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ANALYSIS OF PEPTIDYLARGININE DEIMINASE 4
AND HISTONE CITRULLINATION
IN TRANSCRIPTIONAL REGULATION AND INNATE IMMUNITY

A Dissertation in
Genetics
by
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ABSTRACT

Peptidylarginine deiminase 4 is an enzyme capable of converting both histone arginine and monomethyl-arginine residues into citrulline through reactions termed deimination/citrullination or demethylimination to regulate histone arginine methylation. This histone posttranslational modification has been related to transcriptional regulation. This dissertation first investigated the role of PAD4 and PAD4 catalyzed histone citrullination in the transcriptional repression of p53-target genes, such as p21/CIP1/WAF1. PAD4 is recruited to the p53-target gene promoter in a p53-dependent manner. Paused RNA Pol II and PAD4 are detected at the p21 promoter before UV irradiation, while RNA Pol II activity and PAD4 association at the p21 promoter are dynamically regulated after UV irradiation. We also detected that PAD4 and histone citrullination coordinate with HDAC2 that mediates histone lysine deacetylation in repressing tumor suppressor gene expression. PAD4 and HDAC2 associated with p21 promoter simultaneously, and both dissociated from several p53-target gene promoters after DNA damage. Our data further revealed that PAD4 promoter association and histone citrullination level are dependent on both p53 and HDAC activity, but HDAC2 promoter association and histone Lys acetylation level are only slightly affected by p53 and PAD4 activity. PAD4 inhibitor Cl-amidine and HDAC inhibitor SAHA induced p53-target gene expression and inhibited cancer cell growth additively in a p53-dependent manner. Using knockout PAD4 mice as a genetic model, we found that PAD4 knockout mice cannot form neutrophil extracellular traps, which are highly decondensed chromatin structures that are important in fighting against invading pathogens after stimulated with chemokines and bacteria. These PAD4 knockout mice are more susceptible to bacterial infection due to the lack of NET-mediated anti-bacterial ability, suggesting an essential role of PAD4 and histone citrullination in innate immunity.
# TABLE OF CONTENTS

LIST OF FIGURES ........................................................................................................ viii

LIST OF TABLES .......................................................................................................... xi

LIST OF ABBREVIATIONS ........................................................................................ xii

ACKNOWLEDGEMENTS ............................................................................................... xiii

Chapter 1  Literature Review ..................................................................................... 1

1.1 Histone modifications and their role in transcriptional regulation ...................... 2
  1.1.1 Chromatin structure and regulation ............................................................. 2
  1.1.2 Posttranslational histone modifications and histone code ............................ 2
  1.1.3 Histone arginine methylation and its role in transcription .............................. 4
1.2 PAD family enzymes and citrullination ............................................................... 5
  1.2.1 PAD family enzymes ................................................................................... 5
  1.2.2 Human PAD4 and its citrullination activity ................................................... 8
1.3 Tumor suppressor protein p53 ............................................................................. 9
1.4 Epigenetics and cancer ....................................................................................... 12
1.5 Neutrophil extracellular traps ............................................................................ 14
1.6 Dissertation summary ......................................................................................... 17

Chapter 2  Materials and Methods ....................................................................... 18

2.1 Molecular Cloning .............................................................................................. 19
  2.1.1 Plasmid construction .................................................................................... 19
  2.1.2 Cracking Gel ............................................................................................... 23
  2.1.3 PCR based point mutation .......................................................................... 24
2.2 Cell Culture ........................................................................................................ 25
  2.2.1 Cell culture conditions ................................................................................ 25
  2.2.2 Cell passage ................................................................................................ 25
  2.2.3 Freeze down cells ........................................................................................ 26
  2.2.4 Recover cells from liquid nitrogen .............................................................. 26
2.3 Cells transfection with plasmid DNA and siRNA .......................................... 26
  2.3.1 Cells transfected with plasmid DNA ......................................................... 26
  2.3.2 Cells transfected with siRNA ..................................................................... 28
2.4 Overexpressing stable cell line generation by retroviral transduction ................. 28
2.5 RNA preparation ............................................................................................... 29
  2.5.1 TRIZOL reagent .......................................................................................... 29
  2.5.2 RNeasy Mini Kit ......................................................................................... 30
2.6 Total protein extract preparation from cells ...................................................... 30
2.7 Nuclear extract preparation ............................................................................... 31
2.8 SDS-PAGE ........................................................................................................ 32
2.9 Coomassie blue staining ................................................................................... 33
2.10 Silver staining ................................................................................................. 33
Chapter 3  Regulation of p53 Target Gene Expression by Peptidylarginine Deiminase 4

3.1 Abstract ................................. 60
3.2 Introduction .............................. 61
3.3 Materials and Methods .......................... 64
   3.3.1 Plasmid constructs .................. 64
   3.3.2 Cell treatments with siRNAs, shRNA, Cl-amidine, and UVC ....... 64
   3.3.3 Coimmunoprecipitation ............... 65
   3.3.4 Permanganate footprinting and LM-PCR .......................... 66
3.4.1 PAD4 inhibitor, Cl-amidine, activates the expression of the p53-target gene p21 .................. 67
3.4.2 Depletion of PAD4 increased the expression of a subset of p53-target genes and apoptosis .................. 69
3.4.3 Depletion of PAD4 induced the expression of p21, apoptosis, and cell cycle arrest in a p53-dependent manner in HCT116 cells .................. 71
3.4.4 Interaction of PAD4 and p53 provides a mechanism for promoter targeting of PAD4 .............................................. 72
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.4.5 Dynamic changes in PAD4 association and histone Arg modifications at the p21 promoter following UV irradiation</td>
<td>74</td>
</tr>
<tr>
<td>3.4.6 PAD4 is recruited to the p21 promoter in a p53-dependent manner</td>
<td>76</td>
</tr>
<tr>
<td>3.4.7 Detection of paused and elongating RNA Pol II by ChIP and permanganate footprinting</td>
<td>77</td>
</tr>
<tr>
<td>3.5 Discussion</td>
<td>80</td>
</tr>
<tr>
<td>3.6 Acknowledgement</td>
<td>85</td>
</tr>
<tr>
<td>Chapter 4 Coordination of PAD4 and HDAC2 in the Regulation of p53-Target Gene Expression</td>
<td>109</td>
</tr>
<tr>
<td>4.1 Abstract</td>
<td>110</td>
</tr>
<tr>
<td>4.2 Introduction</td>
<td>111</td>
</tr>
<tr>
<td>4.3 Materials and Methods</td>
<td>113</td>
</tr>
<tr>
<td>4.3.1 Plasmid constructs</td>
<td>113</td>
</tr>
<tr>
<td>4.3.2 HDAC activity assay</td>
<td>113</td>
</tr>
<tr>
<td>4.3.3 Cell treatment with doxorubicin, siRNA, and inhibitors</td>
<td>113</td>
</tr>
<tr>
<td>4.3.4 Re-chromatin immunoprecipitation</td>
<td>114</td>
</tr>
<tr>
<td>4.3.5 Cell growth curve</td>
<td>114</td>
</tr>
<tr>
<td>4.3.6 Cell growth and morphology analysis using phase contrast microscopy</td>
<td>114</td>
</tr>
<tr>
<td>4.4 Result</td>
<td>115</td>
</tr>
<tr>
<td>4.4.1 Protein-protein interactions of PAD4, HDAC2 and p53</td>
<td>115</td>
</tr>
<tr>
<td>4.4.2 PAD4 and HDAC2 dynamically associate with the p53-target gene promoter during DNA damage response</td>
<td>117</td>
</tr>
<tr>
<td>4.4.3 PAD4 or HDAC2 siRNA treatment increases p21 expression</td>
<td>119</td>
</tr>
<tr>
<td>4.4.4 PAD4 and HDAC inhibitors cooperatively affect p53-target gene expression and cancer cell growth</td>
<td>120</td>
</tr>
<tr>
<td>4.5 Discussion</td>
<td>123</td>
</tr>
<tr>
<td>4.6 Acknowledgement</td>
<td>125</td>
</tr>
<tr>
<td>Chapter 5 PAD4 is Essential for Antibacterial Innate Immunity Mediated by Neutrophil Extracellular Traps</td>
<td>142</td>
</tr>
<tr>
<td>5.1 Abstract</td>
<td>143</td>
</tr>
<tr>
<td>5.2 Introduction</td>
<td>144</td>
</tr>
<tr>
<td>5.3 Materials and Methods</td>
<td>146</td>
</tr>
<tr>
<td>5.3.1 PAD4 knockout mouse generation</td>
<td>146</td>
</tr>
<tr>
<td>5.3.2 Mouse genotyping</td>
<td>146</td>
</tr>
<tr>
<td>5.3.3 Mouse neutrophils purification from peripheral blood</td>
<td>147</td>
</tr>
<tr>
<td>5.3.4 Flow cytometry and TEM analyses</td>
<td>148</td>
</tr>
<tr>
<td>5.3.5 Bacterial strains and growth conditions</td>
<td>149</td>
</tr>
<tr>
<td>5.3.6 Bacteria DNase activity assay</td>
<td>149</td>
</tr>
<tr>
<td>5.3.7 Treatment of neutrophils with chemokines or bacteria</td>
<td>149</td>
</tr>
<tr>
<td>5.3.8 Phagocytosis Assay</td>
<td>150</td>
</tr>
<tr>
<td>5.3.9 Bacterial killing assays using neutrophils</td>
<td>150</td>
</tr>
<tr>
<td>5.3.10 Bacteria killing using histones and nucleosomes with or without citrullination</td>
<td>151</td>
</tr>
<tr>
<td>5.3.11 Mouse infection, necrotizing fasciitis and histology assays</td>
<td>151</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 3-1: PAD4 inhibitor Cl-amidine inhibits the activity of PAD4 ........................................ 86
Figure 3-2: Cl-amidine treatment increases p21 expression .................................................. 87
Figure 3-3: Effect of Cl-amidine on p21 expression ............................................................. 89
Figure 3-4: Effect of PAD4 depletion by siRNAs on the expression of p53-target genes and cell growth in U2OS cells ................................................................. 91
Figure 3-5: Increase of apoptosis after PAD4 depletion by siRNA ....................................... 93
Figure 3-6: Depletion of PAD4 increased p21 expression, apoptosis, and cell cycle arrest in a p53-dependent manner in HCT116 cells ......................................................... 94
Figure 3-7: Interaction of PAD4 and p53 .............................................................................. 96
Figure 3-8: Protein purification in studying interaction between p53 and PAD4 ...................... 98
Figure 3-9: Dynamic p53 and PAD4 association and histone Arg modifications at the p21 promoter after UV irradiation ................................................................. 99
Figure 3-10: PAD4 and histone H3 association with the p21 promoter after UV irradiation ................................................................. 101
Figure 3-11: Association of PAD4 with the p21 promoter is p53-dependent ........................... 102
Figure 3-12: Detection of paused and elongating RNA Pol II at the p21 promoter .................. 104
Figure 3-13: A “three-state” model for the role of PAD4 and dynamic histone Arg modifications in the expression of p21 gene after UV irradiation .................................. 106
Figure 3-14: Effects of PAD4 overexpression on p21 expression ........................................... 107
Figure 4-1: Identification of HDAC2 as a PAD4 interacting protein .................................... 126
Figure 4-2: Detection of PAD4 and HDAC2 activities and protein-protein interaction domain mapping of PAD4, HDAC2, and p53 ................................................................. 128
Figure 4-3: PAD4 and HDAC2 dynamically associate with the p21 promoter during DNA damage response ................................................................. 130
Figure 4-4: Dynamic association of PAD4 and HDAC2 with GADD45 and PUMA promoters during DNA damage ................................................................. 132
Figure 4-5: Promoter association of PAD4 and HDAC2 during DNA damage and after depletion of PAD4 or HDAC2.................................................................................................................. 134

Figure 4-6: Inhibition of PAD4 and/or HDACs impacts on histone modifications at p53-target gene promoters.......................................................................................................................... 136

Figure 4-7: PAD4 and HDAC inhibitors cooperatively affect p53-target gene expression in a p53-dependent manner. ............................................................................................................. 138

Figure 4-8: The effects PAD4 and HDAC inhibitors on the growth of U2OS cells and U2OS/p53-shRNA cells.......................................................................................................................... 139

Figure 4-9: Protein-protein interaction study between PAD4 and HDAC1. .......................................................... 141

Figure 5-1: PAD4 knockout mice generation. ........................................................................................................... 163

Figure 5-2: Knockout of PAD4 in mice did not prohibit neutrophil differentiation. .................. 165

Figure 5-3: Histone citrullination activity of mouse PAD4. .................................................................................. 166

Figure 5-4: PAD4 is required for histone citrullination and chromatin decondensation. ............ 167

Figure 5-5: PAD4 is required for chromatin decondensation and NET formation after chemokine treatment. .................................................................................................................. 169

Figure 5-6: Knockout of PAD4 did not alter the expression of other active PAD family members in neutrophils. .................................................................................................................. 171

Figure 5-7: PAD4 is required for bacterial killing mediated by neutrophil extracellular traps. .......................................................................................................................... 172

Figure 5-8: PAD4 is not required for phagocytosis but is important in NET-mediated innate immunity against group A Streptococcus (GAS) bacteria. .................................................. 173

Figure 5-9: Antagonism of PAD4-mediated NET formation and bacterial extracellular DNase-mediated NET destruction. ........................................................................................................... 175

Figure 5-10: PAD4 is important in immune defense against group A Streptococcus (GAS) in a mouse model of necrotizing fasciitis. .......................................................................................... 177

Figure 6-1: PAD4 protein levels in different human cancer cell lines........................................... 189

Figure 6-2: A model of the cooperative regulation of p21 expression by PAD4 and HDAC2. .......................................................................................................................... 190

Figure 6-3: A model of the biological roles of PAD4 in innate immunity and transcriptional regulation. .......................................................................................................................... 191

Figure A-1: p53 acetylation attenuates its interaction with PAD4. .......................................................... 193
Figure B-1: PAD4 represses p53 transcription on naked DNA template. ........................................... 198

Figure B-2: The formation of well-spaced nucleosomes on the pG3ML plasmid DNA by microcococcal nuclease digestion. ................................................................................................................. 199

Figure C-1: p53 citrullination by PAD4 in vitro and in vivo. ................................................................. 202

Figure D-1: PAD4 activity is important for the formation of highly decondensed chromatin. .................................................................................................................................................. 205

Figure D-2: PAD4 activity is important for NET formation after cytokine and bacteria treatment. .................................................................................................................................................. 206
LIST OF TABLES

Table 2-1: SDS-PAGE resolving gel preparation. ................................................................. 32
Table 2-2: List of antibodies. .................................................................................................. 57
Table 2-3: List of primers. ..................................................................................................... 58
Table 5-1: Percentages of mouse neutrophils with positive histone citrullination, increased nuclear size, and NET formation after treatment with LPS, PMA, and H₂O₂. ............................................................................................................................... 180
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAD4</td>
<td>Peptidylarginine Deiminase 4</td>
</tr>
<tr>
<td>PRMT</td>
<td>Protein Arginine Methyltransferase</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone Acetyltransferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone Deacetylase</td>
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<tr>
<td>Arg</td>
<td>Arginine</td>
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<tr>
<td>Cit</td>
<td>Citrulline</td>
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<tr>
<td>Lys</td>
<td>Lysine</td>
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<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
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<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
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<tr>
<td>MBP</td>
<td>Myelin basic protein</td>
</tr>
<tr>
<td>SAHA</td>
<td>Suberoylanilide hydroxamic acid</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin Dependent Kinase</td>
</tr>
<tr>
<td>RNA Pol II</td>
<td>RNA Polymerase II</td>
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<tr>
<td>CTD</td>
<td>C-terminal Domain</td>
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<tr>
<td>IgL</td>
<td>Immunoglobulin-like</td>
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<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
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<tr>
<td>NETs</td>
<td>Neutrophil Extracellular Traps</td>
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<tr>
<td>PMA</td>
<td>Phorbol Myristate Acetate</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharides</td>
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<tr>
<td>GAS</td>
<td>Group A <em>Streptococcus</em></td>
</tr>
</tbody>
</table>
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Chapter 1

Literature Review
1.1 Histone modifications and their role in transcriptional regulation

1.1.1 Chromatin structure and regulation

In eukaryotic cells, 147 base pairs of DNA is wrapped around a histone octamer to form the nucleosome core particle structure, the fundamental repeating unit of chromatin, where the most important genetic information is stored (Kornberg and Lorch, 1999; Luger et al., 1997). Each histone octamer is composed of a tetramer formed from two H3-H4 dimers and two individual H2A-H2B dimers (Davey et al., 2002; Luger et al., 1997). With the participation of linker histone H1, nucleosomes are further packed into higher order chromatin structure (Kornberg and Lorch, 1999; Ramakrishnan, 1997). It is believed that the highly condensed characteristic of chromatin and the presence of nucleosomes restrain the access of multiple DNA- and histone-binding factors to their cognate binding sites (Campos and Reinberg, 2009; Narlikar et al., 2002). Therefore, in order to smoothly go through every DNA-involved metabolic event such as transcription, DNA damage repair, DNA recombination and chromosome rearrangements, the regulation of histone-DNA interaction and the condensation/decondensation of chromatin structure become very essential (Groth et al., 2007; Kouzarides, 2007; Li et al., 2007b). So far several mechanisms have been proposed to mediate chromatin structure, which include chromatin remodeling, histone modifications and histone variant incorporation (Jenuwein and Allis, 2001; Kouzarides, 2007; Li et al., 2007b).

1.1.2 Posttranslational histone modifications and histone code

Nucleosomal histones contain a middle globular domain through which they interact with each other to form the histone octamer, and a flexible N- or C-terminal tail that protrudes from
the surface of the nucleosome to interact with other protein factors or neighbor nucleosomes (Kornberg and Lorch, 1999). These unstructured histone tails are rich in basic residues and subjected to various kinds of posttranslational modifications, including methylation, acetylation, phosphorylation, citrullination (Kouzarides, 2002; Kuo and Allis, 1998; Nowak and Corces, 2004; Wang et al., 2004b).

Previous research suggested that histone modifications affect chromatin structure and activities via three mechanisms: intrinsic effects which occur in a single nucleosome by changing the charge of histones, mostly through acetylation, attenuate histone-DNA interaction and enhance nucleosome mobility (Cosgrove et al., 2004; Garcia-Ramirez et al., 1995; Sterner and Berger, 2000); extrinsic effects which affect the array of nucleosomes thus abrogating the ability of nucleosomes to form higher-order structures (Shogren-Knaak et al., 2006); and effector-mediated effects which histone modifications bring in non-histone proteins, referred as effectors, to regulate chromatin template (Francis et al., 2004; Nielsen et al., 2001; Zhao et al., 2000). Effectors could work either intranucleosomally by recognizing modifications in the same histone tail or different tails of the same nucleosome, or internucleosomally to bring together two nucleosomes that are either adjacent or unconnected (Campos and Reinberg, 2009; Ruthenburg et al., 2007). The combination of different modifications could work either synergistically or antagonistically for effector binding and provide either "on" or "off" signals for gene expression (An et al., 2004; Fischer et al., 2007; Shilatifard, 2006). To explain the contribution and biological output of histone modifications on chromatin structure, a "histone code" hypothesis has been proposed, suggesting that different histone modifications, either working singularly or combinatorially, could be read by multiple effectors and in turn affect various biological functions (Jenuwein and Allis, 2001; Strahl and Allis, 2000; Turner, 2000).
1.1.3 Histone arginine methylation and its role in transcription

Arginine (Arg) methylation is a common modification on histone tails, which is catalyzed by one class of methylating enzymes named protein arginine methyltransferases (PRMTs). Currently, nine PRMTs are known in mammals. Except for PRMT 2 and 9, methyltransferase activities are found in the other seven PRMTs (Bedford and Clarke, 2009). All active PRMTs are able to produce monomethyl arginine, and are divided into two groups according to the type of dimethyl arginine they generate (Bedford and Clarke, 2009; Bedford and Richard, 2005; Gary and Clarke, 1998; Lee et al., 2005). Type I PRMTs includes PRMT 1, 3, 4, 6, and 8, which produce asymmetric dimethyl Arg from monomethyl Arg, while type II PRMTs (PRMT5 and 7) generate symmetric dimethyl Arg (Wysocka et al., 2006).

Arg methylation is involved in multiple cellular processes, including protein transportation, signal transduction, and transcriptional regulation (Bedford and Clarke, 2009; McBride and Silver, 2001). To affect transcriptional outcomes, PRMTs are recruited to the target gene promoter region by interacting with transcription factors, such as p53, YY1, PPARγ, and RUNX1 (An et al., 2004; Rezai-Zadeh et al., 2003; Yadav et al., 2008; Zhao et al., 2008). Histones are common substrates for PRMTs, and histone Arg methylation catalyzed by different PRMTs could either activate or repress transcription: PRMT4 (CARM1) works with the p160 family of transcriptional coactivators of nuclear receptors to activate transcription (Chen et al., 1999); PRMT1 has been shown to methylate histone H4 Arg 3 (H4R3) to activate transcription (Wang et al., 2001); PRMT5, however, could mediate transcriptional repression through methylation at histone H3R8 and H4R3 (Pal et al., 2004). Arg methylation also work coordinately with other histone modifications to regulate transcription. Previous studies have shown that Arg methylation catalyzed by PRMT1 and CARM1 works synergistically with p300/CBP, a histone acetyltransferase to activate p53-mediated target gene transcription (An et al., 2004).
Histone modification enzymes usually work in pairs to counteract each other's effect, such as histone acetylases (HATs) and histone deacetylases (HDACs), kinases and phosphatases (Kuo and Allis, 1998; Nowak et al., 2003). For a quite long time, Arg and Lys methylation were considered stable modifications because the turnover of methylation in chromatin is relatively slow (Bannister et al., 2002). Recently, several Lys demethylases have been discovered, including amine oxidase LSD1 using FAD as a cofactor and several JmjC domain-containing hydroxylases such as JHDM1, which use $\text{Fe}^{2+}$ and $\alpha$-ketoglutarate as cofactors (Shi et al., 2004; Tsukada et al., 2006). Regarding Arg demethylase, JmjC domain-containing 6 (JMJD6) protein has been suggested as a histone H3R2 and H4R3 demethylase that is dependent on $\text{Fe}^{2+}$ and 2-ketoglutarate (Chang et al., 2007). However, another report described JMJD6 as a lysyl-hydroxylase which functions in the regulation of RNA splicing instead of Arg demethylation (Webby et al., 2009). In addition, peptidylarginine deimase 4 (PAD4) was identified in searching for enzymes that can revert Arg methylation. PAD4 was found to be able to convert monomethyl-Arg to citrulline through a biochemical reaction termed demethylimination (Cuthbert et al., 2004; Wang et al., 2004b).

1.2 PAD family enzymes and citrullination

1.2.1 PAD family enzymes

PAD4 is one of the five isotypes of PAD family proteins, which are able to catalyze the conversion of protein-bound Arg to citrulline (Cit) in a calcium dependent manner (Vossenaar et al., 2003b). Citrulline is a nonconventional amino acid, which can only be produced by posttranslational modifications since there is no citrulline tRNA available. PAD protein was first
described in 1977 as the enzyme that converts arginine residues to citrulline in proteins of hair follicle extracts (Rogers and Taylor, 1977).

Currently there are five isotypes of PAD family enzymes discovered in mammals, four of which have citrullination activity (Vossenaar et al., 2003b). No PAD protein has been discovered in prokaryotes, except one enzyme found in Porphyromonas gingivalis that could citrullinate Arg without protein environment (McGraw et al., 1999). However, it is not evolutionarily conserved with peptidylarginine deiminase and its activity is not calcium dependent (Shirai et al., 2001). In non-mammalian vertebrates, such as fish and frog, only one PAD enzyme has been found and they are more closely conserved with each other than any of the five PAD isotypes in mammals (Vossenaar et al., 2003b).

PAD family proteins are highly conserved in mammals. The five isotypes share a relative high homology, either the different isotypes in the same species or the same isotype in different species (Vossenaar et al., 2003b). Compared with the N-terminus part, the C-terminal catalytic domain shows an even higher level of conservation. All of the active PADs possess a conserved Cys residue and a conserved His residue that are important for their catalytic activity (Shirai et al., 2001). A couple of Asp residues are conserved among all PAD enzymes, which is believed to play important roles in substrate recognition (Vossenaar et al., 2003b). The genome organization of PAD genes in chromatin in different mammal species reveals another level of conservation of PAD protein. All PAD genes are located in the same chromosomal locus and arranged in the same order, direction, and relative spacing in human, mouse, and rat, suggesting that PAD genes are derived from gene duplication events in evolution (Vossenaar et al., 2003b).

PAD enzymes show tissue-specificity. PAD1 locates mainly in epidermis and uterus (Rus'd et al., 1999; Terakawa et al., 1991), while later EST data detected PAD1 in some other tissues (Guerrin et al., 2003). PAD2 is the most widely distributed PAD enzyme. It is detected in spleen, brain, and skeletal muscle, and its expression is regulated at both transcriptional and
translational levels (Vossenaar et al., 2003b; Watanabe et al., 1988). The expression of PAD3 is restricted to hair follicles and co-localized with its substrate tricholyalin, a basic structural protein of inner root sheath cells (Kanno et al., 2000; Nachat et al., 2005). PAD4 is detected mainly in white blood cells such as granulocytes and monocytes, and is the only PAD enzyme localized in nucleus. Its targets include nuclear proteins such as histones and nucleophosmin (Hagiwara et al., 2002; Nakashima et al., 1999). PAD6, the PAD protein without detectable citrullination activity, locates in egg cell cytoplasm (Wright et al., 2003).

Because citrullination neutralizes the net positive charge on arginine residue, it could affect the intramolecular and intermolecular interactions (Tarcsa et al., 1996). Numerous studies reveal that citrullination plays a significant role in the structures and functions of the target proteins, and is related to the etiology of several autoimmune diseases, including rheumatoid arthritis (RA) caused by PAD4. RA is a human autoimmune disease in the synovial joint that exists in about 1% of the world population, and occurs in women three more times than men (Vossenaar et al., 2003a). The most specific characteristic of RA is that many autoantibodies generated against self-antigens exist in patient serum (Raptopoulou et al., 2007). Most of them are anti-citrullinated protein antibodies, which could be specifically detected in 80% RA patients at very early stage during disease progression (van Boekel et al., 2002; Vasishta, 2002). The autoantibodies attack self-proteins and finally cause severe damage in the joints. Studies have suggested that the high level of citrulline-containing proteins is produced by the infiltration of a large number of leucocytes that express high level of PAD4 into the inflamed synovial joint (Kinloch et al., 2008; Vossenaar et al., 2004b). PAD enzyme is activated by increased calcium concentration in apoptosis or necrosis of leucocytes and hence produces citrullinated proteins, which gradually lead to this chronic inflammatory disorder (Vossenaar et al., 2004a).

Another PAD protein related disease is multiple sclerosis (MS), occurring in central nervous system. MS is caused by excess citrullination of myelin basic protein (MBP), the major
component of the myelin sheath that covers the axons of nerve cells (Moscarello et al., 1994). Healthy people have about only 18% of MBP citrullinated at 6 out of its 19 arginine residues. However, the percentage of citrullination increases to 45% or more in a MS patient (Wood et al., 1996). Decrease in positive charge by too much citrullination of MBP destabilizes the protein and attenuates its interaction with phospholipids to form the multibilayer structure around the axons, which then inhibits the nerve signal transduction (Boggs et al., 1999; Pritzker et al., 2000).

1.2.2 Human PAD4 and its citrullination activity

Human PAD4 is originally considered as a novel PAD enzyme and named as PAD5 because its SDS-PAGE mobility and relative activities toward artificial substrates are different from rat PAD4 (Nakashima et al., 1999). However, later data in subcellular localization, expression, sequence comparison and genomic organization all suggested that the human PAD5 is actually the homolog of rat PAD4 (Asaga et al., 2001; Nakashima et al., 2002). From then on, human PAD5 was renamed as PAD4 (Vossenaar et al., 2003b).

PAD4 is the only isotype of the PAD family that locates in the nucleus (Nakashima et al., 1999). Structural studies revealed that PAD4 has two immunoglobulin like structural domains at its N-terminus and a catalytic domain at its C-terminus (Arita et al., 2004). A nuclear localization signal at the N-terminal domain is essential for the nuclear localization of PAD4 and five calcium-binding sites are identified critical for its enzymatic activity.

PAD4 and PAD2 are both detected in synovial tissue and generate citrullinated proteins involved in rheumatoid arthritis (Vossenaar et al., 2004a; Yamada, 2005). PAD4 attracts more and more attention recently because its enzymatic ability to use histones as substrate to regulate transcription (Thompson and Fast, 2006). PAD4 can deiminate histones H2A, H3, and H4 at their N-terminal tails (Hagiwara et al., 2002). Several studies have shown that PAD4 could serve as a
transcriptional corepressor to inhibit transcription under the control of estrogen-responsive pS2 promoter by converting methyl Arg to Cit (Cuthbert et al., 2004; Wang et al., 2004b).

1.3 Tumor suppressor protein p53

The tumor suppressor protein p53 is one of the well-studied genes. It was considered an oncogene when first discovered in 1979, but was later determined to be a tumor suppressor gene (Baker et al., 1989; Finlay et al., 1989; Linzer and Levine, 1979). p53 plays a very important role in controlling cell growth and tumorigenesis. Mutation and malfunction in any part of p53 may cause severe cellular defects and p53 mutation has been found in more than 50% of cancers (Levesque and Eastman, 2007; Levine, 1997).

p53 functions at the center of an extremely complicated signaling network in human cells (Laptenko and Prives, 2006; Vogelstein et al., 2000). Multiple cell stress signals such as DNA damage and aberrant growth signals activate p53, which in turn activates the expression of its target genes (Harris and Levine, 2005; Vogelstein et al., 2000). p53 regulates a wide range of genes that are involved in cell growth arrest, apoptosis, and other metabolic processes such as autophagy (Kruse and Gu, 2009; Vousden and Prives, 2009; Vousden and Ryan, 2009).

p21/WAF1/CIP1, which encodes a cyclin dependent kinase inhibitor, is one of the best studied p53 target genes involved in cell growth arrest (Ko and Prives, 1996). The basal level of p21 transcription is observed at normal conditions, and its transcriptional activation is induced by DNA damage such as UV irradiation (Espinosa et al., 2003; Gomes et al., 2006). When cells experience irreversible DNA damage, p53 activates proapoptotic genes such as Bax and Bak to go through apoptosis (Kruse and Gu, 2009). Cytoplasmic p53 also activates Bax in a transcription-independent manner to facilitate mitochondria cytochrome c release and thus apoptosis (Chipuk and Green, 2006). p53 even plays a role in maintaining metabolic homeostasis
(Vousden, 2009; Vousden and Ryan, 2009). For example, a low level of cytoplasmic p53 inhibits autophagy under normal conditions, while p53 could be activated by AMP-activated protein kinase and inhibit mTOR kinase expression under metabolic stress (Vousden and Ryan, 2009). p53 can also regulate autophagy by activating the expression of a lysosomal protein, DRAM (Crighton et al., 2006; Mathew et al., 2007).

p53 is subjected to a wide range of posttranslational modifications, including methylation, phosphorylation, acetylation, and ubiquitination (Brooks and Gu, 2003; Ivanov et al., 2007; Kruse and Gu, 2009). These modifications regulate p53 target gene expression, thereby offering an efficient mechanism for p53 regulation. Phosphorylation at Ser15 and Ser20 by ATM, ATR, chk1, and chk2 is important in destabilizing the interaction of p53 and its negative regulator Mdm2 under stress conditions (Appella and Anderson, 2001; Shieh et al., 2000; Shieh et al., 1997). p53 is the first non-histone protein identified as substrate acetylation and deacetylation (Gu and Roeder, 1997; Luo et al., 2000). Acetylation of p53 by p300/CBP facilitates p53-DNA binding and increases p53 stability (Ito et al., 2001), and acetylation of p53 by Tip60/hMof functions in DNA repair and affects the cell’s decision between cell cycle arrest and apoptosis (Li et al., 2009; Tang et al., 2006). p53 C-terminal domain has several Lys residues, many of which could be methylated by different methyltransferases thereby activating the expression of p53 target genes in cell cycle arrest or apoptosis (Kruse and Gu, 2009).

Structural and functional studies of p53 suggested that p53 contains an N-terminal activation domain (residue 1-73), a proline rich domain (residue 63-97), a DNA binding domain (residue 94-312), a tetramerization domain (residue 324-55), and a C-terminal regulatory domain (360-393) (Kitayner et al., 2006). Mutations in each domain are found in cancers, while mutations in p53 DNA binding domain is most common in tumorigenesis (Hainaut and Hollstein, 2000). The consensus p53 binding sequence contains two copies of head-to-head arranged pentamers, 5’-RRRC(A/T)(A/T)GYYY-3’ (R is purine, Y is pyrimidine) separated by 0-13
nucleotides (el-Deiry et al., 1992). More ChIP studies revealed that the binding of p53 to specific DNA is much more complicated than just utilizing the consensus sequence: neither all p53 binding sites contain the consensus sequence, nor all consensus sequence are bound by p53, suggesting that p53 binding to DNA is also affected by other factors (Imbriano et al., 2005; Vousden and Prives, 2009; Wei et al., 2006). For example, the status of posttranslational modifications at p53 C-terminal domain could affect p53 binding to DNA as a tetramer (Ahn and Prives, 2001).

For a long time it was considered that one of the most important way p53 acquires its transcriptional activities is by recognizing and binding to a specific DNA sequence (Laptenko and Prives, 2006). Recent studies have shown that even in the unstressed cells, a large fraction of intrinsically active p53 is able to bind to DNA (Kaeser and Iggo, 2002; Szak et al., 2001). However, these p53 proteins remain physiological inactive, probably due to the repression of Mdm2 and MdmX (Kruse and Gu, 2009). Mdm2 is an E3 ubiquitinase and serves as a negative regulator of p53 by inducing the degradation of p53 and keeping p53 expression at a low level (Brooks and Gu, 2006). DNA damage such as UV irradiation induces a series of signaling events that inhibit the ability of Mdm2 to ubquitinate p53 (Michael and Oren, 2003). MdmX is also a negative regulator of p53, which can interact with Mdm2 and enhance its E3-ligase activity (Marine and Jochemsen, 2005). Although MdmX does not ubiquitinate p53 directly, it could repress p53-mediated transcription (Linares et al., 2003).

Hence, the updated model of p53 activation includes three steps, disruption in any of which could result in malfunction of p53 and carcinogenesis (Kruse and Gu, 2009). The first step is stabilization of p53, which is induced by various stresses and attenuates the ubiquitinase activity of Mdm2 via different mechanisms; the second and also the key step is release of p53 from a repressed state to a physiologically activated state, which is achieved by several posttranslational modifications on p53 as well as small molecules to inhibit Mdm2 activity (Kruse
and Gu, 2009). After antirepression, the third step is the binding of p53 to specific DNA fragments and the further recruitment of other cofactors or coregulators to the p53 target gene promoters to fine-tune their transcription. To activate gene expression, p53 recruits histone modification enzymes, nucleosome remodelers, or mediators to facilitate transcription (Laptenko and Prives, 2006). For instance, multiple histone acetyltransferases (HATs) have been found as p53 coactivators (Barlev et al., 2001; Espinosa and Emerson, 2001). It has been reported that histone Arg methyltransferases could work synergistically with HAT to activate p53-target gene expression (An et al., 2004). p53 can also directly recruit other transcription factors or help with the reinitiation of transcription (Ko and Prives, 1996; Xing et al., 2001). On the other hand, multiple mechanisms of negative regulation of transcription by p53 have been discovered, including recruiting histone deacetylase to counteract transcriptional activation mediated by HAT (Dannenberg et al., 2005; Luo et al., 2000), preventing activators from binding to the promoter (Liu et al., 1999; Zhu et al., 2005), interfering with preinitiation complex assembly (Ko and Prives, 1996; Subbaramaiah et al., 1999), and binding to a novel p53 recognition element which has a different orientation and dictates a repression site (Johnson et al., 2001). p53 even represses the transcription of some genes through activating other p53 target genes. In sum, p53 plays multifaceted roles in gene regulation (Ho and Benchimol, 2003; Laptenko and Prives, 2006).

1.4 Epigenetics and cancer

Cancer is a major threat to human health and many efforts have been devoted to fight this devastating disease. Previous research emphasized the genetic basis of cancer, especially the mutations that result in oncogene activation or tumor suppressor gene inactivation. Recent research, however, more and more evidence indicates an important role of epigenetic modifications in tumorigenesis (Esteller, 2008; Gal-Yam et al., 2008).
Two major epigenetic alterations found in cancer are DNA methylation and histone modifications. Mammalian DNA methylation is catalyzed by DNA methyltransferases (DNMTs) and occurs in the 5 position of the pyrimidine ring of cytosine that precede guanine (Holliday, 1990). CpG dinucleotides appear in a low frequency in the whole genome but show up at a relative high frequency in the gene promoter or first exon region, which are called CpG islands (Bird, 1986; Craig and Bickmore, 1994). In normal cells, CpG islands are hypomethylated to allow the expression of the gene, but almost all remaining CpG dinucleotides are methylated, especially those located in repetitive regions, for maintaining the genomic stability (Eckhardt et al., 2006; Esteller, 2007b). However, the methylation level is reversed in cancer cells, where a global loss of DNA methylation is observed and CpG islands become hypermethylated with overexpression of DNMT1 and DNMT3b (Esteller, 2007a; Rhee et al., 2002). The decrease of global DNA methylation level could result in chromosome instability, reactivation of transposable elements, and loss of imprinting; while CpG islands hypermethylation would repress the expression of tumor suppressor genes, all of which could cause cancer (Esteller, 2008; Kristensen et al., 2009).

Histone modifications are also found to be involved in cancer. The loss of H4K16 acetylation and H4K20 trimethylation are commonly observed in malignant transformation, which could be due to decreased HATs activity or increased HDACs activity (Fraga et al., 2005). Moreover, DNA methylation and histone modifications can work together in cancer cells. Research has shown that hypermethylated CpG islands at the promoter region facilitates the binding of Methyl-CpG-binding domain protein, which recruits HDACs to decrease acetylation and repress transcription (Wade, 2001). HDACs can even recruit more DNMTs to the promoter region to further inhibit tumor suppressor gene expression (Fuks et al., 2000; Rountree et al., 2000). For instance, the epigenetic silencing of tumor suppressor genes p21 and p16INK4 is due to
the deregulation of histone modifications in conjunction with CpG islands hypermethylation. (Aneja et al., 2007; Kuerbitz et al., 1999).

As such, a new avenue for cancer treatment is to target DNA methyltransferases or histone modifying enzymes so as to epigenetically activate tumor suppressor genes. Currently some DNA demethylating agents and HDAC inhibitors have been approved by the US Food and Drug Administration (FDA) for clinical cancer treatments. For example, one DNA demethylating agent, 5-azacytidine, has been used to treat myelodysplastic syndrome and leukemia (Esteller, 2005; Mack, 2006). p21 is the universal target of HDAC inhibitors, and its expression is greatly increased after inhibitor treatment to induce histone hyperacetylation at its promoter (Richon et al., 2000). Other proapoptotic genes that are silenced in leukemia could also be re-activated by HDAC inhibitor treatment (Insinga et al., 2005). One HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA), is now approved by the FDA to treat T cell cutaneous lymphoma (Marks and Breslow, 2007). Moreover, the combination of demethylating agents and HDAC inhibitors show a synergistic effect in activating tumor suppressor genes in both cancer cells and mouse models (Belinsky et al., 2003; Cameron et al., 1999; Steele et al., 2009; Yang et al., 2001), and administration of demethylating agents and/or HDAC inhibitors prior to chemotherapy or radiation therapy can significantly improve the effects of these traditional cancer therapies (Kristensen et al., 2009). These all suggest the exciting potential of epigenetic therapy in cancer treatment.

1.5 Neutrophil extracellular traps

Neutrophils play an essential role in the innate immune system as the first defense line against pathogen infection (Lekstrom-Himes and Gallin, 2000). For decades, neutrophils have been known to clear invading pathogens through phagocytosis, by which they migrate to the
infection site and engulf pathogens (Groves et al., 2008; Nathan, 2006). Neutrophils can also release a plethora of antimicrobial molecules such as neutrophil elastase and myeloperoxidase at the infection site to kill pathogens (Zasloff, 2002). Recently, a novel mechanism of neutrophils fighting against invading pathogens has been discovered, by which neutrophils release their intracellular components, mainly nuclear contents, to the extracellular space and form a web-like structure called neutrophil extracellular traps (NETs), to trap and kill bacteria (Brinkmann et al., 2004). NETs have been discovered in multiple vertebrates including human, mice, chicken, and zebrafish (Chuammitri et al., 2009; Palic et al., 2007; Wartha et al., 2007b). Functional studies of NETs revealed that they efficiently inhibit the growth of Gram-positive and Gram-negative bacteria, fungi, and parasites (Brinkmann and Zychlinsky, 2007; Guimaraes-Costa et al., 2009; Urban et al., 2006). Besides neutrophils, mast cells and eosinophils may also form NET-like extracellular traps (von Kockritz-Blickwede et al., 2008; Yousefi et al., 2008). This novel cellular process in innate immunity is collectively referred as ETosis (Wartha and Henriques-Normark, 2008).

NETs are composed of both nuclear and granular content of the neutrophil. DNA and histones are regarded as the major constituents of NETs, and chromatin contributes to the backbone of the NET structure in trapping bacteria (Brinkmann et al., 2004). The granular antibacterial peptides and enzymes, such as neutrophil elastase and myeloperoxidase, decorate NETs to facilitate bacterial killing (Wartha and Henriques-Normark, 2008). A recent study of the components of NETs using mass spectrometry suggests that there are at least 24 proteins associating with NETs (Urban et al., 2009). Besides nuclear and granular components, some cytoplasmic proteins are also present. All four types of core histones are confirmed to be present in NETs. Interestingly, the linker histone H1 which is thought to be NET-associated, did not show up in this study (Urban et al., 2009).
NETs are found to efficiently kill pathogens that are hard to eliminate through phagocytosis such as the hyphae form of fungi (Urban et al., 2006). However, the process and the key regulator of NETs formation remain to be elucidated. NET formation is neither cell apoptosis nor necrosis. The formation of NETs does not depend on caspase activation (Brinkmann and Zychlinsky, 2007; Fuchs et al., 2007). It has been discovered that reactive oxygen species (ROS) plays a significant role in NET formation (Fuchs et al., 2007). We and others have found that histone deimination also contributes to chromatin decondensation through neutralizing the positive charge of histones (Neeli et al., 2008; Wang et al., 2009). However, whether PAD4 plays a role in innate immunity has not been formally tested till recently (see Chapter 5).

NETs combat invading pathogens through several ways (Papayannopoulos and Zychlinsky, 2009). The web-like structure of NETs confines the pathogens in a local area and prevents them from escaping. By associating with NETs, the concentrations of antimicrobial agents increase, allowing them to kill pathogens more efficiently (Wartha et al., 2007b). Meanwhile, the restriction of those antimicrobial agents around NETs also minimizes their possible damage to normal tissue through unexpected diffusion (Papayannopoulos and Zychlinsky, 2009).

Immunity is a forever battle between the host and the invading pathogens. In order to escape the killing by NETs, pathogens employ several strategies to counteract NETs. Some bacteria express a secreted DNase to degrade NETs, which would greatly decrease the NET-mediated killing thereby increasing the bacterial virulence (Beiter et al., 2006; Buchanan et al., 2006). Moreover, many bacteria form capsules or change their surface charge to reduce the binding of NETs (Wartha et al., 2007a). The study of NETs in the years to come will unveil many molecular mechanisms underlying innate immunity to eliminate bacterial pathogens.
PAD4 is able to convert both histone arginine and monomethyl-arginine into citruline. Previous studies suggested PAD4 plays an important role in transcriptional regulation at the estrogen-responsive gene promoter (Cuthbert et al., 2004; Wang et al., 2004b) and PAD4 is also overexpressed in various cancer tissues (Chang and Han, 2006). In order to study the mechanisms of carcinogenesis induced by PAD4 deregulation, I firsted investigated the role of PAD4 and PAD4 catalyzed histone citrullination in the transcriptional repression of p53-target genes. Using one of the p53-target genes p21/CIP1/WAF1 as a model gene, I found that PAD4 is recruited to the p21 gene promoter in a p53-dependent manner. PAD4 association and the RNA Pol II activity at the p21 promoter are dynamically regulated after UV irradiation. I further found that PAD4 and histone citrullination coordinate with HDAC2 which is a histone lysine deacetylase in repressing tumor suppressor gene expression. PAD4 inhibitbor Cl-amidine and HDAC inhibitor SAHA induced p53-target gene expression and inhibited cancer cell growth additively in a p53-dependent manner. Finally, using PAD4 knockout mice as a genetic model, I found that PAD4 knockout mice cannot form neutrophil extracellular traps, which is an important mechanism to eliminate invading bacteria. PAD4 knockout mice are more susceptible to bacterial infection due to the lack of NET-mediated anti-bacterial ability, suggesting an essential role of PAD4 and histone citrullination in innate immunity.
Chapter 2

Materials and Methods
2.1 Molecular Cloning

2.1.1 Plasmid construction

2.1.1.1 PCR of cDNA fragment

1) 50 µl reaction system:
   - 5 µl 10x pfu buffer
   - x µl DNA template (0.1-0.5 µg)
   - 4 µl 2.5 mM dNTP mixture
   - 4 µl 5 µM Forward primer
   - 4 µl 5 µM Reverse primer
   - 1 µl pfu turbo (Stratagene, 600259-52)
   - Add ddH2O to 50µl

   Typical PCR program:
   - 94°C for 2 min
   - 35 cycles of:
     - 94°C for 30 sec
     - x°C for 45 sec
   - 70°C for x min (~1kb/min)
   - 70°C for 10 min

2) Run 2 µl PCR product with agarose gel in 1x TAE buffer (50x: 242 g Tris base, 57.1 ml glacialic acid, 100 ml 500 mM EDTA (pH 8.0), add ddH2O to 1 L, adjust pH to 8.0) buffer to check PCR efficiency.
6x Bromophenol blue DNA loading buffer: 0.25% bromophenol blue, 15% Ficoll in H₂O.

6x Xylene blue DNA loading buffer: 0.05% xylene blue, 25% glycerol in H₂O.

2.1.1.2 Restriction enzyme digestion

1) 50 µl system for plasmid vector digestion:
   - 5 µl 10x buffer
   - x µl Plasmid vector (~1 µg)
   - 1 µl Restriction enzyme 1
   - 1 µl Restriction enzyme 2
   - Add ddH₂O to 50 µl

2) 60 µl system for cDNA fragment
   - 6 µl 10x buffer
   - 48 µl cDNA PCR product
   - 1 µl Restriction enzyme 1
   - 1 µl Restriction enzyme 2
   - 4 µl ddH₂O

3) Digest both at 37°C for 2.5 hr

2.1.1.3 Dephosphorylation for vector and gel extraction

1) After digestion, add 5.5 µl 10x antarctic phosphotase buffer, 1 µl antarctic phosphotase to the digested vector sample.
2) Incubate sample at 37°C for 15 min with 5' overhang, transfer to 65°C for 5 min to inactivate enzyme.

3) Run 2 µl vector and cDNA digestion sample in agarose gel to check efficiency.

4) If digestion is good, run the entire sample in agarose gel, perform gel extraction to purify the product using Gel Extraction Kit (Qiagen, 28704). Elutes with 30 µl elution buffer for cDNA fragment, 50 µl for vector.

2.1.1.4 Ligation

1) 40 µl reaction system:
   - 4 µl 10x ligation buffer
   - 28 µl cDNA fragment
   - 7 µl Plasmid vector
   - 1 µl T4 ligase

2) Incubate the reaction system at 16°C overnight.

2.1.1.5 Transformation

2.1.1.5.1 Transformation using chemical competent cells

1) Incubate 20 µl ligation product with 200 µl chemical competent DH5α cells for 30 min on ice.

2) Transfer to 42°C for 90 sec, incubate on ice for another 5 min.

3) Add 1 ml 2x YT (16 g tryptone, 8 g yeast extract, 5g NaCl in 1 L H2O, adjust pH to 7.5 with NaOH) and shake in 37°C shaker (200 rpm) for 45 min.
4) Spin down cells at 5,000 rpm for 1 min, remove most 2xYT and resuspend cells in about 200 µl 2xYT that is leftover.

5) Spread cells on LB agar plate (10 g tryptone, 5 g yeast extract, 5 g NaCl, 16 g agar in 1 L H₂O, adjust pH to 7.5 with NaOH) with desired antibiotics. Invert the plate and incubate at 37°C overnight.

2.1.1.5.2 Transformation using electroporation competent cells

1) Incubate 0.5 µl ligation product (10 pg to 25 ng) with 40 µl electroporation competent cells on ice for 1 min (If more DNA is needed, purify ligation product with PCR Purification Kit (Qiagen, 28104), elute with water and use more for electroporation).

2) Prepare a 2 mm pre-chilled electroporation cuvette, and set the electroporation device to deliver an electrical pulse of 25 µF capacitance, 2.5 kV, and 200 Ω resistance.

3) Transfer the mixture to the cuvette, place the cuvette to the device and start electroporation. Upon completion, check to make sure a time constant of 4-5 milliseconds is achieved with a field strength of 12.5kV/cm.

4) Immediately after electroporation, remove the cells from the cuvette into a room temperature 1.5 ml tube and add 1 ml 2xYT. Leave the cells at room temperature for 5 min and shake in 37°C shaker (200 rpm) gently for 20 min.

5) Spread up to 200 µl on LB agar plate with desired antibiotics. Invert the plate and incubate at 37°C overnight.
2.1.1.6 Colony selection

1) After colonies grow up on the LB plate, pick up several single colonies and inoculate into 3 ml 2x YT with desired antibiotics. Grow up bacteria in 37°C shaker (250 rpm) for 12-14 hours.

2) Select positive colonies by Cracking Gel or plasmid extraction with Miniprep Kit (Qiagen, 27106) followed by restriction enzyme digestion.

3) Send couple of positive plasmids for sequencing to confirm the right sequence.

2.1.2 Cracking Gel

1) Start from the overnight 3 ml 2x YT bacteria culture.

2) Turn heat block to 70°C, prepare a 0.7% agarose gel.

3) Transfer about 50 µl bacteria culture from each culture vials into a 1.5 ml tube, spin at 13,200 rpm for 1 min.

4) Remove supernatant and add 50 µl of 10 mM EDTA into each tube, pipette up and down to resuspend the bacteria.

5) Add 50 µl 2x Cracking Gel buffer (for each 50 ml, 2 ml of 5 M NaOH, 2.5 ml 10% SDS, 10 g sucrose) into each tubes, flick the tube bottom to mix well.

6) Heat the sample at 70°C for 5 min and cool down at room temperature for 10 min.

7) Add 20 µl 6x DNA loading buffer and 2 µl of 3 M KCl to each tube after cooling down, flick the tube bottom to mix well.

8) Leave on ice for 10 min and spin at 13,200 for 5 min.

9) Load 30 µl sample into the agarose gel to compare the plasmid size.
2.1.3 PCR based point mutation

1) 30 µl reaction system to generate the point mutation

3 µl 10x pfu buffer

x µl Plasmid DNA (~ 0.1 µg)

2 µl 2.5 mM dNTP mixture

2 µl 5 µM primer 1

2 µl 5 µM primer 2

0.6 µl pfu turbo

Add ddH2O to 30 µl

PCR program:

94°C for 2 min

20 cycles of:

94°C for 30 sec

55°C for 60 sec

68°C for x min (~1kb/min)

68°C for 10 min

2) After PCR reaction, add 3.4 µl NEB buffer 4 and 1 µl DpnI enzyme to PCR product, digest at 37°C for 2 hr.

3) Transform the sample to DH5α competent cells.

4) Pick up couple of colonies, grow up bacteria, extract plasmids and send for sequencing.
2.2 Cell Culture

2.2.1 Cell culture conditions

1) All mammalian cells are cultured in a 37°C incubator with 5% CO₂. 293T, H1299, U2OS, MCF-7 cells are cultured in Dulbecco Modified Eagle Medium-High Glucose (Invitrogen, 11965) supplemented with 10% fetal bovine serum (Invitrogen, 10438) and 1% penicillin-streptomycin (Invitrogen, 15140). HCT116 p53+/+ and p53−/− cells are cultured in McCoy’s 5A Medium-Modified (Invitrogen, 16600) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. HL60 cells are cultured in RPMI 1640 Medium (Invitrogen, 11875) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin.

2) Spodoptera frugiperda Sf9 cells are cultured at room temperature in Sf-900 II SFM medium (Invitrogen, 10902) supplemented with 5% fetal bovine serum.

2.2.2 Cell passage

1) Warm up medium, DPBS (Invitrogen, 14190), and 0.05% Trypsin-EDTA (Invitrogen, 11875).

2-1) For attached cells, remove medium, wash once with DPBS, digest with Trypsin for 3-4 min, and add same volume of medium to stop digestion, Collect cells into a 15 ml tube, spin at 1,000 rpm for 5 min.

2-2) For suspended cells, collect cells with medium into a 15 ml tube, spin at 1,000 rpm for 5 min.

3) Remove supernatant and resuspend cells with fresh medium.

4) Count cell number with hemacytometer (Hausser Scientific, 1492) if necessary, divide cells into desired number of plates and add fresh medium.
2.2.3 Freeze down cells

1) Follow Cell passage procedure till step 2.

2) Remove supernatant and resuspend cells with medium contains 50% culture medium, 40% FBS, and 10% DMSO.

3) Put cells into stock tubes (1 ml/tube), freeze down in -80°C overnight, and transfer to liquid nitrogen tank next day.

2.2.4 Recover cells from liquid nitrogen

1) Warm the stock tube quickly in 37°C water bath.

2) Add the stock cells into 10 ml pre-warmed medium in a 15 ml tube, mix up, and spin down 1,000 rpm for 5 min.

3) Remove supernatant and resuspend cells in fresh medium and transfer to culture plates.

2.3 Cells transfection with plasmid DNA and siRNA

2.3.1 Cells transfected with plasmid DNA

2.3.1.1 Plasmid transfection with Lipofectamine 2000 (Invitrogen, 11668).

Following procedure is for 24-well plates, scale accordingly for other sizes.

1) One day before transfection, plate cells in culture medium without antibiotics and make cells 90-95% confluent at the time of transfection.

2) Dilute 0.8 μg DNA in 50 μl of Opti-MEM I Reduced-Serum Medium (Invitrogen, 31985). Mix gently.
3) Mix Lipofectamine 2000 gently before use, dilute 2 µl in 50 µl of Opti-MEM Medium. Incubate for 5 min at room temperature.

4) Combine the diluted DNA with diluted Lipofectamine 2000. Mix gently and incubate for 20 min at room temperature.

5) Add the 100 µl complexes to each well. Mix gently by rocking the plate back and forth.

6) Incubate cells for 48-72 hr. Medium may be replaced 6 hr after transfection.

2.3.2.2 Plasmid transfection with Calcium Phosphate.

Following procedure is for 10 cm plate, scale accordingly for other sizes.

1) Change fresh medium prior to transfection.

2) Mix 24 µg DNA with 420 µl 1 mM Tris pH 7.4.

3) Add 60 µl 2 M CaCl₂ drop wise with gentle agitation, mix well.

4) Add the DNA-CaCl₂ solution drop wise to a tube containing 480 µl of 2x HBS (280 mM NaCl, 50 mM HEPES, 1.5 mM Na₂HPO₄, pH 7.0-7.1) with a constant bubbling of air within the 2x HBS solution. The rate of bubble formation should be moderate to vigorous. Mix well. Incubate at room temperature for 25 min.

5) Add the precipitate slowly to the cells. Mix gently.

6) After cells are incubated for 6 hr, wash cells twice with TBS (25 mM Tris-HCl, 137 mM NaCl, 5 mM KCl, 0.7 mM CaCl₂, 0.5 mM MgCl₂, 0.6 mM Na₂HPO₄, pH 7.4).

7) Add fresh medium and incubate for a further 24 hr.
2.3.2 Cells transfected with siRNA

X-tremeGene siRNA Transfection Reagent (Roche, 04476093001) is used. Following procedure is for 6-well plate, scale accordingly for other sizes.

1) One day prior to transfection, plate the cells in culture medium without antibiotics and make cells 30-50% confluent at the time of transfection.

2) Dilute 10 µl of X-tremeGene Reagent with 100 µl Opti-MEM I medium. Mix gently.

3) Dilute 2 µg (~160 pmol) siRNA with 100 µl Opti-MEM I medium. Mix gently.

4) Combine the diluted reagent with diluted siRNA. Mix gently and incubate for 15-20 min at room temperature.

5) Add the 200 µl complexes to the cells. Swirl the wells gently.

6) Incubate cells for 72 hr. There is no need to change to fresh medium.

2.4 Overexpressing stable cell line generation by retroviral transduction

1) Clone target gene into the pMIGR1 vector.

2) In day 1 afternoon, plate ~7.5x10⁵/well 293T cells in a 6-well plate (usually 2 wells for each retroviral construct).

3) In day 2 afternoon 293T cells should be ~60% confluent. Co-transfect 293T cells using Calcium phosphate transfection with 4 µg/well packaging plasmid DNA Ψampho, and 4 µg/well retroviral expression plasmid DNA pMIGR1-target gene.

4) In day 3 morning, remove transfection medium, and overlay transfected cells with 1 ml of fresh culture medium.

5) In day 4 morning, plate 2-3x10⁷/well healthy, log phase target cells with a 750 µl volume in a 6-well plate. Overlay target cells with 0.45 µm filter-filtered retroviral supernatant, add 1 ml fresh culture medium to 293T viral producer cells.
6) The volume of the filtered supernatant should be ~750 µl. Add Polybrene to a final concentration of 8 µg/ml. 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.

7) In day 5 perform the second round of spinoculation after removing the old medium and overlay with new retroviral supernatant and Polybrene. For most cell lines 2 rounds spinoculation is sufficient. With 2 wells of producer 293T cells per each retroviral construct, it is possible to perform 4 rounds of infection (2 per day for 2 days).

8) In day 6 screen for GFP expression. Green fluorescence is usually visible 24 hr after the first round of infection, and reaches maximum 24-48 hr after the last round. Positive cells could be sorted out by flow cytometry.

2.5 RNA preparation

2.5.1 TRIZOL reagent

TRIZOL reagent (Invitrogen, 15596) is used.

1) Homogenization: For tissues, homogenize in 1 ml TRIZOL reagent per 50-100 mg of tissue. Sample volume should not exceed 10% of the volume of TRIZOL reagent. For attached cells, lyse cells directly in a culture dish by adding 1 ml of TRIZOL reagent per 10 cm² area, and passing the cell lysate several times through a pipette. For suspension cells, Pellet cells by centrifugation. Lyse cells in TRIZOL reagent by repetitive pipetting. Use 1 ml of the reagent per 5-10x10⁶ of animal, plant or yeast cells, or per 1x10⁷ bacterial cells.

2) Phase separation: Incubate the homogenized samples for 5 min at 15 to 30°C to permit the complete dissociation of nucleoprotein complexes. Add 0.2 ml of chloroform per 1 ml of TRIZOL reagent. Shake tubes vigorously by hand for 15 sec and incubate at 15 to 30°C for 2 to 3
30 min. Centrifuge at no more than 12,000 x g for 15 min at 2 to 8°C. RNA remains exclusively in the upper aqueous phase, which is about 60% of the volume of TRIZOL reagent used for homogenization.

3) RNA precipitation. Transfer the aqueous phase to a new tube, precipitate RNA by mixing with isopropyl alcohol. Use 0.5 ml of isopropyl alcohol per 1 ml of TRIZOL reagent. Incubate samples at 15 to 30°C for 10 min and centrifuge at no more than 12,000 x g for 10 min at 2 to 8°C. RNA precipitate forms a gel-like pellet on the side and bottom of the tube.

4) RNA wash: Remove the supernatant. Wash the RNA pellet once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml of TRIZOL reagent. Mix the sample by vortexing and centrifuge at no more than 7,500 x g for 5 min at 2 to 8°C.

5) Redissolving RNA: Briefly dry the RNA pellet by air-dry for 5-10 min, avoid drying completely. Dissolve RNA in RNase-free water or 0.5% SDS solution by passing the solution a few times through a pipette tip, and incubating for 10 min at 55 to 60°C. RNA can also be redissolved in 100% deionized formamide and stored at -70°C.

2.5.2 RNeasy Mini Kit

RNeasy Mini Kit (Qiagen, 74104) is used following manufacture’s instructions.

2.6 Total protein extract preparation from cells

1) Collect cells follow cell passage procedure till step 2.

2) Remove supernatant and wash once with DPBS, count cell number. Spin down at 1,000 rpm for 5 min.
3) Remove DPBS and resuspend cells with IP buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM EDTA, 0.2% Triton X-100, 0.2% NP-40) plus protease inhibitors (1 mM PMSF, 10 nM Aprotinin, 10 μM Leupeptin, 1 μg/ml Pepstatin A) at 1x10^7 cell/ml concentration.

4) Samples are sonicated in Bioruptor (Diagenode Inc.) for 5 min (30 sec on, 30 sec off) at high energy level.

5) Spin down at 13,200 rpm for 5 min and collect supernatant as total protein extract.

2.7 Nuclear extract preparation

1) About 1x10^8 cells are collected by scraping to detach the cells from the dishes.

2) Wash cells twice with ice cold PBS and once with Hypotonic buffer (10 mM Tris-HCl, pH 7.3, 10 mM KCl, 1.5 mM MgCl₂ supplemented with protease inhibitors).

3) Spin at 2,000 rpm for 5 min to pellet the cells. Remove supernatant and resuspend cells in 5 ml of Hypotonic solution, put on ice for 10 min.

4) Homogenize cells in a Dounce homogenizer (Wheaton) for 15 strokes with a tight pestle.

5) Check cell lysis under a microscope and collect nuclei by centrifuge at 3,900 rpm for 15 min at 4°C.

6) Resuspend nuclei in same volume of Nuclear extraction buffer (20 mM Tris-HCl, pH 7.3, 600 mM KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol, with 0.7% β-mercaptoethanol and protease inhibitors), homogenize 6 times, rotate for 30 min at 4°C.

7) Spin at 13,200 rpm for 10 min to collect supernatant as nucleus extract.

8) Repeat step 6 and 7 twice more, and combine the supernatant.
9) Nuclear extract could be dialyzed against a medium-salt buffer (20 mM Tris-HCl, pH7.3, 300mM KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol, with 1mM PMSF) overnight at 4°C.

2.8 SDS-PAGE

1) Prepare samples in SDS loading buffer (6x: 300 mM Tris-HCl, 20% glycerol, 6% SDS, 4% β-mercaptomethanol, 0.6% bromophenol blue, pH6.8), heat samples at above 80°C for 5 min. Cool down on ice and briefly spin down.

2) Load samples in SDS-PAGE gel, leave one lane for protein marker (Crystalgen, 65-0671), load the lanes at each side with 1x SDS loading buffer.

3) Run the gel in 1x SDS running buffer (10x: 250 mM Tris-HCl, 1.92 M glycine, 1% SDS) at 210V for ~50 min, stop when the dye runs out of the gel.

Table 2-1: SDS-PAGE resolving gel preparation.

<table>
<thead>
<tr>
<th>(ml)</th>
<th>15%</th>
<th>12%</th>
<th>10%</th>
<th>8%</th>
<th>6%</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acrylamide: Bis (37.5:1)</td>
<td>5.5</td>
<td>4.4</td>
<td>3.65</td>
<td>2.95</td>
<td>2.2</td>
</tr>
<tr>
<td>1 M Tris-HCl, pH8.8</td>
<td>4.1</td>
<td>4.1</td>
<td>4.1</td>
<td>4.1</td>
<td>4.1</td>
</tr>
<tr>
<td>20% SDS</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.005</td>
<td>0.005</td>
<td>0.005</td>
<td>0.005</td>
<td>0.005</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>1.25</td>
<td>2.35</td>
<td>3.1</td>
<td>3.8</td>
<td>4.55</td>
</tr>
</tbody>
</table>

SDS-PAGE stacking gel (5 ml): 0.665 ml 30% Acrylamide: Bis (37.5:1), 0.63 ml 1 M Tris-HCl, pH 6.8, 0.025 ml 20 % SDS, 0.05 ml 10% APS, 0.005 ml TEMED, 3.62 ml ddH₂O.

Water-saturated isobutanol: Shake equal volumes of water and isobutanol in a glass bottle and allow to separate.
2.9 Coomassie blue staining

1) After SDS-PAGE, remove the gel from glass plates, and fix gel for 10 min in the Fixing buffer (50% methanol, 10% HAc).

2) Decant Fixing buffer, stain with Coomassie blue solution (0.1% coomassie blue R-250 in the Fixing buffer) for 10 min.

3) Decant Coomassie blue solution, destain with destaining solution (10% methanol, 5% HAc) until the bands are visible and the background is clean.

2.10 Silver staining

Silver Staining Kit (Invitrogen, LC6070) is used.

1) After SDS-PAGE, fix gel for 20 min in the Fixing buffer.

2) Decant the Fixing buffer, wash the gel in 30% ethanol for 10 min.

3) Decant the ethanol, incubate the gel in the Sensitizing solution (9 ml ethanol, 3 ml Sensitizer, add ddH2O to 30 ml) for 10 min.

4) Decant the Sensitizing solution and wash the gel in 30% ethanol for 10 min.

5) Decant the ethanol and wash the gel in ddH2O for 10 min.

6) Decant ddH2O and incubate the gel in the Staining solution (0.3 ml Stainer, add ddH2O to 30 ml) for 15 min.

7) Decant the Staining solution and wash the gel with ddH2O for 20-60 sec.

8) Decant ddH2O and incubate the gel in the Developing solution (3 ml Developer, ~15 µl Developer enhancer, add ddH2O to 30 ml) for 4-8 min until bands start to appear and the desired band intensity is reached.
9) Once the appropriate staining intensity is achieved, immediately add 3 ml of the Stopper directly to the gel. Gently agitate for 10 min. The change of color from pink to colorless indicates that the development has stopped.

10) Decant the Stopper solution and wash the gel with 30 ml ddH₂O for 10 min.

2.11 Western blotting

1) After SDS-PAGE, transfer protein to 0.2 µm nitrocellulose membrane (Whatman, BA85) in 1x Transferring buffer (25 mM Tris, 192 mM glycine, 20% methanol) using Semi-dry Transferring Unit (GE, TE77).

2) Briefly stain the membrane with Ponceau S solution (0.1% Ponceau S in 5% HAc), wash away the background with ddH₂O, scan the membrane, and cut to desired portions.

3) Wash away Ponceau S with TBST (10xTBS: 1.37 M NaCl, 27 mM KCl, 250 mM Tris-HCl, pH 7.4. Add 0.1% Tween-20 for TBST).

If use α-Modified Citrulline antibody, wash the membrane with water twice. Incubate the membrane in 0.1% Ovalbumin in TBS for 15 min at room temperature with agitation. Wash membrane twice with water. Prepare the Modification buffer by mixing same volume of Reagent A (0.025% FeCl₃ in a solution of sterile, distilled water/98%H₂SO₄/85%H₃PO₄ (55%/25%/20%, store at 4°C or room temperature) and Reagent B (0.5% 2,3-butanedione monoxime, 0.25% antipyrine, 0.5 M HAc, store at -20°C, prevent from light). Immediately add the Modification buffer to membrane. Place the membrane in a lightproof container and incubate at 37°C for at least 3 hr to overnight without agitation. Wash the membrane five times with water, 3 min each. Proceed to following steps.

4) Block the membrane with the Blotting solution (5% nonfat dry milk in TBST) for 30 min at room temperature.
5) Incubate the membrane in the Blotting solution with the appropriately diluted primary antibody overnight (14-18 hr) at 4°C.

6) Next day take the membrane out, wash with TBST three times, 10 min each.

7) Incubate the membrane in the Blotting solution with the appropriately diluted horseradish peroxidase-conjugated secondary antibody for 2-3 hr at 4°C.

8) Take the membrane out, wash with TBST three times, 10 min each.

9) Incubate the membrane with the Lumi-Light\textsuperscript{PLUS} ECL substrate (Roche, 12015196001) for 5 min.

10) Exposure and develop the film in the dark room.

11) To strip the membrane, incubate the membrane in the Stripping buffer (62.5 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM β-mercaptoethanol, 2% SDS) for 30 min at 50°C. Rinse the membrane with TBST.

2.12 Reverse transcription

2.12.1 Reverse transcription-PCR

SuperScript One-Step RT-PCR System with Platinum Taq DNA polymerase (Invitrogen, 10928) is used.

1) Assemble 25 µl reaction system:

12.5 µl 2x Reaction Mixture

x µl Template RNA (10 pg-1 µg, usually use 0.2 µg)

1.2 µl 5 µM Primer 1

1.2 µl 5 µM Primer 2

0.5 µl RT/Platinum Taq Mixture
Add ddH₂O to 25 μl

2) Run the reaction in PCR machine with program:
   - 50°C for 30 min
   - 94°C for 2 min
   - 30 cycles of:
     - 94°C for 30 sec
     - x°C for 45 sec
     - 70°C for x min (~1kb/min)

3) Run 5 μl sample in agarose gel to check product. If the signal is too weak, add 3 more cycles. Gel images are analyzed using the NIH image J program to quantify the amount of signals.

2.12.2 Synthesis first-strand cDNA

SuperScript II Reverse Transcriptase (Invitrogen, 18064) is used.

1) Assemble the reaction:
   - 1 μl 250 ng/μl Random primers
   - x μl total RNA (1 ng-5 μg, usually use 0.2 μg)
   - 1 μl 10 mM dNTP mixture
   - Add ddH₂O to 13 μl

2) Heat the sample at 65°C for 5 min and quick chill on ice. Briefly spin the tube.

3) Add 4 μl 5x First-strand buffer, 2 μl 0.1 M DTT to each tube, incubate at 25°C for 2 min.

4) Add 1 μl of Reverse transcriptase and incubate at 25°C for 10 min

5) Incubate at 42°C for 50 min.
6) Inactivate the reaction by heating at 70°C for 15 min.

7) The cDNA can be diluted appropriately and used as template for PCR.

2.13 Immunostaining

1) Cells are seeded on cover slip by either growing on cover slip (attached cells) or spinning to cover slip at 2,000 rpm for 10 min (suspension cells). Cover slips can be pretreated with 50-100 µl 0.01% poly-L-lysine, air dry, rinse with ddH₂O, and air dry again for 30-60 min.

2) Fix cells with the Fixation solution [1x PBS (10x: 1.37 M NaCl, 100 mM Na₂HPO₄, 20 mM NaH₂PO₄, adjust pH to 7.4 with HCl), 3.7% paraformaldehyde (using 5x PFA: 4 ml ddH₂O, 0.74 g paraformaldehyde, 28 µl 1 M KOH, heat to 65°C to dissolve), 0.1% Triton X-100, 0.2% NP-40] for 10-15 min at room temperature.

3) Wash the cells with PBST (1x PBS with 0.1% Triton X-100) for three times, 10 min each.

4) Block the cells with 2% BSA in PBST for at least 30 min at room temperature.

5) Stain the cells in a dark humid chamber with the appropriately diluted primary antibody in PBST with 2% BSA and 5% normal goat serum (if the secondary antibody is goat source) or with 5% normal donkey serum (if the secondary antibody is donkey source) overnight at 4°C.

6) Next day wash the cells with PBST for three times, 10 min each.

7) Incubate the cells in a dark humid chamber with the appropriately diluted secondary antibody in PBST with 2% BSA and 5% normal goat serum for 2 hr at room temperature.

8) Wash the cells with PBST for three times, 10 min each and PBS for 5 min.

9) Stain the cells briefly with DNA dye Hoechst (1mg/ml) at 1:1000 dilution in PBS.

10) Wash the cells with PBS for 5 min and ddH₂O for 5 min.
11) Mount the cells to slides with the Mounting solution (2% N-proply gallate in 10 mM Tris pH 9.0 and 80% glycerol), air-dry briefly, and seal the edge with nail polish.

12) Images are captured using a Zeiss Axioskop 40 fluorescence microscope.

2.14 Apoptotic cells detection

2.14.1 TUNEL assay

DeadEnd Fluorometric TUNEL system (Promega, G3250) is used.

1) Follow immunostaining procedure till step 3.

2) Wash the cover slip in fresh PBS twice, 5 min each.

3) Equilibrate the cells with 100 µl of the Equilibration buffer for 5-10 min at room temperature.

4) Incubate the cells with a mixture of 45 µl Equilibration buffer, 5 µl Nucleotide mix, and 1 µl rTdT enzyme at 37°C for 60 min in a dark humid chamber. Avoid light from this step.

5) Terminate the reaction by incubate the cells in 2x SSC (2x: 300 mM NaCl, 30 mM Sodium Citric, pH 7.0) buffer for 15 min at room temperature.

6) Wash the cover slip with fresh PBS for three times, 5 min each.

7) If other antibody is going to be used, follow immunostaining procedure from step 4.

2.14.2 Annexin V assay

Annexin V-FITC (Abcam, ab14085) is used.
1) Start with ~5x10^5 cells per sample. Wash cells once with the Binding buffer (10 mM HEPES pH 7.4, 2.5 mM CaCl₂, 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂). Spin down cells and remove supernatant.

2) Resuspend cells in 200 µl Binding buffer, add 10 µl Annexin V-FITC, incubate at room temperature for 20 min in dark.

3) Wash once with the Binding buffer. Resuspend cells in 200 µl Binding buffer.

4) If necessary, add 10 µl 50 µg/ml Propidium Iodide and incubate for 15 min at room temperature.

5) Add another 300 µl Binding buffer and analyze by flow cytometry within 1 hr.

### 2.15 Cell cycle profile analysis

1) Fix cells with cold 70% ethanol for 5 min.

2) Pellet cells by spinning for 5 min at 1,000 rpm. Discard supernatant.

3) Resuspend and rehydrate cells in PBS for 15 min.

4) Treat cells with 10 µg/ml RNase A for 30 min at 37°C.

5) Add propidium iodide at a concentration of 20 µg/ml (Sigma, P4864) and stain for 30 min at room temperature.

6) Wash once with PBS and analyze by flow cytometry.

### 2.16 GST-tagged protein purification from bacteria

1) Transform the recombinant pGEX4T1 plasmid containing the target gene into BL21 competent cells.
2) Pick up couple of single colonies and inoculate 20 ml 2x YT with desired antibiotics.
Incubate overnight at 37°C with vigorous shaking (250 rpm).

3) Put the 20 ml culture into 1 L fresh 2x YT with desired antibiotics, grow at 32°C with
vigorous shaking (250 rpm) until OD<sub>600</sub> reaches 0.6-0.8.

4) Add IPTG to a final concentration of 0.1 mM and continue growing for 2-6 hr at 32°C
with shaking at 200 rpm.

5) Spin down cells at 7,700 x g for 10 min at 4°C.

6) Discard the supernatant and place on ice.

7) Resuspend the cell pellet with 50 ml ice-cold PBS. Spin down cells at 7,700 x g for 10
min at 4°C.

8) Snap freeze the pellet with liquid nitrogen and warm up under tap water.

9) Resuspend the cell pellet with 50 ml ice-cold PBS with protease inhibitors.

10) Sonicate samples in Bioruptor for 5 rounds (30 sec on, 30 sec off) at high energy
level.

11) Add Triton X-100 to a final concentration of 1%. Rotate for 30 min at 4°C.

12) Centrifuge at 12,000 x g for 15 min at 4°C. Save supernatant.

13) Wash 0.5 ml of Glutathione Sepharose three times with PBST.

14) Add the sepharose to the sonicated supernatant, rotate overnight at 4°C.

15) Next day let the sepharose settle down to the bottom of tube by sitting at 4°C for 30
min. Remove supernatant.

16) Wash the sepharose with 40 ml PBST, rotate for 10 min at 4°C. Spin down at 1,000
rpm for 5 min. Remove supernatant. Repeat three more times.

17) Sepharose can now be stored in PBST/50% glycerol at -20°C for future use.
18) To elute protein, incubate the sepharose with same volume of Elution buffer (10mM glutathione in 50 mM Tris-HCl, pH8.0) with protease inhibitors, vortexing at middle speed for 15 min at 4°C. Spin down at 500 x g for 2 min, save supernatant.

19) Repeat step 17 twice more to collect three elutes in total. Samples could be stored at -80°C with 10% Glycerol.

2.17 His-tagged protein purification from bacteria

1) Transform the recombinant pET plasmid containing the target gene into BL21 competent cells.

2) Pick up couple of single colonies and inoculate 20 ml 2x YT with desired antibiotics. Incubate overnight at 37°C with vigorous shaking (250 rpm).

3) Put the 20 ml culture into 1 L fresh 2x YT with desired antibiotics, grow at 32°C with vigorous shaking (250 rpm) until OD$_{600}$ reaches 0.6-0.8.

4) Add IPTG to a final concentration of 0.1 mM and continue growing for 4 hr at 32°C with shaking at 200 rpm.

5) Spin down cells at 7,700 x g for 10 min at 4°C.

6) Discard the supernatant and place on ice.

7) Resuspend the cell pellet with 50 ml ice-cold PBS. Spin down cells at 7,700 x g for 10 min at 4°C.

8) Snap freeze the pellet with liquid nitrogen and warm up under tap water.

9) Resuspend the cell pellet with 50 ml ice-cold Lysis buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole, 0.5% Triton X-100) with protease inhibitors.

10) Sonicate samples in Bioruptor for 5 rounds (30 sec on, 30 sec off) at high energy level.
11) Rotate the sample for 30 min at 4°C.

12) Centrifuge at 12,000 x g for 15 min at 4°C. Save supernatant.

13) Wash 0.5 ml of Ni-NTA agarose three times with Lysis buffer.

14) Add the agarose to the sonicated supernatant, rotate overnight at 4°C.

15) Next day let the agarose settle down to the bottom of tube by sitting at 4°C for 30 min. Remove supernatant.

16) Wash the agarose with 50 ml Washing buffer (50 mM Sodium Phosphate, pH 8.0, 500 mM NaCl, 10 mM imidazole) with protease inhibitors, rotate for 10 min at 4°C. Spin down at 1,000 rpm for 5 min. Remove supernatant. Repeat three more times.

17) To elute protein, incubate the agarose with same volume of Elution buffer (50 mM sodium phosphate, pH 8.0, 100 mM NaCl, 250 mM imidazole, 5% glycerol) with protease inhibitors, vortexing at middle speed for 15 min at 4°C. Spin down at 500 x g for 2 min, save supernatant.

18) Repeat step 17 twice more to collect three elutes in total.

19) The protein can be dialyzed with appropriate buffer (PBS with PMSF for example) overnight at 4°C to remove most imidazole. Protein could be stored at -80°C with 10% glycerol.

### 2.18 FLAG-tagged protein purification from mammalian cell nuclear extract

1) Prepare nuclear extract following the nuclear extract preparation procedure.

2) Wash M2 agarose with 1 ml BC300 (20 mM Tris-HCl, pH 7.3, 300 mM KCl, 0.2 mM EDTA, 20% glycerol) three times.

3) Add M2 agarose to nuclear extract supplemented with protease inhibitor. Rotating overnight at 4°C.
4) Next day let the agarose settle down to the bottom of tube by sitting at 4°C for 30 min. Remove supernatant.

5) Wash with BC300-0.1% NP-40 with protease inhibitors, rotate 10 min at 4°C. Spin down at 1,000 rpm for 5 min, remove supernatant. Repeat three more times.

6) To elute protein, incubate M2 agarose with same volume of BC100-0.05% NP-40 (20 mM Tris-HCl, pH 7.3, 100 mM KCl, 0.2 mM EDTA, 20% glycerol) plus 0.2 mg/ml FLAG peptide with protease inhibitors, vortexing at middle speed for 30 min at 4°C. Spin down at 500 x g for 2 min. Collect supernatant.

7) Repeat step 6 for three more times to collect four elutes in total. Protein can be stored in -80°C.

2.19 Protein expression and purification using baculovirus system

Protein expression and purification in baculovirus system is performed with BaculoDirect Baculovirus Expression Systems (Invitrogen, 12562) following the manufacturer’s instructions.

2.20 Co-immunoprecipitation

Co-immunoprecipitation of p53 by PAD4 antibody is described as an example:

1) Add 5 μl PAD4 antibody into nuclear extract in 1.5 ml tube, bring up total volume to 500 μl if necessary. Mix and rotate overnight at 4°C.

2) Next day take 20 μl Protein A Agarose (Millipore, 16-125) and wash three times with 1 ml IP buffer each time, add the agarose to the mixture and rotate for 4 hr at 4°C.
3) Transfer the mixture to 15 ml tube, let the agarose precipitate to the bottom of tube by sitting at 4°C for 20 min. Remove supernatant. To wash the agarose, resuspend the agarose with 5 ml IP buffer, rotate 10 min at 4°C.

4) Spin down at 1,000 rpm for 5 min. Remove supernatant and repeat wash twice more.

5) After wash, remove supernatant and resuspend the agarose in 10 µl 1x SDS loading buffer, boil the sample and perform Western blotting using p53 antibody.

**2.21 GST pull-down**

1) Wash 20 µl GST agarose with 1 ml PBST for three times.

2) Add GST agarose to nuclear extract in 1.5 ml tube, bring up total volume to 500 µl if necessary. Mix and rotate overnight at 4°C.

3) Next day transfer the mixture to 15 ml tube, let the agarose precipitate to the bottom of tube by sitting at 4°C for 20 min. Remove supernatant. To wash GST agarose, resuspend the agarose with 5 ml PBST, rotate 10 min at 4°C.

4) Spin down at 1,000 rpm for 5 min. Remove supernatant and repeat wash twice more.

5) After wash, GST agarose can be directly boiled in 20 µl 1x SDS loading buffer and subjected to Western blotting.

**2.22 Chromatin immunoprecipitation (ChIP)**

1) Add 37% formaldehyde to the cell culture medium to a final concentration of 1% to fix cells. Agitate gently for 10 min.

2) Add 2 M glycine to a final concentration of 0.125 M to stop cross-linking. Agitate gently for 5 min.
3) Discard medium. Wash three times in cold PBS with protease inhibitors.

4) Harvest cells using a spatula and transfer to 15ml polystyrene tube (BD Falcon, 352099). Pellet down cells by spinning at 1,500 rpm for 5 min at 4°C. Discard supernatant.

5) Resuspend cells with 8 ml SDS-lysis buffer (100 mM NaCl, 50 mM Tris-HCl, pH8.0, 5 mM EDTA, 0.4% SDS) with protease inhibitors.

6) Rotate for 10 min at 4°C, warm up at room temperature until SDS precipitate disappears. Spin down at 1,500 rpm for 6 min at 4°C. Remove supernatant.

7) Resuspend the pellet with 2 ml ice-cold IP buffer (2 parts of SDS-lysis buffer, 1 part of Triton buffer: 100 mM NaCl, 100 mM Tris-HCl, pH 8.0, 5 mM EDTA, 5% Triton X-100).

8) Leave in ice for 10 min, invert occasionally.

9) Sonicate the sample for 5 rounds (30 sec on, 30 sec off) in Bioruptor at high energy level.

To check sonication efficiency, take small aliquot out, reverse cross-linking overnight at 65°C, digest with 2 µl 10 mg/ml Protease K and 1 µl 10 mg/ml RNase A for 1 hr, purify DNA by phenol-chloroform extraction and ethanol precipitation. Check DNA size by agarose gel electrophoresis.

10) Wash Protein A/salmon sperm DNA agarose three times with 1 ml IP buffer.

11) Add Protein A/ssDNA agarose to sonicated sample, use 50 µl agarose per 1 ml sample. Pre-clear sample for 2 hr at 4°C with rotating.

12) 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.

13) Distribute sample to 1.5 ml tubes with desired antibody added, aiming for about 1-5x10^5 cells per antibody with about 2 µg of each antibody. Bring up total volume to 500 µl, rotate overnight at 4°C.
Set up one tube without antibody that will represent the background and the source for the total input.

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

15) 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.

16) Add about 20 µl washed Protein A agarose/ssDNA to each sample, rotate for 1.5 hr at 4°C.

17) Let the agarose settle down by sitting at 4°C for 20 min. Discard supernatant.

18) Wash the agarose in below order and amount. Use 1 ml and rotate for 5 min at 4°C. Spin down the agarose at 3,000 rpm for 1 min at 4°C. Discard supernatant.

- 3 washes of Mixed Micelle buffer (150 mM NaCl, 20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 5.2% sucrose, 1% Triton X-100, 0.2% SDS)
- 2 washes of Buffer 500 (10 mM Tris-HCl, pH 8.0, 50 mM HEPES, pH 8.0, 500 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM EDTA)
- 2 washes of LiCl/Detergent buffer (10 mM Tris-HCl, pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 10 mM EDTA)
- 2 washes of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)

19) Resuspend the agarose in 170 µl fresh prepared Bicarbonate/SDS buffer (100 mM NaHCO3, 1% SDS). Add 120 µl to “Total input” sample.

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

21) Resuspend the agarose in 150 µl Bicarbonate/SDS buffer and repeat step 20.

22) Incubate the samples at 65°C for at least 5 hr to reverse cross-linking.
23) Spin down briefly to collect drops under the lid. Incubate the samples at room temperature for 10 min to cool down.

24) 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.

25) Add 30 µl of 3 M NaAc (pH 5.0) to each sample. Purify DNA with the PCR purification Kit (Qiagen, 28104) and samples are ready for PCR.

2.23 Re-chromatin immunoprecipitation (re-ChIP)

1) Follow ChIP procedure till step 18.

2) Elute twice the DNA/protein complex with 25 µl 10 mM DTT and incubate for 30 min at 37°C with vortexing each 5 min. Pool the supernatants and dilute 10 times with IP buffer.

3) Follow ChIP procedure again from step 13.

2.24 Real-time quantitative PCR

1) Assemble 12 µl real-time qPCR reaction in a 96-well plate:

   6 µl 2x Power SYBR Green PCR Master Mix (Applied Biosystems, 4367659)

   3 µl 1 µM Forward and reverse primer mixture

   3 µl DNA

2) PCR is performed in StepOne Plus Real-time PCR system (Applied Biosystems, 4376598) using below program:

   95°C for 10 min

   40 cycles of:

   95°C for 15 sec
55°C for 30 sec

70°C for 15 sec (Collect data at this step)

Add melt curve after the PCR program to check primer quality.

3) Data is recorded and analyzed using StepOne Software v2.1.

2.25 Histone extraction from tissue culture cells

1) Collect about 5x10^7 cells.

2) Wash cells once with 5 ml ice-cold DPBS.

3) Resuspend cells with 2 ml of the Hypotonic buffer (10 mM Tris-HCl, pH 8.0, 1 mM KCl, 1.5 mM MgCl_2, 1 mM DTT) with protease inhibitors.

4) If the extraction is not very efficient, add 0.5% NP-40 in the Hypotonic buffer.

5) Incubate on ice for 20-30 min.

6) Pellet nuclei by spinning at 4,000 rpm for 10 min at 4°C.

7) Resuspend nuclei with 0.4 ml 0.4 N H_2SO_4, vortex a little while and rotate at 4°C for at least 30 min to overnight.

8) Spin down at 13,200 rpm for 10 min, collect supernatant.

9) Add 100% TCA to a final concentration of 20% to precipitate histone, incubate on ice for at least 30 min.

10) Pellet histone by spinning at 13,200 rpm for 10 min.

11) Wash twice with cold Acetone (-20°C).

12) Air-dry the pellet for at least 20 min.

13) Resuspend histone in appropriate amount of ddH_2O.
2.26 Mononucleosome preparation by micrococcal nuclease digestion

1) Add 37% Formaldehyde to cells to a final concentration of 1% to cross-link cells, agitate gently for 10 min.

2) Add 2 M glycine to a final concentration of 0.125 M to stop cross-linking, agitate gently for 5 min.

3) Wash three times in cold PBS with protease inhibitors. Collect cells by spinning down at 1,500 rpm for 5 min at 4°C. Discard supernatant.

4) Resuspend cells in 1 ml HB buffer (10 mM Tris-HCl, pH 7.3, 10 mM KCl, 1.5 mM MgCl₂, 1% Triton X-100, 1 mM DTT supplemented with protease inhibitors), put on ice for 10 min, invert from time to time.

5) Homogenize cells in a Dounce homogenizer (Wheaton) for 15 strokes with a tight pestle. Spin down at 3,000 rpm for 10 min to pellet nuclei.

6) Resuspend cells in NP-S buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 1 mM CaCl₂, 0.5 mM DTT, 0.1 mM PMSF), aliquot into 500 µl/sample, add CaCl₂ to reach the final concentration of 5 mM. For HL-60 cells, use ~3x10⁷ cell/ml concentration.

7) Add micrococcal nuclease at different concentrations, place at 37°C for ~10 min for digestion. (I used 10 U to digest for 15 min for 10⁷ cells in 500 µl volume).

8) Add 10mM EDTA to stop the digestion.

9) Add SDS to a final concentration of 1%, put on ice for 10 min.

10) Sonicate for 30 sec at high energy level. Spin down at 13, 200 rpm for 15 min at 4°C. Save supernatant for future experiment.

11) To check the digestion efficiency:

   a. Take a small aliquot from each sample, first digest with 10 µl of 10 mg/ml RNase A for 1 hr and then 10 µl of 10 mg/ml Protease K for 2 hr at 37°C.
b. Incubate the sample at 65°C for 5 hr to reverse cross-linking.

c. Purify DNA by Phenol-Chloroform extraction and ethanol precipitation. Add 10 to 20 µg Glycogen before precipitation as carrier if necessary.

d. Spin down at 13, 200 rpm for 10 min at 4°C. Wash twice with 70% cold ethanol. Air-dry at 37°C and resuspend the pellet in 20 µl ddH2O.

e. Run samples on agarose gel electrophoresis to check product size.

2.27 PAD4 activity assay

1) Assemble 20 µl reaction system as follows:

   2 µl 10x PAD buffer (10x: 500 mM Tris-HCl, pH 7.6, 40 mM CaCl2, 40 mM DTT, 10 mM PMSF)

   x µl PAD4 protein (1-2 µg)

   x µl Substrate (Histone or nucleosome, 2-3 µg)

   Add ddH2O to 20 µl

   If Cl-amidine is used to inhibit PAD4 activity, first incubate Cl-amidine with PAD4 for 10 min in the PAD buffer, then add substrate.

2) Incubate at 37°C for 1 to 2 hr.

3) Check result with Western blotting.

2.28 Flow cytometry analysis

Flow Cytometry analysis is performed using the FC500 flow cytometer (Beckman Coulter) and analyzed with the CXP software at the Penn State Flow Cytometry Facility.
2.29 Sucrose gradient ultracentrifugation

1) Sucrose gradient is prepared with SG30 Gradient Maker (GE, 80-6197-80) following the manufacturer’s instructions. Sucrose gradient is made in Ultra-Clear Centrifuge Tubes (Beckman) with different volume.

2) Load sample on top of the sucrose gradient.

3) Centrifuge at desired speed in Beckman Ultra Centrifuge Machine.

4) Carefully take out fractions with desired volume.

2.30 Southern blot

1) Genomic DNA preparation:
   a. Collect cell or tissue sample in 500 µl DNA lysis buffer (100 mM Tris-HCl, pH 8.0, 200 mM NaCl, 10 mM EDTA, 0.5% SDS, 200 µg/ml Protease K). Incubate at 55°C overnight.
   b. Spin at 13,200 rpm for 15 min to get rid of possible precipitate.
   c. Add 500 µl isopropanol to precipitate the DNA, no vortex.
   d. Lift the white DNA pellet from the solution using a blunt pipette tip.
   e. Transfer the pellet to a new 1.5 ml tube filled with 70% ethanol. Spin at 13,200 rpm for 5 min to discard ethanol.
   f. Add 500 µl ddH₂O and incubate at 65°C for 1 to 2 hr to dissolve DNA.
   g. Extract three times with equal volume of Phenol/Chloroform.
   h. Precipitate DNA with 0.1 volume of 3 M NaAc and 2 volume of ethanol.
   i. Lift the DNA pellet from the solution using a blunt pipette tip.
   j. Transfer the pellet to a new 1.5 ml tube filled with 70% ethanol. Spin at 13,200 for 5 min to discard ethanol.
   k. Briefly air-dry the DNA pellet and dissolve DNA in 100 µl ddH₂O overnight at 4°C.
2) Digest ~10 µg genomic DNA sample with restriction enzyme overnight.

3) Load the sample to a 0.8% agarose gel, run gels at 80 V for 3 to 4 hr until the dye runs to the lower 1/3 of the gel.

4) Take a gel picture with short exposure, make holes on the gel at the positions of each ladder.

5) Treat the gel with 0.25 M HCl for about 15 min until the dye changes color to nick the DNA. Rinse twice with ddH₂O.

6) Treat the gel with the Denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 20 min. Repeat again. Rinse twice with ddH₂O.

7) For uncharged membrane, neutralize the gel with the Neutralization solution (1 M Tris, pH 7.5, 1.5 M NaCl) for 20 min. Repeat again. Rinse twice with ddH₂O.

8) Set up the transfer: After each step, remove air bubbles by rolling a pipette over assembly.
   a. Fill tray with 20x SSC (or 0.4 M NaOH) as the Transfer buffer.
   b. Prepare the bridge. Wet 2 pieces of large blotting paper as wick, put on the bridge.
   c. Lay gel on the wick with upside down. Seal each side of the gel with Parafilm to prevent short-circuiting.
   d. Wet the nylon membrane first with ddH₂O, then with Transfer buffer, place on the gel. Mark corner or top.
   e. Wet 2 pieces of small blotting paper, lay them on top of the membrane.
   f. Lay paper towel on top of the blotting paper.
   g. Put heavy weight on top of paper towel to press down.
   h. Seal the tray with Saran wrap to prevent evaporation.
   i. Transfer overnight.
9) Next day mark the ladder on membrane, put the membrane on aluminum foil. Do not let it dry out.

10) Fix DNA by UV cross-linking twice at 1200 J/m².

11) Place membrane in hybridization tube and add 10-20 ml QuikHyb Hybridization solution (Stratagene, 201220). Pre-hyb at 65°C for at least 1 hr.

12) Label probe using Random Primed DNA Labeling Kit (Roche, 1004760):
   a. Add sterilized ddH₂O to 20 -50 ng PCR amplified probe to a total volume of 9 µl.
   b. Heat DNA in boiling water bath for 5 min and quickly chilled on ice to denature DNA. Spin down briefly.
   c. Add 1 µl of each 0.5 mM dATP, dGTP, dTTP to DNA.
   d. Add 2 µl of the Reaction mixture.
   e. Add 5 µl of 50 µCi α-P³²-dCTP.
   f. Add 1 µl of 2U/µl Klenow enzyme.
   g. Mix and briefly spin down to 20 µl reaction system.
   h. Incubate at 37°C for at least 30 min.
   i. Stop the reaction by adding 2 µl of 0.2 M EDTA and/or by heating to 65°C for 10 min.
   j. Use Quick Spin Column (Roche, 1273965) to remove unincorporated deoxyribonucleotide triphosphates.

13) Boil Salmon Sperm DNA for 10 min, add to the pre-hyb at a concentration of 0.5 mg/ml.

14) Add the denatured probe to the Hybridization solution, incubate in rotating oven at 65°C for 2 hr to overnight.

15) Wash the membrane three times for 5 min each at room temperature with 2x SSC and 1% SDS. Then wash with 0.5x SSC and 0.1% SDS for 30 min at 65°C.

16) Wrap the membrane with Saran wrap and expose with a phosphorimage screen.
17) To strip the membrane:

a. Place the membrane in hybridization tube with Denaturation solution and rotate for 30 min at 50°C. Rinse twice with ddH2O.

b. Rotate the membrane in Neutralization solution for 30 min at room temperature. Rinse twice with ddH2O.

c. Rotate the membrane in 2x SSC and 0.1% SDS for 30 min at room temperature.

Membrane is ready for store at 4°C or hybridization.

2.31 Competent cell preparation

2.31.1 Chemical competent cell preparation

1) Prepare sterilized Medium A (for 50 ml, 49 ml 2xYT, 0.5 mM 10 mM MgSO4, 0.5 ml 20% Glucose), Medium B (for 10 ml, 2.98 ml 2xYT, 3.6 ml 100% Glycerol, 3.3 ml 36% PEG, 0.12 ml 1 M MgSO4).

2) Inoculate bacteria in 3 ml 2xYT, grow overnight in 37°C shaker (250 rpm).

3) Add 0.5 ml overnight culture to 50 ml Medium A, grow in 37°C shaker to OD₆₀₀=0.5~0.8.

4) Spin down at 4,000 rpm for 2 min at 4°C. Remove supernatant.

5) Resuspend cells in 0.5 ml Medium A and 2.5 ml Medium B.

6) Aliquot to 0.2 ml/tube.

7) Snap freeze with liquid nitrogen and store at -80°C.
2.31.2 Electroporation competent cell preparation

1) Inoculate bacteria in 3 ml 2x YT, grow overnight in 37°C shaker (250 rpm).

2) Add 3 ml overnight culture to 50 ml fresh 2x YT. Grow in 37°C shaker to OD$_{600}$=0.35-0.4.

3) Rapidly transfer the flask to an ice-water bath for 15-30 min, gently swirling the flask to ensure even cooling. From this point on, always ensure that the cells are kept on ice to avoid temperature increase.

4) Transfer cells into a pre-chilled 50 ml tube, harvest cells by centrifuging at 1000 x g for 15 min at 4°C.

5) Decant the supernatant and resuspend cells in 30 ml pre-chilled ddH$_2$O. Harvest cells by centrifuging at 1000 x g for 20 min at 4°C.

6) Decant the supernatant and resuspend cells in 15 ml pre-chilled 10% Glycerol. Harvest cells by centrifuging at 1000 x g for 20 min at 4°C.

7) Remove the supernatant and resuspend cells in 5 ml pre-chilled 10% Glycerol. Harvest cells by centrifuging at 1000 x g for 20 min at 4°C.

8) Remove the supernatant and any remaining glycerol solution by pipetting. Gently resuspend cells in 0.5 ml pre-chilled GYT broth (for 100 ml, 10 ml Glycerol, 0.125 g Yeast extract, 0.25 g Tryptone, add ddH$_2$O to 100 ml and autoclave).

9) Measure the OD$_{600}$ of a 1:1000 dilution of the cell suspension. Dilute the cell suspension to a concentration of 1x10$^6$ cells/ml (1OD$_{600}$=~2.5x10$^8$ cells/ml) with ice-cold GYT medium.

10) Take 40 µl out to perform electroporation to test whether arcing occurs when an electrical discharge is applied. If so, wash the remaining cell suspension once more with ice-cold
GYT medium to ensure that the conductivity of the bacterial suspension is sufficiently low (<5 mEq).

11) Dispense 40 µl aliquot of the cell suspension into sterile, ice-cold 1.5 ml tubes. Snap freeze with liquid nitrogen and store in -80°C.

2.32 Antibody affinity purification

1) Run about 1 µg/lane of the target protein on SDS-PAGE and transfer to 0.1 µm nitrocellulose membrane.

2) Stain with Ponceau S and cut out the band.

3) Wash the strip briefly with PBS supplemented with 0.5% Tween 20 (PBSTween).

4) Block the strip with PBSTween + 2% BSA + 2% Normal serum for 1 hr at room temperature.

5) Rinse briefly with PBSTween.

6) Add 3 ml serum to a 15 ml tube and put in the strip, rock for 1 hr at room temperature.

7) Elute antibody by adding 3 ml low pH glycine supplemented with Tween 20 (1.5 g glycine, 1ml HCl, 100ml water). Add 0.5% Tween immediately before use. Agitate for 1 hr at room temperature.

   -Neutralize with 1M Tris base (~100 µl/ml glycine).

   -Add an equal volume of PBS + 2% BSA to stabilize the antibody. (Optional) add 2 mM NaN₃.

   -Freeze in aliquot.
2.33 Antibodies used in experiments

Table 2-2: List of antibodies.

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## 2.34 Primers used in experiments

Table 2-3: List of primers.

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Chapter 3

Regulation of p53 Target Gene Expression by Peptidylarginine Deiminase 4
3.1 Abstract

Histone Arg methylation has been correlated with transcriptional activation of p53 target genes. However, whether this modification is reversed to repress the expression of p53 target genes is unclear. We posit that peptidylarginine deiminase 4, a histone citrullination enzyme, is involved in the repression of p53 target genes. Inhibition or depletion of PAD4 elevated the expression of a subset of p53 target genes, including p21/CIP1/WAF1, leading to cell cycle arrest and apoptosis. Moreover, the induction of p21, cell cycle arrest, and apoptosis by PAD4 depletion is p53-dependent. Protein-protein interaction studies showed an interaction between p53 and PAD4. Chromatin immunoprecipitation assays showed that PAD4 is recruited to the p21 promoter in a p53-dependent manner. RNA polymerase II activities and the association of PAD4 are dynamically regulated at the p21 promoter during UV irradiation. Paused RNA Pol II and high levels of PAD4 were detected before UV treatment. At early time points after UV treatment, an increase of histone Arg methylation and a decrease of citrullination were correlated with a transient activation of p21. At later times after UV irradiation, a loss of RNA Pol II and an increase of PAD4 were detected at the p21 promoter. The dynamics of RNA Pol II activities after UV treatment were further corroborated by permanganate footprinting. Together, these results suggest a role of PAD4 in the regulation of p53 target gene expression.
3.2 Introduction

In eukaryotic cells, 147 bp DNA is wrapped around a core histone octamer (including two of each histones H3, H2B, H2A, and H4) to form a nucleosome core particle, the basic structural unit of chromatin (Richmond and Davey, 2003). Posttranslational histone modifications, including methylation, acetylation, phosphorylation, ubiquitination, and citrullination, have been found to play a major role in chromatin functions, such as transcription (Barski et al., 2007; Berger, 2007; Klose and Zhang, 2007; Shilatifard, 2006; Vazgiourakis et al., 2007). Moreover, specialized protein domains, including chromo-, bromo-, and tudor-domains and PHD fingers, have evolved to recognize histone modification “marks” and to regulate nuclear events following histone modifications (Khorasanizadeh, 2004; Kouzarides, 2007; Li et al., 2007c). Histone Arg methylation is catalyzed by members of the protein Arg methyltransferase (PRMT) family (Chen et al., 1999; Strahl et al., 2001; Wang et al., 2001). The methylation of histones H3 and H4 has been correlated with the expression of nuclear receptor target genes and developmentally regulated genes (Bauer et al., 2002; Huang et al., 2005b). In searching for enzymes that can reverse histone Arg methylation, we and others previously reported that a histone Arg demethylimination enzyme, PAD4, can convert both Arg and monomethyl-Arg to citrulline thereby regulating histone Arg methylation (Cuthbert et al., 2004; Klose and Zhang, 2007; Wang et al., 2004b). In contrast to the role of histone Arg methylation in transcriptional activation, the demethylimination and citrullination of histones mediated by PAD4 have been found to play a role in transcriptional repression of nuclear receptor target genes (Balint et al., 2005; Cuthbert et al., 2004; Wang et al., 2004b). Several mechanisms can be envisioned to explain the functions of histone demethylimination and citrullination. First, demethylimination by PAD4 directly decreases the amount of methyl-Arg on histones; second, citrulline residues cannot
serve as the PRMT substrate thereby preventing the PRMT function; third, citrulline can be recognized by effector proteins that regulate chromatin functions.

The tumor suppressor p53 plays a pivotal role in regulating the cell cycle progression and apoptosis in response to various genotoxic and nongenotoxic stresses (Laptenko and Prives, 2006; Pluquet and Hainaut, 2001; Vogelstein et al., 2000). The ability of p53 to function as a sequence-specific transcription factor is critical for its tumor suppressor function. Hundreds of p53 target genes have been identified (Harris and Levine, 2005; Laptenko and Prives, 2006; Vogelstein et al., 2000). These include genes inducing cell cycle arrest, such as p21/WAF1/CIP1 and GADD45, an E3 ubiquitin ligase of p53- MDM2, as well as genes inducing apoptosis, such as PUMA, Bax, and PIG3 (Vogelstein et al., 2000; Wei et al., 2006). To facilitate the activation of its target genes, p53 recruits several histone acetyltransferases (e.g., p300/CBP, Tip60, and PCAF) (Barlev et al., 2001; Brooks and Gu, 2003; Espinosa and Emerson, 2001; Mujtaba et al., 2004; Tang et al., 2006). These acetyltransferases play multiple roles in the activation of p53 target genes by acetylating histones as well as p53 itself (Barlev et al., 2001; Espinosa and Emerson, 2001; Gu and Roeder, 1997). In addition, p53 can repress transcription by recruiting corepressors, such as histone deacetylases 1 and 2, LSD1, and DNA methyltransferase (Gui et al., 2004; Ho and Benchimol, 2003; Huang et al., 2007; Laptenko and Prives, 2006; Le Gac et al., 2006). Consistent with a role of these proteins in the repression of p21, depletion of HDAC1, 2, and LSD1 has been found to increase the expression of p21 in cells without exposure to DNA damage (Harms and Chen, 2007; Huang et al., 2007; Senese et al., 2007).

Recently, protein Arg methyltransferases, PRMT1 and CARM1, were found to regulate the expression of p53-target genes (Fu et al., 2004). An increase of histone Arg methylation was followed by a decrease of this modification on the GADD45 promoter after UV irradiation (Fu et al., 2004). How histone Arg methylation is reversed during p53 target gene expression is unknown. Because PAD4 can convert both monomethyl-Arg and Arg in histones to citrulline, we
investigated whether PAD4 plays a role in the p53 pathway to regulate gene expression. We found that inhibition or depletion of PAD4 induces the expression of p21 in a p53-dependent manner. PAD4 interacts with p53 and is recruited to the p21 promoter to regulate histone Arg methylation and citrullination. Our studies suggest that p53 plays multifaceted roles in gene regulation by interacting with transcriptional coactivators or corepressors to fine-tune the expression of its target genes.
3.3 Materials and Methods

3.3.1 Plasmid constructs

Flag-p53 was cloned into the pIRES vector for expression in human cell lines. GST-PAD4, GST-PAD4\textsuperscript{IL1&2}, GST-PAD4\textsuperscript{IL1}, GST-p53, GST-p53\textsuperscript{1-300}, and GST-p53\textsuperscript{301-393} were cloned in the pGEX4T1 vector for expression in \textit{Escherichia coli} strain BL21. Flag-His6-p53 was cloned into pET11a vector for expression in \textit{E.coli} strain BL21. PAD4 and PAD4\textsuperscript{C645S} mutant were cloned into the pSG5 vector for cell transfection. To establish FLAG-hemagglutinin-PAD4 expressing 293T and MCF-7 cell lines, FLAG-HA-PAD4 was cloned into the pMIGR1 vector.

3.3.2 Cell treatments with siRNAs, shRNA, Cl-amidine, and UVC

PAD4 SMARTpool siRNAs were purchased from Dharmacon Inc. A green fluorescent protein siRNA (Dharmacon Inc.) was used as a control for the siRNA experiments. Cells were incubated in the presence of the siRNAs for 60 hr before analyses. To make the PAD4 short hairpin RNA construct, annealed short hairpin oligonucleotides targeting nucleotides 547 to 565 (5’-GCGAAGACCTGCAGGACAT-3’) of PAD4 mRNA sequence (NCBI access no. NM_012387) were cloned into the pHTP vector (Wang \textit{et al.}, 2003). For the p53 shRNA construct, annealed short hairpin oligonucleotides targeting nucleotides 1026 to 1044 (5’-GACTCCAGTGTAATCTAC-3’) of the p53 mRNA sequence (NCBI access no. NM_000546.3) were cloned into the pHTP vector. At 12 hr after transfection of the shRNA plasmid with lipofectamine 2000, fresh medium containing 3 \(\mu\)g/ml puromycin was added to select transfected cells for 6 days before Reverse transcription-PCR, Western blotting, and flow
cytometry analyses. Due to the cell growth disadvantage after PAD4 depletion, each repeat experiment with the PAD4 shRNA was performed using freshly transformed cells. Cl-amidine was dissolved in H₂O as a 10 mM stock solution and diluted to 200 µM in the complete cell culture medium to treat cells in 6-well plates. Cells were collected at different time points as indicated in the text after Cl-amidine treatment before Reverse transcription-PCR and Western blotting analyses. For UV irradiation, cells were exposed to 50 J/m² of UVC light in a Spectrolinker XL-1000 UV crosslinker (Spectronics Inc.). To analyze cell number changes, 10,000 cells/well were plated in the 6-well plate. The Cl-amidine, PAD4 siRNA, or GFP siRNA treatments were performed in triplicate. At 72 hr after Cl-amidine treatment or 60 hr after PAD4 siRNA treatment, cells were trypsinized and counted using a hemacytometer. The numbers of cells in the wells without Cl-amidine treatment or with GFP siRNA treatment were normalized to 100%, and the percentage of cell number decrease was calculated by dividing the number of cells after Cl-amidine treatment or PAD4 siRNA treatment with that of the respective control groups then multiplying that value by 100. Standard deviations were calculated using the Microsoft Excel program.

3.3.3 Coimmunoprecipitation

For coimmunoprecipitation of p53 with Flag-PAD4, M2 agarose (Sigma, A2220) was incubated with the nuclear extracts at 4°C for overnight, and washed three times with TBST. For coimmunoprecipitation of PAD4 with the p53 antibody, the 293T nuclear extracts were loaded on an 11 ml 5-30% sucrose gradient in PBS buffer supplemented with protease inhibitors, and centrifuged using a Beckman SW28 rotor at 26,000 rpm for 16 hr. Fractions were collected from the top of the sucrose gradient. Fractions containing both p53 and PAD4 were pooled and used
for coimmunoprecipitation. For coimmunoprecipitation of p53 by the PAD4 antibody, 293
nuclear extracts were used.

3.3.4 Permanganate footprinting and LM-PCR

About 2x10^6 U2OS cells were washed with PBS and suspended in 100 µl PBS. Cells
were then treated with permanganate by adding 100 µl of 20 mM KMnO₄ dissolved in PBS. The
permanganate reaction was incubated on ice for 1 min and stopped by the addition of 200 µl Stop
solution (20 mM Tris-HCl, pH 7.5, 20 mM NaCl, 40 mM EDTA, 1% SDS, 400 mM β-
mercaptoethanol). The solution was vigorously shaken until all coloration had vanished. Each
sample was treated with 50 µg of proteinase K for at least 1 hr and then sequentially extracted
with phenol, phenol-chloroform-isoamyl alcohol (49.5:49.5:1), and chloroform. DNA was
precipitated with 0.3 M NaAc (pH 6.0) and ethanol. The DNA pellets were washed with 75%
ethanol and dissolve in 20 µl TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). To determine
the pattern of permanganate reactivity, 500 ng of each DNA sample was diluted in 15 µl TE
buffer. 75µl H₂O and 10 µl piperidine were then added, and each sample was incubated at 90°C
for 30 min. 300 µl H₂O was then added to each sample, and the samples were extracted three
times with 700 µl isobutanol and once with ether. The volume of the DNA was then adjusted to
100 µl with H₂O, and then precipitated with ethanol. The DNA was dissolved in 10 µl TE buffer,
transferred to a fresh siliconized tube and further analyzed by LM-PCR. LM-PCR was performed
with a set of nested primers LM-1, LM-2 and LM-3. These primers span the region +235 to +188
nucleotides downstream from the transcription start site of the p21 gene. The sequence and
annealing temperature used in the PCR for each of these primers are as follows: LM-1: 5’-
TTCACCTGCGCGAGAACC-3’ (61°C); LM-2: 5’-AGAAACACCTGTGAACGCAGCA-3’
(65°C); LM-3: 5’-CAGCACAACCCGCGAAACA-3’ (68°C).
3.4 Result

3.4.1 PAD4 inhibitor, Cl-amidine, activates the expression of the p53-target gene p21

Recently, a PAD4 inhibitor, Cl-amidine (Figure 3-1A), was generated (Luo et al., 2006). Cl-amidine is structurally similar to the PAD4 substrate peptidylarginine (Figure 3-1B), and inhibits PAD4 by covalent modification of a cysteine residue (Cys645) at the active site of the enzyme (Luo et al., 2006). As histone deacetylase (HDAC) inhibitors have been widely used to study HDACs (Gui et al., 2004; Kelly and Marks, 2005), we postulated that Cl-amidine would be useful to study the cellular functions of PAD4. To test the effect of Cl-amidine in PAD4 inhibition, we first analyzed the ability of Cl-amidine to inhibit PAD4 activity using histone H3 as a substrate in vitro and measured the efficacy of citrullination. Cl-amidine inhibited histone H3 citrullination by GST-PAD4 in a concentration-dependent manner (Figure 3-1C and 3-1D). Under the experimental conditions applied, Cl-amidine inhibited more than 50% of the PAD4 activity at the 50 µM concentration, whereas over 90% inhibition was reached with 200 µM of Cl-amidine (Figure 3-1D).

The dosage dependency of Cl-amidine to inhibit PAD4 in cells has been described using a reporter assay system (Luo et al., 2006). To test the effects of Cl-amidine and the inhibition of PAD4 on cell growth and proliferation, we treated the osteosarcoma U2OS cells with 200 µM of Cl-amidine. At 3 days after treatment, the number of Cl-amidine treated cells was decreased to 48.3±3.6% (n=3) of that of the untreated cells (Figure 3-2A). Western blotting found that the doublet bands of PAD4 in U2OS cells were unchanged after treatment with Cl-amidine (Figure 3-2B), suggesting that Cl-amidine treatment did not affect the stability of PAD4. PAD4 was previously showed to convert methyl-Arg residues in histones to citrulline. If Cl-amidine inhibits PAD4 in cells, we postulated that histone citrullination would decrease, while histone Arg
methylation would increase upon Cl-amidine treatment. In agreement, ~2.2 fold increase of histone H3 Arg17 methylation as well as ~50% decrease of histone H3 citrullination were detected (Figure 3-2B).

Because histone Arg methylation has been correlated with the activation of p53-mediated transcription (Fu et al., 2004) and Cl-amidine treatment decreased cell growth (Figure 3-2A), the expression of p21, a p53 target gene encoding a cyclin-dependent kinase inhibitor (el-Deiry et al., 1993; Harper et al., 1993; Xiong et al., 1993), was tested. About a 5.8 fold increase of the p21 protein was detected after Cl-amidine treatment (Figure 3-2B). To test whether the increase of p21 is due to the increase of the p21 transcription, Reverse transcription-PCR were performed and an increase of the p21 mRNA was detected after the treatment of U2OS cells with Cl-amidine for 24 hr (Figure 3-3A). Thus, Cl-amidine may inhibit cell growth by activating the p53 target genes. To test whether p21 induction by Cl-amidine is dependent on p53, Reverse transcription-PCR experiments were performed in the p53−/− lung carcinoma H1299 cells. In the absence of p53, the p21 mRNA was undetectable before and after Cl-amidine treatment in H1299 cells (Figure 3-3B). Further, the expression levels of PAD4 in U2OS cells and H1299 cells were comparable (Figure 3-3C).

Since p53 is important in the expression of p21, we also examined the level of p53 proteins. Intriguingly, treatment with Cl-amidine increased the amount of the p53 protein in U2OS cells (Figure 3-2B). Various genotoxic and nongenotoxic stresses stabilize p53 and often lead to the phosphorylation of multiple serine sites in p53, including the Ser15 site by ATR, ATM, and extracellular signal-regulated kinases (Brooks and Gu, 2003; Pluquet and Hainaut, 2001). To test whether Cl-amidine treatment imposed a stress leading to the activation of p53, the phosphorylation of p53 at Ser15 was tested. An increase of p53 Ser15 phosphorylation in parallel with the increase of p53 was detected (Figure 3-2B), suggesting that the increase of p53 Ser15
phosphorylation may be a result of the increase in the total p53 or the activity of an upstream kinase.

To further test whether the induction of p21 by Cl-amidine depends on p53, we first depleted p53 by shRNA in U2OS cells. The knockdown of p53 by the pHTP/p53-shRNA plasmid was confirmed by Western blotting (Figure 3-2C). After treatment of cells with Cl-amidine, p21 expression was increased ~4 fold (4.0 ± 0.18, n=3) in cells transfected with a control pHTP plasmid (Figure 3-2C, lanes 1 and 2) but not in cells after p53 depletion (Figure 3-2C, lanes 3 and 4). We also examined the induction of p21 by Cl-amidine in the isogenic p53+/+ and p53−/− colorectal carcinoma HCT116 cells, which express similar amount of PAD4 but differ in their p53 expression (Figure 3-3D). Western blotting showed that the p21 expression was increased by 7.7±1.3 fold (n=3) in the p53+/+ HCT116 cells (Figure 3-2D, lanes 1 and 2) but not in the p53−/− HCT116 cells (Figure 3-2D, lanes 3 and 4), suggesting that the activation of p21 by Cl-amidine is p53-dependent. The lower PAD4 band of the PAD4 doublets in the HCT116 cells was not visualized at short exposure. Also noted is that the amount of p53 was not significantly increased in the HCT116 p53+/+ cells after Cl-amidine treatment (Figure 3-2D). To test whether Cl-amidine treatment can increase p21 expression in other cancer cell lines, we tested MCF-7 cells, in which the expression of PAD4 is inducible by estrogen (Cuthbert et al., 2004; Dong et al., 2007). Cl-amidine treatment activated the expression of p21 in the p53+/+ MCF-7 cells without elevating the amount of p53 (Figure 3-3E). Taken together, these results suggest that the inhibition of PAD4 can lead to the increase of p21 expression in a p53-dependent manner.

3.4.2 Depletion of PAD4 increased the expression of a subset of p53-target genes and apoptosis

To inhibit PAD4 functions using an alternative method, we depleted PAD4 from U2OS cells by RNA interference. PAD4 siRNA treatment caused ~60% decrease in PAD4 and ~6 fold
increase in p21 protein relative to that of the control siRNA treatment (Figure 3-4A), suggesting that PAD4 negatively regulates the expression of p21 in U2OS cells. To analyze whether PAD4 depletion affects other p53 target genes, Reverse transcription-PCR experiments were performed. Depletion of PAD4 in U2OS cells had essentially no effect on transcription of MDM2 and CDC25C, target genes directly repressed by p53 (Le Gac et al., 2006) (Figure 3-4B). In contrast, GADD45, PUMA, and p21 exhibited approximately a twofold increase in expression (Figure 3-4B), suggesting that the depletion of PAD4 activates a subset of p53 target genes. To test whether the increase in p21 expression by PAD4 depletion is p53-dependent, PAD4 siRNAs treatment was performed in U2OS cells with stable transfection of the p53-shRNA plasmid or with the control pHTP plasmid. We found that the depletion of PAD4 by siRNA increased the expression of p21 by 4.5 ± 0.4 fold (n=3) in the cells transfected with the pHTP plasmid (Figure 3-4C, lanes 1 and 2) but not in cells with p53 depletion (Figure 3-4C, lanes 3 and 4). These results indicate that the increase in p21 expression after PAD4 depletion in U2OS cells is p53-dependent.

The Reverse transcription-PCR analyses showed that the depletion of PAD4 increased the expression of certain p53 target genes regulating cell cycle progression and apoptosis. In agreement with the gene expression results, we found that the number of the U2OS cells treated with the PAD4 siRNAs was 52.3±4.3% (n=3) of that of the GFP siRNA treated cells (Figure 3-4D), suggesting a decrease in cell growth or an increase in cell apoptosis after PAD4 siRNA treatment. To detect whether PAD4 siRNA treatment led to apoptosis, we carried out fluorometric TUNEL analyses and found that 25.3±3.1% (n=3) of U2OS cell were TUNEL positive after 60 hr of treatment with PAD4 siRNAs. In contrast, only ~1.6% (n=3) of U2OS cells treated with the GFP siRNAs were TUNEL positive. To analyze the relationship of PAD4 with apoptosis, we performed staining with the PAD4 antibody, TUNEL, and the DNA dye Hoechst. After the GFP siRNA treatment, each cell had a comparable amount of PAD4, and TUNEL positive cells were rarely detected (Figure 3-5A, a to c). In contrast, after the PAD4 siRNA
treatment, cells with decreased PAD4 were positively stained by TUNEL (Figure 3-5A, d to f).

Another set of representative images of the PAD4, TUNEL, and Hoechst staining of U2OS cells after PAD4 siRNA treatment is shown in Figure3-5B. These results indicate a correlation between the decrease in PAD4 and the increase in apoptosis.

3.4.3 Depletion of PAD4 induced the expression of p21, apoptosis, and cell cycle arrest in a p53-dependent manner in HCT116 cells

The p21 gene has served as a model for studying gene regulation by p53 (Barlev et al., 2001; Espinosa and Emerson, 2001; Espinosa et al., 2003; Huang et al., 2007; Tang et al., 2006). We used the p21 gene here as a model to characterize the role of PAD4 in gene regulation. To test whether the increase of p21 expression by PAD4 depletion is p53-dependent in the isogenic p53<sup>+/+</sup> and p53<sup>-/-</sup> HCT116 cells, we transfected these cells with the pHTP or the pHTP/PAD4-shRNA plasmid. The depletion of PAD4 by the PAD4-shRNA was confirmed by Western blotting (Figure 3-6A). Compared to cells treated with the pHTP plasmid, Western blotting showed that PAD4 depletion increased the expression of p21 by 2.3±0.3 fold (n=3) in the p53<sup>+/+</sup> HCT116 cells (Figure 3-6A, lanes 1 and 2) but not in the p53<sup>-/-</sup> HCT116 cells (Figure 3-6A, lanes 3 and 4). This increase in p21 protein was accompanied by a 2.5 ± 0.4 fold (n=3) increase in the p21 mRNA in the p53<sup>+/+</sup> HCT116 cells (Figure 3-6B). These results suggest that the increase in p21 expression after PAD4 depletion is p53-dependent in HCT116 cells.

To test whether the apoptosis caused by PAD4 depletion was p53-dependent, flow cytometry experiments were performed. After the transfection of PAD4-shRNA and puromycin selection for 6 days, we found that the percentage of apoptotic cells was increased to 47.4±1.8% (n=3) in the p53<sup>+/+</sup> HCT116 cells transfected with the PAD4-shRNA (Figure 3-6C, column 2) compared to 23.3±3.8% (n=3) apoptotic cells in the p53<sup>-/-</sup> HCT116 cells transfected with the pHTP vector (Figure 3-6C, column 1). The percentage of apoptotic cells was not significantly
increased in the p53+/− HCT116 cells transfected with the PAD4-shRNA compared to cells transfected with the pHTP vector (Figure 3-6C, compare columns 3 and 4). These results show that PAD4 depletion increased the percentage of apoptotic cells in a p53-dependent manner in HCT116 cells. The high levels of apoptotic cells after the pHTP vector transfection in both p53+/+ and p53−/− HCT116 cells were likely due to the puromycin treatment, which kills untransfected cells.

p53 target genes, such as p21, are involved in the regulation of the cell cycle progression (Harper et al., 1993; Xiong et al., 1993). The increase in the expression of p21, a cell cycle inhibitor, prompted us to analyze the cell cycle progression after PAD4 depletion. Flow cytometry analyses showed that the depletion of PAD4 by shRNA increased the population of G1 cells with a concomitant decrease of the S and G2/M cells in the p53+/+ HCT116 cells (Figure 3-6D, top panels) but not in the p53−/− HCT116 cells (Figure 3-6D, bottom panels), suggesting that the depletion of PAD4 caused a G1 cell cycle block in a p53-dependent manner.

3.4.4 Interaction of PAD4 and p53 provides a mechanism for promoter targeting of PAD4

p53 is known to recruit corepressors, such as HDAC1 and 2, to regulate gene expression (Gui et al., 2004; Ho and Benchimol, 2003; Huang et al., 2007; Laptenko and Prives, 2006; Le Gac et al., 2006). Because PAD4 has no distinguishable motif for DNA binding and the inhibition of PAD4 increased p21 expression, we postulated that PAD4 might be targeted to specific gene promoters by transcription factors, such as p53, to modify histones. To test this idea, we first established a FLAG-HA-PAD4 (FH-PAD4) expressing-293T cell line to analyze the possible interaction between PAD4 and p53. We found that p53 was retained by the α-FLAG M2 agarose from 293T cells expressing FH-PAD4 but not from 293T cells without FH-PAD4 expression (Figure 3-7A). Lanes 1 to 4 had 5, 2.5, 1.25 and 0.625% of the input protein samples,
respectively. To further test if endogenous PAD4 and p53 interact, we performed coimmunoprecipitation experiments and found that PAD4 was coimmunoprecipitated from 293T nuclear extracts by a p53 monoclonal antibody but not by the normal mouse IgG (Figure 3-7B). Since p53 is overexpressed in the 293T cells due to the presence of a p53 interacting protein, the simian virus 40 T antigen, we also performed coimmunoprecipitation experiments in 293 cell nuclear extracts in which p53 is expressed in normal level and found that p53 was coimmunoprecipitated by the PAD4 antibody but not by the normal rabbit IgG (Figure 3-8A).

Structural studies showed that PAD4 has two N-terminal immunoglobulin-like domains (IgL1 & 2) and a C-terminal catalytic domain (Arita et al., 2004) (illustrated in Figure 3-7C). To test the domain of PAD4 interacting with p53, we generated constructs expressing GST-PAD4 and its derivatives, GST-PAD4\[^{IgL1}\] and GST-PAD4\[^{IgL1&2}\] (Figure 3-7C). GST-fusion proteins expressed and purified from \(E.\ coli\) (Figure 3-8B) were used in GST pull-down experiments. Both GST-PAD4 and GST-PAD4\[^{IgL1&2}\] but not GST-PAD4\[^{IgL1}\] were able to pull down p53 from 293T nuclear extracts (Figure 3-7D), suggesting that the two IgL domains of PAD4 were sufficient to mediate the interaction of PAD4 and p53, while the IgL1 domain alone was not.

p53 has a N-terminal activation domain, a middle DNA binding domain, and a C-terminal regulatory domain (Kitayner et al., 2006) (illustrated in Figure 3-7E). To map the region of p53 important for its interaction with PAD4, we prepared GST-p53 full length, GST-p53\[^{1-300}\] and GST-p53\[^{301-393}\] fusion proteins (Figure 3-8C). After incubating the GST-fusion protein sepharose with HA-PAD4 expressed in 293T cells, we found that GST-p53 full length and GST-p53\[^{301-393}\] retained PAD4 but GST-p53\[^{1-300}\] did not (Figure 3-7F). Together, the above GST pull-down experiments showed that the C-terminal regulatory domain of p53 interacts with the N-terminal IgL domains of PAD4 (illustrated in Figure 3-7G).

To test whether p53 and PAD4 directly interact, His6-Flag-p53 and GST-PAD4 expressed and purified from \(E.\ coli\) (Figure 3-8D and 3-8E) were used in pull-down experiments.
We found that GST-PAD4 sepharose retained the His6-FLAG-p53 fusion protein (Figure 3-7H), suggesting that PAD4 and p53 can interact directly. Taken together, the above protein-protein interaction studies suggest that p53 could target PAD4 to gene promoters.

### 3.4.5 Dynamic changes in PAD4 association and histone Arg modifications at the p21 promoter following UV irradiation

After genotoxic stress, such as UV irradiation, p53 is stabilized and transported to the nucleus to activate downstream target genes, such as p21 (Espinosa et al., 2003; Laptenko and Prives, 2006; Vogelstein et al., 2000). The induction of p21 as well as the binding of p53 and Pol II to the p21 promoter after UVC treatment in U2OS cells was documented previously (Espinosa et al., 2003). Further, the activation of the p21 promoter was fast and transient, which occurred within the first 2 hr after UVC treatment (Espinosa et al., 2003). We used this established model system to investigate whether histone Arg modifications and PAD4 functions at the p21 promoter at different time points after UV irradiation by ChIP analyses in U2OS cells.

The p21 promoter has two p53 binding sites, p53BS1 and p53BS2 (Figure 3-9A). Before UV treatment, a low amount of p53 was detected on the p53 binding site 2 of the p21 gene, which gradually increased following UV irradiation (Figure 3-9B). In contrast, PAD4 was detected at the 0-hr time point, then decreased at 0.5- and 1-hr time points and increased at 2- and 6-hr time points following UV irradiation. Western blotting showed that the amount of p53 increased, while the amount of PAD4 remained constant after UVC treatment (Figure 3-10A), suggesting the disassociation of PAD4 at 0.5- and 1-hr time points was not caused by the decrease in PAD4. As a control, we found that p53 and PAD4 were not recruited to the housekeeping gene GAPDH promoter before and after UV irradiation (Figure 3-9C), suggesting a gene-specific association of these factors with the p21 promoter. Given that p21 was fast and transiently expressed after UVC...
treatment (Espinosa et al., 2003), we postulated that the disassociation of PAD4 at the 0.5- and 1-hr time points might allow the expression of p21.

To test whether histone citrullination occurs at the p21 promoter, we carried out ChIP experiments using an antibody against citrullinated histone H3 (H3Cit), which was made against a histone H3 peptide (residues 1-20) containing citrulline at positions 2, 8, and 17 (Cuthbert et al., 2004). Histone H3 citrullination at the p53 binding site 2 was detected before UV irradiation (Figure 3-9B), suggesting that PAD4 is active on the p21 promoter in U2OS cells before exposure to genotoxic stress. Following UV irradiation, we detected a decrease in histone H3 citrullination at 0.5 and 1 hr followed by an increase at 2- and 6-hr time points (Figure 3-9B). This decrease in histone citrullination correlated temporally with the decrease in PAD4 on the promoter. In contrast, histone H3R17 methylation first increased (0.5 and 1 hr) and then decreased (2 and 6 hr) after UV irradiation. Thus, there is an inverse correlation between the amount of histone H3R17 methylation and the amount of H3 citrullination at the p21 gene promoter following UV irradiation.

To better quantify changes in histone H3 citrullination and Arg methylation as well as PAD4, we performed real-time qPCR assays of the ChIP samples. Consistent with the results in Figure 3-9B, H3Cit was decreased at 1 hr then increased at 6 hr on both p53 binding site 1 and 2 after UVC treatment (Figure 3-9D and 3-9F), while H3R17 methylation was first increased at 1 hr then decreased at 6 hr time point (Figure 3-9D and 3-9F). An initial decrease of PAD4 at 1 hr followed by an increase at 6 hr on both p53 binding sites was also detected by real-time qPCR (Figure 3-9D and 3-9F). To analyze whether PAD4 preferentially associates to the p53 binding sites, we performed real-time qPCR to analyze the association of PAD4 to the +182-bp region of p21. Consistent with a role of p53 in the recruitment of PAD4, the amount of PAD4 at the p53 binding sites were much higher than that at the +182-bp region (Figure 3-10B).
To monitor whether the nucleosome density at the p53 binding sites changes after UV irradiation, we performed ChIP assays with a histone H3 antibody and found that histone H3 was decreased at both p53 binding sites with different kinetics (Figure 3-10C and 3-10D), suggesting that histone octamers were repositioned or evicted from the p53 binding sites after UVC treatment. To reflect changes in H3, H3 citrullination and H3R17 methylation results were normalized with the total amount of histone H3 (Figure 3-9E and 3-9G). Although the relative values were altered, the trend of changes in H3 citrullination and H3R17 methylation was similar to that before normalization.

3.4.6 PAD4 is recruited to the p21 promoter in a p53-dependent manner

To test whether p53 mediates the recruitment of PAD4 to the p21 promoter, we first performed ChIP experiments in p53−/− H1299 cells with or without the transfection of a p53-expressing plasmid. The expression of p53 was confirmed by Western blotting (Figure 3-11A). The association of PAD4 with the p53 binding site 2 of the p21 promoter was not detected in cells without p53 expression (Figure 3-11B, upper panel) but was detected in cells with p53 expression (Figure 3-11B, middle panel). As a control, we found that neither p53 nor PAD4 was detected on the GAPDH gene promoter (Figure 3-11B, bottom panel). These results indicate that expression of p53 in the p53−/- H1299 cells is sufficient to recruit PAD4 to the p21 promoter.

To further analyze whether the dynamic association of PAD4 to the p21 promoter after UV irradiation is p53-dependent, we performed ChIP assays in U2OS cells without or with p53 depletion using the cell lines as described in Figure 3-2C and 3-4C. In stable U2OS cell line transfected with the control pHTP plasmid, p53 gradually accumulated at the p53 binding site 1 and 2 (Figure 3-11C and 3-11E, columns 1 to 3), while much less p53 was detected at these sites after p53 depletion (Figure 3-11C and 3-11E, columns 4 to 6). Dynamic PAD4 association with
the p53 binding site 1 and 2 was detected in cells without p53 depletion by shRNA (Figure 3-11D and 3-11F, columns 1 to 3). In contrast, after p53 depletion, the amount of PAD4 at the p53 binding sites was dramatically decreased (Figure 3-11D and 3-11F, columns 4 to 6), indicating that the association of PAD4 with the p21 promoter is p53-dependent. Taken together, these results support the hypothesis that p53 facilitates PAD4 recruitment to the p21 promoter.

3.4.7 Detection of paused and elongating RNA Pol II by ChIP and permanganate footprinting

The C-terminal domain (CTD) of the largest subunit of human RNA Pol II is composed of 52 repeats of a heptad sequence (YSPTSPS) and becomes phosphorylated on Ser5 during transcriptional initiation (Phatnani and Greenleaf, 2006; Sims et al., 2004). To relate the changes in histone modifications to the recruitment of the basal transcriptional machinery, we analyzed the recruitment of RNA Pol II to the p21 promoter. ChIP analyses using an antibody against unmodified Pol II CTD showed that Pol II was present at the +182-bp region of p21 before UV irradiation (Figure 3-12A), and was increased at 1 hr then decreased ~10 fold at the 6-hr time point (Figure 3-12A). These kinetic changes suggest that Pol II is associated with the p21 promoter before UV irradiation in a repressed state, and that an additional mechanism of repression occurs 6 hr after UV treatment, leading to the loss of Pol II. The phosphorylation of Ser5 of Pol II CTD has been detected on Pol II in close proximity of transcription start site of active genes or genes with paused RNA Pol II (Espinosa et al., 2003; Phatnani and Greenleaf, 2006; Sims et al., 2004). ChIP with an antibody that recognizes the phosphorylated Ser5 CTD revealed an increase in Pol II Ser5 phosphorylation up to 1 hr after UV irradiation followed by a dramatic decrease at 6 hr (Figure 3-12A). These ChIP data with Pol II antibodies are in agreement with previously reported observations (Espinosa et al., 2003).
The association of RNA Pol II and the Ser5 phosphorylation of RNA Pol II CTD suggest that RNA Pol II might be paused at this promoter before UV irradiation. To obtain more insight into the behavior of Pol II at the p21 promoter in response to UV irradiation, we performed permanganate footprinting. Permanganate preferentially reacts with thymines (Ts) in transcription bubbles associated with RNA Pol II engaged in transcription, thereby detecting both paused and elongating Pol II (Cartwright et al., 1999). This technique detects transcriptionally engaged Pol II and maps the location of these Pol II molecules with a resolution of about 15 bp and is thereby an ideal method to monitor transcriptional activity of Pol II following gene activation.

Permanganate footprinting analyses of U2OS cells before UV irradiation detected hyper-reactive thymines at 9, 11, 36, 38 and 50 positions of p21 (Figure 3-12B, lane 4), suggesting that Pol II is paused in this region. At 30 min and 1 hr after UV treatment, we observed increases in the permanganate reactivity at positions 91 and 152, indicating active transcription (Figure 3-12B, lanes 5 and 6). The permanganate reactivity that persisted in the region proximal to the transcription start site (e.g., +9, +11, etc.) probably reflects reinitiation by Pol II and transient pausing as previously seen on the hsp70 heat shock gene (O'Brien and Lis, 1991). Interestingly, there were significant changes in the permanganate reactivity from the 1-hr to the 2-hr time point following UV irradiation. The permanganate reactivity at +91 and +152 decreased (Figure 3-12B, lanes 7 and 8), suggesting that active transcription had stopped. Even more intriguing, the permanganate reactivity in the promoter-proximal region vanished, suggesting that the association of Pol II with p21 promoter becomes blocked.

The permanganate footprinting results fit very well with the ChIP data. The Pol II detected by ChIP before UV irradiation could be paused Pol II producing permanganate reactivity in the promoter-proximal region. The increase in Pol II detected by ChIP within 1 hr of UV irradiation corresponded to Pol II molecules undergoing active elongation and reinitiation as indicated by the increased permanganate reactivity downstream from the promoter-proximal
region. Finally, at the 6-hr time point, both assays showed a decrease in Pol II below the level observed prior to UV treatment.
3.5 Discussion

In this study, we provide evidence that PAD4 is recruited by p53 to the p21 gene to repress transcription. This conclusion is based on several independent types of experiments. Inactivation of PAD4 with an inhibitor and depletion of PAD4 with siRNA or shRNA both increased the level of p21 transcription. The increase of p21 expression by PAD4 inhibition or depletion was p53-dependent. The results of the siRNA treatment probably underestimate the extent of this increase because immunofluorescence analyses indicate that only a fraction of the cells was depleted of PAD4 by the siRNA protocol. Biochemical assays indicated that p53 interacts with PAD4 both in cells and when combined as purified proteins. ChIP analyses found that the association of PAD4 to the p21 promoter is p53-dependent. Finally, our kinetic analyses of protein association with the p21 promoter following UV induction indicate that PAD4 was associated with p21 when the promoter was repressed but decreased when the promoter was active. The increase in histone Arg methylation and the decrease in histone citrullination occurred when the promoter was active. Conversely, the increase in histone citrullination and the decrease in histone Arg methylation occurred when the promoter was repressed. Interestingly, the permanganate footprinting identified two distinct repressed states. One involved a paused Pol II that was detected in the promoter proximal region before UV induction. The other was at 2-6 hr after UV induction when the promoter seemed to shut off due to the absence of Pol II recruitment to the promoter.

Based on the above results, we propose a model that the expression of p21 is controlled at the “steady”, “on”, and “off” stages after UVC treatment (Figure 3-13). i) In cells without exposure to DNA damage, PAD4 and histone citrullination maintain histone Arg methylation and the transcription of p21 at a low level. RNA Pol II bound to p21 is in a nonproductive and paused
state. ii) Shortly after UV irradiation, histone Arg methylation increases while histone citrullination decreases. These changes in histone Arg modifications correlate with the “on” stage of gene expression. iii) At a later stage following UV treatment, when the expression of p21 is turned “off”, histone citrullination mediated by PAD4 increases with a concomitant decrease of histone Arg methylation and RNA Pol II dissociated with p21. Two factors, the PAD4 activity and the histone dynamics, could contribute to the decrease in histone Arg methylation.

An et al. have first showed that histone Arg methylation mediated by CARM1 and PRMT1 activates p53-mediated gene expression (Fu et al., 2004). In this study, we found that PAD4 is involved in repressing the expression of a subset of p53 target genes, including p21, GADD45, and PUMA. We focused on the p21 gene to analyze the kinetics of PAD4 association, histone Arg methylation and citrullination, and relate chromatin events to the RNA Pol II activities at the promoter. Inhibition or depletion of PAD4 increased p21 expression in cells without DNA damage treatment, suggesting that PAD4 is involved in repressing the p21 expression under regular culture conditions. In addition, both histone Arg methylation and citrullination were detected on the p21 promoter before DNA damage, suggesting that a dynamic balance of histone Arg methylation and citrullination is reached by two opposing enzymatic activities mediated by CARM1 and PAD4, respectively.

Dynamic changes of histone Arg methylation and citrullination were detected on the p21 gene promoter after UV irradiation. In particular, a decrease of PAD4 and histone citrullination as well as an increase of histone Arg methylation temporally correlated with the fast and transient activation of p21. This quick but brief activation of gene expression has been detected by several different approaches, including RNA fluorescent in situ hybridization (Espinosa et al., 2003), permanganate genomic footprinting (this study), and ChIP with the RNA Pol II antibodies [(Espinosa et al., 2003) and this study]. It is unknown what regulates the dissociation of PAD4 from p21 during the “on” stage of gene expression. The C-terminal regulatory domain of p53 is
subjected to many post-translational modifications, including methylation, acetylation, and phosphorylation (Brooks and Gu, 2003; Chuikov et al., 2004; Gu et al., 1997; Huang et al., 2006; Laptenko and Prives, 2006). p53 phosphorylation and acetylation plays a role in recruiting protein factors, such as 14-3-3 (Waterman et al., 1998) and p300/CBP (Barlev et al., 2001; Espinosa and Emerson, 2001; Mujtaba et al., 2004), respectively. Whether p53 modifications negatively regulate the PAD4 and p53 interaction will be explored in our future experiments.

It is very striking that the amount of RNA Pol II on the p21 promoter decreased dramatically following an initial activation within 2 hr after UV treatment. Therefore, under the condition of UV irradiation, there is a decrease in the rate of RNA Pol II reinitiation to repress gene expression. ChIP experiments found that the amount of p53 associated with the p21 promoter remained at a high level even after the decrease of the amount of RNA Pol II, suggesting that the association of p53 with the p21 promoter was not enough to mediate continued transcription. Interestingly, the amount of PAD4 and histone citrullination was detected at a high level around the time points when RNA Pol II reinitiation was decreased. It is possible that p53 recruits a different set of chromatin-modifying factors, such as PAD4, to the p21 promoter to generate a chromatin structure unfavorable for continuing RNA Pol II recruitment and transcription. It remains unknown whether various stress signals affect the expression of p21 by regulating RNA Pol II recruitment. At least, under the condition of stalled DNA replication after hydroxyurea treatment, the lack of p21 induction was found to be due to the decrease of Pol II elongation (Mattia et al., 2007). Thus, the p21 gene expression can be regulated at multiple steps of the transcription cycle, including initiation and elongation.

The expression of PAD4 in MCF-7 cells is induced by estrogen (Cuthbert et al., 2004; Dong et al., 2007), a hormone that increases the cell growth rate. On the other hand, PAD4 is highly expressed in HL-60 cells after retinoic acid treatment (Nakashima et al., 1999), which induces the terminal differentiation of HL-60 cells along the granulocyte lineage, suggesting that
PAD4 may play a unique role in granulocytes/neutrophils. However, whether PAD4 plays cell specific roles in cell growth or differentiation is unclear. Our studies showed that PAD4 might facilitate cell growth by repressing the p53 target genes in MCF-7, U2OS, and HCT116 cells. This is consistent with a report showing the PAD4 is overexpressed in multiple human cancers (Chang and Han, 2006). On the other hand, a recent report showed that the overexpression of PAD4 eventually led to apoptosis of both HL-60 and Jurkat cells (Liu et al., 2006), suggesting that PAD4 has a proapoptotic role in the hematopoietic cells. It was also reported that the overexpression of PAD4 in Jurkat cells increased the amount of p53 and p21 and induced apoptosis (Liu et al., 2006). However, p53 in Jurkat cells carries multiple point mutations (Cheng and Haas, 1990), and Jurkat cells do not respond to DNA damaging drugs to express p21 (Hellin et al., 2000; Mansilla et al., 2003). Further, Jurkat cells do not respond to -irradiation to stabilize p53 (Sandal et al., 2003). Together, these reports suggest that Jurkat cells do not have a functioning p53 pathway. Because Liu et al. reported that PAD4 can induce apoptosis also in HL-60 cells (Liu et al., 2006), which are p53-/-, the proapoptotic function of PAD4 is not p53-dependent in HL-60 cells. Our results showed that the Jurkat cells had very low levels of p53 and undetectable amount of p21 (Figure 3-14A). When PAD4 was overexpressed in Jurkat cells by transient transfection, the increase of p53 or p21 was not observed (Figure 3-14B). Further, breast cancer MCF-7 cells stably expressing FH-PAD4 did not show an increase of apoptosis (data not shown). Western blotting analyses indicated that the amount of p53 and p21 protein in the Flag-HA-PAD4/MCF-7 cells was decreased (Figure 3-14C), suggesting that PAD4 has a repressive role for p53 in MCF-7 cells. Therefore, the mechanism by which PAD4 overexpression induces apoptosis in hematopoietic cancer cells remains to be further explored.

Our current understanding of the PAD4 and histone citrullination regulation is very limited. Histone citrullination has been detected on the estrogen responsive pS2 gene promoter (Cuthbert et al., 2004; Wang et al., 2004b) and the p21 promoter (this study). The interaction of
PAD4 with particular transcription factor, such as p53, likely increases the local concentration of PAD4 around specific promoters and facilitates its modification of histones to regulate gene expression. On the other hand, global histone citrullination is maintained at a very low level in differentiated HL-60 cells (Nakashima et al., 2002; Wang et al., 2004b) and MCF-7 cells (Cuthbert et al., 2004). Multiple mechanisms can be proposed for the metabolic destiny of citrulline in histones, including the replacement of citrullinated histones with histone variants, the eviction of nucleosomes during gene expression, as well as enzyme catalyzed conversion of citrulline to Arg by another yet-to-be-identified protein.
3.6 Acknowledgement

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We thank Dr. Hongjie Yao for contributing Figure 3-9B, Drs. Zhiqiang Zhang and David Gilmour for contributing Figure 3-12B, Dr. Ming Li for contributing Figure 3-7B, Drs. Yuan Luo and Paul Thompson for providing Cl-amidine.

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Figure 3-1: PAD4 inhibitor Cl-amidine inhibits the activity of PAD4.

(A and B) The structure of Cl-amidine (A) is similar to the PAD4 substrate peptidylarginine (B).

(C) The activity of GST-PAD4 in histone citrullination was inhibited by preincubating GST-PAD4 with Cl-amidine.

(D) The amount of histone citrullination signal detected in (C) was quantified using the NIH image J program and graphed, with the concentration of the inhibitor indicated.
Figure 3-2: Cl-amidine treatment increases p21 expression.

(A) The number of U2OS cells treated with 200 μM of Cl-amidine was 48.3±3.6% (n=3) of the number of mock-treated cells, demonstrating that Cl-amidine has a negative effect on cell growth and proliferation.

(B) Western blotting analyses of the levels of PAD4, H3R17Me, H3Cit, p21, p53, and p53 Ser15 phosphorylation in U2OS cells after treatment with Cl-amidine for 24 hr. The level of PAD4 was unaltered (note the doublet of PAD4 detected on Western blotting). Tubulin and histone H3 was monitored to ensure equal protein loading.

(C) The effects of Cl-amidine on the expression of p21 in U2OS cells without or with the depletion of p53 using a pHTP/p53-shRNA plasmid.

(D) Lanes 1 and 2, the changes of PAD4, p53, and p21 in the p53⁺/⁺ HCT116 cells after the Cl-amidine treatment for 24 hr detected by Western blotting. The p21 expression was increased by 7.7±1.3 fold (n=3). Lanes 3 and 4, changes in PAD4, p53, and p21 in the p53⁻/⁻ HCT116 cells after the Cl-amidine treatment for 24 hr. The increase in p21 expression was not observed.
Figure 3-2

A. U2OS cells p53<sup>+/+</sup>

B. U2OS cells p53<sup>+/−</sup>

C. U2OS cells

D. HCT116

E. HCT116

F. Tubulin

G. H3R17Me

H. H3Cit

I. p21

J. p53

K. p53<sup>S15</sup>

L. H3
Figure 3-3: Effect of Cl-amidine on p21 expression.

(A) Reverse transcription-PCR analyses detected an increase in the change of p21 mRNA in U2OS cells after the Cl-amidine treatment.

(B) Cl-amidine did not increase the expression of p21 in the p53<sup>−/−</sup> H1299 cells, suggesting a p53 dependent induction of p21 by Cl-amidine.

(C) H1299, U2OS, and MCF-7 cells expressed roughly equal levels of PAD4. The expression of p53 was not detected in the p53<sup>−/−</sup> H1299 cells. Ponceau S staining showed equal amount of protein loading.

(D) The expression of PAD4 in the p53<sup>+/+</sup> and p53<sup>−/−</sup> HCT116 cells. Note a weaker lower band of the PAD4 doublets.

(E) The treatment of MCF-7 cells by Cl-amidine for 24 hr increased the amount of p21 protein but not the amount of p53, suggesting that p21 can be induced without an increase of the p53 protein.
Figure 3-3

A  U2OS cells p53\(^{+/+}\)
   Cl-amidine    -    +
   p21           
   18S rRNA      

B  H1299   p53\(^{-/-}\)
   Cl-amidine   -    +
   p21           
   18S rRNA      

C  H1299 p53\(^{-/-}\)  U2OS p53\(^{+/+}\)  MCF-7 p53\(^{+/+}\)
   1  2  3
   α-PAD4
   α-p53

D  HCT116 p53\(^{+/+}\)  HCT116 p53\(^{-/-}\)
   1  2  3
   α-PAD4
   α-p53
   α-p21
   Ponceau S

E  MCF-7 cells p53\(^{+/+}\)
   Cl-amidine   -    +
   α-PAD4
   α-p53
   α-p21
   Ponceau S
Figure 3-4: Effect of PAD4 depletion by siRNAs on the expression of p53-target genes and cell growth in U2OS cells.

(A) PAD4 protein was decreased in U2OS cells after the PAD4 siRNA treatment compared to that after the GFP siRNA treatment. Note the doublet of PAD4 in U2OS cells. PAD4 siRNA caused the PAD4 protein level to decrease ~60%. An approximately 6-fold increase in p21 was observed. Tubulin blotting showed equal protein loading.

(B) The levels of GADD45, p21, and PUMA mRNAs were increased in U2OS cells after the PAD4 siRNA treatment over that in the cells treated with the GFP siRNA. In contrast, the levels of other the p53 target genes, such as CDC25C and MDM2, were unchanged.

(C) Effect of PAD4 depletion by siRNAs on the expression of p21 in U2OS cells without or with the depletion of p53 using a pHPT/p53-shRNA plasmid.

(D) Cells were treated with the PAD4 siRNAs or the GFP siRNA as a control for 60 hr. The number of PAD4 siRNA treated cells was 52.3±4.3% (n=3) of that of the GFP siRNA treated cells, suggesting that PAD4 depletion inhibits cell growth and proliferation.
Figure 3-4

A

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α-PAD4  
α-p21  
α-p53  
α-tubulin

B

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<tr>
<td>α-β-actin</td>
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D

Percentage of cell numbers

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<tr>
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<th>PAD4 siRNA</th>
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<td>(%)</td>
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Figure 3-5: Increase of apoptosis after PAD4 depletion by siRNA.

(A) PAD4 staining and TUNEL staining in cells treated with GFP siRNAs (a-c) or PAD4 siRNAs (d-f). Approximately equal amounts of PAD4 were detected in all cells after the GFP siRNA treatment (a). In contrast, a large fraction of cells in (d) exhibited little or no PAD4 staining following PAD4 siRNA treatment (denoted by arrows); TUNEL positive cells were detected in PAD4 siRNA treated cells (e) but not in the GFP siRNA treated cells (b); in the merged images (f), cells with decreased PAD4 were found to be the TUNEL positive.

(B) Another set of representative images showed PAD4, TUNEL, and DNA staining in U2OS cells after 60 hr of PAD4 siRNA treatment. Cells with weak PAD4 staining denoted by arrows (a) were also positively stained by the TUNEL reagent (b). The DNA staining by Hoechst was performed to monitor nuclei positions (c) and merged image is shown in (d).
Figure 3-6: Depletion of PAD4 increased p21 expression, apoptosis, and cell cycle arrest in a p53-dependent manner in HCT116 cells.

(A) Lanes 1 and 2, the changes of PAD4, p21, and p53 proteins after PAD4 depletion by shRNA in the p53^{+/+} HCT116 cells were detected by Western blotting. The expression of p21 was increased by 2.3±0.3 fold (n=3). Lanes 3 and 4, an increase in p21 expression was not detected after PAD4 depletion in the p53^{-/-} HCT116 cells.

(B) The expression of p21 was analyzed by Reverse transcription-PCR experiments. Depletion of PAD4 by the PAD4 shRNA increased the p21 expression in the p53^{+/+} HCT116 cells.

(C) The percentage of annexin V positive cells were analyzed by flow cytometry in the p53^{+/+} and p53^{-/-} HCT116 cells after transfection with the pHTP vector (control) or the PAD4-shRNA and selection with puromycin for 6 days.

(D) Flow cytometry analyses of the effects of the PAD4 shRNA treatment on the cell cycle progression in the p53^{+/+} and p53^{-/-} HCT116 cells.
Figure 3-6

A

HCT116 cells
p53+/+  p53−/−
+ - + -

- + - +

α-PAD4
α-p53
α-p21
α-β-actin

B

HCT116/p53+/−

Fold of change
in p21 expression

n=3

C

Percentage of annexin V
positive cells

D

Ctrl

PAD4 shRNA

G1 S G2/M  G1 S G2/M  G1 S G2/M  G2/M  G1  S  G2/M

HCT116 p53+/+

38% 34% 28%

G2/M 28% G1 38% S 34%

HCT116 p53−/−

22% 43% 35%

G2/M 35% G1 22% S 43%
Figure 3-7: Interaction of PAD4 and p53.

(A) p53 was coimmunoprecipitated by the M2 agarose from the FLAG-PAD4 expressing 293T cells (lane 6), but not from the parental cells lacking FLAG-PAD4 (lane 5). Lanes 1 to 4 had 5, 2.5, 1.25, 0.625% of input, respectively.

(B) Coimmunoprecipitation of endogenous PAD4 from 293T cell nuclear extracts by α-p53 antibody (lane 3), but not by control normal mouse IgG (lane 2). Lane 1 had 2.5% of the input.

(C) Schematic drawing of GST-PAD4 and its derivatives used in the pull-down experiments.

(D) p53 was pulled down by GST-PAD4 and GST-PAD4\textsuperscript{IgL1&2} (lanes 8 and 9), but not by GST, GST-PAD4\textsuperscript{IgL1}, or beads alone. Lanes 1 to 4 had 5, 2.5, 1.25, 0.625% of input, respectively.

(E) Illustration of GST-p53 and its derivatives used in the pull-down experiments.

(F) GST-p53 (lane 5) and GST-p53\textsuperscript{301-393} (lane 7), but not GST-p53\textsuperscript{1-300} or beads alone, were efficient in mediating the p53 and PAD4 interaction. Lanes 1 to 3 had 2, 1, and 0.5% of the input, respectively.

(G) GST pull-down experiments suggest that the N-terminal IgL domains of PAD4 interact with the C-terminal regulatory domain of p53.

(H) GST-PAD4 bound to the glutathione sepharose associated with His6-FLAG-p53, suggesting a direct interaction of p53 and PAD4. Lane 1 to 3 had 5, 2.5, 1.25% of input, respectively.
Figure 3-7

A

Input
- + FLAG-PAD4
IP: α-FLAG

B

Input
Control
p53 IP

PAD4

C

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GST

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D

Input

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WB: α-p53

E

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GST

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WB: α-HA-PAD4

F

G

PAD4

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p53

| AD | DBD | RD |

WB: α-p53
Figure 3-8: Protein purification in studying interaction between p53 and PAD4.

(A) p53 from 293 cell nuclear extracts was coimmunoprecipitated by the PAD4 polyclonal antibody (lane 3) but not by the normal rabbit IgG (Lane 2). Lane 1 had 3% of the input.

(B) The GST-PAD4 and its derivative fusion proteins were purified and analyzed by SDS-PAGE, transferred, and stained by Ponceau S.

(C) GST-p53 fusion proteins were purified and analyzed by SDS-PAGE, transferred, and stained by Ponceau S.

(D) Coomassie blue staining shows that His6-FLAG-p53 was highly purified from E.coli.

(E) Coomassie blue staining shows that GST-PAD4 was highly purified from E.coli.
Figure 3-9: Dynamic p53 and PAD4 association and histone Arg modifications at the p21 promoter after UV irradiation.

(A) Illustration of the p21 gene promoter, including the two p53 binding sites (p53BS1 and p53BS2).

(B) Representative ChIP results of p53 and PAD4 association as well as histone Arg modifications at the p53BS2 region of the p21 promoter after 50 J/m² UVC irradiation.

(C) PAD4 and p53 were not associated with the GAPDH promoter before and after DNA damage treatment.

(D) Real-time qPCR analyses of the H3Cit, H3R17Me, and PAD4 levels on the p53 binding site 1 in U2OS cells at different time points (0, 1, and 6 hr) after UVC treatment (n=6).

(E) The H3Cit and H3R17Me ChIP results in (D) were normalized with that of histone H3.

(F) Real-time qPCR analyses of the H3Cit, H3R17Me, and PAD4 levels on the p53 binding site 2 in U2OS cells at different time points (0, 1, and 6 hr) after UVC treatment (n=6).

(G) The H3Cit and H3R17Me ChIP results in (F) were normalized with that of histone H3.
Figure 3-9

A

-3000 p53BS1 amplicon p53BS2 amplicon

+1 Exon1

ATG Exon2

TAA Exon3

AATAAA

+9000

Intron1

B

UV 0 0.5 1 2 6 (hr)

Input

Beads

α-p53

1.0 2.0 2.3 3.4 3.5

α-PAD4

1.0 0.6 0.8 1.4 2.0

α-H3Cit

1.0 0.4 0.4 0.7 0.8

α-H3R17Me

1.0 2.0 2.5 1.8 1.4

C

GADPH promoter

UV 0 2 6 (hr)

Input

Beads

α-PAD4

α-H3Cit

α-p53

D

p53 binding site 1

(n=6)

ChIP (% of input)

UV (hr) H3Cit H3R17Me PAD4

0 0.1 0.2 0.3 0.4 0.5 0.6 0.7

E

p53 binding site 1

(n=6)

ChIP (normalized with H3)

UV (hr) H3Cit H3R17Me

0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9

F

p53 binding site 2

(n=6)

ChIP (% of input)

UV (hr) H3Cit H3R17Me PAD4

0 0.1 0.2 0.3 0.4 0.5 0.6

G

p53 binding site 2

(n=6)

ChIP (normalized with H3)

UV (hr) H3Cit H3R17Me

0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
Figure 3-10: PAD4 and histone H3 association with the p21 promoter after UV irradiation.

(A) The levels of p53 increased over time, while the amount of PAD4 was unaltered by UVC irradiation in U2OS cells.

(B) Real-time qPCR analyses of the amount of PAD4 associated with the p53 binding site 1, 2, or +182-bp region of p21. PAD4 preferentially associates with the p53 binding sites.

(C) Real-time qPCR analyses of the ChIP samples showed that histone H3 gradually decreased at the p53 binding site 1 of the p21 promoter, resulting in about 2-fold decrease at the 6-hr time point. Standard deviations were indicated (n=4).

(D) Real-time qPCR analyses of the ChIP samples showed that histone H3 rapidly decreased at the p53 binding site 2 of the p21 promoter, resulting in about 2.5-fold decrease of H3 by the 1-hr time point. Standard deviations were indicated (n=4).
Figure 3-11: Association of PAD4 with the p21 promoter is p53-dependent.

(A) The expression of p53 in the p53−/− H1299 cells was restored by transient transfection of a p53-expressing plasmid.

(B) ChIP analyses of the p53 and PAD4 association with the p53 binding site 2 of the p21 promoter in H1299 cells with or without the transfection of a p53-expressing plasmid.

(C-D) ChIP assays of p53 (C) or PAD4 (D) association with the p53 binding site 1 by real-time qPCR in U2OS cells without or with the depletion of p53 by shRNA.

(E-F) ChIP assays of p53 (E) or PAD4 (F) association with the p53 binding site 2 by real-time qPCR in U2OS cells without or with the depletion of p53 by shRNA.
Figure 3-11

A

H1299/p53\(^{-}\)/p53-transfection

α-p53

α-tubulin

B

p21 promoter

H1299/p53\(^{-}\)

p21 promoter

H1299/p53\(^{-}\)/p53-transfection

GAPDH promoter

C

p53 binding site 1

α-p53 (n=4)

D

p53 binding site 1

α-PAD4 (n=4)

E

p53 binding site 2

α-p53 (n=4)

F

p53 binding site 2

α-PAD4 (n=4)
Figure 3-12: Detection of paused and elongating RNA Pol II at the p21 promoter.

(A) The association of RNA Pol II and Ser5 phosphorylated RNA Pol II was monitored at +182-bp region of p21 at different time points after UVC treatment.

(B) Permanganate footprinting was used to monitor RNA Pol II at the p21 promoter region during induction by UVC irradiation. Lane 1 showed a background pattern of bands that accompanied the LM-PCR procedure when DNA was not treated with permanganate. Lane 2 provided markers produced by partial cleavage of DNA at purines (G/A). Lane 3 showed permanganate reactivity that was intrinsic to naked DNA. Despite a high background, one can readily detect permanganate hyper-reactive positions at T9, 11, 36, 38, and 50 that occur in cells (lane 4) but not in naked DNA (lane 3). These reactivities were indicative of paused Pol II. Within 30 min after a brief pulse of UV light, permanganate reactivities at T91 and T152 increased and provided evidence of Pol II undergoing productive elongation at 0.5 and 1 hr after UV treatment. By 2 and 6 hr, transcription appeared repressed since the permanganate reactivity marking paused and elongating forms of Pol II had decreased significantly.
Figure 3-12

A

+182 bp, downstream of transcription start site

(n=6)

UV (hr)

0 1 6 0 1 6

Pol II Pol II pS5

ChIP (% of input)

2.5

2

1.5

1

0.5

0

B

Permanganate treated

DNA G/A marker DNA 0 hr 0.5 hr 1 hr 2 hr 6 hr

G (+9) T (+11) T (+36) C (+50) G (+91)

G T G (+152)
Figure 3-13: A “three-state” model for the role of PAD4 and dynamic histone Arg modifications in the expression of p21 gene after UV irradiation.
Figure 3-14: Effects of PAD4 overexpression on p21 expression.

(A) Compared with U2OS cells, the Jurkat cells expressed low levels of p53. The expression of p21 was undetectable under our experimental conditions, while the p21 protein in U2OS cells was clearly detected. Ponceau S showed equal amount of protein loading.

(B) To express PAD4 in Jurkat cells, we transiently transfected the pSG5-PAD4 and pSG5-PAD4C645S mutant constructs into the Jurkat cells by lipofectamine 2000. Western blotting assays were performed at 48 hr after transfection. The expression of HA-PAD4 and the enzymatic inactive HA-PAD4C645S mutant increased the amount of PAD4 detected in Jurkat cells. Arrow denotes the position of the HA-PAD4 band. The amount of p53 was not changed, while the amount of p21 remained undetectable. Our results indicated that the overexpression of PAD4 in Jurkat cells did not increase p53 or p21.

(C) To analyze the effect of PAD4 overexpression on p53 and p21 in other cells, Western blotting were performed in MCF-7 cells stably express FLAG-HA-PAD4. Arrow indicates the position of FLAG-HA-PAD4. The adding of the FLAG-HA epitopes at the N-terminal of the protein caused the protein to run slower compared to endogenous PAD4. Western blotting indicated the p53 and p21 proteins were not increased but decreased by the PAD4 overexpression. Results shown were representative of three repeatable experiments.
Figure 3-14

A

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C

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<td>α-actin</td>
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Ponceau S staining

1  2  3  4

1  2  3  4
Chapter 4

Coordination of PAD4 and HDAC2 in the Regulation of p53-Target Gene Expression
4.1 Abstract

Histone Arg methylation and Lys acetylation have been found to cooperatively regulate the expression of p53 target genes. Peptidylarginine deiminase 4 is an enzyme that citrullinates histone arginine and monomethyl-arginine residues thereby regulating histone Arg methylation. We have recently found that PAD4 serves as a p53 corepressor to regulate histone Arg methylation at the p53 target gene p21/WAF1/CIP1 promoter. However, it has not been tested whether histone Arg citrullination coordinates with other histone modifications to repress transcription. Here we found that HDAC2 and PAD4 interact with p53 via distinct domains and simultaneously associate with the p21 promoter to regulate gene expression. After DNA damage, PAD4 and HDAC2 dissociate from several p53-target gene promoters (e.g., p21, GADD45, and PUMA) with a concomitant increase in histone Lys acetylation and Arg methylation at these promoters. Furthermore, p53 protein level and HDAC activity regulate PAD4 association and histone Arg modifications at these promoters. In contrast, HDAC2 promoter association and histone acetylation is affected by p53 and PAD4 at minor degrees. Importantly, inhibitors of PAD4 and HDACs, Cl-amidine and SAHA respectively, demonstrate additive effects in inducing p21, GADD45, and PUMA expression and in inhibiting cancer cell growth in a p53-dependent manner. Our results unveil an important crosstalk between histone deacetylation and citrullination, suggesting that a combination of PAD4 and HDAC2 inhibitors could serve as a potential strategy for cancer treatment.
4.2 Introduction

In eukaryotic cells, 146 bp DNA is wrapped around two copies of each histones H3, H2B, H2A and H4 to form a nucleosome – the basic structural unit of chromatin (Kornberg and Lorch, 1999; Luger et al., 1997). Various posttranslational modifications, such as methylation, acetylation, phosphorylation, ubiquitination and citrullination, are believed to play a significant role in chromatin activities, such as transcription (Barski et al., 2007; Berger, 2007; Klose and Zhang, 2007; Li et al., 2007b; Shilatifard, 2006). In light of the biological significance of histone modifications in cellular activities, a “histone code” hypothesis was proposed (Jenuwein and Allis, 2001; Strahl and Allis, 2000; Turner, 2000; Wang et al., 2004a), which suggests that different histone modifications, either working singularly or in combination, fine tune the outcomes of various chromatin-based cellular processes.

Histone modifying enzymes with opposing activities counteract each other’s effect (Fischle et al., 2003). For example, histone acetyltransferases (HATs) catalyze histone acetylation, while histone deacetylases (HDACs) remove the pre-existing acetyl groups. Histone Arg methylation is catalyzed by protein arginine methyltransferases (PRMTs) (Bedford and Clarke, 2009). Conversely, peptidylarginine deiminase 4 (PAD4/PADI4/PADV) was identified as the first mechanism mediating histone Arg demethylation (Cuthbert et al., 2004; Wang et al., 2004b), which converts monomethyl-Arg to citrulline through a biochemical reaction termed demethylimination (Wang et al., 2004b).

The tumor suppressor protein p53 functions at the center of an extremely complicated signaling network in human cells (Laptenko and Prives, 2006; Vogelstein et al., 2000). p53 is a transcription factor containing an activation domain at its N-terminus, a DNA binding domain in the middle and a regulatory domain at the C-terminus (Kitayner et al., 2006).
signals and DNA damaging reagents can activate p53 that in turn activates the expression of various target genes, such as p21, GADD45, PUMA (Harris and Levine, 2005; Vogelstein et al., 2000). The protein products of p53-target genes further result in cell growth arrest and apoptosis. Mutations of p53 cause severe cellular defects in coping with genomic stress. Over 50% of all cancers carry mutant p53 thus attesting to the significance of p53 in genome integrity and cancer prevention (Levesque and Eastman, 2007). p53 plays multifaceted roles in gene expression, regulating transcription by recruiting both coactivators and corepressors to its target gene promoters (Ho and Benchimol, 2003; Laptenko and Prives, 2006). Multiple HATs have been found as p53 coactivators (Barlev et al., 2001; Espinosa and Emerson, 2001), while histone deacetylases (HDACs) function as p53 corepressors by counteracting the function of HATs (Dannenberg et al., 2005; Harms and Chen, 2007; Luo et al., 2000). Recently, we have found that PAD4 functions as a p53 corepressor and counteracts Arg methylation at p21 promoter region to repress gene expression (Li et al., 2008). However, whether histone citrullination and deacetylation are coordinated with each other to mediate the p53 target gene expression remains unknown. Here, we report that HDAC2 interacts with PAD4, and both enzymes interact with p53 to repress gene expression.
4.3 Materials and Methods

4.3.1 Plasmid constructs

GST-HDAC2 was cloned in the pGEX4T1 vector for expression in *E. coli* strain BL21.

4.3.2 HDAC activity assay

GST and GST-PAD4 derivative fusion proteins were used to perform pull-down experiment using 293T nuclear extracts. Proteins retained by sepharose were eluted with the Elution buffer (10mM Glutathione in 50 mM Tris-HCl, pH 8.0). HDAC activities purified with various fusion proteins were analyzed using a Fluorometric Detection Kit (Millipore, 17-356) by following the manufacturer’s instructions.

4.3.3 Cell treatment with doxorubicin, siRNA, and inhibitors.

To analyze the effects of doxorubicin on p21 expression, 0.5 μM of doxorubicin (Sigma-Aldrich) was added to treat cells for 6 hr. To knock down HDAC2 expression, HDAC2 siRNA targeting nucleotides 389 to 407 (5’- TCCGCATGACCCATAACTT -3’) of HDAC2 mRNA sequence (NCBI access no. NM_001527) was purchased from IDT DNA. Cells were incubated in the presence of the siRNA for 60 hr before analyses. PAD4 inhibitor Cl-amidine was prepared via a solution-phase strategy following reported procedures (Causey and Thompson, 2008). The final compound was purified by silica gel flash chromatography. The structure and purity (>95%) was characterized by 1H-NMR and Mass Spectrometry. Cl-amidine was dissolved in H2O as a 10 mM
stock solution and diluted to 200 µM in the complete cell culture medium to treat cells in 6-well plates. HDAC2 inhibitor SAHA is dissolved in DMSO as a 2 mM stock solution and diluted to 1 µM in cell culture medium to treat cells. Cells were incubated in the presence of the inhibitors for 72 hr before analyses.

4.3.4 Re-chromatin immunoprecipitation

The first step ChIP was performed using the M2 agarose beads to recover FLAG-PAD4 associated chromatin fragments. After elution with 200 µg/ml FLAG peptide, the second step ChIP was performed using the affinity purified PAD4 antibody or α-HDAC2 antibody.

4.3.5 Cell growth curve

6x10⁴ U2OS cells or U2OS/p53-shRNA cells were seeded in each well of the 6-well plate. The cell numbers were counted at each time point indicated after inhibitor treatment using a hemacytometer.

4.3.6 Cell growth and morphology analysis using phase contrast microscopy

Cell growth and morphology were analyzed using a VistaVision invert microscope (VWR Inc.) equipped with a Spot Insight digital camera (NDS, Inc.) and a 10x phase contrast lens.
4.4 Result

4.4.1 Protein-protein interactions of PAD4, HDAC2 and p53

In order to identify PAD4 interacting proteins, we affinity purified endogenous PAD4 and its associated protein using PAD4 antibody conjugated to Sepharose from MCF-7 cell nuclear extracts following a previously described method (Baek et al., 2002). Mass spectrometry analyses identified SHARP as a putative PAD4 interacting protein (Data not shown). SHARP is a large transcriptional repressor protein, which was found to interact with HDACs to repress gene expression (Shi et al., 2001). Given that PAD4 (Li et al., 2008) and HDACs (Harms and Chen, 2007) regulate p53-mediated transcription, we postulated that PAD4 coordinates with HDACs to regulate p53 functions. To test this idea, M2 agarose beads were used for affinity purification of FLAG-PAD4 and its associated proteins from 293T and MCF-7 cells. Western blotting analyses found that HDAC2 and p53 were recovered together with FLAG-PAD4 (Figure 4-1A). By comparing the amount of HDAC2 purified by M2 agarose beads with the amount of input protein, we found that about 1% of HDAC2 was retained by M2 agarose beads together with FLAG-PAD4 (Figure 4-1B). To further corroborate PAD4 and HDAC2 interaction, GST pull-down experiments were performed. HA-PAD4 was retained by GST-HDAC2 beads but not by control GST beads (Figure 4-1C). Likewise, HDAC2 was retained by GST-PAD4 beads but not by control GST beads (Figure 4-1D). To further test if endogenous PAD4 and HDAC2 interact, we performed co-immunoprecipitation experiments and found that HDAC2 was co-immunoprecipitated by a PAD4 rabbit polyclonal antibody but not by the control normal rabbit IgG (Figure 4-1E).
The interaction of PAD4 and HDAC2 prompted us to test if histone deacetylase and histone citrullination activities associate with each other. First, GST-PAD4 purified from \textit{E.coli} and FLAG-PAD4 purified from FLAG-PAD4/293T cells showed histone citrullination activities when free histone H3 was used as a substrate (Figure 4-2A). We have previously showed that PAD4 demethyliminates free histone H3 in \textit{in vitro} biochemical assays (Wang \textit{et al.}, 2004b). However, the demethylimination activity has not been tested using nucleosomal substrate \textit{in vitro}. Here, we further tested GST-PAD4 activity using oligonucleosomes purified form Hela cells as substrates. Concomitant with an increase in histone citrullination, a decrease of histone H3 Arg17 methylation was detected in a GST-PAD4 dose-dependent manner (Figure 4-2B), indicating the demethylimination activity of PAD4. Since GST-PAD4 pulled down HDAC2, we further tested if HDAC activity is associated with GST-PAD4. Using a fluorometric HDAC assay method, we found that GST-PAD4 but not GST beads retained HDAC activity (Figure 4-2C, column 10), suggesting that histone citrullination and deacetylation activities can associate with each other via protein-protein interaction.

Neither PAD4 nor HDAC2 has a DNA binding domain, we postulated that these two proteins associate with specific gene promoters by interacting with transcription factors, such as p53. PAD4 has two immunoglobulin-like domains (IgL) at its N-terminus and a catalytic domain at its C-terminus (Arita \textit{et al.}, 2004). We previously found that the two N-terminal IgL domains of PAD4 interact with the C-terminal regulatory domain of p53 (Li \textit{et al.}, 2008). To analyze protein-protein interaction domains between PAD4 and HDAC2, we performed GST pull-down experiments using GST-PAD4 and GST-PAD4\textsuperscript{IgL1} or GST-PAD4\textsuperscript{IgL1&2} fusion proteins purified from \textit{E.coli}. We found that the two IgL domains of PAD4 are required for PAD4 and HDAC2 interaction (Figure 4-2D, lane 8). Moreover, this part of PAD4 was also sufficient to retain the HDAC activity (Figure 4-2C, column 9). To further analyze which part of p53 interacts with HDAC2, we used GST-p53 and its derivatives to pull-down HDAC2, and found that both the C-
terminal regulatory domain of p53 and the N-terminal part of p53 interact with HDAC2 (Figure 4-2E), with a slightly stronger interaction of HDAC2 with the p53 C-terminal regulatory domain detected (Figure 4-2E, compare lane 6 with lane 7). Taken together, protein-protein interaction studies suggest a model of p53, HDAC2, and PAD4 interaction (Figure 4-2F), in which the N-terminal IgL domains of PAD4 interact with the C-terminal regulatory domain of p53 (Li et al., 2008) and HDAC2 (Figure 4-2D), while p53 and HADC2 interaction is mediated by both the p53 C-terminal regulatory domain as well as its N-terminal domain.

4.4.2 PAD4 and HDAC2 dynamically associate with the p53-target gene promoter during DNA damage response

After DNA damage treatment, p53 is stabilized and binds to its target gene p21 via two defined binding sites, PBS1 and PBS2 (illustrated in Figure 3-9A) to regulate transcription. The stabilization of p53 and the induction of p21 expression were detected at 6 hr after DNA damage drug doxorubicin treatment in U2OS cells (Figure 4-3A). To analyze the association of PAD4 and HDAC2 with the p21 promoter during DNA damage response, we performed ChIP experiments after treatment of U2OS cells with doxorubicin for 6 hr. The amount of p53 increased at the two p53 binding sites of p21 after DNA damage (Figure 4-3B). Compared with untreated cells, the amount of PAD4 and HDAC2 decreased after DNA damage (Figure 4-3B). Consistent with a role of PAD4 in mediating histone citrullination and decreasing histone Arg methylation, the dissociation of PAD4 from the p21 promoter after DNA damage was accompanied with a decrease in histone H3 citrullination and an increase in histone H3 Arg17 methylation (Figure 4-3B). Furthermore, the decrease of HDAC2 association correlates to an increase of histone H3 acetylation after DNA damage (Figure 4-3B). These data support a model that PAD4 and HDAC2 associate with p53 at the p21 promoter before DNA damage to inhibit p21 expression, while the two proteins dissociate after DNA damage to allow gene activation.
To test whether the binding of PAD4 and HDAC2 to the p21 promoter is p53-dependent, we performed ChIP experiments in U2OS cells after p53 depletion by shRNA (U2OS/p53-shRNA). The depletion of p53 decreased the amount of p53 and also the induction of p21 by doxorubicin (Figure 4-3A). Compared with the parental U2OS cells, the amount of p53 at the p21 promoter was significantly decreased before and after DNA damage treatment in ChIP assays (Figure 4-3C). Moreover, after p53 depletion, the amount of PAD4, histone citrullination, and histone Arg methylation was greatly decreased (Figure 4-3C), suggesting that p53 is a major factor recruiting histone Arg modifying enzymes to the p21 promoter. In contrast, HDAC2 and histone Lys acetylation were decreased to a less extent (Figure 4-3C), suggesting that additional mechanisms are present to regulate HDAC2 recruitment and histone acetylation.

The interaction of PAD4 and HDAC2 implicates that these two proteins may simultaneously associate with the p21 promoter. To test this idea, we performed re-ChIP experiments, a method to determine if two proteins are on the same promoter at the same time. First, we used M2 agarose beads to perform ChIP from FLAG-PAD4 overexpressing MCF-7 cells or control parental cells. The N-terminal FLAG tag allows efficiently enrichment of PAD4 associated promoters. PCR analyses revealed that the M2 beads recovered p21 promoter from FLAG-PAD4 overexpressing MCF-7 cells but not the parental MCF-7 cells. For the second step ChIP, we used α-HDAC2 monoclonal antibody to perform ChIP using DNA/protein complex eluted from the M2 agarose, while the PAD4 antibody was used as a positive control. The HDAC2 antibody was able to re-ChIP the p21 promoter, while no signal was detected when the control protein A agarose was used for re-ChIP (Figure 4-3D), indicating that PAD4 and HDAC2 are recruited simultaneously to the p21 promoter.

We have previously found that PAD4 inhibits the expression of other p53 target genes, such as GADD45 and PUMA (Li et al., 2008). To further analyze PAD4 and HDAC2 promoter association at other p53 target genes during DNA damage response, we performed ChIP
experiments to measure the recruitment of these factors at GADD45 and PUMA gene promoters in U2OS cells with or without the depletion of p53 (Figure 4-4A and 4-4B). We found that: First, before and after DNA damage treatment, the depletion of p53 by shRNA decreased p53 association with these two promoters in U2OS cells. Second, PAD4 and HDAC2 dissociate from these two promoters after DNA damage with a concomitant increase in histone acetylation and histone Arg methylation and a decrease in histone citrullination. Third, levels of PAD4 promoter association, histone citrullination, and histone Arg17 methylation were decreased after p53 depletion. In contrast, significant amount of HDAC2 associated with these two promoters after p53 depletion. Overall, the dynamics of factor association and histone modifications at GADD45 and PUMA promoters is similar to that of the p21 promoter, suggesting a common theme of p53 target genes regulation by HDAC2 and PAD4.

4.4.3 PAD4 or HDAC2 siRNA treatment increases p21 expression

To further analyze the mechanism of HDAC2 and PAD4 in regulating p21 expression, we used siRNAs to deplete PAD4, HDAC2, or both in U2OS cells. Consistent with our previously published results (Li et al., 2008) and those by others (Harms and Chen, 2007), singular depletion of PAD4 or HDAC2 increased p21 expression as detected by Western blotting (Figure 4-5A, lane 2 and 3 compared with lane 1). Interestingly, when both PAD4 and HDAC2 were depleted (Figure 4-5A, lane 4), we did not detect a consistent further increase in the amount of p21 protein compared to the singular depletion of these two proteins. It is likely that the frequency of complete depletion of both proteins in the same cells was low or that additional factors contribute to the inhibition of p21 expression.

Additionally, we analyzed protein association and histone modifications at the p53 binding sites of the p21 promoter after siRNA treatment of U2OS cells by ChIP (Figure 4-5B).
Strikingly, after the depletion of HDAC2, we found that PAD4 association and histone citrullination were decreased with a concomitant increase in histone Arg methylation and Lys acetylation (Figure 4-5B). On the other hand, depletion of PAD4 affected histone Arg methylation and citrullination but not HDAC2 association or histone H3 acetylation (Figure 4-5B). Above results reveal an interesting crosstalk between histone acetylation and Arg modifications, suggesting a regulatory role of HDAC2 in PAD4 recruitment and function.

4.4.4 PAD4 and HDAC inhibitors cooperatively affect p53-target gene expression and cancer cell growth

To further test the relationship between histone citrullination and deacetylation, we treated U2OS cells with PAD4 inhibitor Cl-amidine and HDAC inhibitor SAHA, singularly or in combination. Inhibition of PAD4 by Cl-amidine led to increased binding of p53 at p21, GADD45, and PUMA promoters (Figure 4-6A and 4-6B) concomitantly with a decrease of histone H3 citrullination and an increase in histone H3 Arg17 methylation at these gene promoters. SAHA is a general inhibitor of the HDAC family proteins (Gui et al., 2004). Histone H3 acetylation was highly elevated at the p21, GADD45, and PUMA promoters after SAHA treatment (Figure 4-6A and 4-6B). Moreover, inhibition of HDAC activity by SAHA not only led to an increase in histone H3 acetylation but also a decrease in histone citrullination and an increase in histone H3 Arg17 methylation at these promoters (Figure 4-6A and 4-6B, bottom four panels). These results support that histone H3 acetylation regulates histone H3 Arg17 modifications.

To analyze the effects of PAD4 and HDAC inhibition on p21 protein expression, we performed western blotting in U2OS cells without or with p53 depletion after Cl-amidine and SAHA treatment. Consistent with previously published results from us (Li et al., 2008) and others (Gui et al., 2004), treatment of with either Cl-amidine or SAHA increased p53 and p21 protein
expression (Figure 4-7A, lanes 2 and 3 compared with lane 1). Interestingly, when both inhibitors were used (Figure 4-7A, lane 4), the amount of p21 was further elevated compared to the singular inhibition treatment. Compared with siRNAs (Figure 4-5B), PAD4 and HDAC inhibitors are more effective in elevating histone Arg methylation and Lys acetylation at the p21 promoter (compare ChIP efficacy of H3 K9/14 acetylation and H3R17 methylation antibodies in Figure 4-6A to that in Figure 4-5B), which may contribute to the increased p21 expression. In contrast, after p53 depletion, the basal level of p21 and its amplitude of expression after PAD4 and HDAC inhibition was dramatically decreased (Figure 4-7A, lanes 5 to 8), indicating that the expression of p21 after Cl-amidine and SAHA treatment is largely if not only dependent on p53. In real-time qPCR analyses, we found that SAHA and Cl-amidine treatment increased the expression of p21, GADD45, and PUMA in a p53-dependent manner (Figure 4-7B to 4-7E). Furthermore, SAHA and Cl-amidine additively increased the expression of these three genes (Figure 4-7B to 4-7E).

The further increase of p53 target gene expression after both Cl-amidine and SAHA treatment suggests that a combination of these two inhibitors may more effectively inhibit cancer cell growth. To test this idea, we analyzed the effect of Cl-amidine, SAHA, or a combination of both inhibitors on the growth of U2OS cells. Compared to the control untreated cells, SAHA and Cl-amidine individually inhibited cell growth at 2 and 3 days after treatment (Figure 4-8A). Consistent with the additive effects of Cl-amidine and SAHA on the expression of p21, GADD45, and PUMA, these two inhibitors cooperatively inhibited U2OS cell growth (Figure 4-8A). In contrast, the cell growth inhibitory effects of these inhibitors were significantly reduced after p53 depletion in U2OS cells (Growth curves in Figure 4-8B compared to those in Figure 4-8A). In particular, after treatment with both inhibitors, U2OS cells grew about 1.3-fold while U2OS/p53-shRNA cells grew about 2.7-fold. The growth inhibitory effect of Cl-amidine, SAHA, or both inhibitors in U2OS cells and U2OS/p53-shRNA cells was also analyzed by phase contrast microscopy (Figure 4-8C). Taken together, above results indicate a combination of PAD4
inhibitor Cl-amidine and HDAC inhibitor SAHA may offer a novel strategy in preventing cancer cell growth by activating the p53 pathway.
4.5 Discussion

We found here that PAD4 and HDAC2 interact with each other, and they are simultaneously recruited to the p21 promoter and serve as corepressors for gene expression. After DNA damage, PAD4 and HDAC2 dissociate with a subsequent association of coactivators to mediate histone Arg methylation and histone acetylation, respectively. Our data favor a model that a dynamic association of PAD4 and HDAC2 ensures a low level of p21 expression before DNA damage. Post-translational histone modifications are thought to regulate gene expression by facilitating the formation of open chromatin structure or by serving as binding platforms for additional effector proteins (Jenuwein and Allis, 2001; Kouzarides, 2007). Covalent histone modifications often cross talk with each other to govern gene expression (Fischle et al., 2003). Histone Arg methylation and Lys acetylation were first shown to cooperatively activate p53-mediated transcription in biochemical analyses (An et al., 2004). In reverse, we recently found that PAD4 functions as a corepressor to regulate p53 target gene expression by counteracting the function of histone Arg methylation (Li et al., 2008; Yao et al., 2008). Here, our ChIP studies at the p21 promoter offered a novel link between histone citrullination and deacetylation. Of particular interest, we found that inhibition of HDAC functions affects PAD4 association, histone citrullination and Arg methylation. On the other hand, HDAC2 association and histone Lys acetylation are largely unaffected by PAD4 inhibition. The ordered cooperative function of the histone H3 acetyltransferase p300 and H3 Arg methyltransferase CARM1 was previously reported where the action of histone H3 acetylation precedes H3 Arg17 methylation during p53-mediated transcription (An et al., 2004). Our studies of PAD4 and HDAC2 inhibition and depletion in U2OS cells are consistent with this previous report, indicating that increased histone H3 Lys acetylation facilitates histone H3 Arg17 methylation, but not vice versa.
HDAC2 is one of the type I HDACs and shares high homology with HDAC1 that is another type I HDAC. HDAC1 and HDAC2 are often found in the same protein complex such as Sin3A/NuRD and have many overlapping functions (Zhang et al., 1999). One recent report suggests that PAD4 works with HDAC1 to repress transcription at the pS2 promoter. I tried to look at PAD4 and HDAC1 interaction in mammalian cell nuclear extract, but Western blot cannot detect interaction between PAD4 and HDAC1 (Figure 4-9). Some previous work using p21 as a model gene suggests that HDAC2 plays a unique role in the repression of p21 (Harms and Chen, 2007; Huang et al., 2005a). So it is possible that PAD4 interacts with different HDACs in order to function at various gene promoters. Other unknown factors may also involve in mediating the interaction between PAD4 and HDACs.

After p53 depletion by shRNA, the association of PAD4 and histone citrullination decreased dramatically while the association of HDAC2 and histone acetylation decreased to a less extent. The largely unaffected HDAC2 and histone acetylation may be due to the presence of other HDAC2 interacting proteins at the p21 promoter. The association of HDAC1/2 is affected at the Sp1 binding sites of the p21 promoter (Lin et al., 2008), which may suggest that Sp1 is another transcription factor that affects histone acetylation level at the p21 promoter.

It has been previously reported that the HDAC inhibitor SAHA (suberoylanalide hydroxamic acid) activates p21 expression by increasing histone acetylation at the p21 promoter (Gui et al., 2004). The US Food and Drug Administration recently approved SAHA for treatment of cutaneous T-cell lymphoma (Marks and Breslow, 2007), which serves as a harbinger for cancer treatment by targeting epigenetic mechanisms (Minucci and Pelicci, 2006). PAD4 is overexpressed in various types of cancers (Chang and Han, 2006; Chang et al., 2009). Our studies showed that PAD4 inhibitor Cl-amidine and HDAC inhibitor SAHA has additive effects in growth inhibition of osteosarcoma U2OS cells, suggesting that targeting these two types of enzymes in cancer treatment is worth of future exploration.
4.6 Acknowledgement

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Figure 4-1: Identification of HDAC2 as a PAD4 interacting protein.

(A) HDAC2 and p53 could be affinity-purified with FLAG-PAD4 by M2 agarose in
FLAG-PAD4 overexpressing 293T or MCF-7 cells, but not from the parental cells lacking
FLAG-PAD4.

(B) Different percentages of input as indicated in lanes 1 to 4 were loaded with affinity
purified proteins by the M2 agarose from FLAG-PAD4 overexpressing 293T cells (lane 6) or the
parental cells (lane 5) to evaluate the efficacy of copurification of HDAC2 with FLAG-PAD4.

(C) GST-HDAC2 but not GST sepharose could pull-down HA-PAD4 from HA-PAD4
overexpressed Cos1 cells. Lane 1 had 2% of the input.

(D) GST-PAD4 but not GST sepharose could pull-down HDAC2. Lane 1 had 2% of the
input.

(E) Coimmunoprecipitation of endogenous HDAC2 by α-PAD4 antibody (lane 3) but not
by control normal rabbit IgG (lane 2). Lane 1 had 2% of the input.
**Figure 4-1**

A

- **IP: α-FLAG**
  - 293T
  - 293T/FLAG-PAD4

- **WB:**
  - PAD4
  - HDAC2
  - p53
  - 1 2

B

- **IP: α-FLAG**
  - MCF-7
  - MCF-7/FLAG-PAD4

- **WB: HDAC2**
  - 1 2 3 4 5 6

C

- **Input**
- **GST**
- **GST-HDAC2**

- **WB:** HA-PAD4
  - 1 2 3

D

- **Input**
- **GST**
- **GST-PAD4**

- **WB:** HDAC2
  - 1 2 3

E

- **Input**
- **Normal IgG**
- **α-PAD4**

- **WB:** HDAC2
  - 1 2 3
Figure 4-2: Detection of PAD4 and HDAC2 activities and protein-protein interaction domain mapping of PAD4, HDAC2, and p53.

(A) GST-PAD4 purified from *E.coli* and Flag-PAD4 purified from 293T cells citrullinated free histone H3 in biochemical analyses *in vitro*. Histone H3 citrullination was detected by H3Cit antibody in Western blotting.

(B) GST-PAD4 citrullinated nucleosomes purified from Hela cells. Concomitant with an increase in histone H3 citrullination, a decrease in histone H3 Arg17 methylation was detected.

(C) HDAC2 activities were retained by full length GST-PAD4 and GST-PAD4<sup>lgL1&2</sup> fusion proteins (lanes 9 and 10), but not by GST, GST-PAD4<sup>lgL1</sup>, or beads alone. Data presented are the averages of fluorometric values from three experiments with standard deviation within 10% of the average.

(D) HDAC2 was pulled down by GST-PAD4 full length and GST-PAD4<sup>lgL1&2</sup> (lanes 8 and 9), but not by GST, GST-PAD4<sup>lgL1</sup>, or beads alone.

(E) GST-p53 and its N- or C-terminal truncation derivatives interacted with HDAC2, suggesting that HDAC2 interacts with multiple parts of p53.

(F) A diagram illustrating the interaction of PAD4, HDAC2, and p53 via different domains.
Figure 4-3: PAD4 and HDAC2 dynamically associate with the p21 promoter during DNA damage response.

(A) The amount of p53, p21, PAD4, and HDAC2 was analyzed by Western blotting in U2OS cells (lanes 1 and 2) or U2OS/p53-shRNA cells (lanes 3 and 4) before and after doxorubicin treatment. β-actin was probed as a control to ensure equal loading.

(B-C) ChIP experiments were performed to analyze the level of p53, PAD4, HDAC2, histone H3 K9/14 acetylation (H3K9/14Ac), H3 citrullination (H3Cit), or H3 Arg 17 methylation (H3R17Me) in U2OS cells (B) or U2OS cells after p53 depletion with p53-shRNA (C) before or 6 hr after 0.5 µM doxorubicin treatment at the two p53 binding sites (PBS1 and PBS2) as well as the transcription start site (TSS). ChIP signals were calculated as percentages of the input (% IP). Protein A agarose was used to measure the background signals. Averages and standard deviations were shown. ChIP experiments were generally performed from over two independent experiments of sample preparation and PCR reactions were performed at least 6 times. For those ChIP results with small fold of changes but key to the conclusion, p-values were analyzed by student t-test. * denotes p<0.02.

(D) Re-ChIP experiments detected simultaneous binding of PAD4 and HDAC2 to the p21 promoter.
Figure 4-3

A

U2OS cells
- + - +
Dox (6h)
α-p53
α-p21
α-PAD4
α-HDAC2
α-β-actin

1 2 3 4

B

U2OS

Ctrl  Dox (6 hr)

Beads

% p

0.3

0.25

0.2

0.15

0.1

0.05

0

PBS1  PBS2  TSS

% p

0.8

0.6

0.4

0.2

0

PBS1  PBS2  TSS

p53

C

U2OS/p53-shRNA

Ctrl  Dox (6 hr)

Beads

% p

0.3

0.25

0.2

0.15

0.1

0.05

0

PBS1  PBS2  TSS

% p

0.8

0.6

0.4

0.2

0

PBS1  PBS2  TSS

p53

D

MCF-7

MCF-7 (Flag-p53)

Input
1st ChIP
M2 agarose
Beads
α-PAD4
α-HDAC2

2nd ChIP

% p

7

5

3

1

0

PBS1  PBS2  TSS

% p

0.08

0.06

0.04

0.02

0

PBS1  PBS2  TSS

H3K9/14Ac

% p

0.08

0.06

0.04

0.02

0

PBS1  PBS2  TSS

H3Cit

% p

0.08

0.06

0.04

0.02

0

PBS1  PBS2  TSS

H3R17Me

% p

0.08

0.06

0.04

0.02

0

PBS1  PBS2  TSS

H3K9/14Ac

% p
Figure 4-4: Dynamic association of PAD4 and HDAC2 with GADD45 and PUMA promoters during DNA damage.

(A-B) ChIP experiments were performed to analyze the level of p53, PAD4, HDAC2, histone H3 K9/K14 acetylation (H3K9/14Ac), H3 citrullination (H3Cit), or H3 Arg17 methylation (H3R17Me) in U2OS cells or U2OS/p53-shRNA cells before or 6 hr after 0.5 μM doxorubicin treatment at the p53 binding site of GADD45 (A) or PUMA (B). ChIP signals were calculated as percentages of the input (% IP). Protein A agarose beads were used to measure the background signals. Averages and standard deviations shown were measured (n=6). p-values were analyzed by student t-test. * denotes p<0.02.
Figure 4-4

A  GADD45 promoter ChIP

B  PUMA promoter ChIP

[Graphs showing data for Ctrl and Dox (6 hr) for each condition]
Figure 4-5: Promoter association of PAD4 and HDAC2 during DNA damage and after depletion of PAD4 or HDAC2.

(A) The effects of PAD4 and/or HDAC2 depletion on the expression of p21 in U2OS cells were analyzed by western blotting. \(\beta\)-actin was probed as a control to ensure equal loading.

(B) ChIP experiments were performed to analyze the level of p53, PAD4, HDAC2, histone H3 K9/K14 acetylation (H3K9/14Ac), H3 citrullination (H3Cit), or H3 Arg 17 methylation (H3R17Me) in U2OS cells after the depletion of PAD4 and/or HDAC2 at the p21 promoter. ChIP signals were calculated as percentages of the input (% IP). Averages and standard deviations were shown (n=6). The depletion of HDAC2 decreased PAD4 binding and histone H3 citrullination with a simultaneous increase of histone H3 acetylation and H3 Arg17 methylation.
Figure 4-5

A

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lane</th>
<th>Image</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP siRNA</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>PAD4 siRNA</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>HDAC2 siRNA</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

B  p21 promoter ChIP

- **GFP siRNA**
- **PAD4 siRNA**
- **HDAC2 siRNA**
- **PAD4&HDAC2 siRNA**

- **Beads**

- **p53**

- **PAD4**

- **HDAC2**

- **H3K9/14Ac**

- **H3Cit**

- **H3R17Me**
Figure 4-6: Inhibition of PAD4 and/or HDACs impacts on histone modifications at p53-target gene promoters.

(A-B) ChIP experiments were performed to analyze the level of p53, PAD4, HDAC2, histone H3 K9/K14 acetylation (H3K9/14Ac), H3 citrullination (H3Cit), or H3 Arg 17 methylation (H3R17Me) in U2OS cells after treatment with the PAD4 inhibitor Cl-amidine and/or the HDAC inhibitor SAHA at the p53 binding sites of p21 (A), GADD45 (B), and PUMA (B). ChIP signals were calculated as percentages of the input (% IP). Averages and standard deviations were shown (n=6).
Figure 4-7: PAD4 and HDAC inhibitors cooperatively affect p53-target gene expression in a p53-dependent manner.

(A) The effects of PAD4 and/or HDAC2 inhibition on the expression of p21 in U2OS cells and U2OS cells after p53 depletion were detected by Western blotting. β-actin was probed as a control to ensure equal loading.

(B-E) mRNA levels of p53 (B) and p53-target genes p21 (C), PUMA (D), and GADD45 (E) in U2OS cells or U2OS/p53-shRNA cells after of Cl-amidine and/or SAHA treatment. cDNA was used for analysis by real-time qPCR. Levels of β-actin gene were monitored to normalize the amount of cDNA input. The expression of each gene in the untreated U2OS cells was normalized to 1. Folds of change in gene expression under various conditions were then compared with that in the U2OS cells without treatment. Averages and standard deviations were shown (n=4).
Figure 4-8: The effects PAD4 and HDAC inhibitors on the growth of U2OS cells and U2OS/p53-shRNA cells.

(A) The growth inhibitory effects of the PAD4 inhibitor Cl-amidine, the HDAC inhibitor SAHA, or both in U2OS cells. The fold of cell number increase and standard deviations at each time point were obtained from three independent experiments.

(B) The growth inhibitory effects of the PAD4 inhibitor Cl-amidine, the HDAC inhibitor SAHA, or both in U2OS/p53-shRNA cells. The fold of cell number increase and standard deviations at each time point were obtained from three independent experiments.

(C) U2OS cells (a-d) or U2OS/p53-shRNA cells (e-h) were untreated or treated with Cl-amidine, SAHA, or both inhibitors for 72 hr. Representative images from three independent experiments were selected to show the changes in cell density and morphology after inhibitor treatment.
Figure 4-8

A  U2OS Cell Growth Curve

B  U2OS p53-shRNA Cell Growth Curve

C

Control  Cl-amidine  SAHA  Cl-amidine

U2OS  U2OS

pHP-p53  pHP-p53
Figure 4-9: Protein-protein interaction study between PAD4 and HDAC1.

Western blot failed to detect HDAC1 after pull-down experiments by GST-PAD4 full length and its derivative proteins.

**Figure 4-9**
Chapter 5

PAD4 is Essential for Antibacterial Innate Immunity Mediated by Neutrophil Extracellular Traps
5.1 Abstract

Neutrophils trap and kill bacteria by forming highly decondensed chromatin structures, termed neutrophil extracellular traps (NETs). We previously reported that histone hypercitrullination catalyzed by PAD4 correlates with chromatin decondensation during NET formation. However, the role of PAD4 in NET-mediated bacterial trapping and killing has not been tested. Here, we use PAD4 knockout mice to show that PAD4 is essential for NET-mediated antibacterial function. Unlike PAD4+/+ neutrophils, PAD4−/− neutrophils cannot form NETs after stimulation with chemokines or incubation with bacteria, and are deficient in bacterial killing by NETs. In a mouse infectious disease model of necrotizing fasciitis, PAD4−/− mice are more susceptible to bacterial infection than PAD4+/+ mice due to a lack of NET formation. Moreover, we found that citrullination decreased the bacterial killing activity of histones and nucleosomes, suggesting that PAD4 mainly plays a role in chromatin decondensation to form NETs instead of increasing histone-mediated bacterial killing. Our results define a role for histone hypercitrullination in innate immunity during bacterial infection.
5.2 Introduction

Peripheral blood neutrophils are the first line of defense after bacterial infection (Nathan, 2006; Segal, 2005). Neutrophils can migrate to sites of infection in response to inflammatory signals, where they engulf and kill bacteria by phagocytosis. Recently, a novel mechanism of extracellular bacterial killing mediated by highly decondensed chromatin structures, termed neutrophil extracellular traps (NETs), was identified (Brinkmann et al., 2004). NET formation is induced by phorbol myristate acetate (PMA), lipopolysaccharides (LPS), and bacteria (Brinkmann et al., 2004). NETs trap and kill pathogenic bacteria, such as *Shigella flexneri* (Brinkmann et al., 2004) and group A *Streptococcus pyogenes* (GAS) (Buchanan et al., 2006). The role of NETs in innate immunity has kindled much attention due to its involvement in human health and diseases and its therapeutic implications (Brinkmann and Zychlinsky, 2007; Nizet, 2007; von Kockritz-Blickwede and Nizet, 2009; Wartha and Henriques-Normark, 2008). For example, impaired NET formation predisposes newborn infants to bacterial infection (Yost et al., 2009). Further, in chronic granulomatous disease (CGD) patients with impaired NADPH oxidase activity and reactive oxygen species (ROS) production, neutrophils cannot generate NETs and possess poor antimicrobial activity (Bianchi *et al.*, 2009; Fuchs *et al.*, 2007). Conversely, gene therapy with the NADPH oxidase gene in a CGD patient to restore both NET formation and antimicrobial functions has offered an effective treatment for this disease (Bianchi *et al.*, 2009).

In the eukaryotic nucleus, 147 bp of DNA is wrapped around a core histone octamer, which includes two of each histone H3, H2B, H2A, and H4, to form nucleosome. Nucleosomes are ~11 nm in diameter and are the basic structural units of chromatin (Richmond and Davey, 2003). The association of linker histone H1 with linker DNA further organizes nucleosomes to form higher order chromatin structures (Brown et al., 2006). During NET formation, chromatin is...
extremely decondensed to form 15 to 25 nm chromatin fibers (Brinkmann et al., 2004). However, the mechanisms regulating this extreme chromatin decondensation are unclear. Post-translational histone modifications play an important role in regulating chromatin structure and function (Jenuwein and Allis, 2001; Kornberg and Lorch, 1999; Kouzarides, 2007), including chromatin decondensation/condensation and transcription regulated by citrullination (Cuthbert et al., 2004; Neeli et al., 2008; Wang et al., 2009; Wang et al., 2004b). We and others have recently found that an increase in histone citrullination is associated with chromatin decondensation during NET formation (Neeli et al., 2008; Wang et al., 2009). The conversion of histone Arg or monomethyl-Arg to citrulline residues is catalyzed by peptidylarginine deiminase 4 (PAD4, also called PADI4 or PADV) (Wang et al., 2004b), a neutrophil enriched nuclear enzyme (Nakashima et al., 2002). Excessive PAD4 function has been related to rheumatoid arthritis (Yamada and Yamamoto, 2007) and cancer (Chang and Han, 2006; Chang et al., 2009). However, whether PAD4 is an important factor for NET-mediated innate immune functions has not been investigated. Here, using PAD4 knockout mice, we show that PAD4 is required for bacterial killing by NETs.
5.3 Materials and Methods

5.3.1 PAD4 knockout mouse generation

1) A 3.1 kb PAD4 intron I fragment and 2.7 kb PAD4 intron II were PCR amplified and cloned the two fragments before and after the neomycin cassette of the pGK-Neo-FRT vector.

2) The knockout construct was sequenced, linearized, and transfected to ES J1 cells by electroporation.

3) ES cell colony screening was performed as previously described (Xiong et al., 2002).

4) ES cells were microinjected into the early embryo fibroblasts to generate chimeric mice in the Transgenic Mouse Facility at Penn State University.

5) To remove neomycin cassette inserted in PAD4 locus, neomycin-containing PAD4 homozygous mice were crossed with flippase-expressing 129S4/SvJaeSor-Gt(Rosa)26Sortm1(FLP1)Dym/J mice from the Jackson Laboratory.

6) The F1 offsprings were sibling crossed.

7) The F2 offsprings were PCR genotyped to identify PAD4 homozygous mice with the neomycin cassette removed from its insertion site at PAD4 exon II.

5.3.2 Mouse genotyping

1) Genomic DNA preparation:

   a. Cut ~5 mm tail and put in 500 μl Lysis buffer (100 mM Tris-HCl, pH 8.5, 200 mM NaCl, 5 mM EDTA, 0.2% SDS, 100 μg/ml Protease K). Incubate at 55°C overnight with rotating.

   b. Next day spin at 13,200 rpm for 10 min to precipitate undigested hair and bone.
c. Pour supernatant to a new 1.5 ml tube with 500 µl Isopropanol. Shake vigorously for 10 min until DNA pellet has formed.

d. Transfer the DNA pellet to a new 1.5 ml tube with 500 µl TE (10 mM Tris-HCl, pH 8.0, 0.5 mM EDTA) using a blunt pipette tip.

e. Dissolve DNA at 55°C for up to 1 hr and incubate at 4°C for another 1 to 2 hr for further dissolve.

2) Set up 15 µl PCR reaction:

1.5 µl 10x buffer
1 µl DNA
1 µl 2.5 mM dNTP mix
0.6 µl 5 µM Primer 1
0.6 µl 5 µM Primer 2
0.3 µl Taq polymerase
10 µl ddH2O

3) Run PCR with normal DNA amplification program for 30 cycles and check results with agarose gel electrophoresis.

5.3.3 Mouse neutrophils purification from peripheral blood

1) Collect mice peripheral blood from mandibular vein using 22G needles (BD, 305155) to EDTA-coated tubes (Terumo, TET-MQK). Collect a total of ~2 ml blood for each purification from several mice.

2) Prepare a Histopaque density gradient by carefully layering 3 ml of Histopaque-1077 (Sigma, 10771) on top of 3 ml Histopaque-1119 (Sigma, 11191) in a 15 ml tube.
3) Load the blood on top of the gradient and centrifuge at 700 x g for 30 min at room temperature.

4) Collect the middle layer between Histopaque-1077 and Histopaque-1119 that contains neutrophils. Pellet cells by spinning at 450 x g for 5 min at 4°C, remove supernatant.

5) To remove contaminating erythrocytes, resuspend the cell pellet in 1 ml of ice-cold 0.2% NaCl solution for 30 sec. Immediately restore the isotonicity by adding 1 ml of ice-cold 1.6% NaCl solution. Spin down cells at 450 x g for 5 min, remove supernatant.

6) Repeat step 5 a couple of times to remove most erythrocytes.

7) Finally resuspend neutrophils in ice-cold PBS containing 10 mM D-Glucose at desired concentration for further experiment.

5.3.4 Flow cytometry and TEM analyses

2x10^5 neutrophils were resuspended in 200 µl PBS supplemented with 3% FBS. Biotin-CD11b antibody was added and incubated for 1 hr on ice. After washing once with PBS, Streptavidin-Phycoerythrin (eBioscience, 12-4317) and fluorescein isothiocyanate-conjugated Ly6G antibody (BD Pharmingen, 551460) were added and incubated for 30 min in dark. At least 1x10^4 positive stained cells were counted. Transmission electron microscopy analyses of PAD4^+/+ and PAD4^−/− neutrophil morphology were performed using the service of the Penn State Electron Microscopy Facility.
5.3.5 Bacterial strains and growth conditions

*Shigella flexneri* strain was grown in Luria-Bertani media. The M1 serotype of Group A *Streptococcus* (GAS) strain and M1 Δ*Sda1* GAS strain were grown in Todd-Hewitt broth (THB) or agar (THA).

5.3.6 Bacteria DNase activity assay

1) M1 and M1 Δ*Sda1* Group A *Streptococcus* bacteria were grown overnight in Todd-Hewitt broth and supernatant are collected.

2) 1 µg U2OS cell genomic DNA was incubated with 3 µl bacteria supernatant for 10 min at 37°C in a total volume of 50 µl buffer (300 mM Tris-HCl, pH 8.0, 3 mM CaCl₂, 3 mM MgCl₂).

3) Add EDTA to a final concentration of 60 mM to stop the reaction.

4) Check result using 1.2% agarose gel electrophoresis.

5.3.7 Treatment of neutrophils with chemokines or bacteria

1) For chemokines treatment, mouse neutrophils were stimulated with 1 µg/ml LPS, 100 µM H₂O₂, and 25 nM PMA, respectively, in the presence of 2 mM calcium at 37°C and 5% CO₂ in PBS supplemented with 10 mM D-Glucose for 3 hr before immunostaining assay to determine NET formation. Enlarged neutrophil nuclei after 3 hr LPS treatment were measured using the NIH Image J program to determine nuclear area changes. For PAD inhibitor treatment, neutrophils were incubated with 200 µM Cl-amidine for 30 min prior to LPS treatment.

2) For IL-8 and bacteria treatment, 2x10⁵ neutrophils were first incubated with 100 ng/ml IL-8 at 37°C and 5% CO₂ for 30 min. 2x10⁷ exponential phase *S. flexneri* or GAS was then added
to neutrophils. Samples were centrifuged at 200 x g for 10 min and further incubated for 2 hr before immunostaining assay.

### 5.3.8 Phagocytosis Assay

Phagocytosis assays were performed using a Vybrant Phagocytosis Assay Kit (Invitrogen, V6694) with modifications for use with flow cytometry essentially as previously published (Siemsen et al., 2007).

### 5.3.9 Bacterial killing assays using neutrophils

1) Neutrophils purified from the peripheral blood of paired PAD4<sup>+/+</sup> and PAD4<sup>-/-</sup> mouse siblings were resuspended in Locke’s solution (10 mM HEPES-HCl, pH 7.3, 150 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 0.18% D-Glucose) at 2x10<sup>6</sup> cells/ml and incubated with 100 ng/ml IL-8 at 37°C in a 5% CO<sub>2</sub> incubator for 30 min.

2) Cytochalasin D (10 µg/ml) and/or DNase I (100 U/ml) were added to each individual group and further incubated for 15 min before infection with bacteria.

3) Exponential phase *S. flexneri*, M1 GAS, or M1 ΔSda1 GAS bacteria were added to the neutrophils to reach 2x10<sup>4</sup> bacteria/ml at multiplicity of infection (MOI=bacteria:cells) of 1:100. Samples were centrifuged at 700 x g for 10 min and further incubated for 30 min at 37°C.

4) Plate samples in LB agar or THA plates at a series of 1:10 dilution and colony formation units (CFUs) were analyzed. Bacterial killing efficacy was calculated as percentages of control values (bacteria incubated alone without neutrophils).
5.3.10 Bacteria killing using histones and nucleosomes with or without citrullination

1) Histone H3 and mononucleosome were citrullinated by PAD4 following PAD4 activity assay procedure.

2) 1x10^6 exponential phase *S. flexneri* were incubated with unmodified or citrullinated histone H3 and mononucleosome at various concentrations for 30 min in a total volume of 100 µl at 37°C with shaking in Hanks’ Balanced Salts Solution (8 g/L NaCl, 400 mg/L KCl, 350 mg/L NaHCO₃, 60 mg/L KH₂PO₄, 48 mg/L Na₂HPO₄, 1000 mg/L D-Glucose) buffered with 10 mM HEPES, pH 7.4.

3) Samples were plated in LB agar plates at a series of 1:10 dilution to determine CFUs. Bacterial killing efficacy was calculated as percentages of control values (bacteria incubated alone without histone H3 or mononucleosome).

5.3.11 Mouse infection, necrotizing fasciitis and histology assays

1) 5x10^6 exponential growth phase M1 GAS or M1 ΔSda1 GAS bacteria were pelleted, washed, resuspended in 50 µl PBS and diluted 1:1 with 2.5 mg/ml Cytodex beads (Sigma, C0646).

2) The mixture was subcutaneously injected into the shaved left and right flanks of 8-10 week-old paired PAD4⁺/⁺ and PAD4⁻/⁻ mouse siblings, respectively. Two pairs of PAD4⁺/⁺ and PAD4⁻/⁻ mouse siblings were simultaneously injected. Repeating experiment was performed for 3 times independently.

4) At three days after infection, mice were euthanized with CO₂ and the lesion areas were pictured alongside with a scale bar. Lesion size was measured using the NIH image J program. The lesion areas were removed by skin biopsy and added to PBS. Bacteria extracted into PBS were plated in THA plates at a series of 1:10 dilution to determine colony formation units.
5) For histological evaluation, the skin lesions were excised at two days after bacterial infection and fixed with 10% neutral buffered formalin, embedded in paraffin, and sectioned. Slides were routinely prepared and stained with hematoxylin and eosin. An assessment of microscopic lesions was made by a veterinarian with training and experience in rodent pathology and blinded to experimental treatment. The presence of neutrophils, edema, necrosis, and ulceration in each lesion were graded subjectively, on a relative scale of 0 to 4, with 0 being normal, 1= mild, 2= moderate, 3= marked, and 4= severe. The pathology score was calculated as the average of the four categories for each infection.

5.3.11 Assurances of animal procedures

All animal experiments were approved by the Penn State Institutional Animal Care and Use Committee and performed by accepted veterinary standards.
5.4 Results

5.4.1 Effects of PAD4 knockout in mice on early development and neutrophil differentiation

To analyze the function of PAD4 in NET formation and bacterial killing, we generated PAD4−/− mice by deleting exon II of the PAD4 gene (Figure 5-1A and 5-1B). The deletion of exon II generates a frame shift close to the 5’ end of PAD4 coding domain sequence thereby terminating PAD4 translation prematurely. PAD4 mutant mice were genotyped using wild type and knockout specific PCR primers (Figure 5-1C-F). The PCR product of the knockout PAD4 locus was sequenced to confirm the replacement of exon II by a residual FRT sequence (Figure 5-1G). PAD4 homozygous mice survived to adulthood and demonstrated no detectable physical abnormality. However, PAD4 heterozygous and homozygous mice were born at a rate lower than predicted by the Mendelian ratio (Figure 5-2A), suggesting that the loss of PAD4 affects embryonic development.

PAD4 expression increases when human leukemia HL-60 cells differentiate along the granulocytic pathway (Nakashima et al., 1999), and PAD4 is detected in the nucleus of peripheral blood neutrophils (Nakashima et al., 2002). To investigate whether PAD4 depletion affects neutrophil differentiation in mice, we used antibodies against neutrophil cell surface markers CD11b and Ly6G to perform flow cytometry analyses. Comparable amounts of neutrophils were detected in peripheral blood from PAD4+/+ and PAD4−/− mice, respectively (Figure 5-2B and 5-2C). Western blotting showed the expression of PAD4 in PAD4+/+ but not PAD4−/− neutrophils (Figure 5-2D). Electron microscopy analyses found that both PAD4+/+ and PAD4−/− neutrophils possess characteristic neutrophil morphology, including lobular nucleus and condensed chromatin
regions under the nuclear envelope (Figure 5-2E). Above results indicate that PAD4 deletion did not affect the generation of mouse neutrophils during hematopoiesis.

**5.4.2 PAD4 is required for histone citrullination and chromatin decondensation**

Next, we analyzed whether PAD4 is important for histone citrullination. First, we found that recombinant mouse PAD4 citrullinated histone H3 in biochemical assays (Figure 5-3). Second, endogenous histone H3 citrullination was detected in neutrophils from PAD4+/+ but not PAD4−/− mice in Western blotting (Figure 5-4A), indicating that the basal level of histone H3 citrullination is PAD4-dependent.

NET formation is induced after LPS and PMA treatment in human neutrophils (Brinkmann et al., 2004). To test whether PAD4 is important for chromatin decondensation and NET formation upon LPS treatment, immunostaining experiments were performed. Before LPS treatment, ~3.69% of PAD4+/+ neutrophils are positively stained for histone H3 citrullination but NET formation was not detected (Figure 5-4B; Table 5-1), while histone H3 citrullination or NET formation was not observed in PAD4−/− neutrophils (Figure 5-4B; Table 5-1). After 3 hr LPS treatment, an increase in histone H3 citrullination was detected in PAD4+/+ but not in PAD4−/− neutrophils by Western blotting (Figure 5-4A) or immunostaining (Figure 5-4C). DNA staining (pseudo colored green) identified chromatin decondensation to various degrees after LPS treatment, including swelling nuclei (Figure 5-4C; Nuclear area quantification with the NIH image J program in Figure 5-5A showed about 3.5-fold increase of these welling nuclei) and elongated chromatin in PAD4+/+ neutrophils (Figure 5-4C; Table 5-1). NETs are composed of highly decondensed chromatin and anti-bacterial granular proteins (Brinkmann et al., 2004). The decondensed chromatin induced by LPS showed positive staining by histone H3 citrullination and neutrophil elastase antibodies (Figure 5-4D), indicating these structures are indeed NETs. PAD
inhibitor Cl-amidine was previously found to repress the formation of NET-like structure in human HL-60 granulocytic cells (Wang et al., 2009). After pretreatment of PAD4+/+ mouse neutrophils with Cl-amidine for 30 min, histone H3 citrullination and chromatin decondensation induced by LPS were much decreased than untreated neutrophils (Figure 5-5B; Table 5-1), indicating that PAD activity is important for NET formation. Furthermore, chromatin decondensation and histone citrullination were detected in PAD4+/+ neutrophils but not in PAD4−/− neutrophils after 3 hr PMA treatment (Figure 5-5C; Table 5-1). These results indicate PAD4−/− mouse neutrophils lack the ability to citrullinate histones or form NETs after LPS and PMA treatment.

Reactive oxygen species (ROS), such as H2O2, efficiently induce the formation of NETs in adult neutrophils (Fuchs et al., 2007; Neeli et al., 2008) but not in newborn infant neutrophils (Yost et al., 2009), suggesting that other cellular mechanisms downstream of ROS are involved in regulating NET formation. Histone H3 citrullination and NET formation were detected in PAD4+/+ but not in PAD4−/− neutrophils after 3 hr H2O2 treatment (Figure 5-5D; Table 5-1), indicating that PAD4 functions downstream of H2O2 stimulus during NET formation. Table 5-1 summarizes the efficacy of LPS, PMA, and H2O2 in inducing chromatin structure changes in neutrophils. Although each of the treatments elicited nuclear morphology changes and NET formation to certain extents, LPS was the most potent inducer for NET formation under current treatment conditions (Table 5-1). Furthermore, reverse transcription-PCR experiments showed that the depletion of PAD4 in neutrophils did not affect the expression of other active PAD family members, including PAD-1, -2, and -3 (Figure 5-6). Taken together, the above results indicate that PAD4 is important for NET formation in mouse neutrophils upon treatment with proinflammatory stimuli.
To analyze whether PAD4 is essential for NET formation after incubation with bacteria, we chose the pathogenic bacteria strain *Shigella flexneri*, which was previously shown to induce NET formation (Brinkmann et al., 2004). After priming neutrophils with IL-8 for 30 min and then incubating with *Shigella flexneri* for 2 hr as previously described (Brinkmann et al., 2004), histone citrullination was detected in 28.1±4.7% of PAD4+/+ neutrophils and formation of NETs was detected in 13.9±1.8% of PAD4−/− neutrophils (Figure 5-7A, arrows denote NETs, and circles denote bacteria), indicating that a fraction of neutrophils commits NET formation to kill bacteria extracellularly. In contrast, histone citrullination or NET formation was not detected in PAD4−/− neutrophils (Figure 5-7B). Immunostaining images at higher magnification showed that histone H3 citrullination antibody stained NETs that are in close contact with bacteria (denoted by open circles) (Figure 5-7C).

To test whether PAD4-mediated NET formation is important for bacterial killing, *Shigella flexneri* bacteria were incubated with IL-8 primed PAD4+/+ and PAD4−/− neutrophils for 30 min, and bacterial killing was analyzed by colony formation assays. Under conditions when both phagocytosis and NET formation were permitted, 54.6±6.6% and 33.6±2.2% of bacteria were killed by PAD4+/+ and PAD4−/− neutrophils, respectively (Figure 5-7D). When phagocytosis was inhibited by cytochalasin D, 33.6±4.5% and 10.5±3.5% of bacteria were killed by the PAD4+/+ and the PAD4−/− neutrophils, respectively (Figure 5-7D), demonstrating a significant decrease (n=4, p<0.001, Student’s t-test) in NET-mediated bacterial killing by PAD4−/− neutrophils than PAD4+/+ neutrophils. In contrast, when DNase I was added to disrupt NETs, PAD4+/+ and PAD4−/− neutrophils showed similar bacterial killing efficacy (Figure 5-7D), suggesting that phagocytosis pathway is not affected by PAD4 deletion. When both cytochalasin D and DNase I were used simultaneously, bacteria killing by both the PAD4+/+ and the PAD4−/− neutrophils was decreased to low and comparable levels (Figure 5-7D). Taken together, these data
indicate that PAD4 is important for *Shigella flexneri* killing mediated by NETs. Further, PAD4+/+ and PAD4−/− neutrophils uptake fluorescent bioparticles at comparable efficacy in phagocytosis analyses (Figure 5-8A-B), indicating that the loss of PAD4 does not affect phagocytosis.

5.4.4 Killing M1 GAS or M1 ΔSda1 GAS bacteria by neutrophils

Certain serotypes of group A *Streptococcus*, such as M1 GAS, cause an invasive infection disease called necrotizing fasciitis (Buchanan *et al.*, 2006). M1 GAS secretes an extracellular DNase (Sda1) that helps these bacteria evade trapping and killing by NETs (Buchanan *et al.*, 2006). We postulated that M1 GAS and M1 ΔSda1 GAS offer a unique system to test the role of PAD4 in mediating NET formation and bacterial killing *in vitro* and *in vivo*. Similarly as previously reported (Buchanan *et al.*, 2006), DNase activity was detected from the culture supernatant of M1 GAS (Figure 5-9A, lane 2) but not from M1 ΔSda1 GAS (Figure 5-9A, lane 3). Consistent with the notion that Sda1 helps M1 GAS disrupt the DNA backbone of NETs, M1 GAS induced histone H3 citrullination in PAD4+/+ neutrophils but NETs were rarely observed (Figure 5-9B, upper panels; Figure 5-9D, gray bars). In contrast, M1 ΔSda1 GAS induced both histone citrullination and NET formation in PAD4+/+ neutrophils (Figure 5-9C, upper panels; Figure 5-9D, black bars). Higher magnification images showed NET structures associated with bacteria after incubation of PAD4+/+ neutrophils with M1 ΔSda1 GAS (Figure 5-9E). Moreover, neither M1 GAS nor M1 ΔSda1 GAS induced histone citrullination or NET formation in PAD4−/− neutrophils (Figure 5-9B and 5-9C, lower panels), indicating that PAD4 is important for histone citrullination and NET formation induced by GAS.

Next, we tested the bacterial killing efficacy of M1 GAS or M1 ΔSda1 GAS by PAD4+/+ or PAD4−/− neutrophils (Figure 5-8C). Wild type M1 GAS bacteria were killed by PAD4+/+ and PAD4−/− neutrophils at low but comparable efficacy, while M1 ΔSda1 GAS was killed more
efficiently by PAD4<sup>+/+</sup> neutrophils than PAD4<sup>-/-</sup> neutrophils (Figure 5-8C, columns 1-4), suggesting that (1) NET-mediated bacterial killing by PAD4<sup>+/+</sup> neutrophils was inhibited by the DNase Sda1, and (2) PAD4<sup>-/-</sup> neutrophils have a decreased ability in killing M1 ΔSda1 GAS due to a lack of NET formation. Furthermore, when phagocytosis was inhibited by cytochalasin D, PAD4<sup>+/+</sup> neutrophils more effectively killed M1 ΔSda1 GAS but not M1 GAS than PAD4<sup>-/-</sup> neutrophils (Figure 5-8C, columns 5-8). In contrast, when NET formation was inhibited by DNase I treatment, PAD4<sup>-/-</sup> neutrophils could not more efficiently kill bacteria than PAD4<sup>-/-</sup> neutrophils (Figure 5-8C, columns 9-12). These results underscore the importance of PAD4 in NET-mediated bacterial killing of GAS and a dynamic contribution of NETs and phagocytosis in bacterial killing.

**5.4.5 PAD4<sup>-/-</sup> mice are more susceptible to infection by M1 ΔSda1 GAS**

To analyze the susceptibility of PAD4<sup>-/-</sup> mice to bacterial infection, we applied the mouse model of necrotizing fasciitis (Buchanan et al., 2006), in which M1 GAS or M1 ΔSda1 GAS bacteria were subcutaneously injected into paired PAD4<sup>+/+</sup> and PAD4<sup>-/-</sup> mouse siblings. In agreement with the in vitro bacterial killing results, PAD4<sup>+/+</sup> and PAD4<sup>-/-</sup> mice developed large lesions at three days after injection of 5x10<sup>6</sup> M1 GAS bacteria (Figure 5-10A, mouse left flanks). In contrast, PAD4<sup>+/+</sup> mice developed small lesions while PAD4<sup>-/-</sup> mice developed much larger lesions at three days after injection of 5x10<sup>6</sup> M1 ΔSda1 GAS bacteria (Figure 5-10A, mouse right flanks). The lesion areas were measured with the NIH Image J program. Compared to that in PAD4<sup>+/+</sup> mice, M1 ΔSda1 GAS formed ~4.03-fold larger lesions in PAD4<sup>-/-</sup> mice (P<0.001) (Figure 5-10B, black bars). Colony formation assays of bacteria recovered from necrotic lesion sites found that M1 ΔSda1 GAS proliferated ~3.97-fold more in PAD4<sup>-/-</sup> mice than in PAD4<sup>+/+</sup> mice (P<0.001) (Figure 5-10C, black bars). Skin lesions were also taken at two days after
infection and prepared for histology analyses. The lesion severity was scored from 0 (normal) to 4 (severe) based on the presence of neutrophils, edema, necrosis and skin ulceration following a previously described method (Mann et al., 2004). The difference was most pronounced with the M1 ΔSda1 GAS infections where the average pathology score for PAD4 /− mice was approximately 4 times greater than that of the PAD4 +/+ mice (data not shown). Representative photomicrographs of lesions from each group were shown (Figure 5-10D). These histopathological findings are in close agreement with the overall findings and colony formation assays.

5.4.6 Histone citrullination mediates NET formation to trap bacteria

Histones are highly enriched for positively charged Lys and Arg residues and have been found to effectively kill bacteria at low concentrations (Hirsch, 1958). To test the effects of Arg-Cit conversion on bacterial killing by histones, we treated free histone H3 or mononucleosomes with PAD4 and confirmed the histone citrullination by Western blotting (Figure 5-11A). In bacterial killing assays, we found that the citrullination of Arg residues of free histone H3 or nucleosomal histones decreased their efficacy of bacterial killing (Figure 5-11B). Taken together with the bacterial killing results by neutrophils, our results indicate that histone citrullination contributes to antibacterial function mainly by facilitating NET formation instead of increasing histone’s antibacterial activity.
5.5 Discussion

Although the role of histone hypercitrullination in NET formation was implicated in previous studies, it is now becoming clear that PAD4 is an important immune factor required for NET formation and NET-mediated anti-bacterial innate immunity. We have shown here that knockout of PAD4 in mouse genome abolishes both basal and inducible histone citrullination in peripheral blood neutrophils. This lack of histone citrullination is likely due to the absence of PAD4 since the depletion of PAD4 does not affect the expression of other active PAD family members. We have also found that PAD4 is necessary for the formation of NETs induced by LPS, PMA, H₂O₂, and bacteria in cell culture, and PAD4⁻/⁻ neutrophils possess significantly decreased activity in killing bacteria in vitro due to a lack of NET formation.

Interestingly, Buchanan et al. showed that certain bacterial pathogens have evolved a mechanism by secreting an extracellular DNase to escape killing and trapping by NETs to enhance their survival within the host (Buchanan et al., 2006). Using the necrotizing fasciitis model with Streptococcus strains of M1 GAS and M1 ΔSda1 GAS in PAD4⁺/⁺ and PAD4⁻/⁻ mice, we demonstrated that PAD4⁻/⁻ mice are more susceptible to bacterial infection than PAD4⁺/⁺ mice due to a lack of NET function.

Many pathological and genetic studies link PAD4 and protein citrullination in synovial fluid with rheumatoid arthritis (Yamada and Yamamoto, 2007). We envision that under chronic inflammatory conditions, PAD4 is released from neutrophils during NET formation inside joints, leading to citrullination of self-proteins thereby generating autoimmune antigens. As such, the decision for neutrophils to form NETs must be deliberately controlled. It has been observed that neutrophils in newborns lack the ability to form NETs (Yost et al., 2009), and only a certain percentage of neutrophils engage NET formation in mice and human neutrophils (Fuchs et al.,
Further study of how NET formation is regulated will help us understand more about this fascinating process of bacterial killing.
5.6 Acknowledgement

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We thank Dr. Ming Li for generating the knockout PAD4 ES cells, Micheal Lindberg for performing GST-mPAD4 activity analyses, Dr. Mary Kennett for performing histology analyses, and Dr. Na Xiong for assisting knockout mouse generation and flow cytometry analyses.

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Figure 5-1: PAD4 knockout mice generation.

(A) Schematic illustration of the replacement of the exon II in the PAD4 gene with FRT-site-flanked neomycin cassette. A Hind III restriction site nearby to exon II was also replaced.

(B) Replacement of exon II leads to an appearance of a 12.5 kb fragment in addition to the 4.5 kb fragment detected by the 5’ probe (upper panel) or the 7.5 kb fragment detected by the 3’ probe (lower panel) in Southern blot after digestion ES cell genomic DNA with Hind III.

(C) The PAD4 wild type and heterozygous mutant mice carrying the neomycin cassette were characterized using the allele specific PCR primers as illustrated.

(D) The wild type allele was detected in the heterozygous and the wild type mice. In contrast, the mutant allele was detected in the heterozygous but not the wild type mice.

(E) Schematic drawing of the PCR primers used to characterize wild type and knockout PAD4 locus with the exon II replaced by an FRT sequence.

(F) The wild type PCR product was detected in PAD4^{+/+} but not PAD4^{-/-} mice. In contrast, the knockout PCR product was detected in PAD4^{-/-} but not PAD4^{+/+} mice.

(G) The PCR product with the PAD4 exon II replaced by the FRT DNA fragment was sequenced. PAD4 intron I sequence, residual FRT sequence, and PAD4 intron II sequence were underlined.
Figure 5-1

A

Exons I II III XVI
(35kb)

(12kb)

III
III
III
III

III

4.5kb

7.5kb

12.5kb

5' probe

3' probe

(12.5kb)

5' probe

3' probe

B

Wild Type Knockout

12.5kb

4.5kb

5' probe

Wild Type Knockout

12.5kb

7.5kb

3' probe

C

WT

exon II

Neo

Neomycin

D

PAD4

+/-

+/

WT PCR

Neo PCR

E

WT

Exons I II III

PCR amplicon 1

KO

Exons I III

PCR amplicon 2

F

+/

-/

WT PCR

KO PCR

G

5'--TTGGTTGCCCAGTTTCCAGGAACGCCTCAATTGATGCATCCCCCACCAGGGG

PAD4 intron I

TGGCGGCCGAAGTTCATTTCTCTGAAAGTATAGGAAGATTCGACCTCGAAT

FRT sequence

AATCTCGATAGCACTAGTTACGAAAGATTTATCGAGGGGGGCCGGTATGTTGC

GGACTACGTGACTGCTAGCCTTTCTGCTCGGCCTCTAG--3'

PAD4 intron II
Figure 5-2: Knockout of PAD4 in mice did not prohibit neutrophil differentiation.

(A) Numbers of PAD4^{+/+}, PAD4^{+-}, and PAD4^{-/-} mice obtained from the PAD4 heterozygous mice cross. Chi-square test indicated that both PAD4^{+-} and PAD4^{-/-} mice were born at lower rate than predicted by the Mendelian ratio.

(B-C) Flow cytometry analyses of peripheral blood neutrophils in PAD4^{+/+} (B) and PAD4^{-/-} (C) mice using antibodies against neutrophil surface markers CD11b and Ly6G.

(D) Western blotting of PAD4 protein in PAD4^{+/+} and PAD4^{-/-} neutrophils. Mouse PAD4 was detected as a ~73 kDa protein in SDS-PAGE.

(E) Representative transmission electron microscopy images show that PAD4^{+/+} and PAD4^{-/-} neutrophils have similar morphologies. Arrows denote condensed chromatin underneath the nuclear envelope.
Figure 5-3: Histone citrullination activity of mouse PAD4.

(A) Mouse PAD4 and human PAD4 have a similar domain structure, including two IgL domains at the N-terminus and a catalytic domain at the C-terminus. Overall, the mouse and human proteins share 73% identity, with different degrees of identity among the three domains.

(B) Coomassie blue staining showed the amount of GST-mPAD4 (mouse) and GST-hPAD4 (human) expressed and purified from *E. coli* strain BL21.

(C) Western blotting of histone H3 citrullination (H3Cit) by GST-mPAD4 (mouse) and GST-hPAD4 (human). General H3 antibody was used to show the amount of H3 in each lane. Control experiments were performed using H3 incubated with GST.
Figure 5-4: PAD4 is required for histone citrullination and chromatin decondensation.

(A) Western blotting of histone citrullination in PAD4+/+ and PAD4−/− neutrophils before and after LPS treatment. General H3 antibody was used to show the amount of H3 in each lane.

(B) Histone citrullination and nuclear morphology in untreated neutrophils. A small number (~3.69%) of PAD4+/+ neutrophils showed robust histone H3 citrullination (H3Cit) staining before stimulation. DNA dye Hoechst staining was pseudo colored green. Decondensed chromatin was not observed before stimulation. Histone H3Cit or chromatin decondensation was not observed in PAD4−/− neutrophils before stimulation.

(C) LPS treatment induced histone citrullination and chromatin structural changes in PAD4+/+ neutrophils. DNA was pseudo colored green. Notice swelling nucleus (top panels) and chromatin elongation (middle panels). In contrast, LPS treatment did not induce histone citrullination or chromatin structural changes in PAD4−/− neutrophils (bottom panels).

(D) Histone citrullination and neutrophil elastase staining colocalized with decondensed chromatin stained by DNA dye in PAD4+/+ neutrophils after LPS treatment.
Figure 5-4

A

Control  LPS
PAD4^{+/+}  PAD4^{-/-}  PAD4^{+/+}  PAD4^{-/-}

WB: 
α-H3Citr
α-H3

B

Control
PAD4^{+/+}

PAD4^{-/-}

20 µm α-H3Citr
DNA
Merge

C

LPS treatment
PAD4^{+/+}

PAD4^{-/-}

20 µm α-H3Citr
DNA
Merge

D

LPS treatment
PAD4^{+/+}

α-H3Citr
α-Neutrophil elastase

DNA
Merge
Figure 5-5: PAD4 is required for chromatin decondensation and NET formation after chemokine treatment.

(A) The nuclear areas of PAD4^{+/+} neutrophils with swelling nuclei after LPS treatment were measured by NIH Image J program and compared with that of the control untreated nuclei.

(B) Effects of Cl-amidine treatment on NET formation. Although histone citrullination and NET formation were routinely observed in PAD4^{+/+} neutrophils after LPS treatment, pretreatment with PAD4 inhibitor Cl-amidine significantly decreased histone citrullination and chromatin decondensation in PAD4^{+/+} neutrophils after LPS treatment.

(C) PMA treatment induced histone citrullination and chromatin structural changes in PAD4^{+/+} neutrophils but not in PAD4^{-/-} neutrophils.

(D) H_{2}O_{2} treatment induced histone citrullination and chromatin structural changes in PAD4^{+/+} neutrophils but not in PAD4^{-/-} neutrophils.
Figure 5-5

A

Areas of PAD4+/+ neutrophils

Fold of Change

Ctrl | LPS

B

50 μm α-H3Cit | DNA | Merge

LPS treatment

PAD4+/+

50 μm α-H3Cit | DNA | Merge

Cl-amidine + LPS treatment

PAD4+/+

50 μm α-H3Cit | DNA | Merge

C

PMA treatment

PAD4+/+

α-H3Cit | DNA | Merge 20 μm

PAD4−/−

α-H3Cit | DNA | Merge 20 μm

D

H2O2 treatment

PAD4+/+

α-H3Cit | DNA | Merge 20 μm

PAD4−/−

α-H3Cit | DNA | Merge 20 μm
Figure 5-6: Knockout of PAD4 did not alter the expression of other active PAD family members in neutrophils.

Of the 5 PAD family members, PAD-1, -2, -3, and -4 encode active enzymes. Only PAD4 encodes a nuclear enzyme. PAD1 expression was undetectable in PAD4+/+ and PAD4−/− neutrophils. The expression of PAD2 and PAD3 was detectable in neutrophils but their levels were unchanged after PAD4 deletion. 18S rRNA was used to normalize the amount of total RNA for Reverse-transcription PCR.

**Figure 5-6**

<table>
<thead>
<tr>
<th>Neutrophil</th>
<th>RT-PCR</th>
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<tbody>
<tr>
<td>PAD4+/+</td>
<td>41 cycle</td>
</tr>
<tr>
<td>PAD4−/−</td>
<td>32 cycle</td>
</tr>
<tr>
<td>PAD1</td>
<td>35 cycle</td>
</tr>
<tr>
<td>PAD2</td>
<td>32 cycle</td>
</tr>
<tr>
<td>PAD3</td>
<td>28S rRNA</td>
</tr>
<tr>
<td>PAD4</td>
<td>18S rRNA</td>
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Figure 5-7: PAD4 is required for bacterial killing mediated by neutrophil extracellular traps.

(A-B) Incubation of IL-8 followed by *Shigella flexneri* bacteria induced histone citrullination and NET formation in PAD4<sup>+/+</sup> (A) but not PAD4<sup>−/−</sup> neutrophils (B). Arrows in (A) denote decondensed chromatin forming NETs. Circles in (A) and (B) highlight bacteria stained by the DNA dye.

(C) Higher magnification images showing H3 citrullination and DNA staining of decondensed chromatin associated with bacteria.

(D) Percentages of *Shigella flexneri* bacteria killed by PAD4<sup>+/+</sup> or PAD4<sup>−/−</sup> neutrophils, or neutrophils treated with cytochalasin D, DNase I, or both cytochalasin D and DNase I before incubation with bacteria. P-values were determined by Student’s t-test.
Figure 5-8: PAD4 is not required for phagocytosis but is important in NET-mediated innate immunity against group A Streptococcus (GAS) bacteria.

(A) The loss of PAD4 does not affect neutrophil phagocytosis. Both PAD4$^{+/+}$ and PAD4$^{-/-}$ neutrophils show similar capabilities in phagocytosis assay.

(B) Percentages of neutrophils that are positive for phagocytosis signals. Standard deviations are obtained from four independent repeat experiments.

(C) Wild type M1 GAS bacteria were killed by PAD4$^{+/+}$ and PAD4$^{-/-}$ neutrophils at comparable levels, while M1 $\Delta Sda1$ GAS bacteria were killed more efficiently by PAD4$^{+/+}$ neutrophils than PAD4$^{-/-}$ neutrophils, indicating that the DNase Sda1 helps GAS escape killing mediated by NETs. After cytochalasin D treatment to inhibit phagocytosis, M1 $\Delta Sda1$ GAS bacteria were killed more efficiently by PAD4$^{+/+}$ than PAD4$^{-/-}$ neutrophils. In contrast, after DNase I treatment to inhibit NET-mediated bacterial killing, PAD4$^{+/+}$ neutrophils failed to kill M1 $\Delta Sda1$ GAS bacteria at higher efficacy than PAD4$^{-/-}$ neutrophils. Standard deviations are obtained from four independent experiments. P-values were determined by Student’s t-test.
Figure 5-8

A

PAD4⁺/⁺

Counts

FITC

PAD4⁻/⁻

Counts

FITC

B

Phagocytosis Assay

Phagocytosis Percentage (%)

PAD4⁺/⁺  PAD4⁻/⁻

C

% Bacteria Killing

M1 GAS  WT  ΔSda1  WT  ΔSda1  WT  ΔSda1

Cytochalasin D  -  -  - -  +  +  - -

DNase I  -  -  - -  -  +  +  +  +
Figure 5-9: Antagonism of PAD4-mediated NET formation and bacterial extracellular DNase-mediated NET destruction.

(A) Genomic DNA (untreated in lane 1) was incubated with cell culture supernatant from wild type M1 GAS (lane 2) or M1 ΔSda1 GAS (lane 3). DNA degradation by DNase Sda1 was observed (lane 2).

(B) Upon incubation of M1 GAS with PAD4+/+ neutrophils, histone H3 citrullination was detected but NETs were rarely observed by immunostaining (top panels). In contrast, histone H3 citrullination or NETs were not detected after incubation of M1 GAS with PAD4−/− neutrophils (lower panels).

(C) Both histone H3 citrullination and NETs were detected after incubation of M1 ΔSda1 GAS with PAD4+/+ neutrophils (top panels, arrows denote NETs). In contrast, histone H3 citrullination or NETs were not detected after incubation of M1 ΔSda1 GAS with PAD4−/− neutrophils (lower panels).

(D) Percentages of PAD4+/+ neutrophils with H3 citrullination staining or with both H3 citrullination and NET formation after incubation with M1 GAS or M1 ΔSda1 GAS were analyzed. The presence of Sda1 decreased NET formation by ~4.2-fold (p<0.003 by Student’s t-test). All NETs were positive for the H3 citrullination antibody staining.

(E) Higher magnification images show NETs formed in PAD4+/+ neutrophils after incubation with M1 ΔSda1 GAS (arrows denote NETs).
Figure 5-9

A

B

C

D

E

**Neutrophils (PAD4+/+)**

<table>
<thead>
<tr>
<th></th>
<th>M1</th>
<th>M1 Δsda1 (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 H3Cit</td>
<td>25</td>
<td>30</td>
</tr>
<tr>
<td>2 H3Cit</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>3 H3Cit &amp; NETs</td>
<td>5</td>
<td>3</td>
</tr>
</tbody>
</table>

p<0.003
Figure 5-10: PAD4 is important in immune defense against group A *Streptococcus* (GAS) in a mouse model of necrotizing fasciitis.

(A) In necrotizing fasciitis assays, M1 GAS but not M1 ΔSda1 GAS induced large lesions in PAD4+/+ mice (two representative PAD4+/+ mice shown in left panels). In contrast, both M1 GAS and M1 ΔSda1 GAS formed large lesions in PAD4−/− mice (right panels).

(B) Lesion size was measured using the NIH Image J program. The size of lesion formed by M1 ΔSda1 GAS in PAD4−/− mice increased ~4.03-fold compared to PAD4+/+ mice (P<0.001 by Student’s t-test).

(C) The number of bacteria recovered from lesion formation site was analyzed by colony formation assays. The number of M1 ΔSda1 GAS bacteria recovered from PAD4−/− mice increased ~3.97-fold compared to PAD4+/+ mice (P<0.001 by Student’s t-test).

(D) Representative photomicrographs of skin lesions from PAD4+/+ and PAD4−/− mice infected with M1 GAS and M1 ΔSda1 GAS. Arrows depict neutrophilic infiltrates and the star highlights intact epithelium that is absent in the other sections. Magnification is 100x. Scale bars, 200 µm.
Figure 5-10

A

<table>
<thead>
<tr>
<th>PAD4 (^{+/+})</th>
<th>PAD4 (^{-/-})</th>
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<tbody>
<tr>
<td>M1</td>
<td>M1 (\Delta Sda1)</td>
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</table>

B

<table>
<thead>
<tr>
<th>PAD4</th>
<th>M1</th>
<th>M1 (\Delta Sda1)</th>
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<tbody>
<tr>
<td>(+/+)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>(-/-)</td>
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<td></td>
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</table>

C

![Bar chart showing colony formation units](chart_c.png)

D

![Histological images](image_d.png)
Figure 5-11: Histone citrullination is important to mediate NET formation to trap bacteria.

(A) Western blotting of histone citrullination of histone H3 or mononucleosome before and after PAD4 treatment. General H3 antibody was used to show the amount of H3 in each lane.

(B) Percentages of Shigella flexneri bacteria killed by histone H3, citrullinated histone H3, mononucleosome, and citrullinated mononucleosome at the concentration indicated.

**Figure 5-11**

A

<table>
<thead>
<tr>
<th>GST-PAD4</th>
<th>Histone H3</th>
<th>Mononucleosome</th>
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<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

17 kDa  
α-H3Cit

17 kDa  
α-H3

Ponceau S

B

[Graph showing percentage of bacteria killing against concentration (µg/ml).]
Table 5-1: Percentages of mouse neutrophils with positive histone citrullination, increased nuclear size, and NET formation after treatment with LPS, PMA, and H$_2$O$_2$.

<table>
<thead>
<tr>
<th></th>
<th>PAD4$^{+/+}$</th>
<th>PAD4$^{-/-}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Histone citrullination</td>
<td>Enlarged nucleus$^{1,2}$</td>
</tr>
<tr>
<td>Control</td>
<td>3.7±0.3%$^5$</td>
<td>U.D.$^6$</td>
</tr>
<tr>
<td>LPS</td>
<td>42.3±3.9%$^5$</td>
<td>23.4±2.7%$^5$</td>
</tr>
<tr>
<td>Cl-amidine$^7$ → LPS</td>
<td>8.1±1.3%$^5$</td>
<td>1.2±0.2%$^5$</td>
</tr>
<tr>
<td>PMA</td>
<td>48.5±3%$^5$</td>
<td>10.9±0.3%$^5$</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>48.3±5.4%$^5$</td>
<td>13.4±1.4%$^5$</td>
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</tbody>
</table>

$^1$Nucleus is scored as an enlarged nucleus if its nuclear diameter is over 1.5-fold larger than a regular neutrophil, which is ~10 μM in diameter.

$^2$Enlarged nuclei were found to be histone citrullination positive.

$^3$NET formation is scored if chromatin has extruded and elongated from the nucleus and is confirmed by neutrophil elastase staining after LPS treatment.

$^4$NETs were found to be histone citrullination positive.

$^5$Averages and standard deviations were shown (three independent experiments, over 500 cells were counted from each experiment).

$^6$U.D., undetectable.

$^7$200 μM Cl-amidine was used to treat neutrophils for 30 min prior to LPS treatment for 3 hr.
Chapter 6

Conclusion and Discussion
PAD4 is an enzyme capable of converting arginine residue to citrulline, an unconventional amino acid. Many reports indicate that PAD4 is involved in the autoimmune disease rheumatoid arthritis, while others and us have found that PAD4 plays a role in transcriptional regulation (Cuthbert et al., 2004; Vossenaar et al., 2003b; Wang et al., 2004b). PAD4 is able to use histones as substrates and produce citrulline from arginine and monomethyl-arginine through reactions termed deimination/citrullination or demethylimination, respectively. Overexpression of PAD4 has been detected in a wide range of cancer tissues, suggesting that PAD4 is involved in tumorigenesis (Chang and Han, 2006; Chang et al., 2009). We obtained several human cancer cell lines in the lab, all of which show high and comparable levels of PAD4 expression (Figure 6-1). However, the mechanism through which PAD4 facilitates cancer cell growth is still poorly understood. This dissertation investigated the role of PAD4 and PAD4 catalyzed histone citrullination in the transcriptional regulation of p53-target genes. PAD4 is recruited to the p53-target gene promoter in a p53-dependent manner, while RNA Pol II activity and PAD4 association at the p21 promoter is dynamically regulated after UV irradiation. It is also found that PAD4 collaborates with HDAC2 in repressing tumor suppressor gene expression. PAD4 and HDAC2 associate with the p21 promoter simultaneously, and they both dissociate from several p53-target gene promoters after DNA damage. Chromain immunoprecipitation experiments further revealed that PAD4 promoter association and histone citrullination levels are dependent on both p53 and HDAC activity, but HDAC2 promoter association and histone Lys acetylation are only slightly affected by p53 and PAD4 activity. Application of both PAD4 inhibitor Cl-amidine and HDAC inhibitor SAHA induced p53-target gene expression and inhibited cancer cell growth additively, suggesting that PAD4 might be a promising target in cancer treatment. Furthermore, in order to study the biological role of PAD4, we generated knockout PAD4 mice as a genetic model. It is found that neutrophils from PAD4 knockout mice cannot form neutrophil extracellular traps after stimulation with chemokines and
bacteria, and lack NET-mediated anti-bacterial ability, supporting the essential role of PAD4 and histone citrullination in innate immunity.

The repression of p53-target gene expression mediated by PAD4 and histone citrullination turned out to be p53-dependent. PAD4 does not contain a DNA binding domain so it has to interact with p53 in order to be recruited to its target gene promoter. After DNA damage such as UV irradiation, a dynamic change of PAD4 binding at the p53-target gene promoter is observed, i.e., PAD4 dissociates from the promoter initially, but returns to the promoter at later time points after DNA damage. Interestingly, the pattern of PAD4 association at the p21 promoter after UV irradiation differs from that of p53, which consistently increases its association with the p21 promoter. One possibility is that p53 recruited to the promoter after DNA damage has a low affinity for PAD4 binding, but a highly affinity for binding transcriptional coactivators, such as histone arginine methyltransferases and lysine acetylases. The dissociation of PAD4 and the recruitment of PRMTs would result in the increase of Arg methylation as well as the activation of p53-target genes. This idea is supported by a ChIP experiment showing that PRMT is recruited to p53-target gene promoter after UV irradiation (An et al., 2004). However, the molecular mechanisms controlling the affinity of p53 with PAD4 or PRMT as cofactors in transcriptional regulation remains largely unknown.

Besides recruiting cofactors to target gene promoters to modify histones, p53 itself could be modified by methylation, acetylation, phosphorylation, etc (Kruse and Gu, 2009). The posttranslational modifications on p53 have been found to play a role in recruiting cofactors to regulate target gene expression (Barlev et al., 2001). Our preliminary results found that when Lys 373 and Lys 382 at p53 C-terminal domain are mutated to glutamine to mimic acetylation, the ability of p53 to bind to PAD4 decreased dramatically (Figure A-1b). Peptide pull-down assay using unmodified or the Lys 373, 382 acetylated p53 C-terminal peptide showed that unmodified peptide has a much higher efficacy in binding PAD4 compared with acetylated peptide (Figure A-
1c), suggesting that acetylation of p53 likely regulates its affinity for PAD4 binding. More in vivo examination such as chromatin immunoprecipitation experiments could be conducted to further test this idea and to analyze whether p53 acetylation plays a negative role in the interaction between PAD4 and p53. Since p53 acetylation has been found to facilitate the binding of coactivators (Barlev et al., 2001), I speculate that p53 acetylation serves as a molecular switch in transcriptional activation by excluding the binding of corepressors while recruiting coactivators to its target gene promoters.

Besides being recruited by p53 to target gene promoters and citrullinating histones, PAD4 showed the ability to citrullinate p53 in in vitro experiments (Figure C-1a). Point mutation study suggested p53 Arg 363 and 379 could be two possible residues that can be citrullinated by PAD4. We generated α-p53 citrullination antibody against a p53 Cit379 peptide. Our preliminary data using this p53 Cit379 antibody found that p53 citrullination also exists in vivo. Thus, citrullination might be another novel posttranslational modification occurring on p53. However, the biological significance of p53 citrullination is still mysterious and worth of further pursue.

Multiple histone modifying enzymes often occupy the same promoter to achieve efficient activation or repression, which is described as “histone modification crosstalk”. PRMTs and HATs have been found to cooperatively regulate p53-mediated transcriptional activation with histone acetlyation catalyzed by p300/CBP precedes histone Arg methylation catalyzed by PRMT4 (An et al., 2004). In reverse, our results suggest that PAD4 and HDAC2 could associate with p53-target gene promoter simultaneously and repress its expression, thus establishing a novel link between citrullination and deacetylation to achieve efficient gene regulation. Interestingly, ChIP experiments found that knockdown or inhibition of HDAC2 greatly decreased the binding of PAD4 and the histone H3 citrullination level at p53-target gene promoters, suggesting that the association of PAD4 with p53-target gene promoters is dependent on HDAC2 activity. On the other hand, depletion or inhibition of PAD4 has little effect on the binding or the
activity of HDAC2 and Lys acetylation. Therefore, it is possible that PAD4 and HDAC2 also possess similar ordered cooperative functions to repress transcription, by which histone deacetylation facilitates histone citrullination, but not *vice versa*. Another possible scenario to explain the HDAC2-dependent PAD4 association could be that HDAC2 also deacetylates p53, which increases the affinity of PAD4 for p53. As mentioned earlier, p53 acetylation decreases p53 and PAD4 interaction. The recruitment of HDAC2 deacetylates p53 thereby promoting the association of PAD4 to p53-target gene promoters through interaction with p53. In sum, we proposed a model for the coordination of PAD4 and HDAC2 in regulating p53-target gene expression (Figure 6-2). In cells without DNA damage, both PAD4 and HDAC2 associate with p53-target gene promoter, maintain histone Arg methylation and Lys acetylation at a low level. When cells receive DNA damage signal, the recruitment of more p53 brings in transcriptional coactivators such as PRMTs and HATs, which would cause the dissociation of PAD4 and HDAC2 and increase of Arg methylation and Lys acetylation, and induce target gene expression.

HDAC2 belongs to the type I HDAC family and shares high homology with another family member HDAC1. HDAC1 and HDAC2 are often included in the same protein complex such as Sin3A/NuRD and have many overlapping functions (Zhang *et al.*, 1999). But some previous work using p21 as a model gene suggests that HDAC2 plays a unique role in the repression of p21 (Harms and Chen, 2007; Huang *et al.*, 2005a). We found that PAD4 collaborates with HDAC2 in the regulation of p53-target genes. Another recent report indicates that PAD4 also works with HDAC1 to repress the estrogen-regulated pS2 promoter (Denis *et al.*, 2009). It is possible that different transcriptional factors may choose a different set of cofactors to regulate their target gene expression, or other cofactors involved in the same protein complex could affect the choice of HDAC enzymes that work with PAD4.

Carcinogenesis is caused by the activation of the oncogenes and the repression of tumor suppressor genes. More evidence has shown that epigenetic modifications play essential roles in
the deregulation of tumor suppressor genes resulting in tumor generation. DNA methylation inhibitors and HDAC inhibitors have been used in cancer therapy (Esteller, 2005; Marks and Breslow, 2007). We have found that the combination of the PAD4 inhibitor Cl-amidine and the HDAC inhibitor SAHA has an additive effect in inhibiting cancer cell growth by inducing the expression of p53-target genes involved in cell growth arrest and apoptosis. SAHA has been approved by US Food and Drug Administration for clinical treatment of T cell cutaneous lymphoma (Marks and Breslow, 2007). Thus, PAD4 could be another epigenetic target to treat cancer through inhibiting its catalytic activity. One concern now is the bioavailability of Cl-amidine. Since Cl-amidine is hydrophilic, it is hard to pass through the cell membrane to its target proteins, which leads to the requirement of high concentration to effectively inhibit cancer cell growth. Compare with SAHA that only needs several micromolar to be effective, we are currently using Cl-amidine at several hundred micromolar concentration. Thus, an improved PAD4 inhibitor with a higher bioavailability would have more potential to become a powerful drug for anti-tumor therapy.

Given the role of PAD4 in repressing p53-target gene expression, it would be perceivable that knockout PAD4 mice would have a shorter life span because of the increased expression of cell cycle arrest and apoptosis genes after PAD4 depletion. However, knockout PAD4 mice do not die earlier than wild type mice and no physical abnormality is detected. This could be due to the tissue specific expression PAD4. PAD4 is highly enriched in white blood cells compared with other types of cells such as fibroblasts. It is very possible that the depletion of PAD4 only affects the function of particular types of cells. Interestingly, heterozygous and homozygous PAD4 knockout mice are born at a lower rate than predicted by the Mendelian ratio, suggesting that PAD4 may play a role in the early development of the mouse embryo.

Previously, neutrophils are known to migrate to the infection site and engulf the invading pathogens through phagocytosis or release multiple antimicrobial molecules to kill pathogens.
The formation of neutrophil extracellular traps (NETs) is a newly discovered mechanism utilized by neutrophils to fight against invading pathogens. NETs provide a fast and efficient way to eliminate pathogens that are too large for phagocytosis by releasing the intracellular contents of neutrophils into the extracellular space so as to trap and kill pathogens. The major component of NETs is extremely decondensed chromatin (Brinkmann et al., 2004). However, the key regulators involved in the chromatin decondensation during NET formation are poorly understood. Using PAD4 knockout mice as a genetic model, we have found that PAD4 and histone citrullination play a key role in NET formation and bacterial killing mediated by NETs. Histone citrullination neutralizes the positive charge on the Arg residue of the chromatin, and was previously implicated in chromatin decondensation (Wang et al., 2009). My work offered the first evidence that PAD4 is also essential in anti-bacterial function of NETs. Comparing the bacterial killing efficacies of citrullinated and uncitrullinated histones or mononucleosomes, we suggest that histone citrullination contributes to antibacterial function by facilitating NET formation but not by increasing the antibacterial activity of histones. A detailed mass spectrometry analysis on the NETs constituents found that the four types of core histones are included in the NETs, but linker histone H1 is not (Urban et al., 2009). Linker histone H1 is believed to participate in further assembly of compact chromatin after the nucleosomes have formed. It is very possible that histone citrullination could work with other yet-to-be identified factors to release H1 from NETs.

There are still many questions related to NETs that need to be answered. For instance, there is only a portion of neutrophils that form NETs. When neutrophils encounter invading pathogens, what is the upstream signal that determines whether an individual neutrophil should fight against pathogens by phagocytosis or NETs? Moreover, the autoimmune diseases (e.g., rheumatoid arthritis) could result from the release of PAD4 during NET formation from neutrophils that enter synovial joints in the chronic inflammatory conditions. Hence, the regulation of NETs would be an interesting question that can be further explored.
To sum up, I propose a model regarding the biological roles of PAD4 in transcriptional regulation and innate immunity, respectively (Figure 6-3). PAD4 and histone citrullination have two separate roles in transcriptional regulation and innate immunity. Overexpression of PAD4 in normal tissue would lead to the repression of tumor suppressor gene expression and carcinogenesis; the absence of PAD4 in neutrophils would result in immunity-defect and severe pathogen infection, and the deregulation of PAD4 in neutrophil may result in autoimmune deseases. As such, the protein level of PAD4 requires exquisite control in order to maintain whole body wellness.
Figure 6-1: PAD4 protein levels in different human cancer cell lines.

Western blot detected high and comparable levels of PAD4 protein expressed in different human cancer cell lines which are used in the study.

**Figure 6-1**
Figure 6-2: A model of the cooperative regulation of p21 expression by PAD4 and HDAC2.

Before DNA damage, PAD4 and HDAC2 are associated with the p21 promoter to catalyze histone citrullination, histone and p53 deacetylation, respectively, thereby maintaining a low level of p21 expression. In contrast, after DNA damage, PAD4 and HDAC dissociate. p53 as well as coactivator (e.g., PRMT1, 4, and p300/CBP) binding increases at the p21 promoter. Histone Arg methylation and Lys acetylation as well as p53 acetylation increase to facilitate p21 activation.
Figure 6-3: A model of the biological roles of PAD4 in innate immunity and transcriptional regulation.

PAD4 expressed in neutrophils is important for host innate immunity, PAD4 is able to deiminate/citrullinate histone Arg to facilitate neutrophil extracellular traps formation for eliminating invading pathogens; deregulation of PAD4 in the joints may result in autoimmune diseases such as rheumatoid arthritis. On the other hand, PAD4 also involves in transcriptional regulation to demethylimize methyl-Arg at promoter histones so as to repress target gene transcription; overexpression of PAD4 in normal tissues could lead to carcinogenesis by repressing tumor suppressor genes.

Figure 6-3
Appendix A

p53 Acetylation Impedes p53 and PAD4 Interaction

Multiple Lys residues in the C-terminus of p53 are acetylated by HAT such as p300/CBP and PCAF, including Lys373 and 382 (Figure A-1a) (Brooks and Gu, 2003; Ito et al., 2001). Previous studies show that p53 acetylation at K382 provides a binding site for the histone acetyltransferase p300/CBP (Barlev et al., 2001; Espinosa and Emerson, 2001; Mujtaba et al., 2004), and that p53 dual acetylation at K373 and K382 provides a binding site for TAF1 (Li et al., 2007a).

Since p53 acetylation is correlated with gene activation, we hypothesize that p53 acetylation may attenuate its interaction with PAD4. To test this idea, we first generated p53 Lys to Gln point mutations (K373, 382Q) in GST-p53301-393 construct. Compared with the wild type fusion protein, GST-p53301-393 (K373, 382Q) mutant had much decreased PAD4 binding (Figure A-1b).

To further test the effects of p53 acetylation on p53 and PAD4 interaction, we performed peptide pull-down experiments with two p53 peptides (p53361-393 and p53361-393 (K373, 382Ac)). These two peptides are both N-terminal biotinylated but differ from their acetylation status at K373 and K382. Unmodified p53361-393 peptide bound PAD4 while the dual acetylated p53361-393 (K373, 382Ac) did not (Figure A-1c).

These results suggest that simultaneous acetylation of K373 and K382 at the p53 C-terminus strongly attenuates the p53 and PAD4 interaction. In fact, multiple Lys residues of the p53 C-terminus, including K370, 372, 373, 381, and 382, are acetylated by p300/CBP. Thus, it is possible that the acetylation of p53 by p300/CBP initiates two events at the same time. One is to dissociate a corepressor PAD4 and the other is to recruit coactivators (e.g., CBP or TAF1). Whether other factors are involved in p53 and PAD4 interaction will be tested in future.
Figure A-1: p53 acetylation attenuates its interaction with PAD4.

(a) Schematic drawing of p53 structure domains and possible acetylation sites at its C-terminus.

(b) GST pull-down experiments showed when K373, 382 in the p53\textsuperscript{301-393} were converted to Gln, the binding of this fusion protein to PAD4 was decreased (compare lane 6 to lane 5).

(c) Peptide pull-down experiments showed that the acetylation at K373 and K382 abolished the interaction of the peptide with PAD4, suggesting that acetylation at these two Lys residues decreases interaction between p53 and PAD4.
Appendix B

Chromatin Reconstitution and In Vitro Transcription

B.1 Materials and Methods

B.1.1 Chromatin reconstitution using the Drosophila S-190 extract

1) Canton-S wild-type flies are grown at 25°C at 70-80% humidity in population cages. Collect embryos on molasses-agar plates covered with yeast.

2) Four batches of embryos [30-50 g, collected (every 6 hr over a 24-hr period) between 0 and 6 hr after fertilization and then stored for less than 18 hr at 4°C] were harvested in nylon mesh with water.

3) Embryos are dechorionated by immersion for 90 sec in 1:1 of bleach (5.25% sodium hypochlorite)/water at room temperature.

4) Embryos are quickly rinsed with 1 L Embryo wash buffer I (0.7% NaCl, 0.04% Triton X-100, store at room temperature). Then washed with water at room temperature and transferred to an 800-ml beaker in an ice bath.

5) Add 500 ml of Embryo wash buffer II (0.7% NaCl, 0.05% Triton X-100, store at 4°C) to the beaker. Suspend embryos with a glass rod and let embryos settle to the bottom of beaker for about 2 min.

6) Remove the cloudy suspension above the embryos, which contains chorion particles and other debris, by aspiration.

7) Repeat step 5 and 6 once with 500 ml Embryo wash buffer II, twice with 500 ml 0.7% NaCl solution (store at 4°C), and once with 500 ml Buffer R (10 mM HEPES-KOH, pH 7.5, 10
mM KCl, 1.5 mM MgCl₂, 0.5 mM EGTA, 10% glycerol, 10 mM β-glycerophosphate, 1 mM DTT, 0.2 mM PMSF, store at 4°C). In the final wash, the embryos take about 10 min to settle.

8) Transfer the embryos to a Dounce homogenizer and homogenize for 15 strokes with loose pestle followed by 40 strokes with tight pestle.

9) Centrifuge the homogenate at 8,000 rpm for 5 min at 4°C. Collect the cloudy, yellow cytoplasmic fraction with a syringe, avoid the white layer at the top and the pellet at the bottom.

10) Add 1 M MgCl₂ to increase the Mg²⁺ concentration to 7 mM.

11) Centrifuge the extract at 192,000 g (45,000 rpm using Beckman SW55 rotor) for 2 hr at 4°C. Remove the white upper layer with a spatula and collect the yellow-brown liquid.

12) Snap freeze the extract in liquid nitrogen, thawed with water at room temperature, and centrifuge again at 192,000 g for 2 hr at 4°C.

13) Collect the supernatant as the Drosophila S-190 extract and snap freeze in liquid nitrogen.

14) Store the S-190 extract at -80°C, it can remain active for more than 1 year.

15) To reconstitute chromatin, Incubate 7 mg (about 275 µl) S-190 extract with 2-3 µg core histones and histone H1 (when desired) in buffer R in a total volume of 450 µl for 30 min at 27°C.

16) Add 5 µg plasmid DNA, 3 mM ATP, 30 mM creatine phosphate, 1 µg/ml creatine kinase to the above mixture. Adjust the MgCl₂ concentration to 7 mM and bring the total volume to 500 µl.

17) Incubate the reaction for 5 hr at 27°C.

18) Optional: To check the efficiency of chromatin reconstitution, adjust the concentration of CaCl₂ in the reaction to 3 mM. Add appropriate amount of micrococcal nuclease to the sample (experiment has been done with 0-80 mU in 250 µl volume), digest for 10 min at 25°C. Terminate the reaction with 20 mM EDTA. Sequentially treat samples with RNase A and
Protease K. Purify DNA with phenol-chloroform extraction and ethanol precipitation.

Analyze the DNA sample by 1.5% agarose gel electrophoresis.

19) Terminate the reaction by adding EDTA to a final concentration of 20 mM.

20) To purify the reconstituted chromatin, load 3-4 ml of sample on top of 30-50% sucrose gradient (10 mM HEPES-KOH, pH 7.5, 1 mM EDTA, 35 mM NaCl, with proteinase inhibitors in 30-50% sucrose). Centrifuge at 26,000 rpm (use Beckman SW41 rotor) for 16 hr at 4°C. Collect every 1 ml as a fraction, use 40 µl to purify DNA and run 1% agarose gel electrophoresis to determine the location of the reconstituted chromatin.

B.1.2 In vitro transcription

In vitro transcription assay is performed following the published protocol (An and Roeder, 2004) with necessary modifications.

B.2 Results

B.2.1 PAD4 represses p53 transcription using a DNA template in in vitro transcription assay

We used naked pG5ML plasmid containing a tandem of five p53 binding sites in front of a TATA box and a G-less cassette (Figure B-1a). This plasmid DNA template has been used previously to study p53 transcription (An et al., 2004). We purified His6-FLAG-p53 from E. Coli strain BL21, and used Hela nuclear extracts for PIC assembly and transcriptional elongation. The scheme in Figure B-1b was used to perform the transcriptional analyses. The addition of p53 increased the transcription by ~15 folds (Figure B-1c). Because PAD4 activity requires calcium, we tested the effects of calcium alone, calcium and PAD4, as well as calcium and the enzymatic
inactive PAD4$^{C645S}$ mutant on p53-mediated transcription. Although adding calcium alone or calcium with the PAD4$^{C645S}$ mutant all had some inhibitory effects, GST-PAD4 treatment reduced the transcription level by three fold compared with p53 alone, suggesting that PAD4 can interfere with other processes of the p53 transcription besides modifying histones.

**B.2.2 Chromatin Reconsitution using the *Drosophila* S-190 extracts**

We successfully reconstitute high quality chromatin using the *Drosophila* S-190 extracts. Micrococcal nuclease digestion indicates the reconstituted chromatin has well-positioned nucelosomes on the plasmid DNA (Figure B-2). *In vitro* transcriptional assay using this reconstituted chromatin to study the effects of histone modifying enzymes (such as PAD4 and HDAC2) on p53-target gene transcription will be pursued.
Figure B-1: PAD4 represses p53 transcription on naked DNA template.

(a) Schematic drawing of the pG3ML plasmid used to perform in vitro transcription assays.

(b) The scheme of the in vitro transcription procedure.

(c) Analyses of the role of PAD4 in p53 transcription on naked DNA template.
Figure B-2: The formation of well-spaced nucleosomes on the pG3ML plasmid DNA by micrococcal nuclease digestion.
Appendix C

p53 Citrullination by PAD4 *in vitro* and *in vivo*

Numerous residues of p53 are post-translationally modified in various ways, including phosphorylation, acetylation, ubiquitination, Lys methylation, etc. The modifications of p53 regulate the stability of p53, the p53 binding to DNA, the interaction of p53 with other protein factors (Brooks and Gu, 2003). There is evidence supports that p53 acetylation by Tip60 is required for p53 to activate proapoptotic gene expression (Tang *et al.*, 2006). Additionally, p53 modifications also regulate each other in a similar way as histone modification “cross-talk” (Ivanov *et al.*, 2007). Together, p53 modifications impact on many aspects of p53 function as a transcriptional regulator and as a tumor suppressor.

Because PAD4 interacts with p53 and represses p53 transcription on a DNA template (Figure B-1c), we tested whether PAD4 directly modifies p53. Because many of p53 modification sites are clustered at the p53 C-terminus, we first tested whether this part of the p53 protein can be citrullinated. His6-p53\(^{301-393}\) fusion protein was purified and treated by GST-PAD4 in the presence of calcium and DTT. Western blotting using α-Mod-Cit antibody, we found that p53 was citrullinated (Figure C-1a). To identify Arg residue(s) that are citrullinated by PAD4, we converted Arg residues to Lys by point mutation (Arg residues showed in Figure A-1a in red). Because Lys cannot be converted to Cit, we expect that deletion of PAD4 target Arg residues will decrease the amount of citrullination signals on Western blotting. Using this strategy, the p53 target sites were narrowed down to two Arg residues (R363 and R379) (Figure C-1b, lane 1). Mutation of either R363 or R379 greatly decreased the amount of citrullination as determined by Western blotting (Figure C-1b, compare lanes 3 and 5 with lane 1), suggesting that these two Arg residues can both be citrullinated by PAD4.
PAD4 is known to target many Arg residues for citrullination in histones (Wang et al., 2004b), which suggests PAD4 has a low site preference. In order to test whether p53 could be citrullinated by PAD4 in vivo, we generated an α-p53 citrullination polyclonal antibody against a p53 Cit379 peptide [KGQSTS(Cit)HKKLMF]. The antibody showed high preference toward the citrullinated p53 peptide but not the unmodified p53 peptide (Figure C-1c). We further used this antibody to test whether the activation of PAD4 with calcium ionophore in MCF-7 cells can increase p53 citrullination. As shown in Figure C-1d lane 2, more p53 citrullination was detected in MCF-7 cells after the calcium ionophore treatment. The biological effects of p53 citrullination will be further investigated.
Figure C-1: p53 citrullination by PAD4 *in vitro* and *in vivo*.

(a) GST-PAD4 citrullination of the p53 C-terminal regulatory domain (lane 3). Histone H3 was used as a positive control (lane 1).

(b) To map the p53 Arg residue(s) that can be citrullinated, we first convert R306, 333, 335, 337, and 342 to K. When only R363 and R379 are present, the His6-p53<sup>301-393</sup> fusion protein was still citrullinated by GST-PAD4 (lane 1). When either R379 or R363 was converted to K, the amount of citrullination was greatly reduced (lane 3 and lane 5).

(c) Generation of the p53 Cit379 antibody. The antibody showed specificity to the citrullinated peptide but not the unmodified peptide in dot blot analyses.

(d) Detection of p53 citrullination in MCF-7 cells after calcium ionophore treatment to activate PAD4. Ponceau S staining showed the amount of protein loaded in each lane.
Appendix D

**PAD4 Activity is Important for Chromatin Decondensation in HL-60 Cells**

To test whether PAD4 activity is important for chromatin decondensation, we treated HL-60 granulocytes with Cl-amidine prior to calcium ionophore treatment. Double staining of HL-60 granulocytes with the H4Cit3 antibody and the DNA dye Hoechst was performed to analyze histone citrullination and the formation NET-like chromatin structure. Without calcium ionophore treatment, each cell had only weak H4Cit3 staining and did not display NET-like chromatin structure (Figure D-1a). After calcium ionophore treatment, ~36% of cells were strongly stained with the H4Cit3 antibody (Figure D-1b). Notably, over 8% of cells formed NET-like chromatin structures (Figure D-1b, denoted by arrows). In contrast, Cl-amidine treatment for 15 min prior to calcium ionophore treatment decreased the number of cells showing positive H4Cit3 staining (Figure D-1c). Moreover, NET-like chromatin structure was not observed in cells treated with Cl-amidine prior to calcium ionophore treatment (Figure D-1c). The effect of PAD4 inhibition by Cl-amidine on histone H3 and H4 citrullination after calcium ionophore treatment was validated by Western blot (Figure D-1d, lane 3 compared with lane 2). These results indicate that PAD activity is important for histone citrullination and chromatin decondensation in HL-60 granulocytes following calcium ionophore treatment.

To further analyze whether PAD4 activity is important for NET formation under more physiologically relevant conditions, we treated HL-60 cells, DMSO differentiated HL-60 cells, or DMSO differentiated HL-60 cells pretreated with Cl-amidine with IL-8 and bacteria *Shigella flexneri*, which was shown to induce NET formation in peripheral blood neutrophils (Brinkmann *et al.*, 2004). Histone H3 citrullination was rarely detected in undifferentiated HL-60 cells treated with IL-8 and bacteria *Shigella flexneri* for 3 hr (Figure D-2a and 2d). Cells occasionally
observed with positive staining may reflect HL-60 cell spontaneous differentiation and PAD4 expression. In contrast, after differentiation along the granulocytic lineage to increase PAD4 expression and IL-8 and bacteria treatment, 9.8±0.7% (n=4) of cells became histone H3 citrullination positive with a concomitant increase of NET formation in 3.2±0.1% (n=4) of cells (Figure D-2b and 2d, cells with decondensed chromatin were denoted by arrows). Further, pretreatment of differentiated HL-60 cells with Cl-amidine inhibited histone citrullination and the formation of NETs (Figure D-2c and 2d). Percentages of cells with positive staining of H3 citrullination and/or chromatin decondensation in four independent experiments were counted and shown in bar graphs (Figure D-2d). Although a histone H3 citrullination positive cell may have a round nucleus or a nucleus with decondensed chromatin forming NETs, each nucleus with decondensed chromatin was stained with the histone H3 citrullination antibody. To quantify the amount of chromatin forming NETs, we performed MNase digestion using previously described methods (Fuchs et al., 2007) with slight modifications. Compared with the parental HL-60 cells, we consistently observed an increase in MNase digestion and nucleosomal DNA ladder formation in DMSO differentiated HL-60 cells after IL-8 and bacteria treatment for 15 hr (Figure D-2e, lanes 4 and 5). Furthermore, MNase digestion was decreased when DMSO differentiated HL-60 cells were pretreated with the PAD4 inhibitor, Cl-amidine (Figure D-2e, lanes 5 and 6). Together, the above immunostaining and MNase analyses indicate that PAD4 activity is required for histone citrullination and NET formation.
Figure D-1: PAD4 activity is important for the formation of highly decondensed chromatin.

(a-c) H4Cit3 and DNA staining of HL-60 granulocytes without treatment (a), with calcium ionophore treatment (b), and with the PAD4 inhibitor Cl-amidine treatment prior to calcium ionophore treatment (c). (d) Western blot assays of histone H3 and H4 citrullination. Histone H3 blot is a loading control.
Figure D-2: PAD4 activity is important for NET formation after cytokine and bacteria treatment.

(a-c) Histone H3 citrullination and DNA staining in undifferentiated HL-60 cells (a), DMSO differentiated HL-60 cells (b), and DMSO differentiated HL-60 cells pretreated with Cl-amidine after treatment with IL-8 and bacteria Shigella flexneri (c). (d) Percentages of cells with positive staining of H3 citrullination and/or chromatin decondensation with standard deviations (n=4, over 3,000 cells counted in each experiment). (e) After 15 hr of IL-8 and bacteria treatment, NET formation was measured by MNase digestion. Numbers denote mono- and poly-nucleosomal DNA.
References


221


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