Fodor et al., 2006; Klose et al., 2006; Whetstine et al., 2006). The core JMJC domain contains a double-stranded β-helix core fold (DSBH) which is formed by eight β-strands (Clifton et al., 2006). DSBH is typical of metalloenzymes (Dunwell et al., 2001). The mechanism is that the DSBH in JMJC domain coordinates di-iron (Fe(II)) and the α-ketoglutarate cofactors to form an active pocket. The two-electron oxidation of the substrate fits in and converse the α-ketoglutarate cofactor into succinate and CO₂ (Schofield and Zhang, 1999).
JMJD6 was first recognized as phosphatidylserine receptor (PSR or Ptdsr), a protein functioning as a cell surface receptor to recognize and engulf apoptotic cells (Fadok et al., 2000). Several groups’ studies showed that the protein located in nucleus (Cikala et al., 2004; Cui et al., 2004; Krieser et al., 2007; Mitchell et al., 2006; Tibrewal et al., 2007), so the hypothesis that the protein functions as a transmembrane receptor is questionable. Also, contradicting to previous paper, PSR-knockout mice show normal engulfment of apoptotic cells both in vivo and in vitro (Bose et al., 2004). PSR-knockout mice have perinatal lethality, growth retardation and severe differentiation defects in brain, eyes, lung, kidney, liver and intestine at different stages of embryogenesis (Botto, 2004; Kunisaki et al., 2004; Li et al., 2003), which demonstrates that PSR plays an important role in embryo development, but its molecular function is still unknown. Because of the still unclear function of the “phosphatidylserine receptor gene”, the name of PSR has been changed to jumonji domain containing gene 6 (JMJD6)

Given that JMJD6 have similarities with asparaginyl hydroxylase in sequence and predicted structure, as well as that several JMJC domain proteins have been found to be histone lysine demethylases, it is likely that JMJD6 have an analogous demethylases activity. In vitro and in vivo data from Chang et al. have shown that JMJD6 demethylates histone H3 at Arg 2 (H3R2) and histone H4 at Arg 3 (H4R3) (Chang et al., 2007). Recent studies raised the doubt on JMJD6-catalyzed Arg demethylation and indicated that lysyl hydroxylation is the dominant oxidative catalytic activity of JMJD6 (Webby et al., 2009). Due to these contradictory results about JMJD6, more research is necessary to investigate the actual function and biochemical activity of JMJD6.
5. Role of p53 in regulating the cell cycle and apoptosis.

The p53 gene was first identified as tumor-suppressor gene in 1979 (DeLeo et al., 1979). At the very beginning, it was studied as an oncogene. But data collected in the following years shows that it was a tumor suppressor gene (Vogelstein et al., 2000). Under stress, p53 can recruit multiple coactivators and corepressors to regulate transcription of gene important for cell cycle and apoptosis, forming a complicate p53 network (Figure 2A).

p53 has three functional domains, an N-terminal transcriptional activation domain, a central DNA binding domain and a C-terminal regulation domain. In normal cells, regulated by MDM2 mediated polyubiquitination and proteolytic degradation, p53 protein is maintained at a low level (Momand et al., 2000). Only when the cells are stressed or damaged, the p53 pathway is activated. Recent studies have showed that at least three independent pathways could activate p53. First, DNA damage such as those caused by ionizing radiation could activate the p53 target gene expression. Two protein kinases ATM and Chk2 are involved in this process (Carr, 2000). Second, growth signals such as those resulting from the expression of the oncogenes Ras or Myc activate the p53 network. This process needs the help of a protein called p14ARF (Lowe and Lin, 2000; Sherr and Weber, 2000). Third, chemotherapeutic drugs, ultraviolet light, and protein-kinase inhibitors also activate the p53 network. The pathway is dependent on kinases called ATR and casein kinase 2. All these pathways inhibit the ubiquitin-mediated proteolysis of p53 and maintain p53 at a high concentration. Then p53 can bind to particular DNA sequences and
Figure 2: P53 network and histone modifications involved in the regulation of p53 target gene expression. (A) p53 network. Stress such as DNA breaks and UV light activate p53, leading to the expression of p53 target genes which are important in growth arrest, apoptosis, etc. (B) Histone modifications involved in the regulation of p53 target gene expression. Before activation, at p53 promoters, histone citrullination and hypoacetylation regulated separately by p53 corepressors PAD4 and HDAC2 maintain target gene expression at a low level. After activation by DNA damage or stress signals, histone acetylation and Arg methylation regulated by p53 coactivators PRMT1, 4 and p300/CBP lead to expression of p53 target gene.
activate the target genes controlling cell cycle or apoptosis such as p21 and puma.

Various post-translational p53 modifications such as phosphorylation, acetylation, methylation and ubiquitination can regulate the stability and function of p53 (Brooks and Gu, 2003). For example, acetylation of lysine residues or phosphorylation of serine residues in the p53 C-terminus can enhance the binding of p53 to DNA, thus affecting the function of p53 (Selivanova et al., 1998). Phosphorylation of p53 N-terminus can change the binding of MDM2 and p53, thus affecting the p53 degradation (Buschmann et al., 2000).

Previous work from our laboratory has found that PAD4 functions as a corepressor of the p53 transcription factor. Histone deacetylases (HDACs) remove the histone acetyl groups, counteracting the function of histone acetyltransferases (Dannenberg et al., 2006), while PAD4 converts monomethyl-Arg to citrulline through demethylimination thereby counteracting Arg methylation. Since histone Arg methylation and Lys acetylation coordinate with each other to regulate the expression of p53-target gene (An et al., 2004), it will be interesting to analyze whether histone deacetylation and histone demethylimination also coordinate with each other (Figure 2B). My work has contributed to the finding that PAD4 coordinates with HDAC2 to repress p53-target gene expression (Li et al., 2010).
Chapter Two  
Materials and methods

1. Materials

1) Antibodies and dilutions:

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2) P21 primers for CHIP-reCHIP
P21-CHIP-5: CCA GCC CTT TGG ATG GTT T
P21-CHIP-3: GCC TCC TTT CTG TGC CTG A

3) JMJD4 H187AD189A primers for point mutation
   Forward: 5’-TGG TCC CCG TTC GCT GCT GCC ATC TTC CGC
   TCC-3’
   Reverse: 5’-GGA GCG GAA GAT GGC AGC AGC GAA CGG GGA
   CCA-3’

4) JMJD4 cloning into PMIGR1 vector
   NotI-JMJD4-long: GGC CGC CGT GCA GGC CCG GAG CCC CAG
   NotI-JMJD4-short: GC CGT GCA GGC CCG GAG CCC CAG
   EcoRI-JMJD4-long: AATT C CTA TGG GGC CGC AGC AGC ATC
   EcoRI-JMJD4-short: C CTA TGG GGC CGC AGC AGC ATC

5) JMJD6 cloning into PMIGR1 vector
   NotI-JMJD6-long: GGC CGC AAC CAC AAG AGC AAG AAG CGC
   NotI-JMJD6-short: GC AAC CAC AAG AGC AAG AAG CGC
   EcoRI-JMJD6-long: AATT C TCA CCT GGA GGA GCT GCG CTC
   EcoRI-JMJD6-short: C TCA CCT GGA GGA GCT GCG CTC

6) His-JMJD6 cloning into Pet11A vector
   Nde1-JMJD6-Long: TATG ATG AAA AAA CAC CAC CAC CAC CAC
   CAC AAC CAC AAG AGC AAG AAG
   Nde1-JMJD6-short: TG ATG AAA AAA CAC CAC CAC CAC CAC
   AAC CAC AAG AGC AAG AAG
   BamH1-JMJD6-Long: GATC C TCA CCT GGA GGA GCT GCG CTC
   BamH1-JMJD6-short: C TCA CCT GGA GGA GCT GCG CTC

7) JMJD4 siRNA (h):
   sc-88226 from SANTA CRUZ BIOTECHNOLOGY, INC

8) JMJD6 siRNA (h):
   sc-36324 from SANTA CRUZ BIOTECHNOLOGY, INC
2. Cell Culture

1) The mammalian cells are cultured in the incubator at 37°C with 5% CO₂. 293T, H1299, U2OS, MCF-7 cells are cultured in DMEM (Dulbecco Modified Eagle Medium-High Glucose) with 10% FBS (fetal bovine serum) and 1% penicillin-streptomycin. HCT116 p53+/+ and p53-/- cells are cultured in McCoy’s 5A Medium-Modified with 10% FBS and 1% penicillin-streptomycin. HL60 cells are cultured in RPMI 1640 Medium with 10% FBS and 1% penicillin-streptomycin.

3. Cells transfection with shRNA or siRNA

1) Cells were transfected with plasmid DNA with Lipofectamine 2000 (Invitrogen) by following manufacturing procedure in 6-well plate.
2) Cells were transfected with siRNA using X-tremeGene siRNA Transfection Reagent. (Roche) by following manufacturing procedure in 6-well plate.

4. Total cell extract preparation

1) Cells are collected from the dishes.
2) Wash cells twice with ice cold PBS and once with IP buffer.
3) Add proper amount of IP buffer, make the concentration be ~10⁷ cells/ml.
4) Sonicate for 5min and spin at 14000 rpm for 10min.
5) Take the supernatant and store at -80°C.

5. Nuclear extract preparation

1) About 1x10⁸ cells are collected from the dishes.
2) Wash cells twice with ice cold PBS and once with Hypotonic buffer.
3) Spin at 2,000 rpm for 5 min to collect the cells. Remove supernatant and resuspend cells in 5 ml of hypotonic solution, put on ice for 10 min.
4) Dounce cells for 15 times with a tight pestle.
5) Check cell lysis by a microscope and spin down nuclei at 3,900 rpm for 15 min at 4°C.
6) Resuspend nuclei in proper amount of IP buffer and sonicate for 5 min.
7) Spin at 13,200 rpm for 10 min to collect supernatant as nucleus extract.

6. Histones extraction from culture cells
   1) 10ml of culture containing ~5×10^6 cells/ml was collected by slow spinning.
   2) Wash cells once with ice cold PBS and spin down.
   3) Resuspended with 5 ml hypotonic buffer. Dounce 15 times with tight pestle.
   4) Check cell lysis under a microscope
   5) Pellet nuclei by spinning at >4000 rpm for 10 min at 4°C
   6) Resuspend nuclei in 0.4 N H₂SO₄, vortex a little bit and incubate with rotating at 4°C over night.
   7) Spin at 13,200 rpm for 10 min to collect supernatant, add 100%TCA to 20% to precipitate histone for >30 min on ice.
   8) Spin down the histones at 13,200 rpm for 10 min.
   9) Wash twice with cold acetone (-20°C).
   10) Air dried for at least 20 min and resuspended in appropriate amount of ddH₂O.

7. SDS-PAGE
   1) Prepare samples with 6× SDS loading buffer, heat samples at above 80°C for 5 min. Put on ice for seconds and briefly spin down.
   2) Load samples in SDS-PAGE gel.
3) Run the gel in 1x SDS running buffer V220 for 45min.

SDS loading buffer (6X)
300 mM Tris.HCl (pH6.8)
20% glycerol
6% SDS
4% beta-mercaptomethanol
0.6% Bromophenol
10xSDS running buffer (1L)
30g Tris
144g Glycine
10g SDS

SDS PAGE (from Johansen's group)

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<th>12%</th>
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<td>4ml</td>
<td>3.3ml</td>
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<tr>
<td>1% Bis</td>
<td>3.25ml</td>
<td>2.6ml</td>
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<td>1.0ml</td>
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8. Silver staining using Silver Staining Kit (Invitrogen, LC6070)

1) After SDS-PAGE, rinse the gel briefly with ddH₂O in a 15cm dish.
2) Fix the gel in the Fixative solution for 20 min to 1h.
3) Wash the gel with 30% ethanol for 10 min.
4) Incubate the gel in the Sensitizing solution for 10 min.
5) Wash the gel with 30% ethanol for 10 min.
6) Remove the ethanol and wash the gel with ddH$_2$O for 10 min.
7) Remove ddH$_2$O and incubate the gel in the Staining solution for 15 min.
8) Remove the Staining solution and wash the gel with ddH$_2$O for 20-60 sec.
9) Remove ddH$_2$O and incubate the gel in the Developing solution for 4-8 min to reach optimized color.
10) Once the appropriate staining intensity is achieved, immediately add 3 ml of the Stopper directly to the gel. Gently incubate for 10 min.
11) Remove the Stopper solution and wash the gel with 30 ml ddH$_2$O for 10 min.

9. Western blotting
1) After SDS-PAGE, transfer protein to Nitrocellulose membrane.
2) Briefly stain the membrane with Ponceau S.
3) Rinse the membrane briefly with water. Scan the membrane.
4) Block the membrane with the 5% milk in TBST for 30 min at room temperature.
5) Incubate the membrane in 5% milk in TBST with appropriately diluted primary antibody overnight at 4°C.
6) Next day wash the membrane with TBST three times, 10 min each.
7) Incubate the membrane in 5% milk in TBST with appropriately diluted secondary antibody for 2-3 hr at 4°C.
8) Wash the membrane with TBST three times, 10 min each.
9) Incubate the membrane with substrate (combine A and B rightly before use) for 5 min.
10) Expose and develop the film.

10. Calcium ionophore treatment
1) Grow cells on 6-well plate.
2) Wash the cells with PBS prewarmed in 37 °C water baths.
3) Add prewarmed Loke’s solution (0.15M NaCl, 5mM KCl, 10mM Hepes.HCl pH 7.3, 2mM CaCl$_2$, 0.1% Glucose) and incubated at 37°C with 4 uM A23187 for 15-20 min.

11. Immunostaining.
1) Grow cells on 6-well plate, with glass coverslips for immunostaining.
2) Make fixing solution containing 1xPBS, 1x PFA, with 1% Triton X-100, 2% NP-40 (for 6ml, 0.6ml 10xPBS, 1ml 5xPFA, 50μl Triton X-100, 100μl NP-40, cut the tip of the pipette for transferring Triton-X-100 and NP-40). 1ml of fixing for each well in a 6 well plate
3) Fix cells with the fixing solution for 10 min at room temperature.
4) Wash the cells with PBST for three times, 10 min each.
5) Block the cells with 2% BSA in PBST for at least 30 min at room temperature.
6) Add primary antibody diluted in PBST with 2% BSA and 5% normal goat serum in a humidified container for overnight at 4°C.
7) Wash the coverslips with PBST for three times, 10 min each.
8) Add secondary antibody diluted in PBST with 2% BSA and 5% normal goat serum for 2 hr at room temperature.
9) Wash the coverslips with PBST for three times, 10 min each.
10) Stain the cells with 1μg/ml DAPI.
11) Mount with the Mounting solution, seal the edge with nail polish, and take pictures on the microscope.

12. Point mutation
   1) Point mutation of JMJD4 is performed by QuikChange Site-Directed Mutagenesis Kit (Stratagene 200518).

   1) GST fusion proteins, including GST-PAD4, GST-JMJD4, GST-JMJD6 were expressed in *E.coli* by transforming the recombinant pGEX4T1 plasmid containing the target gene into BL21 competent cells and induced by IPTG.
   2) Purification of the proteins is conducted by using glutathione agarose beads (sigma-Aldrich) according to manufacturer’s instruction.

14. Purification of His-tagged protein
   1) His-tagged proteins, including His-tagged-JMJD4, His-tagged-JMJD6 were expressed in *E.coli* by transforming the recombinant pet11a plasmid containing the target gene into BL21 competent cells and induced by IPTG.
   2) The cells was collected by spinning and washed once with PBS buffer.
3) The cells were quickly frozen in liquid N\textsubscript{2} and warmed up under tap water. When the cells started thawing, add 50ml lysis buffer (50mM sodium phosphate (pH 8.0), 300mM NaCl, 10mM imidazole, 0.5 % Triton X-100, with protease inhibitors) per liter of cell culture.

4) The cells were then sonicated and the cell lysate was loaded onto Ni-NTA agarose column at 4 °C. The loaded column was washed with 500x bed volume washing buffer (50mM sodium phosphate (pH 8.0), 500mM NaCl, 10mM imidazole). Purified protein was eluted with 10x bed volume elution buffer (50mM sodium phosphate (pH 8.0), 100mM NaCl, 250mM imidazole, 5% glycerol).

15. Generate Flag-Hemagglutinin (HA)-JMJD6 and FLAG-HA-JMJD4 expressing 293T and MCF-7 cell lines by retroviral transduction

1) Clone Flag-HA-JMJD6 and Flag-HA-JMJD4 into the pMIGR1 vector.

2) In day 1 afternoon, plate ~7.5x10\textsuperscript{5}/well 293T cells in a 6-well plate.

3) In day 2 afternoon, When 293T cells reach ~60% confluent, co-transfect 293T cells using Calcium phosphate transfection with 4 μg/well packaging plasmid DNA Ψ\textsubscript{ampho}, and 4 μg/well retroviral expression plasmid DNA pMIGR1-FH-JMJD6 or pMIGR1-FH-JMJD4.

4) In day 3 morning, remove transfection medium, and add 1 ml of fresh culture medium into each well. In day 4 morning, plate 2-3x10\textsuperscript{5}/well healthy, log phase target cells in a 6-well plate, add 750 μl media. Filter the retroviral
supernatant with 0.45 μm filter and add Polybrene to a final concentration of 8 μg/ml. Add the supernatant to the target cells and centrifuge the plate at 1,400 rpm for 45 min at room temperature. Following spinoculation, leave the plate in the 37°C incubator until the next round of infection. Add 1 ml fresh culture medium to 293T viral producer cells.

5) In day 5 perform the second round of spinoculation. 2 rounds is sufficient for most cell lines.

6) In day 6 examine GFP expression. Green fluorescence is usually visible 24 hr after the first round of infection. It will reach maximum 24-48 hr after the last round. Positive cells could be sorted out by flow cytometry.

16. Purification of FLAG Fusion Protein

1) Generate Flag-Hemagglutinin (HA)-JMJD6 and FLAG-HA-JMJD4 expressing 293T and MCF-7 cell lines by retroviral transduction

2) Purification of the proteins is conducted by using ANTI-FLAG M2 Affinity Gel (sigma-Aldrich) according to manufacturer’s instruction.

17. Purification of HA Fusion Protein

1) Purification of the proteins is conducted by using anti-HA conjugated beads (sigma-Aldrich) according to manufacturer’s instruction

18. Demethylation assay
1) 5× buffer (200 μl): 100 μl 0.5M Hepes 8.0, 2μl 0.5M α-KG, 5μl 0.1M ascorbate, 25μl 1mM FeSO₄, 25μl 1mM (NH₄)₂SO₄
2) 20 μl reaction system, add proper amount of JMJD6 or JMJD4 proteins, 4μl 5× buffer and 2 μg histones. Incubate for 1h at 37°C.
3) Examine the result by western blotting.

19. PAD4 inhibitor assays
   1) Prepare 10× PAD buffer containing 50mM Tris HCl pH7.6, 40mM DTT, 40mM CaCl₂
   2) The 20 μl reaction system contains 2 μg histone H3, 2 μg PAD4, 50μM or 200μM inhibitor.
   3) First add GST-PAD4+inhibitor in the reaction buffer system, incubate 15min at 37°C
   4) Add histone H3 and incubate 1h at 37°C
   5) Examine the result by western blotting

20. GST pull-down
   1) Wash 20 μl GST beads with 1 ml PBST for three times.
   2) Add GST beads to nuclear extract in 1.5 ml epp tube, bring up total volume to 500 μl. Mix and rotate for overnight at 4°C.
   3) After incubation, wash GST beads with PBST for 10 min with rotation, 3 times.
   4) Elute the protein with Glutathione Elution buffer and do the Western Blotting to examine the result.

21. Chromatin immunoprecipitation (CHIP)
   1) Add 37% Formaldehyde to a plate of cells to a final concentration of 1%. Shake gently for 10 min.
2) Add 2 M Glycine to a final concentration of 0.125 M to stop cross-linking. Shake gently for 5 min.

3) Wash three times with PBS with protease inhibitors.

4) Spin down cells at 1,500 rpm for 5 min at 4°C.

5) Resuspend cells with 8 ml SDS-lysis buffer with protease inhibitors.

6) Rotate for 10 min at 4°C. Spin down at 1,500 rpm for 6 min at 4°C.

7) Resuspend the pellet with 2 ml ice-cold IP buffer. Leave on ice for 10 min, invert occasionally.

8) Sonicate the sample for 5 min.

9) Pre-clear sample for 2 hr at 4°C with rotating.

10) Spin down at 3,000 rpm for 2 min at 4°C. Collect supernatant and spin again at 13,200 rpm for 15 min at 4°C to discard possible aggregates.

11) Share sample to epp tubes with desired antibody added, rotate overnight at 4°C. Set up one tube without antibody that will represent the background and the source for the total input.

12) Next day spin down the IP samples at 13,200 rpm for 15 min at 4°C. Collect supernatant and discard possible aggregates.

13) Collect 30 µl from the “No antibody” sample and keep on ice; this will be processed at reverse cross-linking step as the “Total input” sample.

14) Add about 20 µl washed Protein A agarose/ssDNA to each sample, rotate for 1.5 hr at 4°C.
15) Wash the beads in below order and amount. Use 1 ml and rotate for 5 min at 4°C. Spin down the beads at 3,000 rpm for 1 min at 4°C. Discard supernatant.
   3 washes of Mixed Micelle buffer
   2 washes of Buffer 500
   2 washes of LiCl/Detergent buffer
   2 washes of TE buffer
   Resuspend the beads in 170 µl fresh prepared Bicarbonate/SDS.
   Add 120 µl to “Total input” sample.

16) Vortex at low speed for 15 min at room temperature. Spin down at 3,000 rpm for 2 min and collect 150 µl supernatant.

17) Resuspend the beads in 150 µl Bicarbonate/SDS buffer and repeat step 20.

18) Incubate the samples at 65°C for at least 5 hr to reverse cross-linking.

19) Spin down briefly to collect drops under the lid. Leave at room temperature until cool.

20) Add 3 µl of 10 mg/ml Protease K and 1 µl of 10 mg/ml RNase A and incubate at 37°C for 2.5 hr.

21) Add 30 µl of 3 M NaAc (pH 5.0) to each sample. Purify DNA.

22. CHIP-reCHIP

  1) Add 37% formaldehyde to a final concentration 0.1%. Incubate with shaking for 10 min, then stop cross linking.
  2) Wash with PBS twice.
  3) Collect cells in 5 ml collection buffer (100 mM Tris-HCl PH9.4, 100 mM DTT)
  4) Incubate on ice for 10 min, 30°C for 10 min.
5) 2000rpm at 4°C for 5 min.
6) PBS wash once
7) Buffer A wash once (10 mM EDTA, 0.5mM EGTA, 10mM HEPES PH6.5, 0.25% Triton X-100)
8) Lysis-buffer 4°C, rotate for 10 min.
9) Centrifuge and get rid of the supernatant.
10) Add IP buffer and sonicate the pellet.
11) Add flag- beads over night.
12) Wash the beads with 4 high salt buffers.
13) Elute with 10mm DTT, 50 μl twice, then dilute 10 time with IP buffer
14) Split to each antibody then do the second round CHIP.

Chapter Three. Results

1. **Release of isocyanic acid (HNCO) is a novel diagnostic marker for mass spectrometric identification of citrullination.**

Citrullination catalyzed by PAD4 can be detected by anti-citrulline antibodies, but the exact site of citrullination can only be detected by Mass Spectrometric approaches. Since the mass shift to convert arginine to citrulline is only 1 Da and isobaric with deamidation on Asn and Gln residues, it is difficult to detect by mass spectrometric approaches. In our published study, we found a gas-phase fragmentation pathway of citrullinated peptides by electrospray-tandem mass spectrometry (Figure 3; Hao et al., 2009). In this pathway, an isocyanic acid (HNCO) is released from the citrulline ureido group, leading to a characteristic neutral loss of 43 Da (Figure 3A). Nucleophosmin (NPM), a molecular chaperon involved in nucleosome core particle assembling, is a reported substrate of PAD4
(Hagiwara et al., 2002), but the exact site was unknown. To test whether the HNCO release could be used to identify an unknown citrullination site, NPM was used. I treated recombinant human His-NPM with GST-PAD4 and confirmed citrullination by Western blot analyses (Figure 3B). Upon electrospray-tandem mass spectrometry, Arg 197 was identified as a target site of PAD4 in NPM (Hao et al., 2010).

2. Chromatin decondensation is associated with PAD4 activation and histone citrullination.

Previous studies showed that in response to stimuli such as pathogen infection, neutrophils could form a highly decondensed chromatin structure to trap the pathogen. This structure is called neutrophil extracellular traps (NETs) (Beiter et al., 2006; Brinkmann et al., 2004; Buchanan et al., 2006; Fuchs et al., 2007). Since we have found the function of PAD4 in histone modification in chromatin, whether PAD4 regulated citrullination also play a role in the chromatin higher order structure is still unknown. We performed immunostaining with HL-60 granulocytes after calcium ionophore treatment for 15 min. A web-like chromatin structure formed by long stretches of extensively decondensed chromatin was found in the extracellular space (Figure 4A-J). Interestingly, histone H4 Arg3 methylation was decreased (Figure 4B) and histone H4 citrullination at Arg 3 (H4Cit3) was increased (Figure 4E&4H) at regions of highly decondensed chromatin. Given that PAD4 converts Arg and monomethyl-Arg to citrulline, these results show that chromatin decondensation is associated with PAD4 activation. The H4 K16 acetylation (H4AcK16), which was tested as a control, didn’t increase at decondensed chromatin region, suggesting that the increased staining of histone H4Cit3 was not caused by increased antibody accessibility. To further confirm the increase of histone citrullination, I performed western
Figure 3: (A) Proposed fragmentation product--isocyanic acid elimination from citrulline peptides. (B) PAD4 treatment leads to marked increase in citrulline level, as detected by anti-Cit antibody in Western blot.
blotting experiments with histones extracted from calcium ionophore treated HL-60 cells. Compared with untreated cells, both H3 and H4 citrullination were significantly increased (Figure 4K).

3. **PAD4 and HDAC2 interact and simultaneously bind to the p21 promoter.**

Since histone Arg methylation and Lys acetylation coordinate with each other to regulate the expression of p53-target gene (An et al., 2004), we analyzed whether histone deacetylation and histone demethylimation also coordinate with each other to repress transcription. PAD4 and HDAC2 interaction was first detected by GST-pull down assay. PAD4 has two immunoglobulin (IgL)-like domains at its N-terminus (Figure 5A) (Arita et al., 2004). GST- pull down assay was performed by GST-PAD4, GST-PAD4-IgL1 containing residues 1-125 and GST-PAD4-IgL1&2 containing residues 1-300. All the GST fusion proteins were purified from *E.coli* (Figure 5C). We found that only full length GST-PAD4 and GST-PAD4-IgL1&2 interacted with HDAC2 (Figure 5D, lane 8&9), indicating that the two IgL-like domains are necessary and sufficient for PAD4 and HDAC2 interaction. Since neither PAD4 nor HDAC2 has a DNA-binding domain, these two proteins may bind to gene promoters by mediation of some transcription factors. Previous studies showed that the two IgL-like domains of PAD4 interacted with the C-terminal domain of transcription factor p53 (Li et al., 2008), there is a high possibility that PAD4 and HDAC2 bind to promoters by interacting with p53. So we tested the interaction between HDAC2 and p53. We purified full length GST-p53, C-terminal domain of p53 (residues 301-393) and N-terminal part of p53 (residues 1-300 containing both the activation domain and the middle DNA-binding domain) from *E.coli* (Figure 5B&5E). GST-pull down
Figure 4: Chromatin decondensation is associated with PAD4 activation and histone citrullination in HL-60 granulocytes. (A-C) After calcium ionophore treatment, there is loss of histone H4Arg3 methylation on the decondensed chromatin (denoted by arrows) (D-F) After calcium ionophore treatment, there is increase in H4Cit3 on the decondensed chromatin (denoted by arrows). (G-J) Grayscale images show that H4K16 acetylation was not elevated on the decondensed chromatin compared with H4Cit3 (denoted by arrows). (K) Western blotting shows the increase in histone H3 and H4 citrullination after calcium ionophore treatment.
experiments showed that both C-terminal domain and N-terminal part of p53 interact with HDAC2, while the interaction between C-terminal domain and HDAC2 is stronger (Figure 5F, lanes 6&7). A model of p53, PAD4 and HDAC2 interaction was drawn from the experiments results (Figure 5G). The IgL-like domains of PAD4 interact with the C-terminal regulation domain of p53 and HDAC2, and both C-terminal domain and N-terminal part of p53 interact with HDAC2. Further, the interaction between PAD4 and HDAC2 suggests the two proteins may simultaneously bind to the same promoter. To test this possibility, I performed CHIP-reCHIP experiment at p21 promoter (Figure 5H). I performed first round CHIP with MCF-7 cells expressing Flag-PAD4 and control MCF-7 cells. M2 agarose beads bound to Flag-PAD4 and enriched PAD4-associated promoters. PCR results showed that the M2 beads recovered p21 promoter from Flag-PAD4 MCF-7 cells but not control MCF-7 cells. Then we eluted the DNA-protein complex from M2 beads by FLAG peptide, and used HDAC2 antibody to perform second round CHIP. PAD4 antibody was used as a positive control and also indicated the efficiency of the CHIP experiments. The HDAC2 antibody could recover the p21 promoter in DNA-protein complex from Flag-PAD4 MCF-7 cells, but not the control MCF-7 cells. This result indicated that PAD4 and HDAC2 bound to the p21 promoter simultaneously.


Since PAD4 is found out to be a potential drug target for cancer treatment and PAD4 inhibitor Cl-amidine is shown to inhibit the growth of MCF-7 cells. We tried to improve the effect of Cl-amidine to find out a practicable cancer
**Figure 5:** PAD4 and HDAC2 interact with each other, simultaneous binding to the p21 promoter. (A) PAD4 contains two immunoglobulin (IgL)-like domains (residues 1-150 and residues 150-300) at its N-terminus and a catalytic domain (residues 301-663) at its C-terminus. (B) p53 contains a C-terminal regulatory domain (residues 301-393) and its N-terminal part (residues 1-300) contains an activation domain and a DNA-binding domain. (C) The amount of GST and GST-PAD4 fusion proteins purified from E. coli BL-21 and used in the pull-down experiments by Ponceau S staining. (D) The pull-down experiments show that the GST-PAD4 full length and GST-IgL1&2 interact with HDAC2. (E) The amount of GST and GST-P53 fusion proteins purified from E. coli BL-21 strain and used in the pull-down experiments. (F) The pull-down experiment showing that the GST-P53 full length, GST-p53\(^{1-300}\) and p53\(^{301-393}\) interact with HDAC2. (G) A Model shows that the (IgL)-like domains of PAD4 interact with the C-terminal regulation domain of p53 and HDAC2, and both C-terminal domain and N-terminal part of p53 interact with HDAC2. (H) CHIP-reCHIP experiment demonstrates simultaneous binding of PAD4 and HDAC2 to the p21 promoter. The first round ChIP was performed by M2 agarose beads that could recover FLAG-PAD4-associated chromatin fragments. Then we collected the chromatin fragments by eluting with 200 mg/ml FLAG peptide, the second round ChIP was performed by the affinity purified PAD4 or HDAC2 antibody.
drug that could be used in the future. More PAD4 inhibitors were synthesized based on the structure of Cl-amidine by Dr. Gong Chen’s group in the Chemistry Department of Pennsylvania State University (Figure 6). Conserved in the core active part, the derivatives have been changed in the length of the side chains at the N- and C-terminal of the haloacetamidine group. The effect of PAD4 inhibitors on PAD4 activity was analyzed by histone H3 citrullination antibody in Western blot analyses (Figure 7). Compared with Cl-amidine, several newly synthesized inhibitors had similar or even better effect, such as YW1-91, BL1-07, BL1-15, YW1-90, YW1-30 and YW1-44. The most promising one was YW1-90 which inhibited the activity of PAD4 even at a lower concentration compared to Cl-amidine (Figure 7).

5. The putative demethylation activity of JMJD4 and JMJD6.

Previous studies showed that JMJD6 was a potential histone Arg demethylase (Chang et al., 2007). However, recent studies indicated that lysyl hydroxylation is the dominant oxidative catalytic activity of JMJD6 (Webby et al., 2009). To reconcile these contradictory results, I have further analyzed whether JMJD6 functions as an Arg demethylase. Furthermore, another JmjC-domain protein JMJD4 has high similarity with JMJD6, it is included in my study as well. To test the demethylase activity of these two proteins, I cloned Flag and HA epitope tagged JMJD6 (FH-JMJD6) and JMJD4 (FH-JMJD4) into the pMIGR1 vector and established 293T and HeLa cell lines expressing these fusion proteins by retroviral transduction. We then purified FH-JMJD4 and FH-JMJD6 from 293T cells by anti-FLAG antibody conjugated M2 agarose beads (Figure 8A). Then I further purified the proteins using anti-HA antibody conjugated beads which could enrich the HA-tagged fusion proteins (Figure 8B). Compared the proteins enriched
Figure 6: Structures of PAD4 inhibitors. The inhibitors are synthesized based on the structure of PAD4 inhibitor Cl-amidine.
Figure 7: PAD4 inhibitor assays. GST-PAD4 and inhibitors of the indicated concentration were incubated for 15 min at 37°C, then histone H3 was added and incubated for 1h at 37°C. The amount of H3 used in the reaction is shown by Ponceau Staining. The level of H3 citrullination was detected by Western blot.
after one or two round of purification, an elimination of unspecific or low affinity binding proteins was observed. I then cut out the bands after second round purification and sent them to Mass spec analysis. The results showed that JMJD4 interacts Hsp70 family of proteins (Hsp70-1 and -2), while JMJD6 interacts with a splicing factor called LUC7L2 (Figure 8B), which has been reported to interact with JMJD6 (Webby et al., 2009).

6. Testing the demethylation activity of JMJD4 and JMJD6

To test the demethylation function of JMJD4 and JMJD6, we first used GST-tagged fusion proteins to treat the histones with the existence of Fe (II), 2-oxoglutarate, and ascorbate. No distinct change was detected (Figure 9A), suggesting that GST-tagged JMJD4 or JMJD6 was not active demethylase. On the other hand, Western blotting experiments of histones extracted from Flag-HA, Flag-JMJD4 and Flag-JMJD6 293T cells showed decreases of methylation level of histone H3 Arg17 asymmetrical dimethylation (H3R17 ADMA) and histone H4 Arg3 symmetrical dimethylation (H4R3 SDMA) in both FH-JMJD4 and FH-JMJD6 expressing 293T cells, and histone H4 Arg3 asymmetric dimethylation (H4R3 AMDA) in FH-JMJD4 expressing 293T cells, suggesting a preference for the substrate methylation status by JMJD6 and JMJD4. No decrease in histone H3 Lys 27 trimethylation (H3K27Me3) was detected, suggesting that JMJD4 and JMJD6 affects Arg methylation but not lysine methylation (Figure 9B). In immunostaining of HeLa cells transiently transfected with Flag-JMJD6 expression vector, a decrease in H4R3SDMA level was detected (Figure 9C).

To analyze the biochemical activity of JMJD4 and JMJD6, I treated nucleosome with Flag-JMJD4 or Flag-JMJD6 proteins purified from 293T
Figure 8: Affinity purification of proteins associated with Flag-HA-JMJD4 and Flag-HA-JMJD6. (A) Purification of Flag-HA-JMJD4 and Flag-HA-JMJD6 by passing through anti-FLAG antibody conjugated M2 Affinity Gel. (B) Further purification by anti-HA antibody conjugated beads.
cells. Consistent with western blotting results above, a decrease of H4R3SDMA level was shown after Flag-JMJD4 treatment (Figure 9D). But surprisingly, no decrease was detected after Flag-JMJD6 treatment. Since mutation of the residues predicted to mediate Fe(II) binding in JMJD6 prevented its demethylation function, we wonder to know whether such kind of mutation could also prevent demethylation function of JMJD4 proteins. The residues 187 and 189 of JMJD4 were mutated (Histidine 187 to Alanine 187 and Aspartate 189 to Alanine 189) and the mutated JMJD4 was expressed in 293T cells. Western blot results showed that these mutations prevent JMJD4 mediated H4R3SDMA demethylation (Figure 9E).

Given that Flag-tag proteins are purified from mammalian cells, some other demethylation enzymes may have been copurified with JMJD4 or JMJD6. So I purified His-JMJD6 protein with a His-tag at its C-terminus expressed in E.coli strain BL21. I found that the 200 mM imidazole eluted most of the proteins (Figure 10A). I confirmed the protein eluted was His6-JMJD6 by anti-his6 antibody and found that His6-JMJD6 (Figure 10B). To determine the purified His-JMJD6 protein concentration, I ran it in parallel with BSA of different concentrations and used coomassie blue staining to estimate the concentration of JMJD6 (Figure 10C). The concentration His6-JMJD6 protein was roughly 100 ng/µl. I then used purified His-JMJD6 to treat histones and mononucleosomes. The levels of H3R17ADMA and H4R3SDMA were detected in Western blot analyses. A significant decrease was shown in the H4R3SDMA level of treated histones sample, but not in the H3R17ADMA level, suggesting a preference of His6-JMJD6 to particular modification sites. Also, no decrease was found in
Figure 9: Demethylation activity analysis of JMJD4 and JMJD6 proteins. 

(A) No change was found in the methylation level of nucleosomes treated with purified GST-JMJD4 or GST-JMJD6. (B) Decreases of H4R3SDMA and H3R17ADMA level are shown in histones extracted from 293T cells stably expressing Flag-JMJD4 and Flag-JMJD6. A decrease of H4R3ADMA level is shown in histones extracted from Flag-JMJD4 but not Flag-JMJD6 293T cells. (C) Transient transfection of Flag-JMJD6 in HeLa cells. Immunostaining shows the decrease of H4R3SDMA in JMJD6 overexpressing cells. (D) A decrease of H4R3SDMA level is shown in Flag-JMJD4 treated nucleosomes but not in Flag-JMJD6 treated nucleosomes. (E) A decrease of H4R3SDMA level is shown in histones extracted from Flag-JMJD4 and Flag-JMJD6 stable expressing 293T cells but not Flag-JMJD4 mut stable expressing 293T cells. No change of H3R17ADMA level is shown.
Figure 10: The purification of His6-JMJD6 protein from *E. coli* and demethylases activity analysis. (A) Purification of His-JMJD6, washing with binding buffer and eluting sequentially by 10mM, 20mM, 200mM, 500mM imidazole (IMD). The proteins are shown by Coomassie Blue Staining. (B) Detection of the existence of His6-JMJD6 in different protein collections by α-His antibody. (C) Determination of the purified His-JMJD6 concentration by running purified protein in parallel with BSA of different concentrations. (D) Demethylation assay. Treat histones and mononucleosomes with His6-JMJD6 and detect the methylation level by western blotting.
treated mononucleosome sample, indicating that His6-JMJD6 might have substrate preference (Figure 10E).

7. Interaction of JMJD4 with Hsp70 p53 JMJD6

Mass spec analysis results showed that Flag-JMJD4 and Hsp70 could be co-purified from Flag-JMJD4 expressing 293T cells. Since previous studies have shown that Hsp70 is highly expressed in many types of cancer cells and interact with p53, playing a role in tumor cell apoptosis (Fourie et al., 1997; Han et al., 2007; Sherman et al., 2007). It will provide an interesting direction to study the function of JMJD4 if it interacts with Hsp70 and is involved in the p53 pathway. We performed GST pull down experiments and detected an interaction between JMJD4 and Hsp70 (Figure 11A). Compared with that of JMJD4 and Hsp70, a weaker interaction was shown between Hsp70 and p53 (Figure 11B). We further tested the interaction between different parts of p53 and Hsp70. We purified full length GST-p53, C-terminal domain of p53 (residues 301-393) and N-terminal part of p53 (residues 1-300) from E.coli. The pull-down experiments showed only the full length and C-terminal domain of p53 could interact with Hsp70 (Figure 11C), indicating the C-terminal domain and not the N-terminal part of p53 could interact with Hsp70. To further confirm the interaction between JMJD4 and Hsp70, co-immunoprecipitation (co-IP) experiments were performed and an interaction of JMJD4 and Hsp70 was detected (Figure 11D). Flag-OKL38, a tumor suppressor gene that regulated by p53 served as a negative control to show the specific binding. Furthermore, co-IP analyses using p53 antibody showed an interaction between JMJD4 and p53 (Figure 11E). Surprisingly, an even stronger interaction between JMJD6 and p53 was detected (Figure 11E).
Figure 11: Interaction of JMJD4, JMJD6, Hsp70 and p53. (A) GST-pull down experiments show the interaction between GST-JMJD4 and Hsp70. (B) GST-pull down experiments show that GST-p53 has weaker interaction with Hsp70. (C) GST-pull down experiments show that the GST-p53 full length and GST-p53(1-300) interact with Hsp70. (D) Copurification of Hsp70 with FLAG-JMJD4 but not FLAG-OKL38. (E) The co-IP results show p53 was copurified with FLAG-JMJD4 and FLAG-JMJD6.
The lack of JMJD6 has been correlated with increased apoptosis in *Drosophila* (Krieser et al., 2007), the interaction of JMJD4 and JMJD6 with p53 prompted us to test whether these two proteins play a role in p53-target gene expression. We first performed shRNA and siRNA treatment to deplete the JMJD4 or JMJD6 and test the change of p53 target genes. The efficiency of shRNA depletion of JMJD6 and JMJD4 in U2OS cells was analyzed by Western blot (Figure 12A). The amount of p53, p21, OKL-38 and puma was analyzed by Western blot. α-actin was probed as a control to ensure equal loading. The amount of OKL38 showed significant increase in both JMJD4-shRNA and JMJD6-shRNA treated cells. Depletion of JMJD6 by shRNA increased the expression of p21 while there was no change in p21 levels after JMJD4 depletion. No change of p53 was found after JMJD4 or JMJD6 depletion. In contrast, the level of puma decreased after JMJD4 or JMJD6 depletion (Figure 12B). We further investigated the amount of p53 and p21 changes after after JMJD4 and JMJD6 depletion using commercially available siRNA reagents in U2OS. The efficiency of JMJD4 and JMJD6 depletion was shown by western blotting (Figure 12C and 12D). Depletion of JMJD4 or JMJD6 increased the H3R17ADMA level, suggesting the demethylation functions of JMJD4 and JMJD6 (Figure 12E). Depletion of JMJD6 by siRNA increased the p21 level while depletion of JMJD4 didn’t make much change, which is consistent with the shRNA experiments (Figure 12F). Depletion of JMJD6 by siRNA also increased the p53 level while there is no change for the depletion of JMJD4 (Figure 12F). Above results suggest that JMJD4 and JMJD6 might be novel regulators of p53.
Figure 12: Effects of JMJD4 and JMJD6 depletion on p53 target gene expression. (A) Western blotting shows shRNA-mediated depletion of JMJD4 and 6. (B) Depletion of JMJD6 by shRNA increases the expression of p21 and OKL38 but not PUMA. In contrast, depletion of JMJD4 increase only the expression of PUMA. (C-D) Depletion of JMJD4 and JMJD6 by siRNAs. (E) Extracted histones from siRNA-JMJD4 or siRNA-JMJD6 treated U2OS cells showed an increase in H3R17ADMA levels. (F) The depletion of JMJD6 increased expression of p53 and p21 while depletion of JMJD4 made no changes.
Chapter Four. Discussion

1. Releasing of isocyanic acid (HNCO) could be used to detect the exact site of citrullination.

Protein citrullination regulated by PAD4 is involved in diverse cellular processed such as gene regulation, apoptosis and so on. Since the mass shift resulted from conversion of arginine to citrulline is small, a more reliable method to detect the protein citrullination is required. In gas-phase fragmentation pathways of citrullinated peptides, an isocyanic acid (HNCO) is released from the citrulline ureido group with an easy detected neutral loss of 43 Da (Figure 3A). We first tested this method by comparison of fragmentation behavior of a small citrullinated peptide and its Arg-containing counterpart. A characteristic neutral loss of 43 Da was shown, indicating the practicability of this method. Since proteins such as histones (H3, H2A, H4), nucleophosmin, myelin and keratin can be citrullinated but the exact site is still unknown (Hagiwara et al., 2005; Hagiwara et al., 2002; Kidd et al., 2008; Kubota et al., 2005; Nachat et al., 2005; Nakayama-Hamada et al., 2005), we used nucleophosmin as a test to investigate whether this method could be used to identify an unknown citrullination site. We treated human His6-NPM with GST-PAD4, showed the deimination effect by western blotting (Figure 3B) and excised the gel band to analyze by LC-MS. The result indicated the citrullination site was Arg 197, suggesting the potential function of the method to identify the exact citrullination site and discover novel ones.

2. Functional connection between demethylimmination and deacetylation of histones.
Post-transcriptional regulation plays an important role in the opening of chromatin structure and also provides binding sites for effector proteins. To regulate gene expression more efficiently, crosstalk happens between different histone modifications. Histone Arg methylation and histone Lys acetylation were reported to coordinate the activation of p53 target gene transcription (An et al., 2004). In our previous study, we reported that the PAD4 could regulate p53 target gene expression by a process called demethylimmination which was to counteract the function of Arg methylation by changing methylated Arg into citrulline. In the other hand, HDAC2 removes the histone acetyl groups through the process of deacetylation. So whether crosstalk also happens between histone Arg demethylimmination and histone Lys deacetylation could be studied. In our study, we report that PAD4 and HDAC2 interact with each other. They are simultaneously recruited to p21 promoter through interaction with p53, serving as p53 corepressors (Figure 5). This result indicates the functional connection between histone Arg demethylimmination and Lys deacetylation. In a recent study, the finding that PAD4 and HDAC1 associate cyclically and coordinately with the pS2 promoter during repression phases also indicates the connection between demethylimmination and deacetylation (Denis et al., 2009). This finding together with our study may provide more support of cross talk between histone modifications. Since PAD4 was a putative drug target for cancer therapy and HDAC inhibitors have been used to treat cancer patients, our study indicate the possibility of combining HDAC and PAD4 inhibitors in cancer treatment.

3. PAD4 inhibitors are served as potential anticaner drugs.
Compared with suberoylanilide hydroxamic acid (SAHA), an inhibitor of histone deacetylases (HDACs) that causes growth arrest, differentiation, and apoptosis in many tumor types (Coffey et al., 2000; Munster et al., 2001; Richon et al., 1996), PAD4 inhibitor Cl-amindine has a weaker activity in inhibiting tumor growth. To investigate a novel potential cancer drug, we studied the synthesized derivatives of Cl-amidine (Figure 6). We work on two aspects: first, we try to increase the inhibition effect, which could be tested in vitro by inhibiting PAD4 citrullination activity. We detected the H3Cit level of PAD4 treated histone H3 with addition of different inhibitors and identified several candidates with similar or better effect compared to Cl-amidine (Figure 7). The most promising one is YW1-90, which is effective at low concentration. Second, we would like to enhance the accessibility of inhibitors to penetrate cell membrane. We will treat the cells by inhibitors with different concentrations to test cell growth inhibition of these novel inhibitors.

4. JMJD6 and JMJD4 have potential Arg demethylation function.

To test the histone Arg demethylases function of JMJD4 and JMJD6 proteins, we tried different kinds of experiments both in vivo and in vitro. We established the stable 293T and HeLa cell lines expressing Flag-JMJD4/6 to analyze their effect on histone Arg methylation level. If JMJD4 or JMJD6 is Arg demethylase, their overexpression will lead to a decrease in histone Arg methylation. Antibodies specific for various methylated sites on histones H3 and H4 were used to assess demethylation. The results showed a decrease of methylation level of H317ADMA, H4R3SDMA in 293T cells expressing Flag-JMJD4 or Flag-JMJD6, and a decrease of H4R3AMDA in 293T cells expressing Flag-JMJD4 (Figure 9B), suggesting a substrate methylation state
preference of these enzymes. These experiments were performed several times and the results were not always consistent, the reason might be that the demethylation activity was weak. To perform the \textit{in vitro} demethylation reactions, we first tried purified GST-tag proteins to treat the histones. No distinct change was shown (Figure 9A). Given that some eukaryotic proteins expressed in prokaryotes might function poorly or lose the activity due to a lack of posttranslational modifications, we expressed and purified Flag-HA-JMJD4/6 proteins in cultured human cells. In contrast to the results with extracted histones and immunostained cells, when we treated histones with purified Flag-JMJD6, the level of H3R17ADMA didn’t decrease (Figure 9C). I think that even if the JMJD4 or JMJD6 is demethylases, the activity should be weak. To directly demonstrate Arg demethylation, I used matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) to analyze the reaction products of treated peptides. Histone H4 peptides synthesized with symmetric dimethyl Arg3 or asymmetric dimethyl Arg3 were incubated with buffer alone, Flag-JMJD6, or Flag-JMJD4. No expected demethylation product was found (data not shown). These results could not be simplified to conclude that JMJD4 and JMJD6 are not Arg demethylases, because either that the demethylation activity is weak or that the peptides are too short to act as substrates will lead to no detection of demethylation activity.

\textit{Drosophila} PSR is also a nuclear protein and share 70% similarity with Homo sapiens JMJD6. Similar with human JMJD6, loss of dPSR also results in apoptosis, indicating a role of PSR in cell survival (Krieser et al., 2007). We considered testing the demethylation function in \textit{Drosophila}. If overexpression of PSR leads to a decrease of methylation or depletion of PSR results an increase, it will provide a clue as to demethylation function
in mammals. We generated flies with PSR overexpressed in saliva glands by crossing UAS-PSR flies with saliva gland specific GAL4-driver flies. Polytene chromosome squash and immunostaining with several histone Arg methylation antibodies were performed. No consistent decrease of methylation level was found compared to control sample, indicating two possibilities. First, different from JMJD6 in mammals, PSR in Drosophila doesn’t have demethylation function. Second, the demethylation function could not be detected by our method.

We further analyzed and the biochemical activity of His6-JMJD6 purified from E.coli. We dialyzed the purified protein with PBS buffer without Fe (II) to get rid of all the metal ions binding to the JMJD6. This process helped a lot and gave us a consistent and visible decrease at H4R3SDMA level (Figure 10D). That no change at H3R17SDMA level served as a negative control, indicating that the decrease did not result from unequal loading. The result that JMJD6 demethylated H4R3SDMA but not H3R17ADMA was consistent with Chang’s paper, making us reconsider our previous results. Histones from U2OS cells treated by siRNA-JMJD4 or siRNA-JMJD4 show increases in H3R17ADMA level. The possibility that other factors in eukaryotic cells might be required in demethylation of H3R17ADMA provides a reasonable explanation to the phenomenon that JMJD4/6 could demethylate H3R17ADMA in vivo but not in vitro. More repeats should be done to ensure the exact site regulated by JMJD4 and JMJD6. The fact that His-JMJD6 could not recognize mononucleosome as a substrate further indicate the substrate preference of JMJD6.

5. A potential function of JMJD4 and JMJD6 in the p53 pathway.
To further understand JMJD4 and JMJD6 proteins, we investigated the pathways involved in the function of JMJD4/6 as well as the proteins interacted with JMJD4/6. Mass Spectrometry analyses showed that JMJD4 interacted Hsp70 family of proteins, while JMJD6 interacted with a splicing factor called LUC7L2 (Figure 8B). The GST-pull down experiments demonstrated the interaction between JMJD4 and Hsp70. Co-IP experiments showed that both JMJD4 and JMJD6 could interact with p53. Recent study used a novel binding assay to show that a Hsp70 binding site existed within the C-terminal domain of p53 (Iosefson and Azem) which was also confirmed by our GST-pull down experiments. Taken together, two possible mechanisms are proposed to explain the interactions. First, as Hsp70 molecular chaperones maintain the p53 native conformation under heat-shock conditions (Walerych et al., 2009), Hsp70 also work as molecular chaperones to regulate the stability of JMJD4. Second, the JMJD4 regulated by Hsp70 involved in the p53 pathway, for example acting as p53 corepressor. Recently, more and more studies suggested that Hsp70 might play an important role in apoptosis by interacting with apoptotic protease-activating factor 1 (Apaf-1) (Matsumori et al., 2006), c-Jun N-terminal kinase (JNK) (Bienemann et al., 2008; Park et al., 2001), and apoptosis-inducing factor (AIF) (Lui and Kong, 2007; Ruchalski et al., 2006). These results lead us to consider more about the possibility of the second hypothesis. It will be interesting if we could show that the JMJD4 and JMJD6 are involved in p53 pathway.

6. Depletion of JMJD4 or JMJD6 affects the expression of a subset of p53 target genes.

To characterize the role of JMJD4 and JMJD6 on p53 activity, we generated U2OS cell lines which expressed shRNA targeting JMJD4 or
JMJD6, respectively. Western blotting showed that JMJD4 and JMJD6 protein levels are significantly decreased upon expression of shRNA targeting JMJD4 or JMJD6, respectively. The depletion of JMJD4 or JMJD6 doesn’t affect the p53 stability. However, when we treated the USOS cells with siRNA-JMJD6, the level of p53 increase a lot but no similar result is got in siRNA-JMJD4 treated U2OS cells. The increase of p21 expression in shRNA-JMJD4 and siRNA-JMJD6 treated U2OS cells showed a promising direction to investigate JMJD6 function in p53 pathway. The depletion of JMJD6 has stronger effects on p53 and p53 target gene, indicating a stronger interaction between JMJD6 and p53 and showing the same way with Co-IP experiments result. In the future, we could further study the function of JMJD4 and JMJD6 in p53 pathway, for example, to analyze JMJD6 association at p21 promoter to test whether JMJD6 and p53 simultaneously bind to the same promoter.
Reference:


